The role of cytokinin in light-dependent seed germination in *Arabidopsis thaliana*

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List of abbreviations

°C	Degree Celsius
ABA	Abscisic acid
ABA1	*ABA-DEFICIENT1*
ABI	*ABSCISIC ACID–INSENSITIVE*
AHK	*ARABIDOPSIS HISTIDINE KINASE*
AHP	*ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN*
AMP	Adenosine monophosphate
APS	Ammonium persulfate
ARR	*ARABIDOPSIS RESPONSE REGULATOR*
ATP	Adenosine triphosphate
BA	Benzyladenine
BSA	Bovine serum albumin
cDNA	Complementary DNA
CK	Cytokinin
CKX	*CYTOKININ OXIDASE/DEHYDROXYGENASE*
CO	*CONSTANS*
Col-0	Columbia-0
Col-3	Columbia-3
COP1	*CONSTITUTIVELY PHOTOMORPHOGENIC 1*
CRF	*CYTOKININ RESPONSE FACTOR*
CUL4	*CULLIN 4*
CYP707A	*CYTOCHROME P450 707A*
CYP735A	*CYTOCHROME P450 MONOOXYGENASE*
cZ	*cis*-zeatin
DAG1	*DOF AFFECTING GERMINATION 1*
DDB1	*DAMAGED DNA BINDING 1*
ddH_2O	Double distilled water
DEG	Differentially expressed gene
DEPC	Diethylpyrocarbonate
DHZ	Dihydrozeatin
DMAPP	Dimethylallyl diphosphate
DOG1	*DELAY OF GERMINATION1*
DW	Dry weight
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EXPA2	*EXPANSIN2*
FDR	False discovery rate
FHL	*FHY1-LIKE*
FHY1	*FAR-RED ELONGATED HYPOCOTYL 1*
GA	Gibberellin
GA20ox	*GA20OXIDASE*
GAI	*GA INSENSITIVE*
GEO	Gene Expression Omnibus
GGPP	geranly-geranyl diphosphate
GID1	*GA INSENSITIVE DWARF1*
GO	Gene Ontology

h	Hour(s)
HDA15	*HISTONE DEACETYLASE*
HFR1	*LONG HYPOCOTYL IN FAR-RED*
HIR	High irradiance response
HR-peroxidase	Horseradish peroxidase
HY5	*LONG HYPOCOTYL5*
HYH	*HY5-HOMOLOG*
IKU	*HAIKU*
iP	Isopentenyl adenine
iPRDP	Isopentenyladenosine diphosphate
iPRMP	Adenosine monophosphate
iPRTP	Isopentenyladenosine triphosphate
IPT	*ISOPENTENYLTRANSFERASE*
JMJ	*JUMONJI*
KAO	ENT-KAURENOIC ACID OXIDASE
kDa	Kilodalton
LAF	*LONG AFTER FAR-RED LIGHT1*
LD	Long day
LFR	Low fluence response
LOG	*LONELY GUY*
mA	Milliampere
MES	2-(N-Morpholino) ethanesulfonic acid
MINI3	*MINISEED3*
MS	Murashige and Skoog
NASC	Nottingham Arabidopsis stock center
NCED	*9-CIS-EPOXYCAROTENOID DIOXYGENASE*
NLS	nuclear-localization
Pac	Paclobutrazol
PAR	Pphotosynthetically active radiance
PCA	Principal component analysis
PCR	Polymerase chain reaction
PIF1	*PHYTOCHROME INTERACTING FACTOR1*
PP2C	*PROTEIN PHOSPHATASES TYPE 2C*
PPDE	Posterior probability of differential expression
PUT2	*POLYAMINE UPTAKE TRANSPORTER 2*
PYL	*PYR1-LIKE*
PYR1	*PYRABACTIN RESISTANCE1*
qPCR	Quantitative real-time PCR
R/FR	red/far-red
RCAR	*REGULATORY COMPONENTS OF ABA RECEPTORS*
RGA	*REPRESSOR OF GA1-3*
RGL1	*RGA-LIKE1*
RIN	RNA integrity number
RMA normalization	Robust multi-array average normalization
RNA-Seq	RNA-Sequencing
ROS	Reactive oxygen species
RRL1	*RETARDED ROOT GROWTH-LIKE*
SDS	Sodium dodecyl sulfate

SnRK2	*SUCROSE NONFERMENTING1-RELATED SUBFAMILY2*
SOM	*SOMNUS*
SPA	*SUPPRESSOR OF phyA-105*
Taq	*Thermus aquaticus* DNA polymerase
TCS	Two-component system
Temed	N,N,N',N'-tetramethylethane-1,2-diamine
TT	*TRANSPARENT TESTA*
tZ	*trans*-zeatin
tZR	tZ riboside
UGP	*UDP-GLUCOSE PYROPHOSPHORYLASE*
UGT	*URIDINE DIPHOSPHATE GLYCOSYLTRANSFERASE*
UVR8	*UV RESISTANCE LOCUS 8*
VLFR	Very low fluence response
WS	Wassilewskija
X-Gluc	5-bromo-4-chloro-3-indolyl-ß-D-glucuronide
ZEP	*ZEAXANTHIN EPOXIDASE*

Summary

Seed germination is a precisely controlled process, involving multiple regulatory pathways. Cytokinin (CK) has been proposed to negatively regulate light-dependent seed germination in *A. thaliana*. Thus, the overall aim of the current study was to provide an in-depth analysis of the role of CK in seed germination induced by red (R) and far-red (FR) light.

In this work, the seed germination phenotype of various CK mutant- and transgenic plants in low and very low fluence R and FR light conditions was studied. Germination rates of CK mutants and transgenic lines were significantly higher than wild-type germination rates, underpinning the negative impact of CK on seed germination in low fluence R light as well as in very low fluence FR light. This study further identified increased germination rates compared to the wild type in seeds (i) impaired in CK biosynthesis, (ii) with an increased CK catabolism, (iii) with a reduced signal perception at the level of histidine kinase receptors (AHKs) and (iv) seeds impaired in CK signal transduction via histidine phosphotransfer proteins (AHPs). Consequently, the signaling components in the respective mutants may well contribute to the repressive effects of CK on germination. A putative role of signaling components downstream of AHPs, such as A- and B-type response regulators (ARRs) in very low fluence germination need further clarification.

The photoreceptor phytochrome A (phyA) is an essential part of the regulatory pathway controlling the onset of germination in very low fluence FR light. Germination assays analyzing CK biosynthesis- and CK receptor mutants lacking functional phyA led to the conclusion that phyA is essential for the induction of germination in very low fluence FR light both in the wild type and in CK mutants. Quantification of phyA protein levels did not confirm a repressive effect of CK on phyA abundance in seeds.

To dissect the hormonal pathways which may influence germination of CK mutant seeds in very low fluence FR light conditions, the contribution of abscisic acid (ABA) and gibberellins (GA) was analyzed into more detail. Hormone measurements indicated neither elevated GA level nor decreased ABA level in CK deficient seeds, suggesting a CK-independent regulation of bioactive GA- and ABA levels in imbibed seeds. Interestingly, CK negatively influenced GA sensitivity, which may be an additional mechanism for CK to suppress germination. However, CK had no measurable effect on ABA sensitivity.

A multitude of maternal effects are known to shape the germination response of the offspring. The present thesis revealed, that a lower CK status in maternal seed tissues led to increased germination rates of the respective seeds in FR light. However, a reduction of the CK status exclusively in the testa or the endosperm was not sufficient to increase germination rates significantly in FR light compared to wild-type seeds. These results exemplify the prominent role of CK as a negative regulator of germination in seed tissues with a higher maternal genome dosage.

In the present thesis, also the gene regulatory network underlying the negative effects of CK on the germination processes in FR light was studied. Although the seeds' CK status had only a minor effect on transcriptomic changes during imbibition, a major reprogramming of the transcriptome during FR light-induced germination dependent on the seeds' CK status was evident.

Overrepresented GO categories revealed that lipid-associated, seed maturation-associated and cell wall organization-associated transcripts were differentially regulated in *ahk2 ahk3* seeds in response to FR light. These results indicate that the aforementioned pathways might be relevant for the negative impact of CK on seed germination in very low fluence light.

Additionally, environmental factors such as the light environment of parental plants during seed development affect the germination phenotype of their offspring. This thesis demonstrated, that growth of parental plants in shade light conditions (enriched in FR light) did not affect the germination response of their F1 offspring in FR light. However, when parental plants were grown for two subsequent generations in shaded conditions, germination rates of F2 seeds were increased in very low fluence FR light conditions. These effects were independent of the seeds' CK status.

Since CK is a prominent regulator of seed size, in the last part of this work the connection between CK, seed size and seed age in FR light-induced germination was investigated. The current study found smaller seeds to germinate better when germination was induced by FR light, again this effect was similar in wild-type seeds and seeds with a reduced CK signal transduction. Regarding seed age, the repressive effect of CK on germination in FR light was retained in aged seeds during long-term storage.

Overall, the presented results improve the understanding how seed germination in non-optimal low and very low fluence light conditions is regulated by CK. CK exerts a negative influence on germination, which is not dependent on altered GA or ABA hormone levels, but seems to involve seed tissues with a higher maternal genome dosage.

Zusammenfassung

Pflanzen regulieren den Prozess der Samenkeimung mit Hilfe verschiedener Phytohormone, unter anderem durch Cytokinin (CK). In der vorliegenden Arbeit wurde die Rolle von CK als negativem Regulator der Samenkeimung im Niedrigfluenzbereich von rotem (R) und dunkelrotem (DR) Licht in *A. thaliana* untersucht.

Im ersten Teil der Arbeit wurde die Keimungsantwort verschiedener Cytokininmutanten und transgener Linien bei niedriger oder sehr niedriger Belichtungsintensität getestet. Cytokininmutanten und transgene Linien mit einem geringeren CK Status zeigten erhöhte Keimungsraten und wiesen damit auf einen negativen Einfluss von CK nach Induktion der Keimung durch Rotlicht und Dunkelrotlicht hin. Im Rahmen dieser Arbeit konnten erhöhte Keimungsraten verglichen zum Wildtyp in Samen (i) mit verringerter Cytokininbiosynthese, (ii) mit erhöhtem Cytokininkatabolismus, (iii) mit verringerter Signaltransduktion über Cytokininrezeptoren und (iv) verringerter Signalweiterleitung über Histidin-Phosphotransferproteine (AHPs) nachgewiesen werden. Dies deutet auf einen negativen Effekt der untersuchten Komponenten des Cytokininmetabolismus- und signalwegs in der Keimung hin. Ob weitere Komponenten des Signaltransduktionsweges, wie beispielsweise A- und B-Typ Antwortregulatoren (ARRs), wichtig für die Samenkeimung im Niedrigfluenzbereich sind, ist nach heutigem Stand dieser Arbeit nicht abschließend geklärt.

Der Fotorezeptor Phytochrom A (phyA) ist ein essentieller Teil der Signalkaskade, die die Initiation der Keimung bei niedriger oder sehr niedriger Belichtungsintensität bedingt. Analysen der Keimungsraten von Cytokininbiosynthese- und Cytokininrezeptormutanten, die mit *phyA* gekreuzt wurden, zeigten, dass phyA, unabhängig vom Cytokininstatus der getesteten Samen, essentiell wichtig für die Keimung ist. Allerdings konnte eine Reduktion der phyA-Proteinlevel durch CK im Rahmen einer Proteinquantifizierung nicht bestätigt werden.

Um die Beteiligung anderer Phytohormone an der Regulation der Keimung in Cytokininrezeptormutanten abzuklären, wurden Abscisinsäure (ABA) und Gibberellin (GA), zwei in der Keimung ausführlich beschriebene Phytohormone, näher untersucht. Hormonmessungen ergaben weder erhöhte GA Level noch verringerte ABA Level in cytokinindefizienten Samen, was auf eine von cytokininunabhängige Regulation der Hormonlevel schließen lässt. Allerdings konnte gezeigt werden, dass CK einen negativen Effekt auf die GA-Sensitivität hat. Dies könnte auf einen zusätzlichen Mechanismus hinweisen, der die Repression der Keimung durch CK begünstigt. Auf die Sensitivität der Samen gegenüber exogen appliziertem ABA hatte CK keinen messbaren Einfluss.

Samenkeimung wird durch eine Vielzahl maternaler Effekte beeinflusst. Im Rahmen der vorliegenden Arbeit konnte nachgewiesen werden, dass ein verringerter CK Status in der Samenschale zu erhöhten Keimungsraten nach Belichtung mit dunkelrotem Licht führte. Allerdings war die Reduktion des CK Status' ausschließlich in der Testa oder dem Endosperm nicht ausreichend, um erhöhte Keimungsraten im Dunkelrotlicht zu induzieren. Diese Ergebnisse weisen auf eine wichtige Rolle der Samenschale im Zusammenhang mit der Regulation der Keimung durch CK hin.

Im Rahmen der vorliegenden Arbeit wurden zudem transkriptionelle Änderungen in cytokinindefizienten- und wildtypischen Samen im Verlauf der Samenkeimung untersucht, um neue Hinweise zur Regulation der Keimung durch CK zu gewinnen. Dabei konnte nachgewiesen werden, dass der Cytokininstatus das Transkriptom quellender Samen kaum beeinflusste. Nach Induktion der Keimung durch Dunkelrotlicht waren allerdings erhebliche Veränderungen auf transkriptioneller Ebene abhängig vom Cytokininstatus der Samen nachweisbar. Differentiell regulierte Gene konnten in „gene ontology" Kategorien klassifiziert werden. Dabei zeigte sich, dass nach einem Dunkelrotlichtpuls Gene, die mit Lipiden, der Samenreifung und der Zellwand assoziiert sind, in Cytokininrezeptormutanten überrepräsentiert waren. Dies könnte ein Hinweis sein, dass diese Prozesse an die Regulation der Keimung durch CK beteiligt sein könnten.

Zudem beeinflussen Umweltfaktoren, wie beispielsweise die Lichtbedingungen, denen Elternpflanzen während der Samenentwicklung ausgesetzt sind, die Keimung der Nachkommen. Eine Anzucht der Elternpflanzen im Schatten beeinflusste in Untersuchungen allerdings nicht das Keimungsverhalten der F1 Nachkommen im Dunkelrotlicht. Wurden allerdings zwei aufeinanderfolgende Generationen im Schatten angezogen, zeigten die resultierenden F2 Samen erhöhte Keimungsraten im Dunkelrotlicht. Dieser Effekt war unabhängig vom Cytokininstatus der getesteten Pflanzen.

Da CK auch die Samengröße reguliert, wurde im letzten Teil dieser Arbeit der Zusammenhang zwischen CK, der Samengröße, des Samenalters und der durch Dunkelrotlicht induzierten Keimung untersucht. Dabei konnte gezeigt werden, dass kleinere Samen höhere Keimungsraten im Dunkelrotlicht aufwiesen als größere Samen. Dieser Effekt war unabhängig vom Cytokininstatus der untersuchten Samen. Untersuchungen zum Einfluss von CK auf die Samenkeimung nach länger Lagerungszeit ergaben, dass CK auch nach langer Lagerungszeit die Keimung im Dunkelrotlicht unterdrückte.

Abschließend ist festzuhalten, dass die in dieser Arbeit gewonnenen Erkenntnisse einen wichtigen Beitrag zum Verständnis der Rolle von CK in der durch Dunkelrotlicht induzierten Samenkeimung leisten.

1 Introduction

Despite the multitude of known influences on almost all aspects of a plant's life, the role of CK in the regulation of the seed germination process is still largely unknown. Thus, the overall aim of this work was to unravel the functional relevance of CK during seed germination.

1.1 The phytohormone cytokinin

CK was discovered in the early 1950s as compound stimulating cell divisions in callus cultures (Miller *et al.*, 1955). Since then, multiple functions of CK in plants have been uncovered, including positive regulation of the shoot apical meristem activity, apical dominance, negative regulation of the root meristem activity, impacts on shoot and root branching, organ size, developmental transitions such as flowering and seed germination (Werner and Schmülling, 2009; Kieber and Schaller, 2018). Additionally, CK was recognized as regulator of plant stress responses both in abiotic and biotic stress situations (Choi *et al.*, 2011; O'Brien and Benková, 2013; Cortleven *et al.*, 2019a).

1.1.1 CK biosynthesis

CKs are N^6-substituted adenine derivatives. Chemically, CKs can be classified by the chemical structure of the substituted side chain in isoprenoid and aromatic CKs. The most abundant CKs in plants have isoprenoid side chains, such as N^6-isopentenyladenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ) and dihydrozeatin (DHZ). In *Arabidopsis*, iP- and tZ-type CKs are the most abundant CKs and display the highest bioactivity (Sakakibara, 2006) while cZ and DHZ are less relevant (Spíchal *et al.*, 2004; Romanov *et al.*, 2006; Heyl *et al.*, 2012). Free CK bases are regarded as major active CK isoforms. Apart from them, CK exists as conjugates of free CK bases with ribose sugar, named CK ribosides, or with phosphorylated ribose sugar, named ribotides (Kieber and Schaller, 2014). Both CK ribotides and ribosides can be converted to free CK bases. tZ riboside (tZR) for example is a major CK transport form through the xylem to the shoot via the action of the ABCG14 transporter (Zhang *et al.*, 2014).

Isopentenyltransferases (IPTs) catalyse the initial, rate-limiting step of CK biosynthesis, using dimethylallyl diphosphate (DMAPP) as prenyl donor and either tRNA or adenosine phosphate (ATP/ADP/AMP) as substrates. Both biosynthesis pathways require the action of IPTs (Figure 1). In *Arabidopsis*, *IPT*s are encoded by a gene family consisting of nine family members. IPT1, IPT3 and IPT4 to IPT8 utilize adenosine phosphate as substrate, ultimately leading to the formation of tZ and iP (Takei *et al.*, 2004; Miyawaki *et al.*, 2006) while IPT2 and IPT9 use tRNA as substrate and catalyze the biosynthesis of cZ (Figure 1) (Takei *et al.*, 2001; Kakimoto, 2001; Miyawaki *et al.*, 2006). The production of cZ depends on the prenylation of tRNA, what is catalyzed by IPT2 and IPT9. Prenylation of adenosine phosphate results in the formation of isopentenyladenosine di- or triphosphate (iPRDP and iPRTP) (Kakimoto, 2001; Takei *et al.*, 2004; Sakano *et al.*, 2004). In the next step, cytochrome P450 monooxygenases (CYP735A1 and CYP735A2) catalyze the hydroxylation of the isoprenoid side chain of the produced iP-ribotides to tZ-ribotides (Takei *et al.*, 2004).

Ribotides are converted to active free CK bases primarily by the action of Lonely guy (LOG) enzymes (Figure 1), which catalyze the production of free bases in a single step in *A. thaliana* using monophosphate-ribotides as substrates (Figure 1) (Kurakawa *et al.*, 2007; Kuroha *et al.*, 2009).

1.1.2 CK catabolism

To regulate the amount of active CKs, plants control both CK biosynthesis and CK degradation and inactivation by conjugation. CK inactivation by conjugation with sugars is mediated either by *N*- or by *O*-glycosylation and is catalyzed by uridine diphosphate glycosyltransferases (UGTs) (Sakakibara, 2006). While CK *O*-glucosides can be converted back to active CK bases, N-glycosylation irreversibly inactivates them (Brzobohaty *et al.*, 1993; Sakakibara, 2006).

CK degradation is catalyzed by cytokinin oxidases/dehydrogenases (CKX) in a single step reaction (Figure 1) (Brownlee *et al.*, 1975; McGaw and Horgan, 1983). CKXs irreversibly cleave unsaturated N^6-isoprenoid side chains of tZ and iP-type (Brownlee *et al.*, 1975; McGaw and Horgan, 1983). Preferred CKX substrates are iP- and tZ-type CK bases and their ribosides, while DHZ-type CKs and benzyladenine (BA) are poor CKX substrates (Armstrong, 1994; Jones and Schreiber, 1997). CK *O*-glycosylation prevents cleavage by CKX (Armstrong, 1994; Werner *et al.*, 2006). In *A. thaliana*, seven *CKX* encoding genes were identified (Bilyeu *et al.*, 2001; Schmülling *et al.*, 2003; Werner *et al.*, 2003). To achieve tissue-specific action, *CKX* vary in their expression pattern throughout different plant tissues and in their localization (Werner *et al.*, 2003). CKX2, CKX4, CKX5 and CKX6 are secreted in the apoplast (Bilyeu *et al.*, 2001; Werner *et al.*, 2001; Werner *et al.*, 2003). CKX1 and CKX3 are located primarily in the ER and the secretory system (Werner *et al.*, 2003; Niemann *et al.*, 2015). CKXs also vary in their activity, while CKX2 and CKX4 are highly active enzymes, CKX1, CKX3, CKX5 and CKX7 are less active (Galuszka *et al.*, 2007). CKX enzymes vary in their substrate preferences as well, also depending on the pH value (Galuszka *et al.*, 2007). All CKX enzymes are able to use free bases as substrates, while secreted CKX prefer free tZ and iP, CKX1 for example prefers iPR over iP (Galuszka *et al.*, 2007).

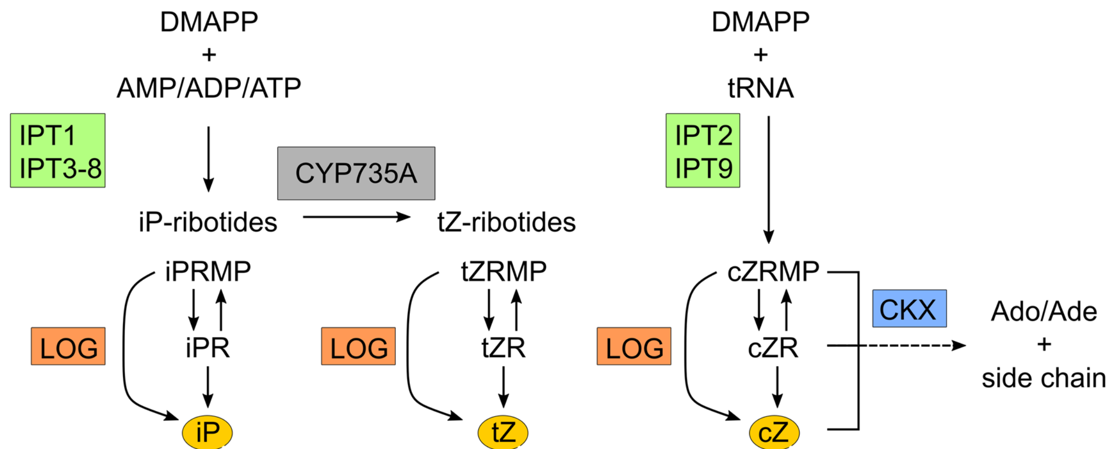

Figure 1. Simplified scheme of cytokinin biosynthesis and catabolism in plants.
IPTs catalyze the biosynthesis of CK ribotides from DMAPP and either adenosine phosphate or tRNA. iP ribotides can be converted to tZ ribotides by the action of CYP735As. iP ribotides can be either converted to the active free bases in a two-step process or in a single step reaction catalyzed by LOGs. CKX degrade CKs.
DMAPP, dimethylallyl diphosphate; adenosine monophosphate, AMP; iPRMP, isopentenyladenosine monophosphate; iP, isopentenyl adenine; tZ, *trans*-zeatin; cZ, *cis*-zeatin; IPT, isopentenyltransferase; CYP735A, cytochrome P450 monooxygenases; LOG, lonely guy; CKX, cytokinin oxidases/dehydrogenases. Modified from Cortleven *et al.* (2018).

1.1.3 CK signaling

In plants, the CK signal is perceived and transduced in a multistep phosphorelay system, which is a more complex version of bacterial two-component systems (TCS). CK perception is mediated via binding of CK to the CHASE domain of the sensor histidine kinase, this results in autophosphorylation of the histidine residue in the kinase domain. The phosphate signal is then relayed to a conserved aspartate residue in the receiver domain of the CK receptor. In the *A. thaliana* genome, three CK-perceiving histidine kinase receptors are encoded, AHK2, AHK3 and AHK4/CRE1 (Figure 2). Crucial for the autophosphorylation of the receiver domain is the dimerization of two AHK monomers (Dortay *et al.*, 2008; Hothorn *et al.*, 2011). Their role as CK receptors have been demonstrated on multiple levels, embracing genetic, biochemical and molecular evidence (Mähönen *et al.*, 2000; Ueguchi *et al.*, 2001a; Yamada *et al.*, 2001; Inoue *et al.*, 2001; Hwang and Sheen, 2001). Reflecting their essential role in CK perception, *AHK* genes are expressed throughout the plant (Nishimura, 2004; Higuchi *et al.*, 2004). While *AHK2* and *AHK3* are mainly expressed in above-ground plant organs (Nishimura, 2004; Higuchi *et al.*, 2004), *AHK4/CRE1* is expressed preferentially in the root (Mähönen *et al.*, 2000; Higuchi *et al.*, 2004). On a subcellular level, AHKs are predominantly localized at the ER membrane, but there is also evidence for a plasma-membrane localization (Caesar *et al.*, 2011; Wulfetange *et al.*, 2011; Lomin *et al.*, 2011). The three AHK receptor perceiving CK vary in their substrate affinities. iP- and tZ-type CKs are the most abundant CKs in plants, what is mirrored in the high binding affinity of AHK2 and AHK4 towards iP and tZ. AHK3 displays a high affinity towards tZ, but the affinity towards iP is drastically reduced (Spíchal *et al.*, 2004; Romanov *et al.*, 2006; Stolz *et al.*, 2011). Adenine itself and inactive CK conjugates were bound by the receptors only with low affinity (Spíchal *et al.*, 2004).

If plants lack all three CK receptors they display strong dwarfism phenotypes, infertility or reduced fertility and altered germination phenotypes (Nishimura, 2004; Higuchi *et al.*, 2004; Riefler *et al.*, 2006).

After phosphorylation of AHKs the phosphate signal is relayed in the TCS from AHKs to AHPs (Figure 2) (Suzuki *et al.*, 2000). AHPs in *A. thaliana* are encoded by a family of six genes. After phosphorylation, AHP1 to AHP5 transmit the phosphate signal to B-type response regulators (B-type ARRs), thus positively feeding into the CK signal transduction pathway. AHP6 is a pseudo-phosphotransfer protein, which lacks a conserved histidine residue essential for signal transmission (Bishopp *et al.*, 2011). Consequently, AHP6 is a negative regulator of the CK signal transduction pathway (Mähönen *et al.*, 2006) (Figure 2).

B-type ARRs are transcription factors, which are encoded by eleven genes in the *A. thaliana* genome, *ARR1*, *ARR2*, *ARR10* to *ARR14*, *ARR18 to ARR21.* They modulate the expression of CK primary response genes after their phosphorylation (Sakai *et al.*, 2001; Heyl and Schmülling, 2003; Brenner *et al.*, 2005; Kieber and Schaller, 2014). In plants, ARR1, ARR10 and ARR12 are regarded as the most important B-type ARRs, since they predominantly regulate multiple responses to CK (Mason *et al.*, 2004; Mason *et al.*, 2005; Ishida *et al.*, 2008; Kieber and Schaller, 2014). *Arr1 arr10 arr12* mutants exhibit CK deficiency symptoms such as defects in shoot development, enlarged seed size, abortion of primary root growth and defects in gametophyte development (Argyros *et al.*, 2008; Ishida *et al.*, 2008; Cheng and Kieber, 2013).

Target genes of B-type ARRs are termed primary response genes. The best known primary response genes in this signaling pathway are A-type *ARR*s and *CYTOKININ RESPONSE FACTOR* (*CRF*) genes (Rashotte *et al.*, 2006) (Figure 2). Their expression in *Arabidopsis* is strongly and rapidly induced after CK treatment (D´Agostino *et al.*, 2000; Rashotte *et al.*, 2003; Brenner *et al.*, 2005). The promoters of A-type *ARR* genes contain binding sites for B-type ARRs which contribute to the induction of A-type *ARR* genes after CK treatment (Taniguchi *et al.*, 2007; Ramireddy *et al.*, 2013). A-type ARRs are encoded by a gene family consisting of ten members in *A. thaliana*, *ARR3* to *ARR9*, *ARR15* to *ARR17* (Heyl and Schmülling, 2003; Kieber and Schaller, 2014). A-type ARRs accept the phosphate signal from AHPs but in contrast to B-type ARRs, they lack a DNA binding domain to induce expression of target genes. Since they compete with B-type ARRs for phosphorylation from AHPs (To *et al.*, 2007), they are regarded as negative feedback regulators of the CK signaling pathway (Werner and Schmülling, 2009) (Figure 2). Despite their high functional redundancy, the expression pattern of individual *ARR* genes may point to a more specific function or a functional relevance in a certain tissue. In seeds for example, *ARR19* and *ARR21* are highly and specifically expressed in the chalazal endosperm (Day *et al.*, 2008).

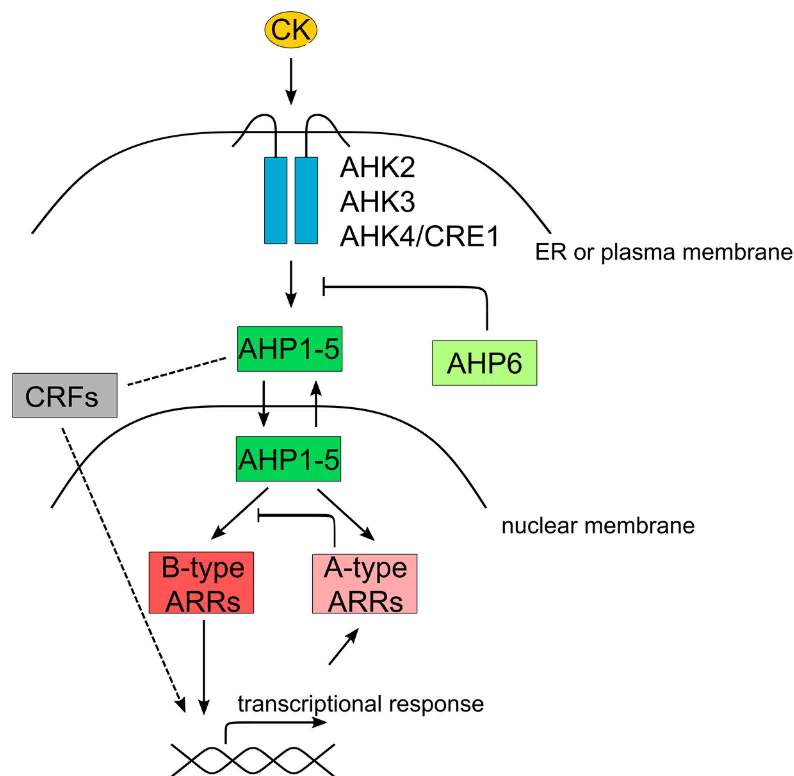

Figure 2. Simplified scheme of the CK signal transduction pathway.
CK is perceived by membrane-bound histidine kinase receptors (AHK2, AHK3 and AHK4/CRE1) thus triggering the phosphorelay of the phosphate signal to histidine phosphotransfer proteins 1-5 (AHP1-5). AHP6 is a pseudo-phosphotransfer protein which does not transduce the phosphate signal, thus negatively regulating the pathway. AHP1-5 translocate to the nucleus upon phosphorylation, there they phosphorylate A-and B-type response regulators (ARRs). B-type ARRs bind to the DNA and induce a transcriptional response. A-type ARRs lack a DNA binding domain thus negatively affecting the transcriptional response. Cytokinin response factors (CRFs) have been shown to interact with AHPs, are translocated to nucleus and regulate CK-dependent gene expression, but their role in the CK signaling pathway is still not clear (dashed lines). Modified from El-Showk *et al.* (2013).

1.2 Seed development

Flowering plants are diploid and reproduce via meiosis and the subsequent union of female and male germ cells within the flowers. The ovules are the female reproductive tissue, which are enclosed in the ovary. After meiosis, each ovule contains a haploid egg cell (n) and a central cell, comprising two nuclei (2 n). In the course of the fertilization, the pollen grain delivers two sperm cells to the female gametophyte in the ovule. One of the sperm cells fuses with the egg cell to form a zygote, the other sperm cell fuses with the diploid central cell to form a triploid cell, the predecessor of the endosperm. This double fertilization is common in angiosperms (Raghavan, 2003).

1.2.1 Embryo development

After double fertilization, the zygote elongates along the apical-basal axis before undergoing an asymmetrical cell division resulting in a small apical cell, from which the embryo originates and a larger basal cell, which develops into the extra-embryonic

suspensor (ten Hove *et al.*, 2015). Seed development is concluded with mature green seeds, the entire developmental process takes typically 20 days in *A. thaliana* (Baud *et al.*, 2002). Three distinct phases of seed development have been postulated (Baud *et al.*, 2002; Chahtane *et al.*, 2017): (1) early embryogenesis, (2) maturation phase and (3) late maturation phase.

Phase 1: In the early embryogenesis stage, seed weight and lipid content are low, and a transient starch accumulation occurs, which is important for later stages of development (Baud *et al.*, 2002). During early embryogenesis, the embryo develops from the pre-globular stage to the globular stage (Figure 3). Four days after flowering, the globular stage *A. thaliana* embryos develops into the heart stage.

Phase 2: Five to six days after flowering, the second developmental phase named maturation phase is initiated. It is characterized by a rapid increase in seed dry weight, due to the accumulation of storage compounds as for example storage lipids and proteins (Baud *et al.*, 2002; Holdsworth *et al.*, 2008b). Around seven days after flowering the embryo develops into the linear cotyledon stage, followed by the mature green stage (Figure 3) (Baud *et al.*, 2002). The onset of maturation is regulated individually between seeds of an ovary and is determined by the developmental stage of the embryo (O'Neill *et al.*, 2019).

Phase 3: The third phase of seed development is the late maturation phase. It is characterized by a constant seed dry weight, and water loss at the end of this phase in the desiccation process (Baud *et al.*, 2002).

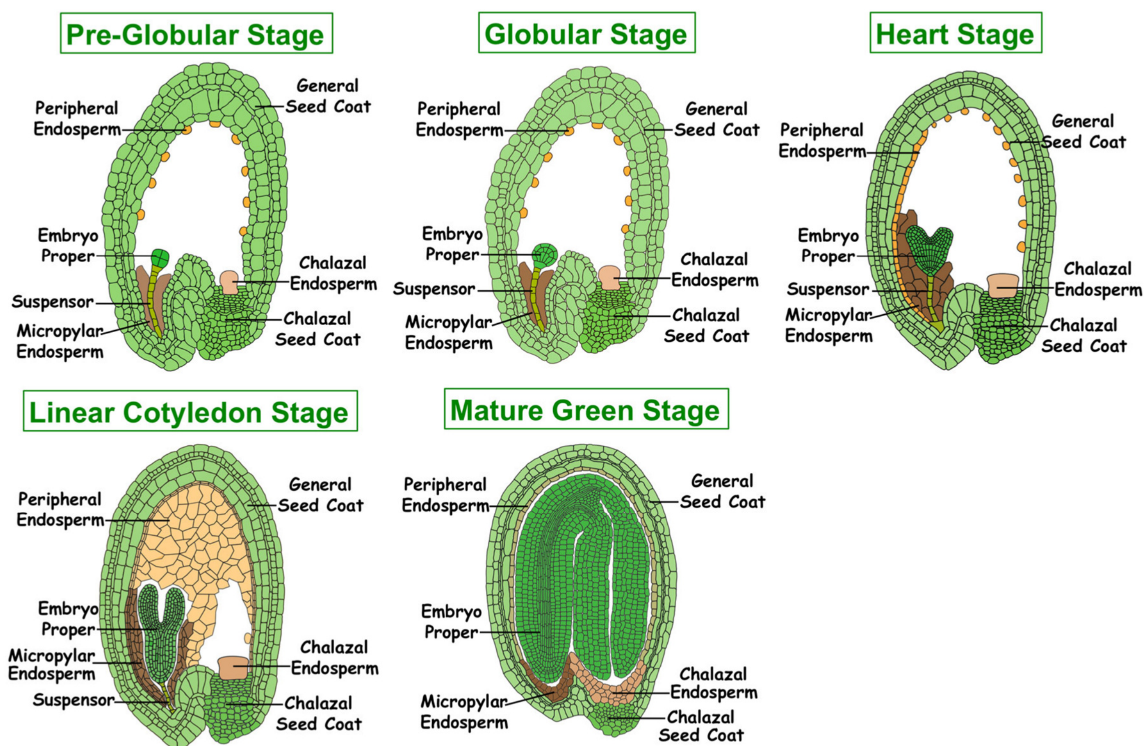

Figure 3. Seed development in *A. thaliana*.
Depicted are five distinct developmental stages of embryo and endosperm development after fertilization. Modified from Belmonte *et al.* (2013).

1.2.2 Endosperm development

Comparable to embryo development, endosperm development is also initiated in the double fertilization process. The triploid central cell, containing two third maternal genome dosage, starts to develop into the endosperm tissue (Olsen, 2001). The first step in this process is the formation of the endosperm coenocyte. This is mediated by repeated rounds of mitosis lacking the following cytokinesis. As a result, a rapidly expanding multinucleate cell is formed (Olsen, 2001; Soerensen *et al.*, 2002). This syncytial endosperm can be separated in different regions according to their position (Brown *et al.*, 1999; Soerensen *et al.*, 2002). The micropylar endosperm is located in close vicinity to the micropyle, surrounding the developing embryo. At the posterior pole the chalazal endosperm is formed. Besides these two specialized endosperm regions, the remaining endosperm is referred to as peripheral endosperm (Soerensen *et al.*, 2002).

The second step in endosperm development is the cellularization of the coenocyte. Cellularization is initiated at the micropylar endosperm and progresses through the peripheral endosperm towards the chalazal endosperm pole (Brown *et al.*, 1999; Soerensen *et al.*, 2002). Embryo and endosperm development is closely connected to each other, although endosperm cellularization is not required for embryo maturation (O'Neill *et al.*, 2019). Embryo cytokinesis and endosperm cellularization share common components on a molecular level but are clearly distinguishable from each other (Soerensen *et al.*, 2002). In *A. thaliana*, the last step of endosperm development is the consumption of the cellularized endosperm by the embryo. Only a peripheral single cell aleurone-like endosperm layer is retained in mature *A. thaliana* seeds (Olsen, 2004).

1.2.3 Testa development

Besides the embryo and the endosperm, mature *A. thaliana* seeds are covered by a layer of brown, dead tissue, named testa. The testa is derived from the the outer and inner maternal integuments that surround the female gametophyte. These layers of solely maternal tissue differentiate and develop into the seed coat, which is responsible for mucilage production (Windsor *et al.*, 2000). The differentiation of the integument cell layers is initiated after fertilization of the central cell. A signal, probably auxin, is transmitted via the action of the AGAMOUS-LIKE 62 transcription factor from the developing seeds towards the seed covering tissue, and initiates the differentiation of the integument to the seed coat (Roszak and Köhler, 2011; Figueiredo *et al.*, 2016; Coen *et al.*, 2017).

During seed coat development, an apoplastic, maternally-derived cutin barrier, produced by the maternal inner integument layer is deposited between maternal and zygotic tissue (De Giorgi *et al.*, 2015; Loubéry *et al.*, 2018). Both deposition and maintenance of the cuticle is regulated by TRANSPARENT TESTA (TT) transcription factors (Loubéry *et al.*, 2018; Coen *et al.*, 2019).

1.2.4 Communication between the seed tissues

Communication among the diploid zygote, the triploid endosperm and the surrounding maternal seed coat is essential for the development of viable seeds (Figueiredo *et al.*, 2016). The regulation of germination is mediated by a coordinated action of all seed tissues. In *Arabidopsis*, crosstalk between the endosperm and the testa is required for rupture of the seed coat, the micropylar endosperm resistance is significantly decreased prior to testa rupture (Lee *et al.*, 2012a). The embryo regulates germination via its growth potential and the elongation of hypocotyl cells in the course of germination. The endosperm is a highly

metabolic active tissue, providing resources and hormonal signals for germination. The testa sets mechanical constrains to the germinating embryo and regulates the light spectrum and intensity that reaches the embryo by its pigmentation (Donohue, 2009; Steinbrecher and Leubner-Metzger, 2018).

1.2.5 Regulation of seed development by CK

Seed development is regulated by a multitude of internal and external factors, such as environmental factors and phytohormones, including auxin, ABA, GA, CK and brassinosteroids. But also epigenetic effects may affect seed development and seed germination (Locascio *et al.*, 2014; Narsai *et al.*, 2017). It has been demonstrated that components of the CK pathway are involved in the regulation of seed development. So the ARRs *ARR8*, *ARR18*, *ARR19* and *ARR21* are expressed in the endosperm, together with *CRF2* and *CRF3* (Day *et al.*, 2008), suggesting a function of CK in the endosperm. Additionally, *IPT4* expression is located in the chalazal endosperm region (Miyawaki *et al.*, 2004) which is induced by ARR21 as has been shown in an *ARR21* overexpression experiment (Kiba *et al.*, 2005). This may indicate that the CK status in the chalazal endosperm is regulated by the coordinated action of ARR21 and possibly ARR19 with IPT4 early in seed development (Day *et al.*, 2008).

Also, epigenetic mechanisms are coupled to CK in seed development. The expression of the CK-catabolizing *CKX2* gene in the endosperm is activated by a member of the IKU pathway and promotes endosperm growth (Li *et al.*, 2013). *HAIKU* (*iku*) mutants phenocopy the increased endosperm growth of seeds harboring an excess of maternal genome dosage. The three genes belonging to the IKU pathway are *IKU1*, encoding a protein of yet unknown function, *IKU2*, a transmembrane kinase and *MINISEED3* (*MINI3*), a transcription factor(Luo *et al.*, 2005). *CKX2* is a transcriptional target of the IKU pathway, *CKX2* expression is induced by IKU1 and IKU2 (Li *et al.*, 2013).

The impact of epigenetic modifications on germination is under investigation. At the end of germination for example, a large-scale demethylation occurs (Narsai *et al.*, 2017). A recent study conducted by Gu *et al.* (2019) identified a histone H3 lysine methyltransferase as a positive regulator of light-dependent seed germination. Moreover, the histone deacetylase HDA15 interacts with PHYTOCHROME INTERACTING FACTOR1 (PIF1), to suppress phyB-mediated seed germination (Gu *et al.*, 2017).

1.3 Seed germination

The seed germination process, from initiation of germination to its completion, is described as *sensu stricto* seed germination in the literature (Fait *et al.*, 2006). This term defines physiological events occurring in non-dormant seeds from the start of imbibition until radicle emergence (Nonogaki, 2006; Nonogaki, 2014).

Mature, dry seeds still contain a residual water content of around ten percent, therefore metabolic processes, such as after-ripening, may still occur in these low-hydrated seeds (Linko and Milner, 1959; Weitbrecht *et al.*, 2011). However, water is not distributed evenly throughout dry seeds.

Nuclear magnetic resonance microimaging of water distribution in tobacco seeds suggests local water accumulation sites (Manz *et al.*, 2005), which provide the basis for biochemical reactions, allowing low-level transcription, post-transcriptional processing and translation also in dry seeds (Holdsworth *et al.*, 2008a; Weitbrecht *et al.*, 2011).

1.3.1 Stages of the germination process

In *A. thaliana*, seed imbibition and germination can be separated in three distinct phases on the basis of the triphasic water uptake of seeds (Bewley, 1997; Manz *et al.*, 2005). These phases are referred to as phase I, phase II and phase III.

Phase I: During imbibition phase I (Figure 4), the low water potential of dry seeds triggers a rapid water influx along the micropyle, independent of the seeds' viability (Weitbrecht *et al.*, 2011). The micropyle is the major entry point of water into the seeds, because there an endospermal lipid gap in the lipid-rich aleurone-layer is located which is established during testa development (Munz *et al.*, 2017; Loubéry *et al.*, 2018). During water uptake, a variety of cellular solutes leak from imbibing seeds. Responsible for this are membrane phase transitions in the re-hydration phase of dry seeds (Crowe *et al.*, 1989). Leakage of solutes from seeds also depends on the characteristics of the seed covering layers (testa and endosperm), as has been shown in *Pisum sativum* (Powell, 1989). In phase I, the seed translatome encompasses transcripts reflecting the embryonic maturation program, before switching to a germination metabolism program which involves reserve mobilization and antioxidation processes (Galland *et al.*, 2014). This is supported by the notion, that seeds are able to complete germination after addition of a transcriptional inhibitor, but not after application of a translational inhibitor (Rajjou *et al.*, 2004). At the end of phase I, reserves are mobilized and endosperm weakening occurs (Bradford and Nonogaki, 2007).

Phase II: After the rapid water influx in phase I, the water content in the seeds remains relatively stable in phase II, ('plateau phase', Weitbrecht *et al.*, 2011). In species with a two-step germination process, as *A. thaliana*, testa- and endosperm rupture are two separate events (Figure 4). The water uptake phase II comprises the testa rupture (Kucera *et al.*, 2005; Müller *et al.*, 2006; Bradford and Nonogaki, 2007). DNA repair mechanisms are activated in the embryo, accompanied by a degradation of stored mRNAs and transcription and translation. The duration of phase II can vary widely (Weitbrecht *et al.*, 2011), since imbibed dormant seeds would also complete water uptake phase I, but remain in phase II until primary dormancy is released (Bradford and Nonogaki, 2007).

Phase III: After phase II non-dormant seeds enter water uptake phase III, in course of which endosperm rupture (Figure 4) and radicle protrusion occurs and seeds complete germination (Weitbrecht *et al.*, 2011). Germination is completed by the protrusion of the radicle through the seed covering cell layers (Kucera *et al.*, 2005; Finch-Savage and Leubner-Metzger, 2006). Furthermore, the transition from water uptake phase II to phase III, which is characterized by late embryo cell elongation, is negatively influenced by ABA, while phase I, II and testa rupture are not affected by ABA (Manz *et al.*, 2005).

1.3.2 Biomechanics of germination

Prior to imbibition, a mechanical balance between the embryo and the endosperm is established. To shift this balance towards the onset of germination, signaling from the embryo towards the endosperm is required (Yan *et al.*, 2014; Steinbrecher and Leubner-Metzger, 2018). Testa rupture is as well a coordinated process, the testa has pre-defined breaking points that facilitate tissue rips (Finch-Savage and Leubner-Metzger, 2006; Steinbrecher and Leubner-Metzger, 2017). The rupture of testa and endosperm is mainly mediated by cell elongation in the embryo rather than by cell division events (Bewley, 1997). In the embryo, the expansion of cotyledon cells, hypocotyl cells and elongation of cells in the radicle adjacent transition zone provide an uni-directional force that allows the radicle to pass through the endosperm cell layer (Penfield *et al.*, 2006; Sliwinska *et al.*, 2009).

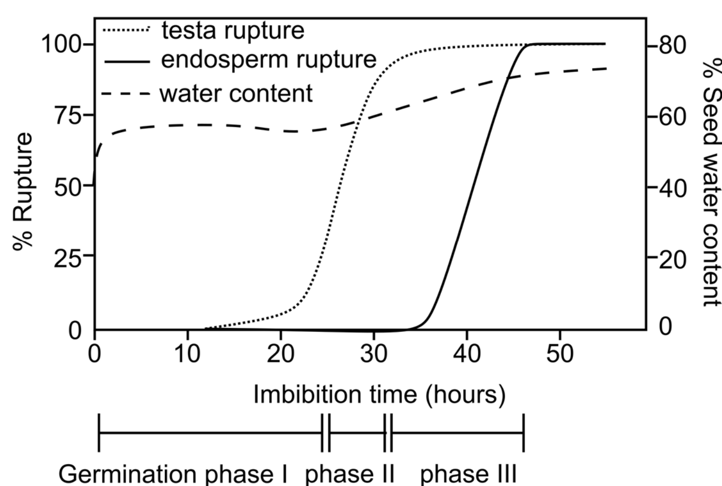

Figure 4. Seed imbibition and germination phases in *A. thaliana*.
In *A. thaliana*, testa rupture and endosperm rupture are temporally separated processes, testa rupture precedes endosperm rupture. Based on the dynamics of testa rupture, endosperm rupture and seed water content, three distinct germination phases may be defined. Modified from Dekkers *et al.* 2013.

Pre-requirement for the onset of germination is endosperm weakening. This endosperm weakening is discontinuous among the tissue, weakening at the micropylar endosperm region is a pre-requisite for successful germination and more pronounced compared to chalazal endosperm weakening (Leubner-Metzger, 2003; Müller *et al.*, 2006; Lee *et al.*, 2012a). Both the onset and the rate of endosperm weakening is controlled by balanced ABA and GA levels. While ABA negatively influences endosperm weakening in a dose-dependent manner and lowers the embryo growth potential by preventing cell wall loosening (Schopfer and Plachy, 1985; Müller *et al.*, 2006; Penfield *et al.*, 2006), GA biosynthesis facilitates endosperm rupture in *A. thaliana* and *L. sativum* seeds (Müller *et al.*, 2006).

In dormant seeds endosperm weakening is blocked, as has been demonstrated in *L. sativum* seeds by overexpressing the dormancy-associated *DELAY OF GERMINATION1* (*DOG1)* gene.
Notably, while endosperm weakening is dependent on *DOG1*, the embryo growth potential is not blocked (Graeber *et al.*, 2010). Furthermore, *DOG1* regulates temperature-dependency of testa- and endosperm rupture. Overexpression of *DOG1* shift the optimal germination temperature of the seeds to 18 °C, germination is delayed at 24 °C (Graeber *et al.*, 2014).

1.3.3 Seed dormancy influences germination

The classical definition of seed dormancy was introduced by Vleeshouwers *et al.* (1995). Dormancy was defined as a seed characteristic, the degree of which defines what conditions should be met to make the seed germinate (Vleeshouwers *et al.*, 1995). More precisely, dormancy is defined as a seed characteristic that hinders the completion of germination of viable seeds under environmental conditions favorable for germination (Finch-Savage and Leubner-Metzger, 2006; Finch-Savage and Footitt, 2017). Through mechanisms such as dormancy, seeds germinate only when environmental conditions are suitable for both germination and seedling establishment. This increases the chances for future growth and reproduction of the next plant generation. Also, variation in dormancy among siblings (temporally separated germination) can reduce competition among siblings or prevent the complete loss of the offspring generation when environmental conditions irregularly switch between favorable and unfavorable states (Nonogaki, 2014).

Dormancy may be classified by morphological, physiological and environmental characteristics important for germination. In *Arabidopsis*, dormancy is physiologically non-deep. In physiologically non-deep dormant species (i) excised embryos develop into seedlings, (ii) GA promotes germination and (iii) dormancy can be broken by stratification and dry after-ripening (Baskin and Baskin, 2004; Bentsink and Koornneef, 2008).

In *A. thaliana*, two types of dormancy occur, namely primary dormancy and secondary dormancy. Primary dormancy develops during seed maturation at the maternal plant and prevents freshly matured seeds from germinating. This type of dormancy is tightly coupled to the ABA signaling pathway (Hilhorst, 1995; Bewley, 1997; Baskin and Baskin, 2004). Using ABA-deficient *A. thaliana* mutants it could be shown that defects in ABA synthesis during seed development resulted in the formation of non-dormant seeds (Karssen *et al.*, 1983; Bradford and Nonogaki, 2007). *Vice versa*, overexpression of ABA biosynthesis genes resulted in highly dormant seeds (Frey *et al.*, 1999). In the case of primary dormancy, the environmental conditions the parental plants experience during vegetative growth and the reproductive phase directly influence seed characteristic and dormancy of their offspring (Roach and Wulff, 1987; Blödner *et al.*, 2007; Donohue, 2009). Environmental cues that directly affect dormancy are for example the plant photoperiod (Donohue *et al.*, 2005; Donohue, 2009), the N supply of maternal plants (Alboresi *et al.*, 2005) and the parental growth temperature (Donohue *et al.*, 2005).

Secondary dormancy refers to a type of dormancy, where non-dormant seeds re-enter dormancy after dispersal (Baskin and Baskin, 2004). Secondary dormancy is induced by unfavorable environmental conditions, as for example high temperature, darkness or low soil water content (Finch-Savage and Leubner-Metzger, 2006; Ibarra *et al.*, 2016). Secondary dormancy is also linked to ABA. Comparison of ABA levels in seeds revealed that the ABA content initially decreases in the imbibition phase (Ali-Rachedi *et al.*, 2004).

While non-dormant seeds maintain a low ABA content and start germinating, in dormant seeds ABA *de novo* synthesis occurs, resulting in increasing ABA levels and a block of germination (Ali-Rachedi *et al.*, 2004).

As for seed development, an interplay between the embryo and the seed covering layers is important for the establishment and maintenance of dormancy (Penfield, 2017). The endosperm is a metabolically active tissue and is capable to release ABA in the direction of the embryo to prevent germination (Lee *et al.*, 2012b). The endosperm responds to ABA, GA and NO and directly affects dormancy (Bethke *et al.*, 2007). While ABA positively influences seed dormancy, the latter components release seeds from the dormant state.

In order to induce germination, seeds have to overcome dormancy. The most prominent mechanisms for dormancy release are after-ripening, cold stratification and GA supply. After-ripening refers to a prolonged period of dry storage of freshly matured seeds at room temperature, which ultimately results in the acquisition of the competence to germinate in these seeds (Bewley, 1997; Leubner-Metzger, 2003; Weitbrecht *et al.*, 2011). After ripening occurs in dry seeds and is sensitive to time- and environmental cues (Finch-Savage and Leubner-Metzger, 2006; Carrera *et al.*, 2008). An alternative way to relief seed dormancy is cold stratification. Cold stratification is tightly coupled to the GA pathway, *de novo* GA biosynthesis occurs by induction of the GA biosynthesis genes gibberellin 3-oxidases *GA3ox1* and *GA3ox2* (Yamauchi, 2004). But not only absolute GA levels, also a stratification-dependent increase in GA sensitivity mediates the relief of dormancy (Derkx and Karssen, 1993; Debeaujon and Koornneef, 2000; Bentsink and Koornneef, 2008).

1.4 The role of light in seed germination

The onset of the germination process is influenced by various environmental cues, which are sensed by imbibed seeds, such as light, water availability, temperature and the neighboring vegetation (Seo *et al.*, 2009). All of these environmental cues are integrated on a molecular level via complex signaling networks. One of the most important environmental cues for plants in general and especially seeds is light. Light is crucial for the regulation of numerous processes during a plants' life, such as seed germination, photomorphogenesis, the regulation of flowering and the entrainment of the circadian clock (Chory *et al.*, 1996; Sullivan and Deng, 2003). A series of photoreceptors allow plants to monitor their light environment. The R/FR proportion of the light (600 - 750 nm) is perceived by phytochromes, while cryptochromes sense UV-A and blue light (320 - 555 nm). Phototropins and ZEITLUPE proteins sense blue light, and UV RESISTANCE LOCUS 8 (UVR8) senses UV-B light (Sullivan and Deng, 2003; Di Wu *et al.*, 2012; Christie *et al.*, 2012).

1.4.1 Phytochromes as molecular red/far-red light switches

In seeds, phytochromes are the most important photoreceptors. The *Arabidopsis* genome encodes five phytochromes, PHYA to PHYE (Quail, 1991; Casal *et al.*, 1998). Phytochromes are chromoproteins synthesized as apoproteins (Terry *et al.*, 1993; Terry, 1997). After covalent binding of the chromophore phytochromobillin in the cytoplasm they form a holoprotein (Terry, 1997).

Phytochromes form homo- and heterodimers, which reside in the cytoplasm in their inactive form, after activation by light they undergo a conformational change, which allows their translocation into the nucleus.

Phytochromes are reversible light-activated molecular switches, since illumination of the inactive Pr form (absorption peak 660 nm) triggers the conversion to active Pfr phytochrome (absorption peak 705- 730 nm), while subsequent illumination with FR light or dark reversion triggers the conversion back to the inactive Pfr form (Mancinelli, 1994; Eichenberg *et al.*, 2000; Hughes, 2013; Sheerin and Hiltbrunner, 2017).

For the induction of seed germination, phyA and phyB are assumed to play a major role, they have been extensively studied in *A. thaliana* (Casal *et al.*, 1998; Chen *et al.*, 2004). Phytochromes C to E are less well characterized, although they also regulate multiple aspects in a plants' life. phyC, phyD and phyE were described as weak R light sensors (Aukerman *et al.*, 1997; Devlin *et al.*, 1999; Monte *et al.*, 2003; Ádám *et al.*, 2013); out of these only phyD was sufficient to induce germination in continuous white light (WL) conditions (Sánchez-Lamas *et al.*, 2016). None of these phytochromes was capable to induce germination in response to FR or R light pulses (Sánchez-Lamas *et al.*, 2016).

The light-stable phytochrome phyB is already present at detectable levels in seeds (Sharrock, 2002). PhyB can reversibly be transferred in an active Pfr state by low fluence R light and converted back to an inactive Pr state by FR light (Neff *et al.*, 2000). Since low R light fluences are sufficient for the onset of germination by active Pfr phyB, this response is referred to as “low fluence response” (LFR) (Neff *et al.*, 2000; Bae and Choi, 2008). However, in case the germination-inducing R light pulse is followed by a FR light pulse, the progression of germination is inhibited (Borthwick *et al.*, 1952; Franklin and Quail, 2010).

PhyA is the only known light-labile phytochrome, the active Pfr phyA form is rapidly degraded in light via ubiquitination followed by 26S proteasomal degradation (Hennig *et al.*, 1999). PhyA accumulates in imbibed seeds after a prolonged imbibition phase in darkness over time, reaching maximum levels 24 h to 48 h after the onset of imbibition (Lee *et al.*, 2012b). The activation maximum of phyA is shifted towards FR light wavelengths, among plant phytochromes phyA is the only receptor activated by FR light (Mancinelli, 1994). Tight control of phyA levels is required in plants to allow a dynamic regulation of plant responses to changing light conditions.

1.4.1.1 Very low fluence response

The activation spectrum of phyA is not limited to FR light. The activation of accumulated phyA in dark-imbibed seeds can be achieved by a wide range of light qualities including blue, R and FR light even in very low light quantities (“very low fluence response”, VLFR) (Shinomura *et al.*, 1996; Botto *et al.*, 1996). In the VLFR, phyA activated by a single illumination event irreversibly induces germination (Shinomura *et al.*, 1996).

Ecologically, the VLFR promotes survival of seeds that face non-optimal environmental light conditions, as can be found under canopy shade, where the FR proportion of light is enriched (Botto *et al.*, 1996). Also burial of dispersed seeds underneath a layer of soil is frequent in nature (Scopel *et al.*, 1991; Botto *et al.*, 1998). The permeability of the soil layer towards light may differ, based on the water content and soil composition (Ciani *et al.*, 2005). Out of the full spectrum of WL, only the FR light wavelengths penetrate deeper soil layers effectively (Ciani *et al.*, 2005).

On a molecular level, phyA targets numerous interaction partners of the light signaling pathway for degradation and additionally has a kinase activity itself, thereby

phosphorylating and inactivating PIF3 (Shin *et al.*, 2016). Because of its regulatory function, tight control of phyA levels is required in plants to allow a dynamic regulation of plant responses to changing light conditions. Therefore, the active Pfr phyA form is rapidly ubiquitinated, followed by 26S proteasomal degradation in light (Hennig *et al.*, 1999).

1.4.1.2 High irradiance response

Apart from the VLFR, a second mode of action is known for phyA, the high irradiance response (HIR). HIR is most effectively induced by continuous irradiation with FR light, which induces formation of long-lasting nuclear speckles of phyA (Kim *et al.*, 2000; Nagatani, 2004). Apart from phyA, also phyE has been described to perceive continuous FR light and trigger seed germination under HIR conditions (Hennig *et al.*, 2002). From an evolutionary perspective, HIR responses have evolved already in ancestors of modern seed plants and are ecologically relevant for seedling establishment in shade light conditions (Possart and Hiltbrunner, 2013).

It is important to note, that the categorization of a light response as either LFR, VLFR or HIR does not solely depended on the light intensities applied. As a rule of thumb, the VLFR requires light intensities of 0.001 to 1 $\mu mol/m^2$, the LFR 1 to 1000 $\mu mol/m^2$ and the HIR more than 1000 $\mu mol/m^2$ (Neff *et al.*, 2000). Also the duration of illumination and the light quality applied has a major impact on the respective light response. So in the literature, FR light intensities to induce the VLFR range from 0.001 to 75 $\mu mol/m^2$, the duration of illumination ranges from 1 min to 30 min (Wang and Deng, 2003; Kneissl *et al.*, 2009; Ibarra *et al.*, 2013).

1.4.2 Light signaling

Active phytochromes translocate to the nucleus in a light-dependent manner (Sakamoto and Nagatani, 1996). For nuclear import of phyB, a C-terminal PAS-related domain functions as nuclear-localization (NLS)-like motif (Chen *et al.*, 2005). Also interactions with NLS-containing proteins such as PIF3 facilitate nuclear translocation of phyB (Pfeiffer *et al.*, 2012).

In contrast to phyB, phyA requires additional proteins to mediate light-dependent nuclear import (Li *et al.*, 2011). In *A. thaliana*, FAR-RED ELONGATED HYPOCOTYL 1 (FHY1) and FHY1-LIKE (FHL), which both contain a NLS, a phyA binding site and a nuclear export signal sequence (Desnos *et al.*, 2001; Hiltbrunner *et al.*, 2005; Genoud *et al.*, 2008) mediate phyA nuclear import.

Following nuclear localization, phytochromes interact with multiple partners in complex signaling pathways to modulate light-dependent target gene expression (Quail, 2010). Two distinct pathways mediating phytochrome-dependent responses are well-described in *A. thaliana*. One pathway is based on the inactivation of the CULLIN 4 (CUL4)/ DAMAGED DNA BINDING 1(DDB1)/ CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1)/ SUPPRESSOR OF phyA-105 (SPA) (CUL4/DDB1 $^{COP1/SPA}$) E3 ubiquitin-ligase complex, which targets positive regulators of photomorphogenesis and light-related responses for degradation by the proteasome (Osterlund *et al.*, 2000; Saijo *et al.*, 2003). Light-activated phyA and phyB suppress the COP1/SPA function and thereby stabilize positive regulators of seed germination and photomorphogenesis. Phytochrome-mediated COP1/SPA suppression occurs via direct binding of phyA to SPA1, SPA2 and COP1, hindering the formation of a functional E3 ubiquitin ligase complex (Sheerin *et al.*, 2015). Another pathway to prevent ubiquitylation of COP1/SPA targets is the phyA/phyB-

dependent exclusion of COP1/SPA from the nucleus (Osterlund and Deng, 1998; Pacín *et al.*, 2014).

Downstream targets of COP1/SPA which are labeled for degradation include various transcription factors as LONG HYPOCOTYL5 (HY5), HY5-HOMOLOG (HYH), LONG HYPOCOTYL IN FAR-RED (HFR1), LONG AFTER FAR-RED LIGHT1 (LAF) and CONSTANS (CO) (Osterlund *et al.*, 2000; Ballesteros *et al.*, 2001; Duek and Fankhauser, 2005; Jang *et al.*, 2008).

In seedling photomorphogenesis the transcription factors HY5, HFR1 and LAF are positive regulators of photomorphogenesis and act largely independent from each other (Jang *et al.*, 2013). Although HY5 is a positive regulator of photomorphogenesis, HY5 negatively regulates germination. HY5 binds to the *ABA INSENSITIVE 5* (*ABI5*) promoter with high affinity and induces the expression of *ABI5* and ABI5 target genes, thus positively influencing the ABA signaling pathway to suppress germination (Chen *et al.*, 2008; Yang *et al.*, 2018). Due to the action of COP1, also HFR1 is ubiquitinated and degraded in the dark, but accumulates in light (Yang *et al.*, 2005; Jang *et al.*, 2007). HFR1 promotes light-dependent germination by binding to and forming heterodimers with PIF1 (Figure 5). The interaction with HFR1 prevents binding of PIF1 to the DNA (Shi *et al.*, 2013), so HFR1 is a positive regulator of seed germination.

The other pathway to integrate phytochrome-dependent responses is based on the interaction of phytochromes with PIF family members (Duek and Fankhauser, 2005). PIFs represses seed germination and photomorphogenesis, while promoting shade avoidance responses (Leivar and Quail, 2011). In darkness, PIF1 repressed germination by repressing the GA biosynthesis genes *GA3ox1* and *GA3ox3*, and simultaneously upregulating the GA catabolism-associated *GA2ox2* gene (Oh *et al.*, 2006; Shi *et al.*, 2013) (Figure 5). Additionally, PIF1 prevented germination by promoting the expression of the DELLA protein-encoding *GAI* and *RGA* genes (Oh *et al.*, 2007) (Figure 5). In response to light, PIFs interact with the active Pfr form of phyA and phyB. This results in phosphorylation and degradation of the PIF proteins (Al-Sady *et al.*, 2006; Lorrain *et al.*, 2008; Leivar and Quail, 2011). PhyA specifically interacts with and inhibits PIF1 and PIF3 to promote germination (Oh *et al.*, 2004; Oh *et al.*, 2006).

Together with the germination-promoting activity of HFR1, a derepression of germination-promoting genes, as for example GA biosynthesis genes *GA3ox1* and *GA3ox2* (Figure 5) allow the onset of germination. Also histone demethylases, encoded by *JUMONJI* (*JMJ*) genes (Cho *et al.*, 2012), which remove repressive epigenetic marks from GA-biosynthesis genes (Figure 5) promote germination. Recently, in a genetic screen a *POLYAMINE UPTAKE TRANSPORTER 2* (*put2*) mutant was identified. *Put2* has increased polyamine (PA) levels and germinated earlier when germination was induced by FR light. Additionally, *put2* showed higher germination rates as compared to the wild type in FR light. In *put2* mutants the effective induction of germination by a FR light pulse is already possible after twelve hours of dark incubation. Wild-type seeds require 48 h of dark imbibition to acquire the competence to germinate in response to a FR light pulse (Lee *et al.*, 2012b; Kim *et al.*, 2019). The higher germination rates were coupled to PIF1, which suppressed PA accumulation after a first FR light pulse. PAs might also contribute to the germination of aged seeds in FR light. Kim *et al.* (2019) observed increased germination rates of older seeds in FR light, this might be due to a higher accumulation of PAs.

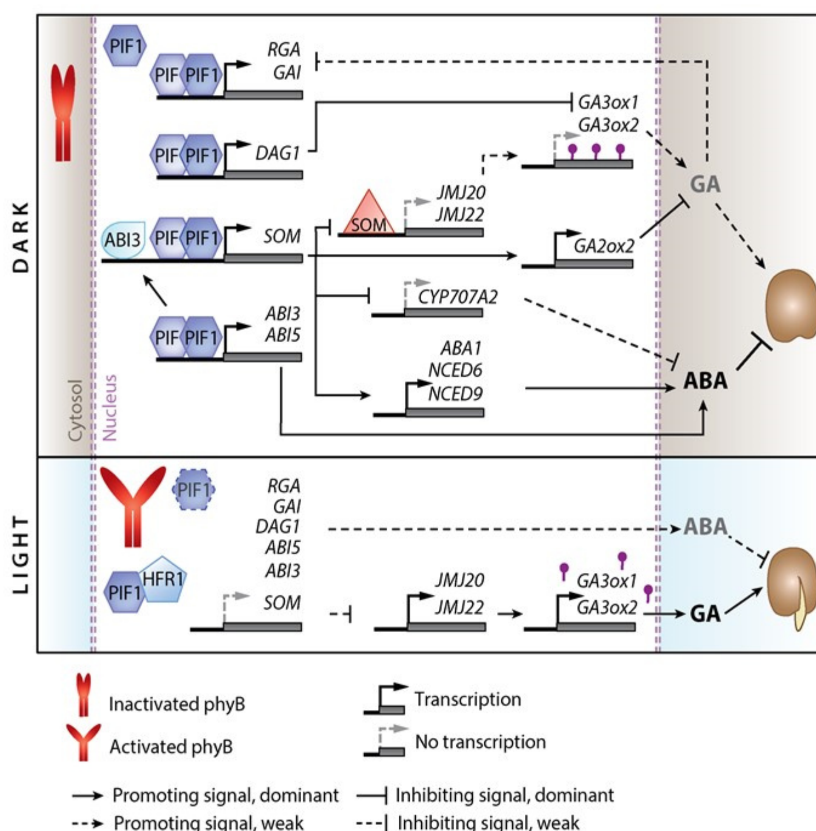

Figure 5. Light-regulated germination signaling in the dark and in the light.
In the dark, inactive phytochrome resides in the cytosol, upon illumination phyB is translocated to the nucleus. In darkness, PIF1 accumulates in the nucleus and induces expression of its target genes. This result in an ABA accumulation and inhibition of germination. In the light upon phyB localiztion to the nucleus, PIF1 is degraded and additionally prevented from binding to the DNA by HFR1. This results in derepression of *JUMONJI* (*JMJ*) genes, encoding a histone demethylase to remove repressive epigenetic marks from GA-biosynthesis genes.
ABI3 and *ABI5, ABSCISIC ACID–INSENSITIVE* 3 and 5; *DAG1, DOF AFFECTING GERMINATION 1; GAI, GIBBERELLIC ACID–INSENSITIVE;* HFR1, LONG HYPOCOTYL IN FAR RED 1; *JMJ, JUMONJI; NCED6* and *NCED9, 9-CIS-EPOXYCAROTENOID DIOXYGENASE 6* and *9;* phyB, phytochrome B; PIF1, PHYTOCHROME INTERACTING FACTOR1; *RGA*, *REPRESSOR OF GA1-3; SOM, SOMNUS.* Modified from de Wit *et al.* (2016).

1.5 CK is coupled to multiple traits related to seed germination and light responses

Numerous physiological and developmental processes are regulated by CK, including cell division, shoot growth, seed size and seed yield, root growth -and architecture and leaf senescence (Werner and Schmülling, 2009; Kieber and Schaller, 2014; Chang *et al.*, 2015; Jameson and Song, 2016). Interestingly, unlike the extensive studies on ABA and GA, the role of CK in seed germination remained largely unknown up to present.

Nevertheless, there are several hints connecting CK and light signaling pathways. Ibarra *et al.*, 2013 found a direct connection between FR light signaling in germination and the regulation of CK-related genes. The authors showed that in FR light, phyA upregulated the expression of *CKX5/CKX6*, *CRF1, 2* and *3*. CK related genes were also direct targets

of PIFs. PIF1 directly downregulated *CRF1, CRF2* and *CRF3*, *AHP5* was indirectly upregulated by PIF1 (Oh *et al.*, 2009).

Another example for a functional connection between light and CK signaling is the regulation of phyB by the A-type response regulator ARR4. Interestingly, the active Pfr form of phyB was stabilized by binding to phosphorylated ARR4 which caused hypersensitivity towards R light in hypocotyl elongation (Sweere *et al.*, 2001; Mira-Rodado *et al.*, 2007). Notably, the regulation of seed germination and hypocotyl elongation differ greatly, although the molecular signaling pathways utilized might be similar. One prominent example are the different functions of HY5 as a positive regulator of seedling photomorphogenesis and the negative impact on germination, as described above (Chen *et al.*, 2008; Yang *et al.*, 2018).

A direct connection between CK and seed-related traits, such as seed size, was evident in CK receptor mutant seeds. The weak *ahk2-5 ahk3-7 cre1-2* triple mutant is capable to self-fertilize and produce viable seeds (Riefler *et al.*, 2006). These seeds show a striking seed size phenotype, they were enlarged by 30% compared to wild-type seeds. Also plants overexpressing *CKX1* or *CKX3* produce larger seeds (Werner *et al.*, 2003), as well as plants impaired in CK signal transduction via AHKs (*ahk2 ahk3 ahk4/cre1*) and B-type ARRs (*arr1 arr10 arr12*) (Riefler *et al.*, 2006; Argyros *et al.*, 2008).

Regarding the effect of CK on seed germination, seeds lacking two of three functional CK receptors showed increased germination rates in R and FR light, as well as earlier germination in WL conditions compared to wild-type seeds (Riefler *et al.*, 2006). These effects were even more pronounced in *ahk2 ahk3 cre1* seeds. Only in FR light conditions, *ahk2 ahk3 cre1* seeds germinated comparable to double receptor mutant seeds (Riefler *et al.*, 2006). This negative effect of CK on seed germination is supported by the finding, that external application of CK (tZR) suppressed germination in *Aconitum heterophyllum* and *Aconitum balfourii* in a concentration-dependent manner (Pandey *et al.*, 2000).

On the other hand, germination of seeds overexpressing *IPT8* in an estradiol-inducible manner was insensitive to ABA (Wang *et al.*, 2011). In these seeds, the A-type ARRs ARR4, ARR5 and ARR6 negatively regulated *ABI5* expression by physically interacting with the ABI5 protein (Wang *et al.*, 2011). In accordance with these findings, it has been shown that *ARR6*, *ARR7* and *ARR15* were upregulated in ABA-deficient seeds and ABI4 directly bound to the promoters of these A-type *ARRs* to repress germination (Huang *et al.*, 2017). Concluding from this, A-type ARRs which feedback inhibit the CK signal transduction pathway, promote seed germination by antagonizing ABA signaling and *vice versa* ABA antagonizes CK by negatively affecting A-type *ARR* expression.

The interaction between CK and ABA seems to be highly context-specific, as for example the simultaneous application of ABA and BA rescued ABA-mediated inhibition of cotyledon greening in *A. thaliana* (Guan *et al.*, 2014). But application of CK did not rescue the ABA-induced repression of seed germination (Guan *et al.*, 2014).

1.6 Phytohormones regulate seed germination

Seed germination is regulated by various phytohormone-associated pathways, involving ABA, GA, ethylene, nitric oxide, auxin, karrikin and CK (Nelson *et al.*, 2010; Wang *et al.*, 2011; Arc *et al.*, 2013; Liu *et al.*, 2013; Corbineau *et al.*, 2014; Shu *et al.*, 2016). ABA and GA are the most prominent phytohormones modulating seed germination. While ABA is a central negative regulator of germination, GA positively influences germination.

1.6.1 ABA negatively influences seed germination

In dry seeds, stored transcripts are enriched in ABA-responsive elements in the promoters of the corresponding genes (Nakabayashi *et al.*, 2005), consistent with the role of ABA in supporting the establishment of primary and secondary dormancy that prevents seeds from premature germinating (Kimura and Nambara, 2010).

In plants the early steps of ABA biosynthesis occur in plastids using carotenoids as precursors. Early ABA precursors are metabolized to zeaxanthin (Cutler and Krochko, 1999; Seo and Koshiba, 2002). The conversion from zeaxanthin to violaxanthin is catalyzed by the activity of ZEAXANTHIN EPOXIDASE (ZEP) (Figure 6). The gene encoding ZEP is named *ABA-DEFICIENT1* (*ABA1*) in *A. thaliana*, *aba1* mutants produce only residual amounts of ABA compared to the wild type but show high zeaxanthin accumulation (Rock and Zeevaart, 1991). From violaxanthin, neoxanthin and subsequently xanthoxin is produced. The latter step is catalyzed by the action of 9-CIS-EPOXYCAROTENOID DIOXYGENASEs (NCEDs) (Figure 6), from which NCED2, NCED3, NCED5, NCED6 and NCED9 are involved in ABA biosynthesis (Tan *et al.*, 2003; Finkelstein, 2013; Yan and Chen, 2017). For seed germination, *NCED6* and *NCED9* are specifically important due to their seed-specific expression (Lefebvre *et al.*, 2006). During seed development, *NCED6* is expressed exclusively in the endosperm, while *NCED9* is expressed both in the embryo and in the endosperm (Lefebvre *et al.*, 2006). While loss-of-function of either *NCED3* or *NCED6* does not affect dormancy, *nced3 nced6* double mutant seeds show reduced dormancy levels compared to single mutant seeds (Lefebvre *et al.*, 2006). After xanthoxin production by NCEDs, ABA2 catalyzes the synthesis of the direct ABA precursor abscisic aldehyde, which is oxidized to bioactive ABA by an ABSCISIC ALDEHYDE OXIDASE (AAO3) (Figure 6). In order to control ABA levels tightly, plants are capable to reduce the ABA content effectively. To do so, members of the CYTOCHROME P450 707A (CYP707A) family catalyze the hydroxylation and inactivation of ABA (Figure 6). Comparable to *NCED*s, also *CYP707A* display spatially and temporally distinct expression patterns during seed development (Okamoto *et al.*, 2006) which allows the precise regulation of ABA levels.

The effective reduction of ABA levels is crucial for the onset of germination. Several mechanisms have evolved in seeds to reduce ABA levels prior to the onset of germination. One of them is the initial leakage of solutes from imbibing seeds (Matilla *et al.*, 2005). Furthermore, in non-dormant seeds R light induces downregulation of the ABA biosynthesis genes *NCED6, NCED9* and *ABA1* and the simultaneous upregulation of ABA catabolism genes like *CYP707A2* (Okamoto *et al.*, 2006; Oh *et al.*, 2007).

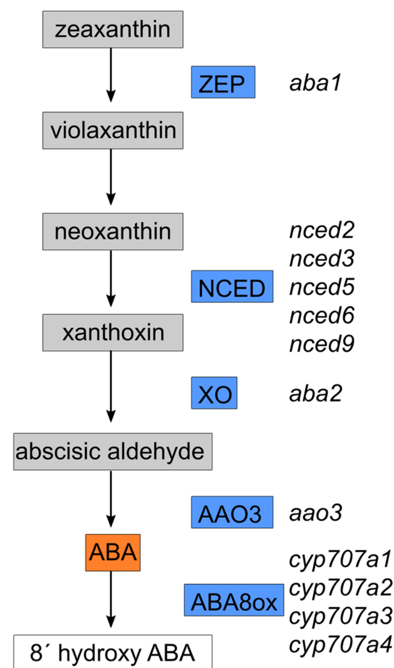

Figure 6. Simplified scheme of the ABA biosynthesis pathway in *A. thaliana*.
ABA precursors are depicted in grey boxes, active ABA is indicated by orange color. 8´-hydroxy-ABA is an inactive ABA breakdown product. Enzymes catalyzing the respective step are indicated in blue, the respective mutants depicted in italics next to the boxes. ZEP, zeaxanthin epoxidase; NECD, 9-cis-epoxycarotenoid dioxygenases; XO, xanthoxin oxidase; AAO3, aldehyde oxidase 3; ABA8ox, ABA 8´ hydroxylase; ABA, abscisic acid; *aba1*, *ABA-DEFICIENT1*; *aba2*, *ABA-DEFICIENT2*; *cyp707a*, *cytochrome P450 707A*. Modified from Seo *et al.* (2002, 2009).

Apart from ABA biosynthesis, ABA signaling is important to regulate the seed germination process. The uptake of ABA via the plasma membrane is mediated by ABCG family transporters and has been shown to be relevant for seed germination, as *abcg* mutant seeds germinate earlier than wild-type seeds and are less sensitive to externally applied ABA (Kang *et al.*, 2010). ABA binds to the intracellular PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL) /REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) receptor both in the nucleus and in the cytoplasm (Ma *et al.*, 2009; Park *et al.*, 2010). This results in the inhibition of PROTEIN PHOSPHATASES TYPE 2C (PP2C), which are negative regulators of ABA signaling (Park *et al.*, 2010). Thereby, dephosphorylation of SUCROSE NONFERMENTING1-RELATED SUBFAMILY2 (SnRK2s) by PP2Cs is prevented, so SnRK2 kinases are able to phosphorylate downstream ABA signaling components, such as the transcription factor ABI5 (Fujita *et al.*, 2009; Umezawa *et al.*, 2009).

ABA-related transcriptional activation or repression is crucial for ABA downstream signaling. Various mutants insensitive to ABA have been described. While *ABI1* and *ABI2* encode PP2Cs, *ABI3*, *ABI4* and *ABI5* loci encode transcription factors responsible for ABA-mediated gene expression and are important for the regulation of dormancy and seed germination (Finkelstein, 1994; Leung *et al.*, 1994).

1.6.2 GA is a positive regulator of seed germination

More than 130 GA isoforms are described nowadays, but only for a few GAs the biological activity in plants is known. The most important bioactive GAs in *Arabidopsis* are GA_1, GA_3, GA_4 and GA_7 (Yamaguchi, 2008). Among them, GA_4 is of outstanding importance for the regulation of seed germination (Yamaguchi, 2008).

GA in plants is produced in a complex metabolic pathway via several biosynthesis intermediates from geranly-geranyl diphosphate (GGPP) to GA_{12}-aldehyde (reviewed in Hedden *et al.*, 2015; Salazar-Cerezo *et al.*, 2018). GA_{12}-aldehyde serves as precursor for both 13-hydroxylated and 13-non hydroxylated GAs (Yamauchi *et al.*, 2007; Hedden and Thomas, 2012) (Figure 7). In the next step, GA_{12}-aldehyde is oxidized to GA_{12} with the help of an ENT-KAURENOIC ACID OXIDASE (KAO). By 13-hydroxylation GA_{53} is synthesized from GA_{12} (Hedden and Sponsel, 2015) (Figure 7). From non-hydroxylated GA_{12}, with the help of GA_{20}-oxidases (GA20ox), GA_9 is produced via the biosynthesis intermediates GA_{15} and GA_{24} (Figure 7). From 13-hydroxylated GA_{53}, GA_{20} is biosynthesized via GA_{44} and GA_{19} (Figure 7). The 13-non hydroxylated GA_9 serves as precursor for GA_{51}, GA_7 and bioactive GA_4, the latter biosynthesis step is catalyzed by GA_3ox (Hedden and Sponsel, 2015; Salazar-Cerezo *et al.*, 2018). Similarly, 13-hydroxylated GA_{20} serves as precursor for GA_{29}, GA_5 and GA_1 (Figure 7), again the biosynthesis of bioactive GA_1 is catalyzed by GA_3-oxidases. From GA_5, the bioactive GA_3 is produced via the action of GA_3-oxidases (Hedden and Sponsel, 2015; Salazar-Cerezo *et al.*, 2018). To inactivate the bioactive GA_1 and GA_4, GA_2-oxidases (GA2ox2) converts them in the inactive forms GA_8 and GA_{34}, respectively (Hedden and Phillips, 2000) (Figure 7).

Due to the multitude of different GA metabolites, the exact side of GA production or signal perception is hard to define (Binenbaum *et al.*, 2018). It has been shown that the GA precursor GA_{12} is the major transport form travelling through the vasculature (Regnault *et al.*, 2015). But also spatially separated expression of early and late GA biosynthesis genes as for example in developing embryos suggest that the localization of GAs is relevant for the regulation of GA responses (Yamaguchi *et al.*, 2002; Binenbaum *et al.*, 2018).

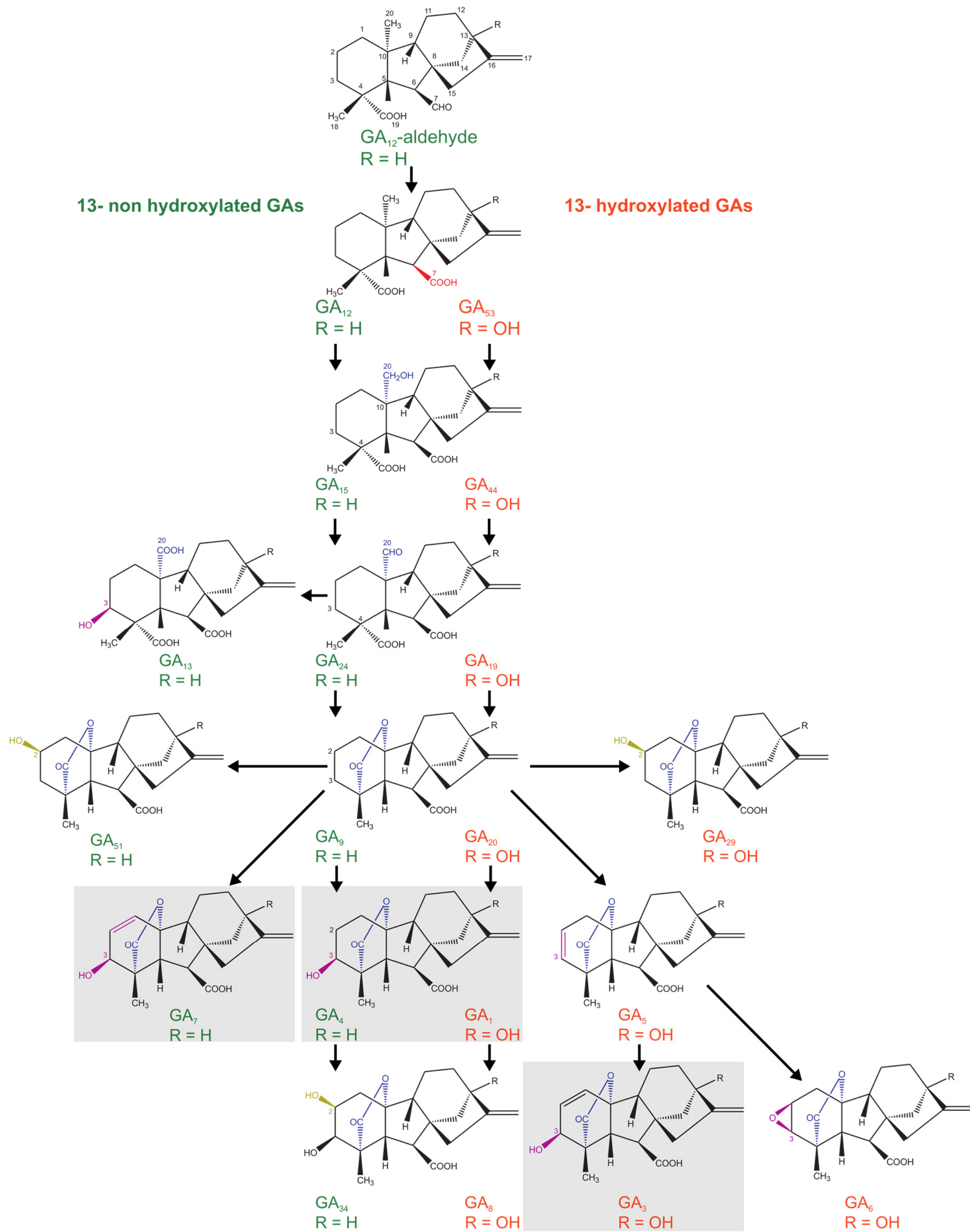

Figure 7. Scheme of GA biosynthesis and GA biosynthesis intermediates.
Depicted are the late steps of the GA biosynthesis pathway in plants. Biosynthesis intermediates are classified in two parallel pathways, 13-non hydroxylated GAs (green) and 13-hydroxylated GAs (red). The main bioactive GAs in plants, GA_1, GA_3, GA_4 and GA_7 are highlighted in grey boxes. Modified from Hedden *et al.* (2015).

In order to exerts its biological function, bioactive GA binds to the GA INSENSITIVE DWARF1 (GID1) receptor. This induces a conformational change that allows GID1-GA to bind DELLA proteins, which are well-known negative regulators of germination (Ueguchi-Tanaka *et al.*, 2005; Nakajima *et al.*, 2006; Shimada *et al.*, 2008; Murase *et al.*, 2008). The *Arabidopsis* genome encodes five DELLAs, *GA INSENSITIVE* (*GAI*), *REPRESSOR OF GA1-3* (*RGA*), *RGA-LIKE1* (*RGL1*), *RGL2* and *RGL3* (Peng *et al.*, 1997; Silverstone *et al.*, 1998; Dill and Sun, 2001; Lee *et al.*, 2002). DELLAs repress almost all known GA-dependent processes, ranging from seed germination to growth (Achard and Genschik, 2009; Daviere and Achard, 2013). In the seed germination process RGL2 is the most important DELLA. Other DELLAs play a less important role in the regulation of germination (Lee *et al.*, 2002; Tyler *et al.*, 2004; Cao *et al.*, 2005; Ariizumi and Steber, 2007; Ariizumi *et al.*, 2013). To release DELLA-mediated repression of GA-associated processes, the GID1-GA complex binds DELLAs, what promotes the interaction with the E3 ubiquitin ligase $SCF^{SLY1/GID2}$ (Ueguchi-Tanaka *et al.*, 2005; Willige *et al.*, 2007; Hirano *et al.*, 2010). As a result of this interaction, DELLAs are polyubiquitinated and degraded via the 26S proteasome pathway (Fu *et al.*, 2002; McGinnis *et al.*, 2003).

1.6.3 Antagonistic interaction between ABA and GA influences germination

ABA and GA regulate seed dormancy and germination antagonistically as recently reviewed in Liu *et al.* (2018). Light mediates the upregulation of GA biosynthesis genes *GA3ox1* and *GA2ox2* while simultaneously repressing the transcription of the GA catabolizing *GA2ox2* gene (Yamaguchi *et al.*, 1998; Oh *et al.*, 2007). At the same time, ABA metabolism is phytochrome-regulated in an opposite fashion to GA metabolism (Seo *et al.*, 2006). This response majorly depends on phyB, since the effect was most pronounced after irradiation with R light (Seo *et al.*, 2006). *NCED6* was downregulated while the ABA catabolizing *CYP707A2* was upregulated six hours after R light illumination (Seo *et al.*, 2006).

In mature seeds ABA from the seed coat is released towards the embryo and thereby shapes the germination phenotype of seeds (Lee *et al.*, 2012b). This finding demonstrates that the spatial distribution of hormones plays and important role in the regulation of germination. The seed coat is responsive to hormones such as ABA, GA and NO (Bethke *et al.*, 2007), but for a long time the ability of the embryo to produce ABA has been regarded as decisive for the prevention of germination and the maintenance of dormancy (Bewley, 1997). With the help of seed-coat bedding assays it was found that besides embryonic ABA, also endosperm-derived ABA opposes phyA signaling in the embryo in FR light (Lee *et al.*, 2012b). Also GA was proposed to be released from the endosperm towards the embryo, to suppress *ABI3* expression in dissected tomato seeds (Bassel *et al.*, 2006), but experimental evidence is lacking.

To investigate the direct effect of ABA and GA levels on the transcriptional changes in these pathways, ABA- and GA-deficient mutants were studied. These analyses revealed high GA levels in ABA-deficient mutants and *vice versa*, indicating a direct negative antagonistic function (Seo *et al.*, 2006; Oh *et al.*, 2007). SOMNUS (SOM) is a negative regulator of seed germination, *som* mutants display lower levels of ABA and higher GA levels compared to wild-type seeds. SOM acts downstream of PIF1, as PIF1 binds to the promoter of *SOM* and activates *SOM* expression (Kim *et al.*, 2008) (Figure 5).

1.7 Priming

The concept of priming against stress is defined as a temporally limited environmental priming stimulus, which prepares and modifies the response of the organism to a future triggering stress (Hilker *et al.*, 2016). Priming acts on the level of individual organisms and does not encompass alteration of the DNA sequence, but may include epigenetic, hormonal or cellular changes (Hilker *et al.*, 2016).

Apart from this stress concept of priming, the term seed priming refers to a commercially used pre-sowing technique to achieve uniform and fast germination of the treated seed lots (Paparella *et al.*, 2015). Seed priming is achieved by rehydration of the seeds with water to allow pre-germinative metabolism but preventing the transition of the seeds to complete germination (Paparella *et al.*, 2015). Pre-germinative metabolism includes *de novo* synthesis of nucleic acids and proteins, ATP production and the activation of DNA repair and antioxidant mechanisms (phase I in the imbibition process) (Paparella *et al.*, 2015). Consequently, the priming treatment is stopped before the seeds lose their desiccation tolerance (Paparella *et al.*, 2015).

In *Brassica napus*, this so-called osmopriming results in enhanced water uptake of primed seeds in the imbibition phase, what is due to microcracks in the seed covering layers and enhanced expression of aquaporin genes (Lechowska *et al.*, 2019). Seed priming may also be achieved by imbibing seeds in chemical-containing solution. For example, KNO_3 and urea were used to prime maize and chinese cabbage seeds in order to achieve increased germination rates under drought stress conditions (Anosheh *et al.*, 2011; Yan, 2015).

The germination capacity of a seed population is strongly dependent on environmental conditions both at the site of seed germination as well as the conditions their parental plants encountered during seed development. Information on environmental conditions parental plants perceive are passed to the next generation, even without the requirement for changes in the genome, this is referred to as intergenerational priming. Information transfer is mainly achieved by maternal effects or via epigenetic inheritance (Holeski *et al.*, 2012).

Parental plants influence the germination phenotype on multiple levels. On the one hand, they directly affect seed characteristics, for example by seed provisioning during development (Alboresi *et al.*, 2005). On the other hand, intergenerational priming effects transmitted via epigenetic marks shape the response of the offspring at a given environment (Paszkowski and Grossniklaus, 2011; Hilker *et al.*, 2016). So plants are capable to inform their offspring about the environmental conditions at the site of seed dispersal. The most important conditions include the light environment, temperature, nutrient availability but also information about pathogens may be transmitted to the following plant generation via epigenetic modifications and site-specific chromatin modifications (Blödner *et al.*, 2007; Galloway and Etterson, 2007; Donohue, 2009; Spoel and Dong, 2012).

In plant species where seeds are dispersed in close vicinity to their maternal plants, such as *A. thaliana* or *Campanulastrum americanum*, seeds show higher germination rates when subjected to light conditions (in terms of light fluence and R/FR ratio) similar to their maternal plants (Galloway, 2001; Galloway and Etterson, 2007). Additionally, high light intensity of parental plants in the flowering phase increases seed longevity and germination, but the parental photoperiod does not affect seed performance (He *et al.*, 2014).

Parental growth temperature during reproduction strongly affects seed germination. In *A. thaliana*, parental plants grown in warm temperatures produced seeds with a higher nitrogen content compared to plants grown under low temperature. The seeds from warm-grown parents showed faster germination and increased seed production themselves (higher reproductive success) compared to seed matured at 15° C (Blödner *et al.*, 2007).

Apart from temperature-induced changes in nitrate content, also the nutritional status of parental plants and consequently of the offspring seems to be critical for the germination of the next generation. Progeny of plants subjected to high nitrate conditions during their growth phase are less dormant compared to seeds produced under a standard nitrate regime (Alboresi *et al.*, 2005).

But not only endogenous nitrate provided by maternal plants, also externally supplied nitrate affects seed germination. So the nitrate availability of seeds, either by external supply of imbibing seeds or after supply to parental plants (endogenous nitrate) reduced ABA levels in the respective seeds, directly linking the higher nitrate status to the lower level of the germination-inhibiting phytohormone (Matakiadis *et al.*, 2009).

1.8 Seed storage, seed longevity and seed aging influence germination

Seed longevity is defined as the total time span during which seeds remain viable (Sano *et al.*, 2016). The notion that longevity of seeds is already established in developing seeds during maturation (Yazdanpanah *et al.*, 2017), is concluded from the reduced seed longevity phenotype of *A. thaliana* mutants defective in seed development, such as *lec1*, *lec2*, and *fus3* mutants, but also *abi3* and *dog1* mutant seeds exhibit reduced seed viability (Meinke *et al.*, 1994; Dekkers *et al.*, 2016).

Additional to genetic factors, environmental conditions determine the time span a seed keeps the competence to germinate. This is not only relevant for seeds dispersed in wet soils under natural conditions, also during dry seed storage environmental cues have a major impact on the capacity of seeds to complete germination. Among them, moisture content, relative humidity, temperature, and oxygen pressure affect seed longevity (Walters, 1998; Sano *et al.*, 2016). During dry seed storage, seed aging processes gradually reduce the longevity of seeds (Sano *et al.*, 2016) until the seeds irreversibly lose the ability to germinate. On a molecular level, this is mainly associated with oxidation of nucleic acids, proteins and lipids by reactive oxygen species (ROS) (Bailly, 2004; Rajjou *et al.*, 2008; Groot *et al.*, 2012). So seed storage proteins and antioxidant systems protecting and maintaining the translation machinery are essential for seed longevity in dry storage conditions (Nguyen *et al.*, 2015). Both the embryo and the testa contribute to seed longevity after storage. The embryo gradually loses viability, and analysis of testa mutants (e.g. *tt* mutants) revealed that a modified content of flavonoids in the testa tissue also affects the ability to germinate after dry storage (Debeaujon *et al.*, 2000).

1.9 Research objectives

Previous research discovered that CK is important for the regulation of germination in different light conditions (Riefler *et al.*, 2006; Wang *et al.*, 2011; Huang *et al.*, 2017). Riefler *et al.* (2006) demonstrated a negative role of CK in the germination process since CK receptor double and triple mutant seeds germinated earlier and displayed higher germination rates in R and FR light compared to the wild type.

In addition, Wang *et al.* (2011) and Huang *et al.* (2017) provided evidence for an antagonistic relationship between CK and ABA via A-type ARRs in the regulation of seed germination.

In the first part of this work, I aimed to characterize the germination phenotype of various CK mutants. In order gain a deeper understanding of the mechanisms that lead to the observed germination phenotypes I used a combination of physiological, transcriptional as well as proteomic methods. My specific focus was set to study the role of CK in the phyB-mediated, R light-induced LFR and the phyA-mediated, FR light-induced VLFR. With the help of germination assays, I identified components of the CK metabolism and signaling pathway involved in the seed germination response. On a molecular level, I assessed the processes underlying the altered germination phenotype by using a combination of transcriptomic and proteomic approaches. To further dissect the hormonal pathways which may influence germination of CK mutant seeds in VLFR conditions, I analyzed the contribution of ABA and GA in CK-regulated seed germination.

In the second part of this work, I aimed at investigating CK as a priming agent in the context of seed germination in VLFR conditions. I set the focus on the intergenerational transfer of information about environmental stimuli from parental plants to their offspring, especially with regard to the light environment and nutrient availability. In addition, I analyzed the impact of seed size and seed age on VLFR germination.

Thus, the overall aim of this study was to provide an in-depth investigation of the role of CK in the regulation of the seed germination process.

2 Material & Methods

2.1 Databases and software

The databases and software used in this work are listed in Table 1.

Table 1. Databases and software.

Name	Company, Reference, Resource	Purpose of use
Araport	Arabidopsis information portal, https://www.araport.org	Search for *Arabidopsis* gene information
AxioVision Rel.4.8	Zeiss	Processing of light microscopy pictures
CFX Manager	Bio-Rad	Quantitative real-time PCR
Cyber-T	Kayala *et al.*, 2012; http://cybert.ics.uci.edu	Identification of differentially expressed genes (*in silico* microarray data)
BAR eFP browser	Nakabayashi *et al.*, 2005; Winter *et al.*, 2007, http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi;	Analysis of gene expression patterns
G Power 3	Faul *et al.*, 2007; Heinrich-Heine-University Düsseldorf; http://www.gpower.hhu.de	Calculation of experimental sample sizes for statistics
Gene Expression Omnibus	National Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov/geo	Transcriptome-data mining (*in silico* microarray data)
Genevestigator	Hruz *et al.*, 2008; https://genevestigator.com	Transcriptome-data mining (*in silico* microarray data)
GraphPad Prism Version 8	GraphPad Software, San Diego, USA	Statistical analysis, figure design
Image Studio™	LI-COR Biosciences	Fluorescence Western blot analysis and quantification
ImageJ 1.52a	Abramoff *et al.*, 2004; https://imagej.nih.gov/ij	Image processing and analysis
Inkscape Version 0.92.2	https://inkscape.org	Figure design
Mendeley	Elsevier; https://www.mendeley.com	Managing and citing of references and literature
MEV Version 4.9.0	Multiple Experiment Viewer; Howe *et al.*, 2011; http://mev.tm4.org	Visualization of RNA sequencing (RNA-Seq) data
NASC	The European Arabidopsis Stock Centre; http://arabidopsis.info	Order of *Arabidopsis thaliana* seeds
NCBI	National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov	Literature (PubMed), BLAST
Netprimer	PREMIER Biosoft	Assessment of primer quality
Office (Excel, Powerpoint, Access)	Microsoft	Data analysis, visualization, RNA-Seq analysis
Olympus cellSense	Olympus Life Science	Processing of stereomicroscope pictures
Panther	Thomas *et al.*, 2003; http://pantherdb.org	GO term analysis
Primer-BLAST, Primer3	Untergasser *et al.*, 2012; Jian *et al.*, 2012; www.ncbi.nlm.nih.gov/tools/primer-blast/	Primer design
R Version 3.5.2	The R project; https://cran.uni-muenster.de	Microarray data analysis, RNA-Seq data analysis
TAIR	The Arabidopsis Information Resource, https://www.arabidopsis.org	Search for *Arabidopsis* gene information
T-DNA Express	Salk Institute Genomic Analysis Laboratory; http://signal.salk.edu/cgi-bin/tdnaexpress	Search for *Arabidopsis* T-DNA insertion mutants
Venny	BioInfoGP; http://bioinfogp.cnb.csic.es/tools/venny	Venn diagram creation
Exon/Intro model creator	http://wormweb.org	Visualization of gene exon/intro structure

2.2 Chemicals and consumables

Chemicals and consumables used in this work were distributed by the following companies: AppliChem (Darmstadt, Germany), Bioline (London, UK), Bio-Rad (Hercules, USA), Carl Roth (Karlsruhe, Germany), Duchefa Biochemie (Haarlem, NL), Eppendorf (Hamburg, Germany), Fluka Analytical (München, Germany), GeneAll (Seoul, Korea), Greiner Bio-One (Kremsmünster, Austria), Invitrogen/Thermo Fisher Scientific (Waltham, USA), Macherey-Nagel (Düren, Germany), Merck (Darmstadt, Germany), Peqlab/VWR (Erlangen, Germany), Qiagen (Hilden, Germany), Roche (Mannheim, Germany), Sarstedt (Nümbrecht, Germany), Sigma-Aldrich (St. Louis, USA).

2.3 Kits, enzymes, nucleic acid- and protein ladders

The kits, enzymes and DNA ladders used in this work are listed in Table 2.

Table 2. Kits, enzymes, DNA and protein ladders.

Name	Manufacturer and catalogue number	Purpose of use
DNase I (RNase-Free)	Fermentas/Thermo Fisher Scientific, Cat.no. EN0521	DNA digestion during RNA purification
SuperSignal West Pico PLUS	Thermo Fisher Scientific, Cat.no. 34580	Chemiluminescent substrate
GeneRuler 100 bp DNA Ladder	Thermo Fisher Scientific, Cat. no. SM0243	Estimation of DNA fragment sizes
GeneRuler Express DNA Ladder	Thermo Fisher Scientific, Cat. no. SM1551	Estimation of DNA fragment sizes
Immolase™ DNA Polymerase	Bioline, Cat.no. BIO-21047	quantitative real-time PCR
NucleoSpin RNA Plant Kit	Macherey-Nagel, Cat. no. 740949.50	Isolation and purification of RNA
PageRuler	Thermo Fisher Scientific, Cat. no. 26616	Estimation of protein size
Riboclear Plus	GeneAll, Cat.no. 313-150	Purification of RNA from seeds
Ribospin™ Seed/Fruit	GeneAll, Cat.no. 317-150	Isolation and purification of RNA from seeds
SuperScript III Reverse Transcriptase	Invitrogen/Thermo Fisher Scientific, Cat.no. 18080-044	complementary DNA (cDNA) synthesis
Taq DNA Polymerase, recombinant	Prof. Dr. Wolfgang Schuster, Applied Genetics, FU Berlin	PCR

2.4 *Arabidopsis thaliana* plants

2.4.1 Seed sterilization

2.4.1.1 Liquid sterilization

For plant *in vitro* cultivation, *A. thaliana* seeds were surface sterilized by liquid sterilization. Seeds were transferred to Eppendorf tubes and incubated in 70 % ethanol for 5 min under continuous shaking. This process was repeated twice. Afterwards, seeds were transferred to a dry, sterile Whatman filter paper under a clean bench and allowed to dry thoroughly. Sterilized seeds were sown on the same day and not stored after sterilization.

2.4.1.2 Gas sterilization

Seeds intended for light-sensitive experiments which require sterile seeds, were sterilized with chloride gas to avoid unwanted seed imbibition during liquid sterilization. Seeds were transferred to Eppendorf tubes, these tubes were placed in a rack inside a desiccator, the tube lids were left open. The desiccator was placed inside a fume hood. Next, a glass vessel containing 100 ml bleach (1 tablet of calcium hypochlorite) was placed in the desiccator and 3 ml 37 % HCl was added to the bleach. The desiccator was sealed, and seeds were incubated for one and a half hours in chloride gas. Afterwards, the desiccator was opened carefully, and the seed-containing tubes were immediately closed. Seeds were not subjected to further storage after sterilization.

2.4.2 Plant growth conditions

Arabidopsis thaliana plants were grown under long day (LD) conditions (16 h light/8 h dark) at 22 °C and 40-60 % relative humidity either in a greenhouse or in Percival plant growth chambers (CLF, Wertingen, Germany). Light intensities ranged from 120 µmol m^{-2} s^{-1} to 160 µmol m^{-2} s^{-1} in the greenhouse. In the Percival growth chambers, white light intensity was kept constant at 120 µmol m^{-2} s^{-1} or 150 µmol m^{-2} s^{-1}, the amount of red light (around 6 µmol m^{-2} s^{-1}), far-red light (around 3 µmol m^{-2} s^{-1}) and blue light (around 9 µmol m^{-2} s^{-1}) were kept constant for the period of cultivation of a respective plant set.

When plants were grown in shade light, Percival growth chambers were used for plant cultivation. With their custom-made combination of cool white fluorescent lamps and dimmable red and far-red LEDs, the light composition could be manipulated according to the experimental setup. For one part of this thesis, the influence of different R/FR ratios perceived by maternal plants was investigated. For this setup, plants were grown in shade light conditions, see Table 3. As a control, plants were grown in parallel under non-shaded conditions (Table 3). Because of the reduced light quantity in naturally occurring shade, the light quantity applied to parental plants was reduced to 55-56 µmol m^{-2} s^{-1} as an additional control, referred to as weak shade light or weak light, respectively (Table 3).

Table 3. Light conditions for plant cultivation in regular white light (non-shade conditions) and shade light.
PAR, photosynthetically active radiance; R/FR ratio, red/far red ratio.

Light condition	PAR [µmol m^{-2} s^{-1}]	blue light [µmol m^{-2} s^{-1}]	red light [µmol m^{-2} s^{-1}]	far-red light [µmol m^{-2} s^{-1}]	R/FR ratio
non-shade light	118	9	13	11.5	1.1
shade light	119	10	4	12	0.3
weak light	55	6	7	6	1.1
weak shade light	56	6	8	26	0.3

2.4.2.1 Plant *in-vitro* cultivation

For plant cultivation under sterile conditions, seeds were surface sterilized using the liquid sterilization method with ethanol as described above. For *in vitro* culture, dry, surface-sterilized seeds were transferred on petri dishes containing solid half strength Murashige and Skoog (MS) medium (2.15 g/l MS basal salt mixture, 0.25 g/l MES; pH 5.7; 10 g Agar/l) (Murashige and Skoog, 1962). Seeds were distributed on the plates with the help of sterilized wooden toothpicks. Plates with seeds were stratified for 1 day at 4 °C, then transferred to LD conditions in plant growth chambers.

2.4.2.2 Plant cultivation on soil

For plant cultivation on soil, seeds were evenly sown on watered “sowing soil” (2:2:1, P soil : T soil : sand) and stratified at 4 °C for two days. If intergenerational effects were analyzed, the stratification treatment was skipped. Following stratification, the pots were transferred to the greenhouse or the plant growth chambers to LD light conditions. After the transfer to light, the trays were covered with a clear plastic dome to ensure appropriate humidity for the germinating seeds. Ten days after sowing the seedlings were singled out and transferred to “cultivation soil” (2:2:1, P soil : T soil : Perligran). Again, the trays were covered with a clear plastic dome which was removed three days after singling out.

For experiments analyzing transgenerational effects of an altered soil nutrient content, soil with a reduced amount of fertilizer was used. Initially, seeds were sown on “sowing soil”. 10 days after sowing the seedlings were singled out in pots containing the cultivation soil with reduced fertilizer contents (2:2:1, P soil : T0 soil : sand). Before use, the cultivation soil with reduced amounts of fertilizer was sent to Agrolab (Institut Koldingen GmbH, Visselhövede Germany) for analysis of its pH, as well as its nitrogen, phosphate, potassium, magnesium and sodium content. Based on this analysis, the fertilizer treatment was adapted. Plants were fertilized by watering with a fertilizer solution (5 mM KNO_3; 0.5 mM K_2HPO_4) or a control solution (0.5 mM KNO_3; 0.5 mM KH_2PO_4). Watering with fertilizer or control solution was conducted for the first time three days after singling out. After that, plants were fertilized every 10 days, 5 times in total before bagging of the plants.

2.4.2.3 Preparation of matched seed sets

For the analysis of germination, only seeds derived from matched seed sets were analyzed. In order to generate a matched seed set, the parental plants of the respective seed sets were grown in parallel in a similar growth environment (either in the greenhouse or in a growth chamber). All seeds were sown at the same time, singling out was conducted with a maximum of three days difference between the genotypes. After sowing, the plants were grown for six to seven weeks until they were bagged. This was followed by a two-week phase of seed after-ripening on the bagged plants. Watering of the plants was continued in this phase. Thereafter, parental plants were not watered anymore and allowed to dry thoroughly. Environmental conditions were kept constant during this process, both light regime and humidity were maintained according to the respective plant growth conditions. After the plants had dried, bags were collected and stored at 20 °C and 60 % relative humidity until sieving of the seeds, which was conducted to remove remaining tissue from the parental plants (such as flowers, dry siliques etc.). Seeds were sieved as soon as possible and stored dry in glass vials enclosed with a lid and stored at 20 °C and 55 % relative humidity. The minimum storage time was 90 days, starting from the day the bagged seeds were collected from their parental plants.

2.4.3 Wild type, transgenic and mutant lines

In this work, the *A. thaliana* accession Columbia-0 (Col-0) was used as the wild type, exceptions are indicated accordingly. Apart from Col-0, also Columbia-3 (Col-3) and Wassilewskija (WS) were used as wild type controls for mutant lines in Col-3 or WS background. In Table 4 all mutant and transgenic lines used in this work and the respective references and sources of the seeds are listed.

Table 4. Plant mutant and transgenic lines used in this work.

	Name	Background	References	Source
CK biosynthesis	*ipt2 ipt9*	Col-0	Miyawaki *et al.*, 2006	Dr. Ireen Schwarz
	ipt4 ipt8	WS	Miyawaki *et al.*, 2006	Dr. Anne Cortleven
	ipt3 ipt5 ipt7	Col-0	Miyawaki *et al.*, 2006	Dr. Anne Cortleven
	ipt1 ipt3 ipt5 ipt7	Col-0	Miyawaki *et al.*, 2006	Dr. Anne Cortleven
	rock4	Col-0	Jensen, 2013	Dr. Helen Braun
CK receptors	*ahk2-2tk*	Col-0	Higuchi *et al.*, 2004	Dr. Michael Riefler
	ahk3-3	Col-0	Higuchi *et al.*, 2004	Dr. Michael Riefler
	cre1-2	Col-0	Inoue *et al.*, 2001	Dr. Michael Riefler
	cre1-12	Col-0	Higuchi *et al.*, 2004	Dr. Michael Riefler
	ahk2-5	Col-0	Riefler *et al.*, 2006	Dr. Michael Riefler
	ahk3-7	Col-0	Riefler *et al.*, 2006	Dr. Michael Riefler
	ahk2-2tk ahk3-3	Col-0	Higuchi *et al.*, 2004	Dr. Michael Riefler
	ahk2-5 ahk3-7	Col-0	Riefler *et al.*, 2006	Dr. Michael Riefler
	ahk2-5 cre1-2	Col-0	Riefler *et al.*, 2006	Dr. Michael Riefler
	ahk3-7 cre1-2	Col-0	Riefler *et al.*, 2006	Dr. Michael Riefler
	ahk2-2tk cre1-12	Col-0	Higuchi *et al.*, 2004	Dr. Michael Riefler
	ahk3-3 cre1-12	Col-0	Higuchi *et al.*, 2004	Dr. Michael Riefler
	rock2	Col-0	Jensen, 2013; Bartrina *et al.*, 2017	Dr. Helen Braun
	rock3	Col-0	Jensen, 2013; Bartrina *et al.*, 2017	Dr. Helen Braun
	rock2 rock3	Col-0	Jensen, 2013	Dr. Helen Braun
CK signal transduction	*ahp6-1*	Col-0	Mähönen *et al.*, 2006	Dr. Elisabeth Otto
	ahp6-3	Col-0	Mähönen *et al.*, 2006	Dr. Elisabeth Otto
	ahp2 ahp5-2	Col-0	Hutchinson *et al.*, 2006	Stefanie Zintl
	ahp2 ahp3 ahp5-2	Col-0	Hutchinson *et al.*, 2006	Stefanie Zintl
CK catabolism	*ckx2-1*	Col-0	Bartrina *et al.*, 2011	Dr. Isabel Bartrina
	ckx3-1	Col-0	Bartrina *et al.*, 2011	Dr. Isabel Bartrina
	ckx4-1	Col-0	Bartrina *et al.*, 2011	Dr. Isabel Bartrina
	ckx5-1	Col-0	Bartrina *et al.*, 2011	Dr. Isabel Bartrina
	ckx6-2	Col-0	Bartrina *et al.*, 2011	Dr. Isabel Bartrina
	ckx7-G1	Col-0	unpublished	Dr. Ireen Schwarz
	ckx2 ckx4	Col-0	Bartrina *et al.*, 2011	Dr. Isabel Bartrina
	ckx3 ckx5	Col-0	Bartrina *et al.*, 2011	Dr. Isabel Bartrina
	ckx2 ckx4 ckx5 ckx6	Col-0	Bartrina, 2006	Dr. Isabel Bartrina
	35S:CKX1	Col-0	Werner *et al.*, 2003	Prof. Dr. Tomáš Werner
	35S:CKX2	Col-0	Werner *et al.*, 2003	Prof. Dr. Tomáš Werner
	35S:CKX4	Col-0	Werner *et al.*, 2003	Prof. Dr. Tomáš Werner
A-type ARRs	*arr4*	Col-0	To *et al.*, 2004	Prof. Dr. Klaus Harter
	35S:ARR4	Col-3	Sweere *et al.*, 2001	Prof. Dr. Klaus Harter
	arr7 arr15	Col-0	unpublished	Prof. Dr. Bruno Müller
	arr3,4,5,6,8,9	Col-0	To *et al.*, 2004	Dr. Anne Cortleven

Table 4 continued

B-type ARRs	*arr2*	Col-0	Mason *et al.*, 2005	Dr. Eva Hellmann
	arr14-1	Col-0	Ishida *et al.*, 2008	Dr. Eva Hellmann
	arr19	Col-0	this work	SALK_206924C, NASC Id.no: N696778
	arr21	Col-0	this work	SALK_150381C, NASC Id. no: N664321
	arr1-3 arr10-5	Col-0	Mason *et al.*, 2005	Dr. Anne Cortleven
	arr1-3 arr12-1	Col-0	Mason *et al.*, 2005	Dr. Anne Cortleven
	arr10-5 arr12-1	Col-0	Mason *et al.*, 2005	Dr. Anne Cortleven
	arr19 arr21	Col-0	this work	
Other	*rock1-2*	Col-0	Dobritsa *et al.*, 2011; Niemann *et al.*, 2015	Dr. Michael Niemann
	phyA-211	Col-0	Reed *et al.*, 1994	NASC Id. no: N6223
	phyA ahk2-5 ahk3-7	Col-0	this work	
	phyA ahk2-2tk ahk3-3	Col-0	this work	
	phyA ipt3,5,7	Col-0	this work	
	ARR21:CKX1	Col-0	Harder, 2009	
	ARR21:GUS	Col-0	Harder, 2009	
	GILT:CKX1	Col-0	Harder, 2009	
	GILT:GUS	Col-0	Harder, 2009	

Plant lines specifically expressing *CKX1* in the testa or the endosperm of developing seeds and their corresponding promoter:GUS lines were generated by Harder (2009), supervised by Stefanie Zintl. The tissue-specific promoters were chosen according to Tiwari *et al.* (2006) and expression analysis patterns published in the eFP browser (Winter *et al.*, 2007). Activity of the promoters chosen for cloning was expected to be exclusive for - and restricted to - the expected seed tissue. Furthermore, promoters should not be influenced directly by varying CK levels. Expression analysis of the promoter:*CKX1* construct via qPCR was accomplished by using a *CKX1*-specific forward primer and a reverse primer specific for the Gateway-cloning associated *attB* recombination site (Harder, 2009). All lines used here expressed the construct both in siliques, flowers and whole plants (Harder, 2009). Analysis of seed tissue-specificity of *CKX1* expression was not possible using this method.

2.4.4 Genetic crossings

For genetic crossings, parental plants were raised together according to the protocol described earlier, until they reached the flowering stage. Three to four flowers of each maternal plant were used. First of all, the shoot branch chosen for crossing was prepared by removing open flowers and mature siliques, usually six to eight siliques were removed counting in basipetal direction from the inflorescence. The number of removed flowers and siliques was kept equal between all plants crossed, to avoid differences in seed nutrition by unequally reducing the number of siliques and thereby increasing sink strength of the remaining seeds. Also the younger, closed flower buds in the center of the inflorescence were removed, except for the three to four flower buds chosen for crossing. The flower buds were opened under the binocular with precision tweezers and the flower was emasculated by removing all stamina. The pistil, sepals and petals were kept intact. After preparation, the plants were kept under a clear plastic dome overnight, the pollination was

performed the following day. This two-day crossing protocol allows to identify flowers, that were accidentally pollinated when stamina were removed. Accidentally pollinated flowers were removed from the maternal plant. Non-pollinated pistils developed a sticky stigma overnight, these flowers were used for hand pollination. For pollination, a newly opened flower of the paternal parent was chosen and plucked from the plant. Then the anthers were brushed over the stigma of the emasculated flowers on the maternal plant. After pollination the maternal plants were kept under a clear plastic dome for two to three days, successful crosses developed visible siliques from the pollinated flowers. Crossings were performed in both directions (reciprocal crosses). Seeds of the F1 generation were either tested for their germination phenotype after storage or they were sown out again in order to generate the F2 and F3 generation. In all generations the plants were genotyped.

2.5 Nucleic acid extraction methods

2.5.1 Extraction of RNA from *Arabidopsis* seedlings or adult plants

RNA isolation from leaves or seedling material was conducted using the NucleoSpin RNA Plant Kit (Macherey-Nagel, Düren, Germany) including an on-column DNase digestion, according to the manufacturer’s instructions. RNA was dissolved in 20 µl RNAse-free, diethylpyrocarbonate (DEPC)-treated and autoclaved water.

2.5.2 Extraction of RNA from *Arabidopsis* seeds and siliques

For RNA isolation from seed material, seeds were harvested, transferred to 1.5 ml safe-lock microcentrifuge tubes and shock frozen in liquid nitrogen. Frozen seed material was ground to fine powder with pre-cooled, sterilized plastic pestles in the presence of liquid nitrogen or in pre-cooled porcelain mortars with matching pestles. RNA isolation was conducted according to the protocol published by Vicient *et al.*, 1999. In the following, critical steps and alterations of the published protocol are described.

The first step of the protocol, the grinding of seed material, was performed without addition of quartz powder. RNA extraction buffer (Table 5) was precooled to -20 °C before use and prepared freshly prior to sample grinding. The 4 s centrifugation step following the overnight incubation of the samples at 4 °C proved to be indispensable for the successful RNA isolation. After washing and air drying the RNA pellet, the resuspension of the RNA in 1 ml solubilization buffer (Table 6) was performed at room temperature, in order to prevent sodium dodecyl sulfate (SDS) precipitation. To remove proteins and purify the isolated RNA, the samples were extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform:isoamyl alcohol (24:1). The last aqueous phase was collected in one 1.5 ml microcentrifuge tube, then RNA was precipitated by addition of 3 M sodium acetate (0.1 vol) and 100 % ethanol (1.5 vol). After centrifugation, the RNA pellet was dissolved in 0.5 ml of 3 M sodium acetate and centrifuged again. After washing, the isolated RNA was dissolved in 20 µl RNAse-free, DEPC-treated water.

Table 5. RNA extraction buffer according to Vicient, Delseny (1999)

Component	Concentration
LiCl	8 M
β-mercaptoethanol	2 %
always prepared freshly	
pre-cooled to -20°C	

Table 6. RNA solubilization buffer according to Vicient, Delseny (1999)

Component	Concentration
SDS	0.5 %
NaCl	100 mM
EDTA	25 mM
Tris-HCl pH 7.6	10 mM
β-mercaptoethanol	2 %
always prepared freshly	

In order to meet the requirements for RNA-Sequencing (RNA-Seq), RNA samples were prepared using the kit “Ribospin seed/fruit” (GeneAll, Seoul, Korea) (Table 2) instead, according to the manufacturer's specifications. Additional RNA purification was achieved using the kit “Riboclear plus!” (GeneAll, Seoul, Korea) (Table 2) directly after RNA extraction. For further details for RNA-Seq sample preparation please refer to section 2.9.8 “RNA-Seq”.

After isolation, extracted RNA was subjected to DNAse I (Thermo Fisher Scientific) treatment, in order to digest residual single and double stranded DNA contained in the RNA sample. DNAse I was used according to the manufacturer's specifications.

To assess RNA quality and quantity, RNA samples were measured photometrically using a spectrophotometer (NanoDrop ND-1000, PEQLAB). The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of the isolated RNA. Since nucleotides, RNA and DNA absorb at 260 nm, sample purification as included in the RNA isolation protocol described above is required. Contaminants as proteins or phenol absorb at 280 nm, therefore a 260/280 ratio exceeding 1.8–2.0 was chosen as a quality criterium for the RNA samples. Phenol also absorbs highly at 230 nm, as well as other contaminants such as ethylenediaminetetraacetic acid (EDTA) and carbohydrates, therefore the 260/230 ratio was used as second measure of sample quality. 260/230 ratios exceeding a value of 2.0 were considered as adequate.

2.5.3 Extraction of genomic DNA from *Arabidopsis*

For extraction of genomic DNA from *A. thaliana* leaves or seedlings, plant material was harvested freshly. For genomic DNA extraction from adult plants, one younger leaf or one third of a mature leaf was harvested and transferred into a 2 ml microcentrifuge tube. For genomic DNA extraction from seedlings, 3-5 seedlings were collected together and transferred to a 2 ml microcentrifuge tube. For genomic DNA isolation from seeds, seed material was shock frozen and ground to fine powder using a pre-cooled mortar and a pestle before transfer to a 2 ml microcentrifuge tube. 400 µl DNA extraction buffer (200 mM Tris-HCl pH 7.5; 250 mM NaCl; 25 mM EDTA pH 8.0; 0.5 % (w/v) SDS; in H_2O) and 3 steel beads were added, and the plant material was homogenized using a Retsch mill (Retsch, Haan, Germany). The sample was centrifuged at room temperature for 3 min. at 13000 rpm. 300 µl of the supernatant was transferred to a new 1.5 ml microcentrifuge tube.

For DNA precipitation, 300 µl isopropanol was added to the sample, thoroughly mixed and incubated at room temperature for at least ten min to precipitate the DNA. After a 5 min centrifugation step at room temperature and 10000 rpm, the supernatant was discarded, and the DNA pellet was washed with 350 µl 70 % EtOH. Afterwards, the extracted DNA was air-dried, then resuspended in 50-80 µl double distilled water (ddH_2O) and stored at -20 °C. DNA concentration was analyzed with the help of a spectrophotometer as described for RNA.

2.5.4 Polymerase chain reaction

Standard polymerase chain reaction (PCR) was applied for genotyping *A. thaliana* seeds, seedlings or adult plants. For DNA amplification, thermostable *Thermus aquaticus* DNA polymerase (Taq) was used. Both Taq polymerase and matching Taq buffer (10x Taq buffer: 500 mM KCl, 100 mM Tris-HCl pH 9.0 and 1 % Triton X-100) were generated and provided by Prof. Dr. Wolfgang Schuster. The PCR reaction (20 µl) was composed as shown in Table 7.

Table 7. Constituents of a standard PCR reaction.

Component	Volume [µl]	Final concentration
10x Taq PCR buffer (including $MgCl_2$)	2.0	1x
5 mM dNTP Mix	0.6	150 µM
5 µM primer forward	2.0	0.5 µM
5 µM primer reverse	2.0	0.5 µM
Taq DNA polymerase	0.3	
water	ad 19	
DNA extract (template)	1.0	

A list of primer sequences used for genotyping can be found in Table A1. The standard PCR protocol, summarized in Table 8, was adjusted according to the length of the amplification product and the optimal annealing temperature of the primer pairs. PCR steps 1 and 5 were performed once, while PCR steps 2-4 were cyclical repeated 25-35 times, depending on the PCR reaction. PCR was carried out using a thermocycler (Biometra, Analytik Jena, Jena, Germany).

Table 8. Standard PCR program.

PCR step	Temperature	Duration
1. Initial denaturation	95 °C	2 min
2. Denaturation	95 °C	30 sec
3. Primer annealing	55-58 °C	30 sec
4. Elongation	72 °C	1 kb/min
5. Final elongation	72 °C	5 min

2.5.5 DNA sequencing

Sequencing of amplified PCR products was conducted by Eurofins GATC Biotech (Ebersberg, Germany).

2.5.6 Semi-quantitative reverse transcriptase PCR

Semi-quantitative RT-PCR was used in this work to assess transcript abundance of the gene of interest in selected T-DNA insertion lines. For semi-quantitative PCR, RNA was extracted from siliques of the respective plants using the protocol described previously. This tissue was chosen in accordance to the high expression levels of the genes of interest

predicted by BAR eFP browser. After DNAse digestion, cDNA was synthesized from 100 ng of the RNA template via reverse transcription (see section “cDNA synthesis”). The synthesized cDNA was multiplied by PCR. Primer sequences used for semi-quantitative PCR are listed in Table 9. As a control, a housekeeping gene, *ACTIN7* in this case, was amplified and visualized in every sample.

Table 9. Primers used for semi-quantitative RT-PCR.

Gene	ATG	Primer forward (5´ → 3´)	Primer reverse (5´ → 3´)
ACTIN7	AT5G09810	AATGGTGAAGGCTGGTTTTG	TCCACATCTGTTGGAGGGTG
ARR19	AT1G49190	CGCCTCCTTTGCCCTATTTG	ACTCACAAGGGAAGTCGCA
ARR21	AT5G07210	GTTTGATTCCGAGCCAACCG	CGCTTCTCCTTGTTGATGCTT

After PCR, samples were supplied with 6x DNA loading buffer, loaded on an agarose gel as described in section “agarose gel electrophoresis” and documented thereafter.

2.5.7 Agarose gel electrophoresis

In order to separate DNA fragments according to their size, or check RNA quality, nucleic acids were separated by size using agarose gel electrophoresis. Depending on the size of the DNA/RNA fragments, 1 % or 2 % agarose gels were prepared. Agarose was dissolved in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA pH 8, in ddH_2O) by boiling with the help of a microwave. After preparation, gels were either used freshly or stored at 60 °C to prevent solidification. Before use, 0.2 µg/ml ethidium bromide was added to the liquid agarose gel, before pouring the mixture into a gel tray for solidification. 1x TAE was used as running buffer. Prior to loading on the agarose gel, samples were supplied with 6x loading buffer (15 % Ficoll-400, 0.1 % Bromophenol blue sodium salt, 0.1 % xylene cyanol FF, in ddH_2O), final concentration in the samples was 1x loading buffer. For determination of the nucleic acid fragment sizes, pre-stained nucleic acid ladder 5000-100 bp or 1000-100 bp (GeneRuler DNA ladder, Thermo Fisher Scientific) was loaded on the gel in parallel, depending on the expected nucleic acid fragment size. For separation of the nucleic acids, an electric field was applied using 80-120 V for 30-60 min. Visualization and picture documentation was carried out using the GenoPlex UV-transilluminator gel documentation system in combination with the GenoCapture software (VWR, Darmstadt, Germany).

2.5.8 Genotyping of plants

In order to verify their identity, plants derived from external sources (donations, NASC stock center) were always genotyped to ensure their correct and declared identity prior to use. Also, new mutant lines generated via crossing were genotyped in the F1, F2 and F3 generation. Mutant lines derived from in-house sources were genotyped infrequently to ensure the identity of the respective mutants. Genotyping of plants was performed by harvesting plant material and extracting genomic DNA as described earlier, followed by gene- and mutant-specific PCRs. Genotyping of insertional T-DNA mutants was conducted using two primer pairs, one pair flanking the site of predicted T-DNA insertion in order to amplify the wild-type allele, the second pair consisting of a gene-specific primer (either in forward or reverse orientation) and a T-DNA specific primer for amplification of the mutant allele. Primer sequences used for genotyping are listed in Table A1. PCR and agarose gel electrophoresis were performed as described above.

2.5.9 cDNA synthesis

For semi-quantitative PCR and real-time PCR, cDNA was synthesized from isolated RNA via reverse transcription. For real-time PCR, 1 µg of RNA was transcribed into cDNA using SuperScript III (Invitrogen/Thermo Fisher Scientific, Waltham, US), according to the manufacturer's specifications. 1 µg RNA (in 9.7 µl) was mixed with 2 µl dNTPs (5 mM), 1.8 µl N_9 primer (50 µM) and 1 µl oligo-dT primer (50 µM), ddH_2O was added ad 14.5 µl. These reactions were incubated at 65 °C for 5 min. Afterwards, the samples were incubated on ice for 1 min before adding 4 µl 5x first strand buffer, 1 µl DTT (100 mM) and 0.5 µl SuperScript III Reverse Transcriptase (200 U/µl). Samples were placed in the thermal cycler again and incubated at 25 °C for 5 min and at 50 °C for 60 min for cDNA synthesis. Finally, the reverse transcriptase was inactivated at 70 °C for 15 min. Afterwards, the samples were cooled to 12 °C. The synthesized cDNA was diluted 1:10 with ddH_2O and stored at -20 °C.

2.5.10 Primer design for qPCR

Primers used for qPCR were designed using the Primer-BLAST online tool, which is based on Primer3 (Table 1). Primers were designed to meet the following requirements: 300 bp was set as maximal length of the resulting PCR product, the optimal melting temperature of the primer was set to 60 °C. The primers returned from Primer-BLAST were analyzed using NetPrimer (Table 1). Primers were considered suitable, if the NetPrimer rating exceeded 90 %, and the probability of the primers to form hairpin structures or self- and cross-dimerize was low. Identified primers were ordered from Thermo Fisher Scientific. Primers were dissolved in autoclaved ddH_2O, the concentration was adjusted to 50 µM.

2.5.11 Primer efficiency test

All primers used for qPCR are listed in Table A2. Before use, all primer pairs were tested for primer efficiency. To do so, cDNA derived from imbibed wild-type seeds was utilized to create a cDNA dilution series. cDNA was diluted 1:10, 1:20, 1:40, 1:80 and 1:160. Additionally, a water control was included. The diluted cDNA samples were measured in three technical replicates. The relative amount of cDNA was plotted on the x-axis as the log of the cDNA concentration, the mean of the three Ct measurements was plotted on the y-axis accordingly. Based on that, a regression curve was computed using Excel. The primer efficiency E (given in %) was calculated based on $E = (10^{-1/slope} - 1) * 100$. Primer pairs were considered suitable, if the efficiency was between 85 % and 125 % and if the qPCR did not show amplification of more than one product with the respective primer pair, based on melting curve analysis.

2.5.12 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was used to measure and compare transcript abundances of genes-of-interest in the germination process. For all qPCR studies the CFX96 Real-Time PCR detection System (Bio-Rad, Hercules, USA) in combination with CFX manager software (Bio-Rad) was used. The composition of one qPCR reaction sample is given in Table 10. The qPCR thermal cycler program is listed in
Table 11.

Table 10. Constituents of a qPCR reaction sample.

Component	Volume [µl]	Final concentration
10 x Immolase buffer	2	1x
50 mM $MgCl_2$	0.8	2 mM
20 mM dNTP Mix	0.4	0.1 mM
10 x SYBR Green I	0.2	0.1 x
50 µM primer forward	0.12	300 nM
50 µM primer reverse	0.12	300 nM
Immolase (5 U/µl)	0.04	0.01 U
water	14.32	
cDNA template	2	

Table 11. qPCR thermal cycler program.

qPCR step	Temperature	Duration
1. Initial denaturation	95 °C	15 min
2. Denaturation	95 °C	5 sec
3. Primer annealing	55 °C	15 sec
4. Elongation	72 °C	10 sec
5. Plate read and repeat steps 2 - 4 39 times		
6. Melt curve 60-95 °C, increment 0.5 °C in 5 sec		
7. End	25 °C	1 sec

2.6 Protein methods

2.6.1 Protein extraction

For extraction of total protein from *A. thaliana* seeds, seed material was treated according to the standard protocol for germination assays (see section "standard germination assay"). Seeds were harvested in the imbibition phase, 1 h, 6 h, 12 h, 24 h or 48 h after the first FR light pulse. A second FR light pulse was not applied. Seeds were harvested in 1.5 ml microcentrifuge tubes in dim green safe light. Exposure time to the green light prior to freezing was kept at a minimum, harvesting was conducted in a time slot of 25 to 45 s per sample. All samples were kept in darkness prior to harvest. After transfer of the seed material, microcentrifuge tubes were centrifuged in darkness for 5 seconds and shock frozen in liquid nitrogen. Seeds were ground to fine powder using pre-cooled porcelain mortars and pestles. After that, protein extraction buffer (Table 12), which is optimized for protein extraction from seeds (Arana *et al.*, 2017), was added to each sample and mixed.

Table 12. Protein extraction buffer according to Arana *et al.* (2017)

Component	Concentration
Tris-HCl pH 7.5	50 mM
NaCl	150 mM
Glycerol	10 %
EDTA pH 8.0	
Sarcosyl	0.1 %
following components were always added freshly	
β-mercaptoethanol	10 mM
Protease inhibitor cocktail	1 mM
Mg132	

The mixture was incubated on ice for eight min and centrifuged for ten min at 13000 rpm. The supernatant was transferred to a fresh microcentrifuge tube.

2.6.2 Determination of protein concentrations

Protein concentrations were determined using the Bradford method (Bradford, 1976). For Bradford analysis, Bradford reagent (Bio-Rad) was diluted 1:4. 200 µl of the diluted Bradford reagent was pipetted into the wells of a microtiter plate, 10 µl of the 1:10 diluted protein sample was added. Samples were mixed and incubated for 10 min at room temperature, before photometrical measurement at 595 nm using a microplate reader (Microplate spectrophotometer, Synergy2, BioTek Instruments, Winooski, USA). As a reference, a Bovine serum albumin (BSA) calibration series was prepared. To do so, 1 mg/ml BSA was dissolved in protein extraction buffer (without β-mercaptoethanol, protease inhibitor cocktail and Mg132) and diluted with extraction buffer to the final concentrations 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.05 mg/ml, 0.025 mg/ml. 10 µl of the sample was measured, thereby BSA standards were measured in triplicate, protein samples in duplicate. Additionally, a blank sample containing only protein extraction buffer was measured. To calculate protein concentration, linear regression analysis was performed in Excel, plotting the concentrations of the standards on the x-axis against the respective absorption on the y-axis. With the help of the equation, protein concentrations in the samples were calculated. For the following electrophoresis, samples containing 10 µg of protein per sample were prepared. In order to optimize loading of the samples on the gel and minimize protein degradation while storing the samples at -20 °C, all samples were supplied with 1x SDS loading buffer (Table 13).

Table 13. 4x SDS loading buffer for protein samples.

Component	Concentration
Tris-HCl pH 6.8	250 mM
Orange G	0.01 %
SDS	8 %
β-mercaptoethanol	10 %
Glycerin	40 %

2.6.3 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

For protein analysis, protein extracts were loaded onto a denaturing SDS polyacrylamide gel, to allow protein separation according to their size and the resulting retention in the gel when an electrical field is applied. To do so, first SDS polyacrylamide minigels consisting of stacking and separating gel were prepared. The upper stacking gel contained 4 % acrylamide, the lower separating gel was prepared using 8 % acrylamide. The receipts for both gels are given in Table 14.

Table 14. Reaction mixture for 8 SDS polyacrylamide minigels.

Gel type	Component	Volume
8 % separating gel (50 ml)	ddH_2O	26.725 ml
	40 % Acrylamide	10 ml
	1.5 M Tris-HCl pH 8.8	12.5 ml
	10 % SDS	500 µl
	10 % Ammonium persulfate (APS)	250 µl
	N,N,N',N'-tetramethylethane-1,2-diamine (Temed)	25 µl
4 % stacking gel (20 ml)	ddH_2O	14 ml
	40 % Acrylamide	2 ml
	0.5 M Tris-HCl pH 6.8	3.75 ml
	10 % SDS	150 µl
	APS	75 µl
	Temed	12 µl

After polymerization, the gels were either used freshly or stored under moist conditions at 4 °C. The gels and the following electrophoresis were conducted using the Hoefer Mighty Small II Mini Vertical Electrophoresis System (Thermo Fisher scientific). Prior to loading the protein samples onto the minigel, all samples were boiled for 3 min at 95 °C. Meanwhile, the electrophoresis apparatus was set up, using one or two pre-prepared SDS polyacrylamide minigels per electrophoresis apparatus and 1x SDS running buffer (for receipt of 10x SDS running buffer see Table 15).

Table 15. 10x SDS running buffer for SDS-PAGE

Component	Concentration
Glycin	1.92 M
Tris base	250 mM
SDS	2 %

After cooling, the samples were loaded onto the stacking gel. As a marker for molecular weight, 3 µl of a pre-stained protein ladder (PageRuler, Thermo Fisher Scientific) was loaded onto the gel. Electrophoresis was conducted at 20 Milliampere (mA) (constant) per gel for at least 1.5 h. After electrophoresis, electrophoretically separated proteins were blotted on a membrane by western blotting.

2.6.4 Western Blot

Before proteins were transferred from the SDS polyacrylamide minigel onto a membrane, the electrophoresis apparatus was carefully dismounted, and the separation of the protein ladder and the dye front of the samples were examined. For overnight protein blotting, the Mini Trans-Blot Electrophoretic Transfer Cell System (Bio-Rad) was used. Proteins were transferred to an Immobilon-FL 0.45 µm PVDF membrane (Merck Millipore, Burlington, USA). The membrane was activated before use by incubation in MeOH for 1 min, rinsed with water for 1 min and incubated in 1x blotting buffer, which was diluted 1:10 from 10x blotting buffer (1.92 M Glycin, 250 mM Tris base). The transfer sandwich was prepared, starting with a sponge pad soaked in 1x blotting buffer, one layer of Whatman filter paper, protein gel (facing to the cathode), the activated membrane, one layer of Whatman filter paper and the second sponge pad. The transfer sandwich was inserted into the blotting chamber, which was filled with 1x blotting buffer. Blotting was conducted

overnight at 4 °C with 40 mA (constant). After blotting, the transfer sandwich was carefully dismounted. Transfer was considered successful, if the pre-stained protein ladder was visible on the membrane.

2.6.5 Chemiluminescence-based immunodetection

For protein detection using the classical chemiluminescence-based method, the protein-containing PVDF membrane was cut carefully with a razor blade along the horizonal axis just above the 70 kilodalton (kDa) band of the pre-stained protein ladder. First of all, the membrane pieces were incubated separately in 5 % skim milk in 1x T-PBS buffer (1:10 diluted from 10x PBS; 0.1 %Tween 20) for 60-90 min in a rotator (HAG FinePCR, Gunpo, Korea) at room temperature. The receipt of 10x PBS is given in Table 16.

Table 16. 10x PBS buffer for protein immunodetection.

Component	Concentration
NaCl	1.37 M
KCl	27 mM
Na_2HPO_4	100 mM
KH_2PO_4	18 mM
pH 7.4	

The skim milk solution was discarded, and the primary antibody solution was applied. The upper part of the membrane was incubated with phyA primary antibody. The primary antibody used for detection of phyA was donated by Akira Nagatani (Kyoto University, Japan), as published (Shinomura *et al.*, 1996). phyA is a monoclonal IgG anti-Arabidopsis phyA antibody, host organism is mouse. PhyA primary antibody was diluted 1:1000 in 4 ml T-PBS buffer. The second, lower part of the membrane was subjected to housekeeping protein detection. For detection of housekeeping proteins, either actin (polyclonal antibody, host organism rabbit, cat. no. AS13 2640 Agrisera, Vännäs, Sweden) or UDP-glucose pyrophosphorylase (UGPase) (polyclonal antibody, host organism rabbit, cat. no. AS05 086, Agrisera) primary antibodies were diluted 1:2000 in T-PBS buffer and applied to the membrane.

The membrane was incubated for 1.5 h on a rotator at room temperature with the primary antibody. Alternatively, primary antibody was incubated overnight at 4 °C. After that, the antibody solution was collected in a separate tube and stored at 4 °C. After three washing steps with T-PBS buffer for 5 min, the secondary horseradish peroxidase (HR-peroxidase) coupled antibody was applied to the membrane. For detection of phyA, goat anti-mouse antibody (IgG-HRP, polyclonal antibody, host organism goat, cat. no. sc-2005, Santa Cruz Biotechnology, Dallas, USA) was used in a 1:2000 dilution in 4 ml T-PBS and applied directly to the membrane pieces. Actin and UGPase were detected using bovine anti-rabbit secondary antibody (IgG-HRP, polyclonal antibody, host organism bovine, cat. no. sc-2370, Santa Cruz Biotechnology) in a dilution of 1:3000. Secondary antibodies were removed after 1.5-2 h incubation at room temperature. Membrane pieces were washed four times for 5 min with T-PBS. For visualization of the blotted proteins on the membrane, the enhanced chemiluminescence (ECL) system (Thermo Fisher Scientific, Table 2) was used according to the manufacturer's instructions. The HR-peroxidase, which is coupled to the secondary antibody, catalyzes the oxidation of its substrate, luminol, in the presence of signal enhancers, to detect and visualize the emitted light at 428 nm. For visualization of the luminescence signal, a film (Kodak X-Omat, Sigma-Aldrich/Merck) was incubated on

the membrane. Exposure time was chosen dependent on the intensity of the luminescence signal, usually between 5 sec and 5 min. After film exposure, the film was developed and fixed. For analysis of protein levels, the developed films were digitalized and analyzed using ImageJ (Table 1).

2.6.6 Fluorescence-based immunodetection

For quantification and comparison of protein levels between genotypes, fluorescence-labeled secondary antibodies were used in combination with the laser-based near-infrared LI-COR Odyssey Fc Imaging System (LI-COR Biosciences, Lincoln, USA). This technique combines classical western blotting with fluorescence-labeled secondary antibodies targeting the primary antibodies of the housekeeping protein and the protein of interest. The simultaneous detection of both proteins with fluorescently labeled antibodies allows sensitive signal integration for the quantification of the protein of interest (Derkacheva *et al.*, 2013), rendering the cutting of the membrane before antibody application unnecessary. For immunodetection, both primary antibodies were applied on the membrane and handled as described above. After removing primary antibody solution and washing, fluorescence-labeled secondary antibodies were applied on the membrane. For detection of phyA, the IRDye 680 RD (red, host organism goat, anti-mouse, IgM, cat. nr. 926-68180, LI-COR Biosciences) was used in 1:7500 dilution, for UGPase detection IRDye 800 CW (green, host organism goat, anti-rabbit, IgG, cat. nr. 926-32211, LI-COR Biosciences) was diluted 1:15000. During the two hours of incubation at room temperature, the centrifuge tubes containing the membrane and the antibodies were covered by aluminum foil. After that the membrane was removed and washed four times for 15 min with LI-COR washing solution. Thereafter, the membrane was dried completely. Detection of the fluorescence signal was conducted using the Odyssey Imaging System (LI-COR Biosciences), according to the manufacturer’s instructions. IRDye 680 RD was detected at 700 nm, IRDye 800 CW at 800 nm. Protein quantification was conducted using the Image Studio software (LI-COR Biosciences). For quantification of the fluorescence signals, the signal intensity of the target protein (phyA, red channel) and the housekeeping protein (UGPase, green channel) were normalized by calculating a lane normalization factor, then the target protein fluorescence intensity was divided by the fluorescence intensity of the housekeeping protein and expressed as relative fluorescence intensity.

2.7 Histochemical analysis of GUS expression

To analyze promoter activity in distinct seed tissues, histochemical analysis using the promoter:GUS reporter system (Jefferson *et al.*, 1987) was performed. For GUS staining, promoter:GUS plant lines were grown under standard growth conditions (see “Plant growth conditions”). For GUS staining, one to two green siliques per plant were harvested from the main stem of five-week-old plants. Siliques were cut open with a syringe using a stereomicroscope, and vacuum-infiltrated twice with pre-cooled 90 % acetone. After one hour of incubation in 90 % acetone at -20 °C, siliques were washed twice with 50 mM sodium phosphate buffer (pH 7) and GUS staining solution (1 mM 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (X-Gluc), dissolved in 50 µl DMSO; 10 mM potassium-ferricyanide; 10 mM potassium-ferrocyanide; 0.1% Triton X-100; 50 mM sodium phosphate buffer pH 7) was applied. After vacuum infiltration, siliques were incubated overnight at 37 °C with GUS staining solution to allow the enzymatic reaction. After destaining the siliques with 70 %

ethanol for three hours, the GUS staining pattern was documented with a microscope (Axioskop 2 plus, Zeiss, Jena, Germany) equipped with an AxioCam ICc3 (Zeiss) photographic device.

2.8 Quantification of phytohormone levels

For quantification of GA, ABA and CK levels, seed samples containing 100 mg of dry or dark imbibed *Arabidopsis* seeds were harvested, frozen in liquid nitrogen and stored at -80 °C prior to analysis. The hormone contents were measured using ultra-performance liquid chromatography-electrospray tandem mass spectrometry as described (Novák *et al.*, 2008; Turečková *et al.*, 2009; Urbanová *et al.*, 2013). All samples were measured in triplicate for each genotype and each timepoint.

2.9 Plant experiments

2.9.1 Standard germination assay

For standard germination assays, only matched seed sets were used. For every experiment corresponding control experiments were conducted in parallel. These control experiments included the testing of the seed batches (every biological replicate) for their germination rates either in darkness without an inducing light pulse, or in WL with a saturating WL pulse. At least four biological replicates per genotype and condition were tested. In addition, quality control criteria for germination assays were defined. In darkness, germination rates should not exceed 1.5 %, in order to confirm that seeds were handled in true dark conditions without exposure to scattered light prior to the germination-inducing light pulse. In WL conditions, a germination rate ≥ 75 % was defined as a quality criterium, to make sure the seeds are viable and non-dormant. In case these pre-requisites were not met, seeds were excluded from the analysis. As an additional internal control, *phyA* mutant seeds were included in every experiment, which do not germinate after illumination with FR light. Additionally, germination rates in FR light were measured in at least two technical replicates of every biological replicate.

Under sterile conditions, two layers of autoclaved and dried Whatman filter paper were placed in 145 mm x 20 mm Petri dishes and labeled. In the dark room, the plates were supplied with 8 ml of autoclaved ddH_2O. Sowing and singling out of the seeds with a toothpick was conducted in darkness, only a dim green safe light lamp was used to illuminate the workplace. For one experiment, sowing took three to seven hours. On one plate a maximum of seven to eight different genotypes or biological replicates were sown, using 70-100 seeds per genotype/replicate. If possible, on every plate at least one wildtype and one *ahk2 ahk3* biological replicate was contained.

After four plates were sown, the plates were placed in a custom-made monochromatic light chamber (CLF plant instruments, Wertingen, Germany). The plates were illuminated for 3 min with 90.6 µmol m^2 s^{-1} far-red light to inactivate phyB in imbibed seeds. Following FR light illumination, the monochromatic light chamber was opened in complete darkness, plates were wrapped in two layers of aluminum foil and stored for 48 h in a cabinet. To prevent unintended illumination during the dark imbibition phase, the foil-wrapped plates were additionally covered by a cotton cloth.

After 48 h of dark incubation, plates were subjected to a second light pulse. If not stated otherwise, the respective plates were placed into the monochromatic light chamber, and a 5 min FR light pulse of 18 µmol $m^{-2}s^{-1}$ was applied to induce germination. Germination-inducing light pulses varied depending on the experiment. To test WL

germination rates, seed-containing plates were pulsed with 120-150 µmol $m^{-2}s^{-1}$ WL for 1-2 h in a Percival chamber at 22 °C and 60 % RH. The control experiments in darkness were conducted by unpacking the plates in darkness at the site of handling. After that, all plates were wrapped in two layers of foil in darkness and incubated for another 96 h. Evaluation of germination was conducted using a stereomicroscope. Germinated seeds were defined as seeds, where the radical protruded the seed coat.

2.9.2 Assessment of seed dormancy

To analyze primary dormancy, four to five siliques were harvested from each parental plant, both from the main stem and from a lateral stem. Seeds were tested for their germination response directly after harvest (0 days) and after 4-28 days of dry storage. After sowing the seeds on filter paper, as described in the standard germination assay protocol, the plates were incubated for two hours in darkness, but without any monochromatic light pulse. Then the plates were illuminated with white light (WL, specified in section 2.4.2 "plant growth conditions") for 15 min. Germination was evaluated after another five days of dark incubation.

2.9.3 Germination assay for testing seed germination after fertilization

To test the response of germinating seeds to fertilizer treatment, seed germination assays in FR light were conducted. Seeds derived from parental plants grown in differential fertilized soil (see "2.4.2.2 Plant cultivation on soil") were tested for their germination response in FR light (see 2.9.1 "Standard germination assay"). The water used for wetting the plates was replaced by 0.1 mM KNO_3 or 1 mM KNO_3 solution.

2.9.4 Germination after exogenous hormone application

In order to test the sensitivity of germination to exogenous hormone application in WL and in FR light, the well-known germination inhibitor ABA and the germination-promoting GA were selected. Additionally, sensitivity of germination after CK application was tested. Experiments were conducted using the "Standard germination assay" protocol described previously. First of all, ABA (Sigma-Aldrich) was dissolved in 10 mM 2-(N-Morpholino) ethanesulfonic acid (MES) buffer (pH 5.8). For germination in WL, 0.5-5 µM ABA was added to the plates prior to sowing the seeds in darkness. Germination in FR light was analyzed after application of 0.05-0.25 µM ABA. Using similar ABA concentrations in WL and in FR light was not feasible due to the low wild type germination rates in FR light. As a control, plates were supplied with 10 mM MES buffer only. To test the effect of exogenously applied GA (GA_{4+7}, Duchefa Biochemie), endogenous GA biosynthesis was repressed by application of Paclobutrazol (Pac, Sigma-Aldrich), an effective inhibitor of GA biosynthesis. Pac inhibits the oxidation of kaurene to the GA precursor kaurenoic acid. Germination assays were conducted using the standard germination assay protocol. GA was dissolved in water and a 200 µM GA_{4+7} stock solution was prepared. Additionally, a 50 µM Pac stock solution was prepared by dissolving Pac in DMSO, so the stock solution contained 0.1 % DMSO. Prior to sowing, the plates were supplied with control solution (0.01 % DMSO in water), 10 µM Pac solution, 0.1 µM GA_{4+7} with 10 µM Pac, 1 µM GA_{4+7} with 10 µM Pac and 10 µM GA_{4+7} with 10 µM Pac.

For germination experiments testing the effect of exogenously applied CK, 2-BA dissolved in 1 mM KOH was used. 10 mM MES buffer with 0.1 % KOH (pH 6) was applied to the plates prior to sowing as control treatment. 0.1 µM, 0.5 µM and 1 µM 2-BA were

prepared using 10 mM MES buffer. Sowing and analysis of germination was again conducted following the protocol of the standard germination assay.

2.9.5 Seed size

To determine seed size, two different methods were used in this work. On one hand, seeds were classified in arbitrary size classes, *A. thaliana* seeds range from minimum size class of 15 to maximum of 132. On the other hand, 1000 corn weight was determined by weighing samples of 250 seeds- and calculating the weight of 1000 seeds based on these measurements. For size class and 1000 corn weight determination, a minimum of three biological replicates were measured in three technical replicates. Measurements were done with two independently grown seed sets. The measurements were conducted using the Elmor C1 seed counter (Elmor, Schwyz, Switzerland).

In order to test the influence of seed size on seed germination characteristics, seeds were sieved in different size classes. For sieving, metal sieves with various mesh sizes were combined in a stack. After sieving the seeds were classified in several size classes, three of them were used for germination assays: > 300 µm, > 250 µm and < 200 µm. The seed germination rates in FR light were tested according to the “Standard germination protocol”.

2.9.6 Seed coat dissection

For seed coat dissection, seeds were imbibed on wet filter paper for at least 2 h in darkness. The dissection procedure was conducted according to the protocol published by Lee *et al.*, 2010. In contrast to the published protocol, dissection was conducted in dim green safe light using a stereomicroscope. Instruments used during the procedure were surface sterilized in order to prevent contamination of the dissected seeds with mold spores.

2.9.7 qPCR – Experimental design

To verify and analyze expression of candidate genes predicted to be relevant for germination by *in silico* microarray data analysis, seeds were incubated on wet filter paper (see “Standard germination assay”) for one hour in darkness and harvested. These samples served as untreated control samples. After 48 h of incubation, a germination-inducing WL pulse was applied. After the light pulse, the seeds were incubated in darkness for another twelve hours, then they were harvested (and named 61 h samples, 1 h+ 48 h+ 12 h = 61 h). For analysis of gene expression in FR light, seeds were imbibed for one hour in darkness as control as in the WL assay. After one hour, a first FR light pulse was applied to inhibit active phyB in the imbibed seeds. After the first FR pulse, seeds were incubated in darkness for another 48 h, then a second FR light pulse was applied. Samples were harvested in the middle of the dark imbibition period (1 h+ 27 h= 28 h after sowing). The next harvesting was conducted one hour after the second FR light pulse (1 h+ 48 h+ 1 h= 50 h after sowing) and six hours after the second FR light pulse (1 h+ 48 h +6 h= 55 h after sowing). For analysis, Col-0, *ahk2 ahk3* and *phyA* seeds were harvested in three biological replicates per condition. To collect the necessary amount of seed material, seeds of two plants were pooled for each biological replicate.

To analyze expression of genes well-described to be functionally relevant in seed germination, three experiments were conducted. Each of them was focused on a different time scale of germination, the imbibition phase, early (1 h and 6 h after the second FR

pulse) and late (12 h and 24 h after the second FR light pulse). Four biological replicates were harvested, Col-0, *ahk2 ahk3* and *phyA* were included in every experiment, *ipt3,5,7* seeds were only included in the last experiment (sample harvesting 12 h and 24 h after the second FR light pulse). Samples were subjected to RNA extraction, DNase digestion, cDNA synthesis and qPCR as described above.

2.9.8 RNA-Seq analysis

For RNA-Seq analysis, all samples comprised 20 mg of seed material. Since CK receptor double mutant plants have lower seed yield compared to wild-type plants, samples were prepared as pools. For every biological replicate, seed material of eight plants was pooled, which equals 2.5 mg seed material from each plant. To qualify for the experiment, seeds of the different plants were tested for their ability to germinate in constant WL. Only if germination rates exceeded 90 %, the seeds of the respective plant were included in the experiment. After preparation of the seed material, seeds were surface-sterilized as specified in section “Gas sterilization” and sown on plates directly after sterilization. For every 20 mg of seed sample, an individual plate was used. Sowing and handling of the plates was conducted according to the protocol described under “Standard germination assay”. Seeds were harvested 1 h after sowing, prior to the first FR light pulse in dim green safe light. Harvesting was conducted by a minimum of two persons in parallel, to ensure temporal comparability. After harvest, the samples were frozen in liquid nitrogen and stored at -80 °C. The rest of the plates were treated with a phyB inhibiting first FR light pulse. Next timepoint for harvesting was set to the end of the imbibition phase, 49 h after sowing, but prior to the second germination-inducing light pulse. The respective light pulses, either WL or FR light, were applied as specified in the section “Standard germination assay” and the plates were incubated in darkness until harvesting. 6 h and 12 h after application of the second light pulse, seed samples were harvested as described.

Five biological replicates per timepoint and genotype were harvested. For each biological replicate, seeds of eight plants of the respective genotype were pooled. RNA extraction was carried out using 20 mg of seed material with the help of the “Ribospin seed/fruit” and the “Riboclear plus!” kit (Table 2). After measuring RNA concentration with a spectrophotometer (see section 2.5.2 ”Extraction of RNA from *Arabidopsis* seeds and siliques“), four samples per genotype and timepoint were selected based on the RNA concentration. Samples were treated with DNAse I as described above. In total, 1-5 µg RNA per sample were sent to BGI (Hongkong, China) for RNA quality control, high-throughput RNA sequencing and mapping of the reads to a reference genome.

The quality and quantity of total RNA was analyzed by Nanodrop and Bioanalyzer Agilent 2100 (Agilent Technologies, Santa Clara, CA, USA). Based on RNA quality and quantity measurements, 34 of 36 samples were fully qualified for sequencing. RNA integrity number (RIN) was used as a measure of RNA quality, RIN values exceeded 8.6 in all samples, so based on RIN value, all samples were considered to be qualified for sequencing. In two samples the RNA concentration was lower than 1 µg/sample, 0.4 µg WL_WT_T3_3 and 0.6 µg in sample WL_*ahk2 ahk3*_T3_1. The samples were included in the analysis, both principal component analysis (PCA) and heatmap clustering, presenting the euclidian distance, revealed no differences to the respective biological replicates (Figure 37, Figure A5). After quality and quantity measurement, samples were treated with DNAse I to remove DNA contaminations. Next, mRNA harboring a polyA tail was enriched using oligo dT magnetic beads. This purified mRNA was fragmented and subjected to

reverse transcription for the synthesis of cDNA with N6 random primer. After end repair with phosphate at the 5' end and adenine at the 3' end, sequencing adapters were ligated. Sequencing was conducted using the “BGISEQ-500RS RNASeq” (Quantification) pipeline. Sequencing generated in average 24 million raw sequence reads per sample, after filtering of low-quality reads about 23.9 million clean reads per sample were identified. Clean reads were mapped to the TAIR10 reference genome using HISAT/Bowtie2 (Kim *et al.*, 2015).

Differentially expressed genes were identified using the Bioconductor DESeq2 package for R (Love *et al.*, 2014). For analysis, the raw count data was used as an input. The adjustment of p values for false discovery rate was optimised for the p value cut off, in this analysis alpha was set to 0.05. Both the False Discovery Rate (FDR) detection using the Benjamini-Hochberg procedure and the Bonferroni correction were utilized to statistically correct for multiple testing. Since the Bonferroni correction is more conservative, exclusively differentially expressed genes (DEGs) identified to be significantly regulated ($p \leq 0.05$) according to the Bonferroni corrected p values were analyzed in this work.

In this work it was assumed that functionally relevant DEGs should show a minimum of two-fold up- or downregulation (log_2-fold change ≥1 or ≤-1) compared to the respective control sample. Principal component analysis and sample distance visualization in a heatmap required the use of the Bioconductor packages RColorBrewer and pheatmap. Gene Ontology (GO) annotation was performed using the Panther classification system (Table 1).

2.10 Bioinformatics

2.10.1 Statistical power analysis

In order to to generate meaningful and conclusive results, effect sizes and the appropriate number of individual study objects, seeds in this case, were estimated using the tool G*Power 3 (Faul *et al.*, 2007). According to the analysis, a minimum of 70-80 seeds per biological replicate are sufficient.

2.10.2 Statistical tests

Statistical analysis was performed using the GraphPad Prism Version 8 software. As a first step, column statistics were performed for every dataset in order to define, if the group means of a data set were normally distributed. Column statistics included tests for data normality, namely the D’Agostino and Pearson test, Shapiro-Wilk normality test and the Kolmogorov-Smirnov test. If these tests revealed significant p values ($p \leq 0.05$), the null hypothesis, proposing normal distribution among a data set, had to be rejected and consequently a significant difference between the data was assumed.

If data was normally distributed, a One- or a Two-Way ANOVA was used to test the data for statistically significant differences between the group means of a predefined data set. The selection of either One- or Two-Way ANOVA is based on the number of independent variables in the data set. If only one variable was considered, for example genotype or treatment as source of variance, a One-Way ANOVA was selected. If two independent variables may alter the variance of the dataset, a Two-Way ANOVA was conducted. If significant differences between the groups were discovered by the ANOVA, a post-hoc test for multiple comparisons was used, in order to define the groups which were significantly different from each other. In this work, One-Way ANOVA followed by Tukey´s

post-hoc test or Two-way ANOVA followed by Tukey's post-hoc test was calculated. If the data was not normally distributed according to the tests for normality, the non-parametric Kruskal-Wallis-Test, followed by a Dunn's post-hoc test was conducted. Differences revealed by the respective analysis were defined as significant, if the p value was smaller than 0.05, or highly significant if the p value was smaller than 0.01 or 0.001.

2.10.3 *In silico* identification of marker genes for germination in WL

For *in silico* identification of marker genes for germination in WL, publicly available datasets were screened using the Genevestigator database. The publicly available data should fulfill the following criteria: 1) the experimental setup should resemble the setup of germination experiments in this work, 2) the data should be generated using seed tissue for analysis, 3) the ecotype should be Col-0, 4) the time frame covered in the experiment should include timepoints up to 24 h after the application of a germination-inducing light pulse and 5) the seeds should not be subjected to a stratification treatment prior to the experiment. The dataset GSE30223, published by Narsai *et al.* (2011) on the Gene Expression Omnibus (GEO) platform, fulfilled four of the pre-defined criteria. The samples for the microarray were collected as follows: Freshly harvested seeds were after-ripened for 15 days, before a 48 h stratification treatment at 4 °C was applied. After stratification, the seeds were transferred to WL for 48 h. In this work, only samples exposed to WL were considered for analysis, samples harvested during stratification were not analysed further. The respective numbers of the individual samples are listed in Table 17.

Table 17. List of datasets from Narsai *et al.* (2011) used in this work.
Datasets belong to experiment GSE30223. SL means stratification and light, the time indicates the number of hours after transfer of the seeds to white light.

Gene Expression Omnibus identifier	Sample
GSM748481	1 h SL; 1. biological replicate
GSM748482	1 h SL; 2. biological replicate
GSM748483	1 h SL; 3. biological replicate
GSM748484	6 h SL; 1. biological replicate
GSM748485	6 h SL; 2. biological replicate
GSM748486	6 h SL; 3. biological replicate
GSM748487	12 h SL; 1. biological replicate
GSM748488	12 h SL; 2. biological replicate
GSM748489	12 h SL; 3. biological replicate
GSM748490	24 h SL; 1. biological replicate
GSM748491	24 h SL; 2. biological replicate
GSM748492	24 h SL; 3. biological replicate
GSM748493	48 h SL; 1. biological replicate
GSM748494	48 h SL; 2. biological replicate
GSM748495	48 h SL; 3. biological replicate

Bioinformatic data processing was conducted using the Bioconductor affy package (Gautier *et al.*, 2004), including Robust Multi-array Average (RMA) Normalization. RMA is a quantile normalization method with Median polish to correct Affymetrix data for background signals and ensure comparability between the different microarrays. Normalized data sets were used for identification of differentially expressed genes.

Two different methods were used in this work to determine DEGs, the Cyber-T method combined with the posterior probability of differential expression (PPDE) for false discovery rate calculation and the $\log_2$ fold change analysis (Brenner *et al.*, 2005).

The Cyber-T approach is based on a Bayesian probabilistic framework, normalized microarray data were analyzed via the software Cyber-T (see Table 1). The PPDE analysis uses the p values of all genes, analyzes them and calculates a PPDE value. The distribution of PPDE value is either uniform (when the respective gene is not differentially expressed) or non-uniform (indicating differential expression). Also, the rate of false positives and false negatives may be estimated (Hatfield *et al.*, 2003). Differentially expressed genes were selected by a PPDE value greater than 0.96 (false discovery rate, FDR) and a Bonferroni-corrected p value of ≤ 0.05.

Additionally, another approach was taken to identify DEGs in the microarray dataset. The log_2-fold changes were calculated in Excel as the ratio between the mean of the control treatment (1 h imbibed seeds) and the mean of the respective treatment (6 h, 12 h, 24 h and 48 h, respectively). Statistical significance was calculated using a t-test statistic (Witten and Tibshirani, 2007; Brenner and Schmülling, 2012). Differentially expressed genes were defined by a log_2-fold change expression of ≥ 1 (2-fold upregulated) or ≤ -1 (2-fold downregulated) and p value ≤ 0.05. Since the aim of this study was to identify marker genes for germination, downregulated genes were not analyzed into more detail here.

To ensure comparability between the two methods for DEG calculation, the list of DEGs identified by the log_2-fold change method was compared to the DEGs identified by Cyber-T and PPDE.

3 Results

3.1 Light sensitivity of seed germination is altered by the seeds' CK status

In order to germinate and establish the next plant generation, it is indispensable for seeds to do so even in non-optimal light conditions. A previous study analyzing the germination phenotype in *A. thaliana* of various AHK receptor mutant seeds provided evidence, that CK receptors play a role in regulating germination in different light environments (Riefler *et al.*, 2006).

3.1.1 phyB-dependent germination is increased in CK receptor mutant seeds

In order to study the connection between light sensitivity of germination and CK, the phyB-dependent low fluence response was investigated in seeds with a lower CK status or signal transduction.

After sowing, seeds were illuminated with a FR light pulse to shift active Pfr phyB to an inactive Pr state. After imbibition in darkness the seeds were illuminated with a low fluence R light pulse which induces the conversion of inactive Pr phyB into active Pfr phyB, which triggers the onset of germination. Initially, the germination of CK receptor double and single mutant seeds in low fluence red light was tested by Stefanie Zintl (see light treatment in Figure 8 A). The experiments focused on CK receptor single and double mutants due to the dwarfed growth, the limited number and increased size of *ahk2 ahk3 cre1* triple mutant seeds (Riefler *et al.*, 2006), that might affect the germination response.

In phyB-activating red light conditions, *ahk2-5 ahk3-7* seeds germinated significantly higher compared to wild-type seeds, 29.7 % and 3.7 %, respectively when a low photon fluence (6 µmol m^{-2}) was applied (Figure 8 B). This effect was abolished when illumination was conducted with higher red light fluences, at 15 µmol m^{-2} *ahk2-5 ahk3-7* germinated with 89 % compared to wild-type seeds (60 % germination). Low amount of red light triggered the onset of germination in CK receptor double mutant seeds more effective than in wild-type seeds, this phenomenon was abolished when higher red light fluences were applied. Germination assays testing *ahk2-2 ahk3-3* showed, albeit not significantly, increased germination rates of 39.8 % compared to wild-type seeds from which 7.1 % completed germination at the lowest applied red light fluence (Figure 8 C). In contrast, *ahk2-5 cre1-2* and *ahk3-7 cre1-2* did not differ in their germination rates from wild-type seeds (Figure 8 B). So the loss of the AHK2 and the AHK3 receptor promotes phyB-dependent germination of Arabidopsis seeds in low fluence red light conditions.

To evaluate more precisely the contribution of AHK2 and AHK3, single and double receptor mutants harboring different *AHK2* and *AHK3* alleles were tested. None of the tested single receptor mutants exhibited significantly higher germination rates than wild-type seeds (Figure 8 C). This suggests that in case of *ahk* single receptor mutants the reduction of CK signal transduction is not sufficient to alter the germination rates of the respective seeds in red light conditions. So AHK2 and AHK3 receptors appear to play redundant roles in germination under phyB-dependent light conditions.

To test whether a reduced endogenous CK content in seeds would result in increased germination rates seeds derived from plants overexpressing CK catabolizing *CKX* genes were analyzed. Strong CK deficiency strikingly alters the plants' morphology, including dwarfed growth, delayed flowering time and reduced seed yield (Werner *et al.*, 2003).

The CK-deficient transgenic lines *35S:CKX2* and *35S:CKX4* were chosen for analysis, since they show, in contrast to *35S:CKX1*, neither dwarfed growth nor retardation of shoot growth or delayed flowering time and therefore allow to study the effect of lower endogenous CK content without studying morphological side effects that may influence germination. Overexpression of *CKX2* resulted in slightly but increased germination rates compared to wild-type seeds (19.4 % compared to 1.3 % germination, respectively), whereas seeds overexpressing *CKX4* germinated 9 % in low-fluence red light (Figure 8 D). Since both CK deficiency and impaired CK signal perception enhanced germination under low red light fluences, one may conclude that CK has a negative impact on seed germination in low fluence red light conditions.

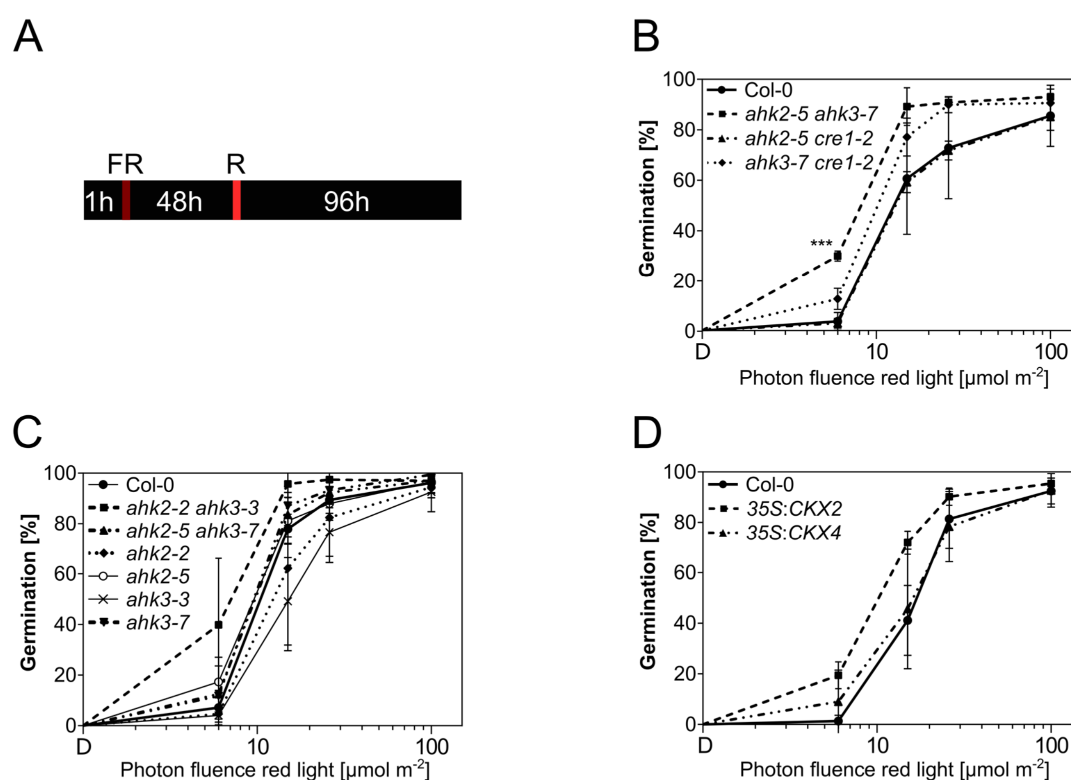

Figure 8. Reduced CK perception or increased CK catabolism results in higher seed germination rates under phyB-dependent germination conditions.
A) Schematic overview indicating the experimental setup in B-D. The 2nd germination-inducing red light pulse was applied using different red light photon fluences. Germination rates were examined after a 96 h dark incubation phase. For this analysis seeds defective in CK perception (*ahk*) (B and C) and seeds overexpressing CK catabolizing *CKX* genes (D) were tested. Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0, as calculated by One-Way ANOVA, post-hoc Dunnett's test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. FR, FR light pulse, first pulse FR for 3 min. with 90.6 µmol m^{-2} s^{-1}, second pulse very low fluence red light (R), 20 seconds 6 -100 µmol m^{-2} s^{-1}. Experiments were planned and conducted by Stefanie Zintl.

A characteristic feature of phyB-dependent germination is the possibility to arrest germination progression by a FR light pulse following the germination-inducing R light pulse (Figure 9 A). On a molecular level, a subsequent light pulse shifts the phyB equilibrium from active Pfr phyB towards inactive Pr phyB, thus arresting germination. The applied R light pulse induced high germination rates in wild-type as well as in CK receptor mutant seeds (Figure 9 B and 2 C, red). The induction of germination was significantly more

effective in *ahk2-2 ahk3-3* and *ahk2-5 ahk3-7* mutants, where germination rates of 96 % and 94 % were measured respectively, compared to wild-type seeds, germinating 82 % after a R light pulse (Figure 9 B). Other *ahk* double mutant combinations as well as *ahk* single mutants germinated like wild-type seeds (Figure 9 B and C, red). These results are consistent with the R light fluence experiments and indicate that AHK2 and AHK3 are of particular importance for germination under low-fluence R light conditions. Seeds with a lower endogenous CK content germinated indistinguishable from wild type after R light illumination to 100 % (Figure 9 D), the slightly increased germination rates of *35S:CKX2* seeds in R light (Figure 8 D) obtained in the previous experiments was not reproducible here.

The reduction of the active Pfr phyB pool by a subsequent FR light pulse strongly decreased germination rates in wild-type seeds in all experiments (Figure 9 B, C and D, FR light). Comparing different CK receptor double mutant seeds, *ahk2-2 ahk3-3* and *ahk2-5 ahk3-7* showed significantly higher germination rates of 46 % and 56 % respectively, after a subsequent FR light pulse compared to wild-type seeds (7 %) (Figure 9 B, FR). Among the CK receptor single mutants, *ahk3-7* germinated with 67 % significantly better than wild-type seeds, of which 46 % completed germination, after a FR light pulse. This indicates a more pronounced role of the AHK3 receptor in this respect (Figure 9 C, FR).

Additionally, seeds overexpressing *CKX2* showed significantly increased germination rates of 74 % after a phyB inactivating FR light pulse (Figure 9 D, FR) compared to 51 % wild-type germination. Interestingly and in accordance to previous data, seeds overexpressing *CKX4* do not show higher germination rates (41 %) after a phyB-inhibiting FR light pulse (Figure 9 D, FR), yet again pointing to distinct roles of CKX2 and CKX4 in germination.

Taken together, mutants with a lower endogenous CK status showed higher germination rates in low fluence R light conditions and after a FR light pulse, that reduces the active phyB pool in the seeds. Concluding from this, CK is regarded as negative regulator of the phyB-dependent LFR.

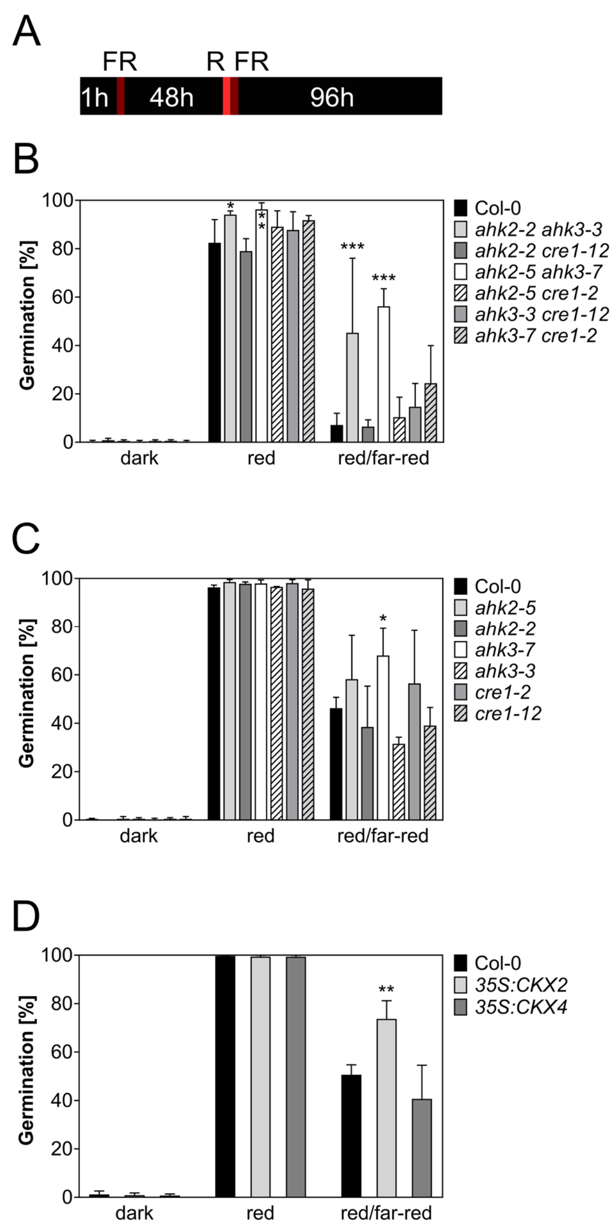

Figure 9. Arrest of seed germination following a FR light treatment is less pronounced in seeds with reduced CK perception or increased CK catabolism.
A) Schematic overview indicating the experimental setup in B-D. Germination percentages were examined in seeds defective in CK perception (*ahk*) (B, C) and in seeds overexpressing CK catabolizing *CKX* genes after subsequent R/FR light treatments (D). Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0, as calculated by One-Way ANOVA, post-hoc Dunnett's test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. FR, FR light pulse, first pulse FR for 3 min. with 90.6 µmol m^{-2} s^{-1}, second pulse R, red light, 5 min. with 30 µmol $m^{-2}s^{-1}$, third pulse FR 1 min. 10 µmol m^{-2} s^{-1}. Experiments were planned and conducted by Stefanie Zintl.

3.1.2 phyA-dependent germination is drastically increased in CK receptor mutant seeds

Since in *A. thaliana* the phytochromes A and B are the most prominent regulators of germination, it was hypothesized that the seeds' CK status may also impact the phyA-dependent induction of germination. To test this, seeds were sown in darkness and a germination-inhibiting FR light pulse was applied to shift active phyB to its inactive state. After imbibition in darkness for two days, the seeds were illuminated with a FR light pulse of different photon fluences, which triggered the onset of germination via phyA. Germination rates were scored 96 h after the germination-inducing light pulse (see scheme Figure 10 A).

A reduced CK perception in *ahk2-2 ahk3-3*, *ahk2-5 ahk3-7* and *ahk3-7 cre1-2* positively affected seed germination, as indicated by significantly higher germination rates compared to respective wild-type seeds after exposure to various FR light fluences (Figure 10 B and C). Thus, impaired CK signaling enhanced phyA-dependent seed germination. Interestingly, CK receptor double mutant combinations containing a non-functional AHK3 receptor (*ahk2-2 ahk3-3*, *ahk2-5 ahk3-7* and *ahk3-7 cre1-2*) tended to germinate better than mutants lacking AHK2 or CRE1 (*ahk2-5 cre1-2*). Despite that, mutation of single AHK receptors was not sufficient to significantly increase seed germination rates (Figure 10 C), what argues against a prominent role of single AHK receptors in this process but rather for an additive effect that could not be compensated by the remaining AHK receptor.

The negative impact of CK on phyA-dependent germination was further supported by the finding that *35S:CKX2* exhibited significantly increased germination rates at very low amounts of FR light (Figure 10 D). The beneficial effect of the reduced CK status was restricted to low and very low fluences of FR light. *35S:CKX4* expressing seeds germinated comparable to wild type (Figure 10 D).

Collectively, a decreased CK status as well as an impaired CK signal transduction positively influences seed germination, both in phyB associated low fluence R light conditions and in phyA associated low and very low fluence FR light conditions. Surprisingly, the effects of FR light were more pronounced compared to the effects of R light, therefore the focus of this work has been set on phyA-dependent germination.

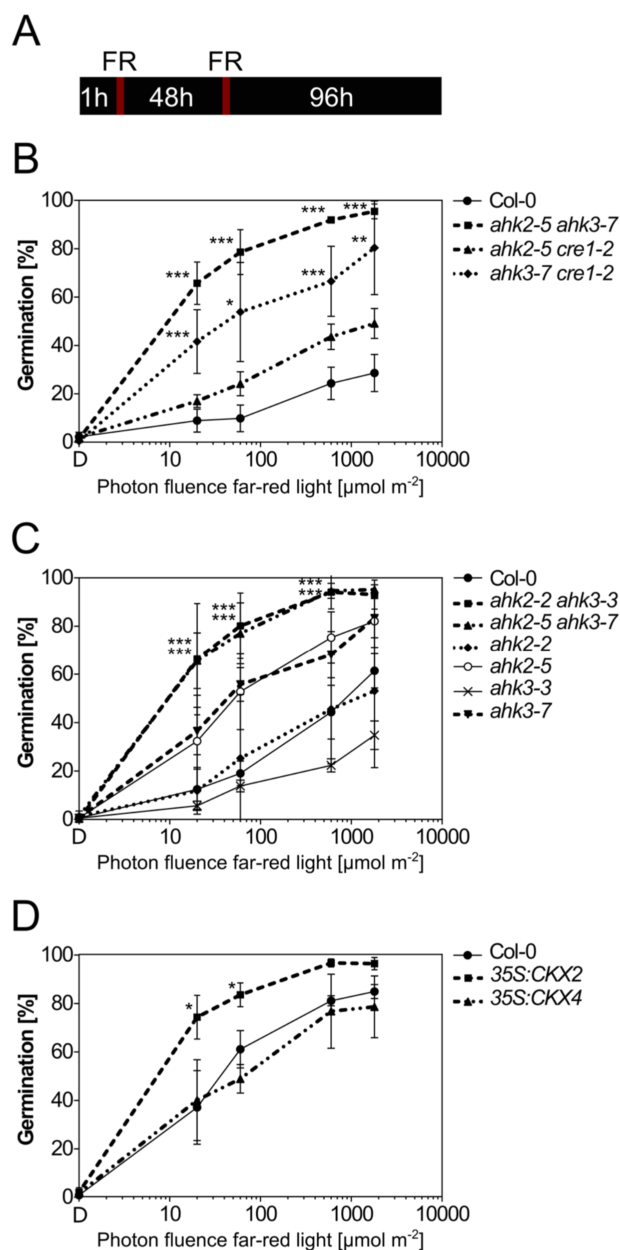

Figure 10. Reduced CK perception or increased CK catabolism enhances light sensitivity of germination under phyA-specific light conditions.
Germination percentages in FR light were examined in wild-type (Col-0) seeds as well as in seeds defective in CK perception (*ahk*s) (B, C) or seeds with an increased CK catabolism (35S:*CKX* expressing seeds) (D). Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0, as calculated by One-Way ANOVA, post-hoc Dunnett's test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. FR, FR light pulse, first pulse FR for 3 min. with 90.6 µmol m^{-2} s^{-1}, second pulse FR 20 seconds 2-150 µmol $m^{-2}s^{-1}$. Experiments were planned and conducted by Stefanie Zintl.

To determine and compare the minimum light fluence required for the induction of germination exclusively via phyA in wild-type and seeds impaired in CK signal transduction, germination was induced after a dark imbibition period with FR light of various photon fluence rates. Strikingly, already an illumination of five seconds with 2 µmol m^{-2} s^{-1} FR light was sufficient to induce 48 % of the CK receptor double mutant seed population to germinate, while wild-type seeds exhibited low germination rates of 5 % in these light conditions (Figure 11). With increasing illumination intensity, wild-type seeds reached a maximum of 11 % germination. Dark imbibed Col-0, *ahk2 ahk3* and *phyA* mutant seeds did not germinate (Figure A1). These results provided an additional proof that the differences in germination based on the seeds' CK status are most pronounced under very low fluence light conditions.

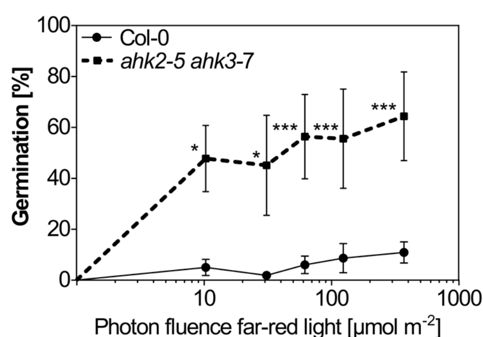

Figure 11. Illumination with very low amounts of FR light is sufficient to induced high germination rates in seed with a reduced CK perception.
Germination rates were scored 96 h after the germination-inducing light pulse. Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0, as calculated by Two-Way ANOVA, post-hoc Sidak's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Germination inducing FR light pulses with 2 µmol m^2 s^{-1} for 5 s, 15 s, 30 s, 1 min and 3 min were applied.

3.1.3 Very low fluence blue light effectively induces germination in seeds with impaired CK perception

PhyA perceives very low fluence light of various light qualities, so the question arose whether the increased light sensitivity of CK receptor double mutant seeds is specific to a certain light quality. To investigate this, seed were illuminated with blue light of different low photon fluences to induce the onset of germination and germination rates were determined. As in FR light, *ahk2-5 ahk3-7* seeds showed significantly higher germination rates of 13 % already at 79 µmol m^2s^{-1} compared to wild-type seeds, germinating 0.5 % in very low fluence blue light (Figure 12). These findings strengthen the hypothesis that a reduced CK perception alters seed germination under VLFR conditions irrespective of the light quality of the germination-inducing pulse.

Notably, in the experiments where very low fluences of light, either FR or blue light, were applied, wild-type germination rates were low throughout different experiments. This may reflect the biological importance of this phenomenon as an emergency germination program in non-optimal germination conditions only a certain proportion of wild-type seeds is capable of.

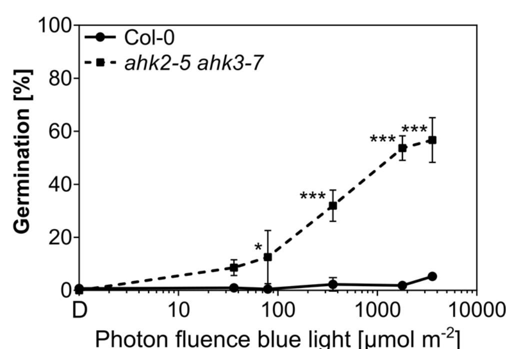

Figure 12. Induction of germination by very low fluence blue light triggers high germination rates in CK receptor double mutant seeds.
Induction of germination by very low fluence blue light triggers high germination rates in CK receptor double mutant seeds. Germination rates were scored 96 h after the germination-inducing light pulse. Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0 at the respective photon fluence as calculated by multiple t-tests, post-hoc test Bonferroni-Dunn. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Following blue light pulses were applied: 0.6 µmol $m^2 s^{-1}$ for 1 min; 1.32 µmol $m^2 s^{-1}$ for 1 min; 5.96 µmol $m^2 s^{-1}$ for 1 min, 5 min and 10 min.

3.1.4 The seeds' CK status alters the timing of germination

To investigate the process of germination into more detail, not only final germination rates are interesting, also the timing of germination may give valuable indications for a better understanding of the role of CK in seed germination. The germination rates of CK receptor mutant seeds showed a slightly earlier completion of germination in WL 36 h after illumination, *ahk2-5 ahk3-7* mutant seeds showed significantly increased germination rates of 78 % compared to wild-type seeds (23 %) (Figure 13 A). This result was congruent with the findings of Riefler *et al.* (2006) where earlier germination of CK receptor mutant seeds has already been described.

In FR light, a significantly higher proportion of CK receptor mutant seeds completed germination 36 h after the germination-inducing FR light pulse (Figure 13 B). The difference manifested in the course of time, 48 h, 72 h and 96 h after the pulse *ahk2-5 ahk3-7* seeds showed significantly higher germination rates compared to wild-type seeds (Figure 13 B). Interestingly, germination rates remained constant 48 h and 96 h after the germination inducing light pulse both in wild-type and in *ahk2-5 ahk3-7* seeds.

This demonstrates, that 48 h after the inducing light pulse the proportion of the seed population which acquired the potential to germinate completed germination, the remaining seeds of the population do not acquire the ability to germinate over time.

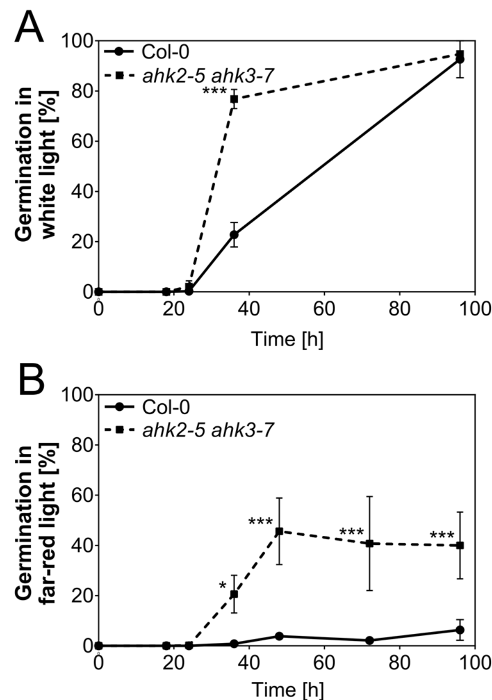

Figure 13. Seeds impaired in CK perception show alterations in the timing of germination. Seeds impaired in CK perception show alterations in the timing of germination. Both in WL (A) and in FR light (B) conditions, *ahk2-5 ahk3-7* seeds germinated significantly earlier compared to wild-type seeds. After application of the germination-inducing light pulse, germination rates were determined at the indicated time. Error bars indicate standard deviations. These data need repetition for verification. Asterisks represent statistically significant differences to Col-0 at the indicated times as calculated by (A) Two-Way ANOVA, post-hoc test Bonferroni-Dunn and (B) Mann-Whitney test. * adjusted $p < 0.05$; ** adjusted $p < 0.01$; *** adjusted $p < 0.001$.

3.1.5 Primary dormancy is lost over time

Freshly harvested mature seeds are said to have primary dormancy (Finch-Savage and Leubner-Metzger, 2006), that is dependent on parental plants, seed characteristics and environmental conditions. Dormant seeds are characterized by low germination rates even when environmental conditions for germination are optimal (Vleeshouwers *et al.*, 1995). Primary dormancy is released subsequently, for example by after-ripening or by stratification (Finch-Savage and Leubner-Metzger, 2006). In order to analyze and compare seed dormancy among the different CK mutants, seeds were harvested and, after different periods of dry storage, tested for their ability to germinate after application of a germination-inducing 15 min. WL pulse.

Ahk2-5 ahk3-7 CK receptor double mutant seeds showed higher germination rates of 26 % to 53 % compared to wild-type seed that germinated 13 % to 42 % until 11 days after harvest (Figure 14 B). After that, *ahk2-5 ahk3-7* tended to germinate better (67 %) compared to wild-type seeds (55 %) until 18 days after harvest, this trend was not significant (Figure 14 A). Unlike *ahk2-5 ahk3-7*, *ahk2-2 ahk3-3* mutant seeds, as well as *ahk2-5 cre1-2* and *ahk3-7 cre1-2* germinated comparable to wild-type seeds (Figure 14 A and B). Germination rates of CK receptor single mutants revealed wild-type like germination rates of *ahk2-5* and *ahk3-7* seeds, while *cre1-2* seeds germinated significantly lower (mean 37 %) than wild-type seeds (mean 55 %) in WL (Figure 14 B). Noteworthy are the observed differences in germination rate of seeds with an increased CK catabolism.

While *CKX2* overexpressing seeds showed significantly higher germination rates compared to wild type, germination rates of *CKX4* overexpressing seeds were reduced either significantly ten and 30 days after harvest or as a tendency (Figure 14 A and B).

Interestingly, differences in WL-induced germination levelled out in the course of time. 30 to 49 days after harvest, germination rates exceeded 80 % germination in all genotypes, no significant differences in germination were measurable anymore. These findings argue in favor of a loss of primary dormancy in the dry storage process over time (Figure 14). Based on the results presented here, an after-ripening protocol including a 90 days dry storage period was established and used throughout this work to ensure seed germination capacity is not altered due to the influence of seed dormancy. Seed were stored in glass vials at room temperature, to allow after-ripening but prevent exaggerated protein oxidation in seeds and the resulting loss of viability.

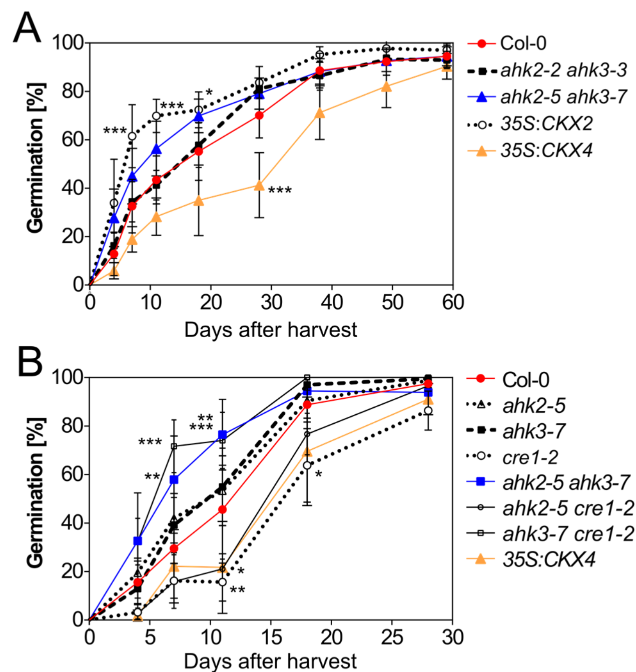

Figure 14. Loss of primary dormancy over time.
Seeds were harvested and subjected to dry storage at room temperature for the indicated period of time. To evaluate germination rates, seeds were sown in darkness and dark incubated for two hours before the application of a 15 min. white light pulse. Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0 at the indicated times as calculated by Two-Way ANOVA, post-hoc Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.1.6 Functional relevance of different CK metabolism- and signaling components for germination in FR light

As described above, seeds with a lower CK status, such as seeds overexpressing *CKX2* and seeds lacking two of three functional CK receptors showed higher germination rates compared to wild type in FR light. In order to investigate the contribution of an altered CK status to seed germination in these specific light conditions, components of the CK biosynthesis and catabolism pathways were studied for their relevance in very low fluence FR light-induced seed germination.

3.1.6.1 CK metabolism

First, the germination response of CK biosynthesis mutants was examined. All analyzed mutants showed, in full-spectrum WL conditions, germination rates close to 100% (Figure A1). Seeds did not germinate without an inducing light pulse, dark germination was only obvious in *ipt1,3,5,7* in one of the seed batches and could not be reproduced in any other seed set (data not shown). As a positive control for enhanced germination in FR light, *ahk2-5 ahk3-7* seeds were included in every experiment.

In *A. thaliana*, isopentenyltransferases (IPTs) are crucial for the biosynthesis of CK precursors (see introduction section 1.1.1). IPT2 and IPT9 catalyse the initial steps of the cZ-type CK biosynthesis via the tRNA pathway, while IPT1 and IPT3-8 biosynthesise iP and tZ precursors using adenosine phosphates as substrates (Miyawaki *et al.*, 2006). In order to evaluate if impaired biosynthesis of CK precursors via these pathways affect phyA-dependent seed germination, higher order *ipt* mutant seeds were tested. Impaired biosynthesis of CK precursors via the tRNA pathway does not influence seed germination in FR light, *ipt2 ipt9* seeds germinated comparable to wild type, where 15 % of the tested seed population germinated (Figure 15 A). Higher order *IPT* mutants (*ipt3,5,7* and *ipt1,3,5,7*) displayed significantly higher germination rates of 97 % and 81 %, respectively compared to wild-type seeds, even exceeding the germination of *ahk2-5 ahk3-7* seeds (41 %) (Figure 15 A). It is concluded, that impaired CK biosynthesis via the adenosine phosphate pathway positively influences seed germination in VLFR conditions. The role of IPT4 and IPT8 in germination could not be clarified in this work, the *ipt4 ipt8* mutant in the Wassilewskija (WS) background did not germinate in FR light, like the respective WS ecotype seeds (data not shown).

To further analyze the negative effect of CK on seed germination in VLFR light, seeds with a reduced CK catabolism, resulting in increased CK contents were tested for their germination phenotype in FR light. *ckx* single mutants germinated comparable to wild-type seeds, only *ckx7* germinated significantly less frequent (1.4 %) than wild type (5.6 %) (Figure 15 B, C and D). Due to partial functional redundancy of the seven *CKX* gene family members, higher order mutants were analyzed. The functional relevance of *CKX2* and *CKX4* could not be determined conclusively, *ckx2 ckx4* showed no difference from wild-type seeds (Figure 15 B) or germinated significantly lower (1 %) than the respective wild type (5.6 %) (Figure 15 D). Further, germination rates of *ckx3 ckx5* seeds revealed no functional relevance for these components in regulating germination (Figure 15 C and D). Even higher order mutants revealed only subtle effects, *ckx2,4,5,6* showed no (3.8 %, wild-type seeds 2 %) (Figure 15 C) or only slight (1.4 %, wild-type 5.6 %) reduction of germination (Figure 15 D). The variations between the experiments may be explained by diverging wild-type seed germination rates in the individual germination assays. Due to already low germination rates of wild-type seeds in FR light, a possible further reduction of germination rates in *ckx* mutant seeds was difficult to measure and a calculation of statistically significant differences was impossible under these conditions.

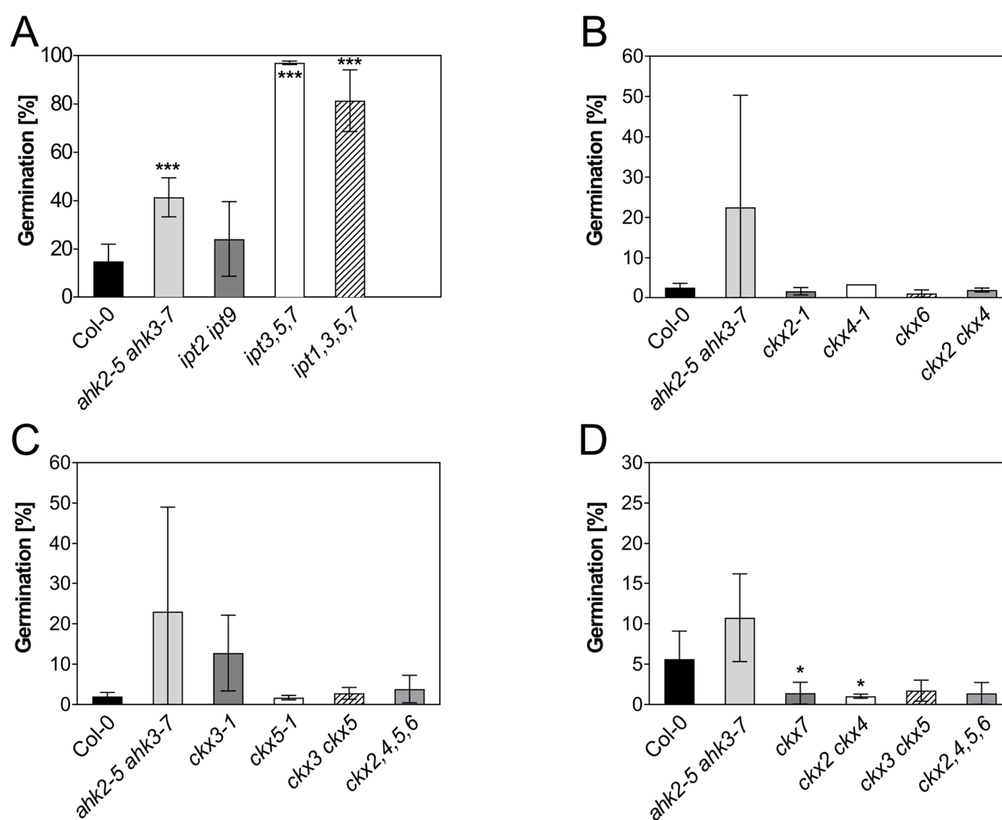

Figure 15. Increased germination rates of mutant seeds defective in CK biosynthesis and decreased germination rates of seeds with impaired CK catabolism under phyA-specific germination conditions.
Germination in FR light was tested in higher order *IPT* mutant seeds (*ipt3,5,6* and *ipt1,3,5,7*) (A) and seeds with a decreased CK catabolism (*CKX* loss-of-function mutants, *ckx*) (B-D). Germination rates were scored 96 h after the germination-inducing light pulse. Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0 as calculated by (A, C, D) One-Way ANOVA, post-hoc Dunnett's test or (B) Kruskal-Wallis-Test, post-hoc Dunn's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.1.6.2 CK signaling components are relevant for the seed germination response in FR light

To shed light on the connection of signaling components downstream of CK biosynthesis and catabolism impacting seed germination, various signaling components were tested by analyzing the germination of the respective mutant seeds in FR light.

At the level of CK receptors, *rock*-mutants (repressor of CK deficiency) which harbour constitutive active gain-of-function variants of the *AHK2* and *AHK3* genes (Bartrina *et al.*, 2017), thus displaying an increased CK status, were analyzed. *rock2* single and *rock2 rock3* double mutant seeds displayed a non-significant tendency to germinate to higher rates (17 % and 19 % respectively) than the wild type (6 %) in very low fluence FR light (Figure 16 A) while the *rock3* single mutant germinated comparable to wild type.

Besides *rock2 rock3*, also *rock1* and *rock4* seeds were tested for their capacity to germinate in FR light. ROCK1 was identified as an ER localised nucleotide sugar transporter influencing CKX1 activity (Niemann *et al.*, 2015). *rock1* mutant seed reached germination rates of 21 % compared to 6 % wild type germination, this difference was

calculated as not significant (Figure 16 A). This result was unexpected given the decreased *CKX1* activity in these mutants. *rock4* mutants express a missense mutation in the CK synthesis gene *IPT3*, resulting in a gain-of-function version of IPT3 (Jensen, 2013), this had no effect on seed germination (Figure 16 B).

AHPs are important components of the CK signaling pathway downstream the CK receptors (see introduction section 1.1.3). Mutant seeds lacking two functional phosphotransfer proteins germinate like wild type (24 % germination) in VLFR conditions, while a higher-order mutant (*ahp2, 3, 5*) displayed significantly enhanced germination rates (72 %) in these conditions, comparable to *ahk2 ahk3* mutant seeds (68 % germination) (Figure 16 C). In contrast to AHP2, AHP3 and AHP5, AHP6 lacks the ability to transfer phosphate to response regulators and thereby acts as a negative regulator of CK signaling. For AHP6, different mutant lines were tested in this work. The suppressor screen derived *ahp6-1* mutant harbours a shorter, non-functional AHP6 version, the *ahp6-3* line is a T-DNA insertion line. Both *ahp6-1* and *ahp6-3* are phenotypically similar (Mähönen *et al.*, 2006; Otto, 2013). Germination experiments in FR light indicated that AHP6 does not affect seed germination, *ahp6* mutant seeds germinated indistinguishable from wild-type seeds (Figure 16 D).

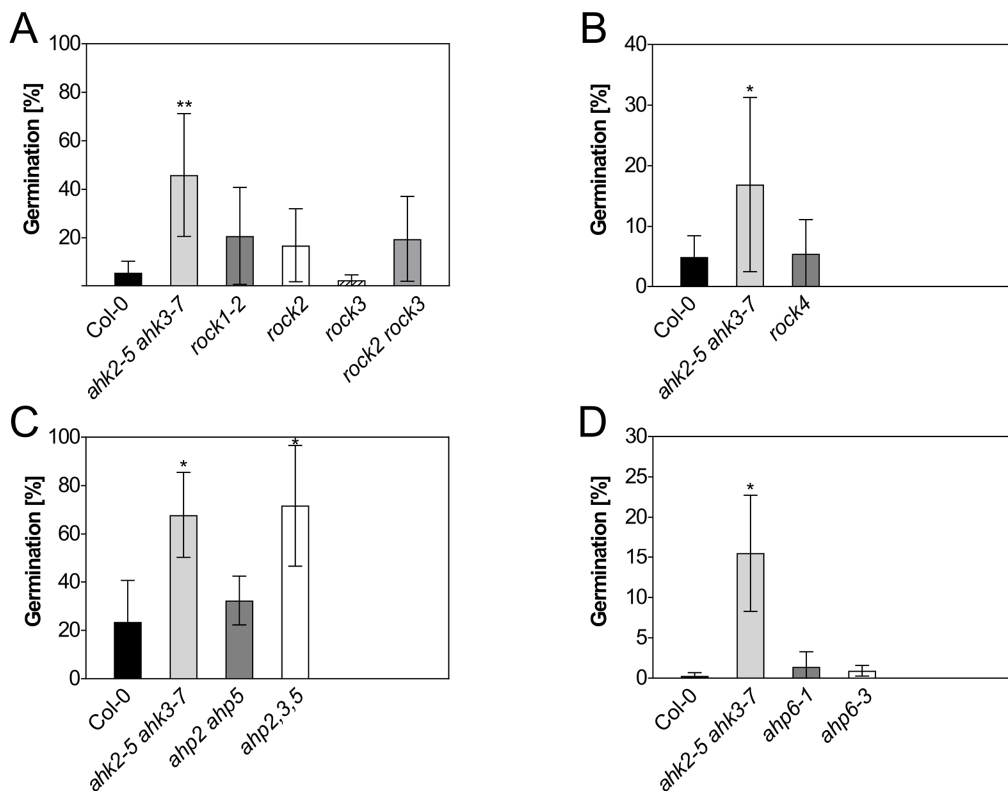

Figure 16. CK signaling components contribute to the altered germination response in phyA-specific light conditions.
Constitutive active AHK receptors (*rock2*, *rock3*) (A) and a gain-of-function IPT3 version (*rock4*) (B) had no effect on seed germination. (C -D) Higher order *ahp* mutants showed increased germination rates in FR light conditions. Germination rates were scored 96 h after the germination-inducing FR light pulse. Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0 as calculated by (A) One-Way ANOVA, post-hoc Dunnett's test or (B) Kruskal-Wallis-Test, post-hoc Dunn's test, (C-D) Kruskal-Wallis-Test, post-hoc uncorrected Dunn's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.1.6.3 The role of A- and B-type ARRs in germination remained unclear

These experiments aimed to investigate the role of A- and B-type response regulators in seed germination in VLFR conditions. First, the contribution of the well-characterized CK output components, the B-type ARRs ARR1, ARR10 and ARR12 was analyzed (Argyros *et al.*, 2008; Ishida *et al.*, 2008). For this purpose, *arr1-3 arr10-5*, *arr1-3 arr12-1* and *arr10-5 arr12-1* double mutants were tested for their germination response in FR light. The data revealed no influence of these response regulators in the seed germination process under very low fluence light conditions, although *arr1 arr12* seeds showed slightly increased germination rates compared to wild-type seeds (Figure 18 A). Triple *arr1 arr10 arr12* mutants were not tested in this work. The reason for excluding them was the phenotype described in the literature for *arr1 arr10 arr12* mutants (Ishida 2008).

Also, seed germination rates of B-type ARR single loss-of-function mutants (*arr2* and *arr14-1*) did not significantly differ from wild-type seeds (Figure 18 B). Furthermore, the B-type ARRs *ARR19* and *ARR21* were included in this work, because of their highly specific expression profiles in the chalazal endosperm of developing seeds (Day *et al.*, 2008). Because of this specific expression pattern, they represent promising candidates to investigate the influence of B-type response regulators on seed germination in very low fluence light conditions and a possible tissue-specificity of the CK response. Phenotypically *arr19* and *arr21* plants were indistinguishable from the wild type (Figure 17 A). Both *arr19* and *arr21* did not harbor aborted seeds in their siliques, thus, a pre-mature seed abortion due to the impaired function of *ARR19* or *ARR21* was excluded (Figure 17 B). The absence of the respective transcript in *arr19* and *arr21* knock-out lines was verified (Figure 17 B).

In seed germination assays under very low fluence FR light conditions, germination rates of *arr19* and *arr21* single mutants were not altered compared to wild-type seeds (Figure 18 C). To study a possible functional redundancy of these two response regulators, *arr19 arr21* double mutants were generated via genetic crossing of the confirmed homozygous single loss-of-function mutants. Phenotypical analysis of *arr19 arr21* revealed, as for the single mutants, no obvious growth phenotype or aborted seeds (Figure 17 A and D). As for the single mutants, also a loss of both seed-specific B-type ARRs in the chalazal endosperm did not result in seed abortion in the siliques (Figure 17 C). Germination assays in FR light showed no increased germination rates of *arr19 arr21* double mutant seeds (Figure 17 C), indicating no functional relevance or redundancy of these B-type ARRs in seed germination under very low fluence light conditions.

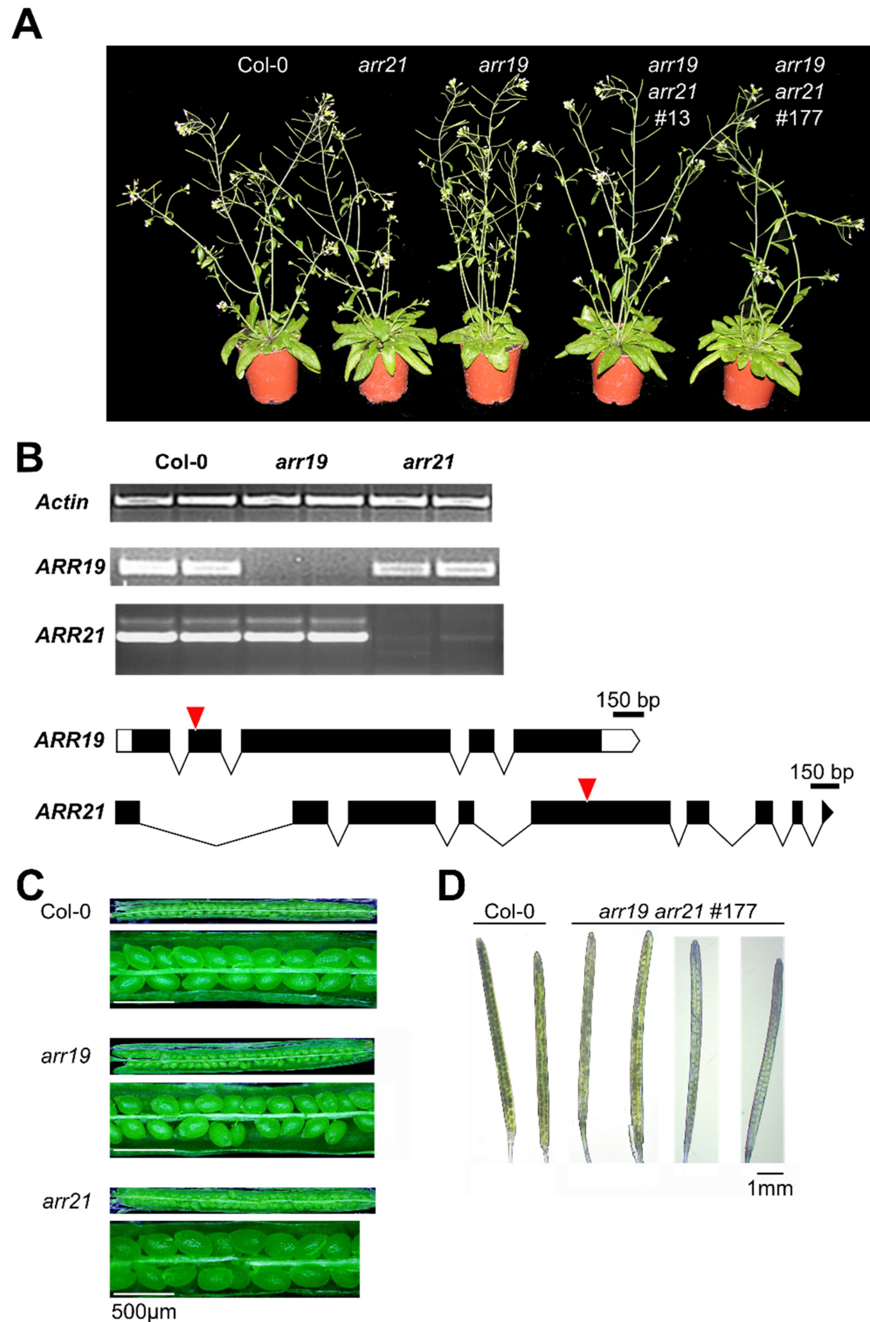

Figure 17. Phenotype of *arr19, arr21* and the respective double mutant plants grown under long day conditions.
(A) *Arr19* single mutant, *arr21* single mutant and two independent *arr19 arr21* double mutant lines, specified with #13 and #177. (B) Semi-quantitative PCR revealed the absence of *ARR19* and *ARR21* transcript in the respective single mutant lines which were used from generating the *arr19 arr21* double mutants. Gene models indicate exons (black boxes), introns (black lines), untranslated regions (white boxes) and the position of the respective T-DNA insertion (red triangles). (C) Freshly dissected wild-type, *arr19* single and *arr21* single mutant siliques, (D) fixed and cleared wild-type and *arr19 arr21* siliques.

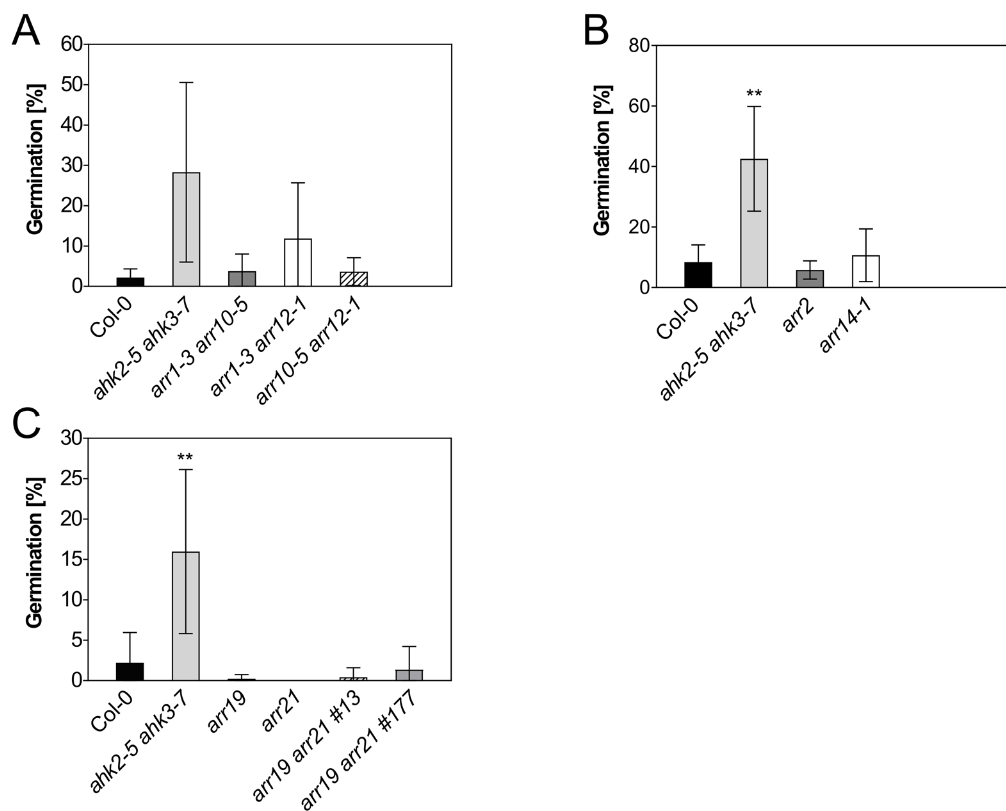

Figure 18. Reduction of CK signal transmission does not alter germination in phyA-specific light conditions in the tested B-type ARRs mutants.
Germination rates were analyzed 96 h after the germination-inducing FR light pulse for *arr2* and *arr14-1* (A, One-Way ANOVA, post-hoc Dunnett's test), for double mutant combinations of *arr1-3*, *arr10-5* and *arr12-1* (B, One-Way ANOVA, post-hoc Dunnett's test) and for *arr19* and *arr21* single and two *arr19 arr21* double mutant lines (labeled as #13 and #177)(C, Kruskal-Wallis-Test, post-hoc Dunn's test). Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0 as calculated by the above-mentioned statistical tests, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Germination assays analyzing *arr2*, *arr14-1*, *arr19* and *arr21* single mutants were conducted by Stelz (2016).

Besides B-type ARRs also A-type ARRs are important CK output components and were investigated for their function in seed germination. In the literature, several A-type ARRs as ARR4, ARR5, ARR6, ARR7 and ARR15 were linked to seed germination via the ABA pathway (see introduction section 1.5).

Additionally, ARR4 has been connected to red-light signaling (Sweere *et al.*, 2001), therefore the germination characteristics of both loss-of-function (*arr4*) and seeds overexpressing *ARR4* have been tested in this work. Neither *arr4* loss-of-function nor the overexpression line significantly differed from respective wild-type seeds, not supporting a functional relevance of ARR4 in VLFR germination (Figure 19 A). Furthermore, germination experiments revealed no significant effect of ARR7 and ARR15 on germination in very low fluence light, although *arr7 arr15* seeds exhibited repeatedly, but not significantly, lower germination rates (0–1 %) compared to wild-type seeds (8-14 %) (Figure 19 B).

The role of other A-type ARRs in germination was tested by analyzing the germination response of the *arr3,4,5,6,8,9* mutant in FR light, because of a proposed high functional redundancy of the respective response regulators (To *et al.*, 2004).

The loss of six A-type ARRs did not lower the germination rates of respective mutant seeds (Figure 19 C).

Summarizing these results, germination in very low fluence FR light was not altered in the tested A- and B-type ARR mutants. To allow a broader statement on the contribution of ARRs on phyA-dependent seed germination, the remaining members of the gene family have to be tested. In order to refine the contribution of A- and B-type ARRs to the regulation of seed germination, studies testing higher order mutants would be necessary.

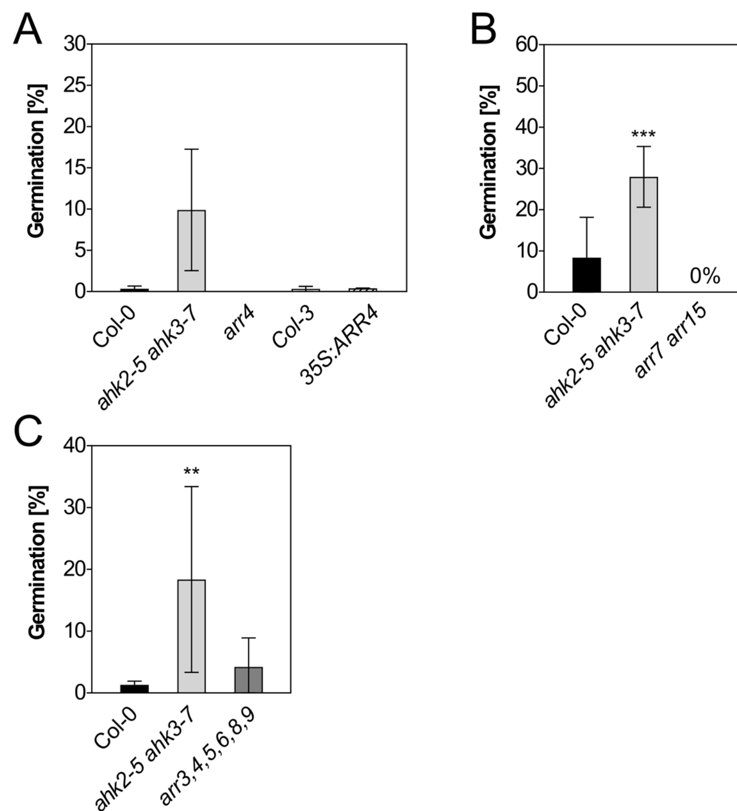

Figure 19. Impaired negative feedback regulation of the CK signaling pathway by A-type ARRs does not affect seed germination in phyA-specific light conditions.
Germination rates were scored 96 h after the germination-inducing FR light pulse. Seeds of the wild-type (Col-0 and Col-3), *ARR4* loss-of-function, *ARR4* overexpression (A) and *arr3,4,5,6,8,9* (B) were germinated in FR light. *arr7 arr15* seeds germinated sparsely in FR light (C). Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0 as calculated by Kruskal-Wallis-Test, post-hoc Dunn's test (A and C) or One-Way ANOVA, post-hoc Dunnett's test (B). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Germination assays analyzing *arr4* and 35S:*ARR4* seed sets were conducted by Schmidt (2018)

3.1.7 External application of CK does not inhibit seed germination in FR light

To test whether externally applied CK impacts germination, increasing concentrations of BA, a synthetic CK analogue, were supplied to the germination medium. BA application did not inhibit seed germination in FR light (Figure 20). As expected, *ahk2 ahk3* seeds did not respond to increased CK concentrations in the medium (Figure 20), germination rates were significantly increased compared to wild-type seeds irrespective of the applied CK concentration. Interestingly, in wild-type seeds a reproducible, non-significant tendency towards higher germination rates with increasing CK concentrations was apparent (Figure 20). Based on these findings, CK application to fully matured seeds did not decrease germination rates, so CK may play a role earlier in seed development rather than inducing a block of germination in fully matured seeds.

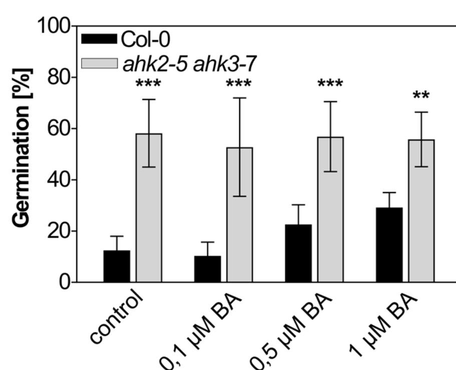

Figure 20. Exogenous CK application does not alter germination rates in FR light.
Seeds were sown on filter paper soaked with 0,1-1 µM BA, dissolved in KOH, in 10 mM MES solution. As a control, seeds were sown on 1 µM KOH in 10 mM MES solution. Plates were illuminated with FR light to induce seed germination according to the standard protocol. Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0 as calculated by Two-Way ANOVA, post-hoc Sidak's test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.2 phyA is essential for light perception and induction of germination in FR light in CK mutants

According to the results presented in the previous chapter, CK negatively regulates the onset of seed germination, with most pronounced effects in very low fluence light conditions. Very low fluence light is perceived specifically by the phyA photoreceptor, which is characterized by its light-lability upon illumination and its activation spectrum, that is shifted towards FR light wavelengths. The following section aimed to elucidate the functional relationship between phyA and CK in the regulation of VLFR seed germination in *A. thaliana*.

3.2.1 Characterization of *phyA ahk2 ahk3* and *phyA ipt3,5,7* plants

In the first place, the relevance of functional phyA for the germination phenotype of CK mutant seeds in FR light was tested. To achieve that, genetic crosses between plants lacking a functional version of the phyA photoreceptor and plants with a decreased CK biosynthesis or signal perception were conducted. The respective CK mutant lines for crossing were chosen based on their drastically increased germination rates in FR light

(refer to results section 3.1.2). The *phyA* mutation was introgressed into *ahk2-2 ahk3-3*, *ahk2-5 ahk3-7* and *ipt3,5,7* via crossing, plants homozygous for all respective loci could be isolated and tested.

The phenotypical analysis of homozygous *phyA ahk2-5 ahk3-7* plants and *phyA ahk2-2 ahk3-3* mutant lines showed a growth phenotype similar to ahk2 ahk3, but this was not studied in more detail. Regarding seed appearance, introgression of the *phyA* mutation into *ahk2 ahk3* plants had no obvious effect on seed morphology (Figure 21 E).

Two homozygous *phyA ipt3,5,7* quadruple mutants were identified in this work. While one of the lines resembled the reduced rosette size of *ipt3,5,7* plants (Figure 21 C), the second line displayed a rosette size similar to *phyA* mutant plants (Figure 21 D). Regarding the seeds, offspring of *phyA ipt3,5,7* line 49 resembled phenotypically seeds derived from the parental lines (Figure 21 F).

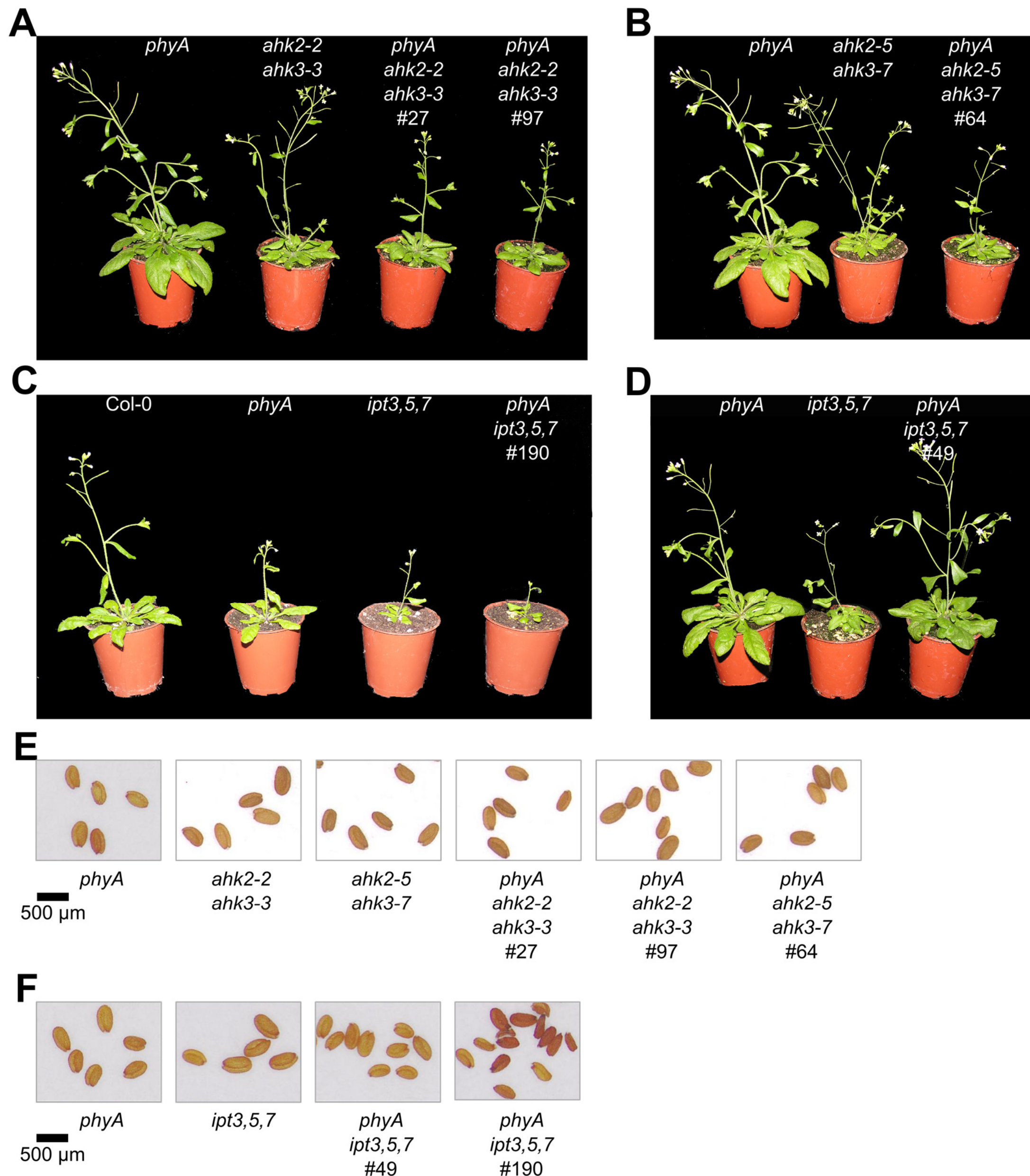

Figure 21. Phenotype of *phyA ahk2 ahk3* and *phyA ipt3,5,7* plants grown under long day conditions.

Figure 21 continued

(A) *phyA ahk2-2 ahk3-3* homozygous, independent lines (numbers 27 and 97), (B) *phyA ahk2-5 ahk3-7* homozygous line number 64, (C, D) *phyA ipt3,5,7* homozygous lines numbers 190 and 49. (E, F) Phenotype of mature dry seeds derived from the respective lines.

3.2.2 The photoreceptor phyA is required for FR light-dependent germination of CK-deficient seeds

To elucidate the functional relevance of phyA for seed germination in very low fluence FR light in seeds with an altered CK status, germination rates of *phyA ahk2 ahk3* and *phyA ipt3,5,7* were investigated in different light conditions.

Germination in WL was tested to exclude dormancy and ensure viability of the seeds. Both *phyA ahk2-2 ahk3-3* and *phyA ahk2-5 ahk3-7* lines exhibited a slight, non-significant decrease in germination rates compared to *phyA* single mutants (Figure 22 A). Defects in the seeds' perception of WL, which comprises the entire spectrum of the photosynthetically active radiance, were not expected in the tested mutant lines, because *phyA ahk2 ahk3* seeds still harbor functional phyB, which is able to perceive the red light spectrum and would consequently induce the onset of germination.

In FR light, neither *phyA ahk2-2 ahk3-3* nor *phyA ahk2-5 ahk3-7* mutant seeds germinated, thus mimicking the phyA single mutant phenotype (Figure 22 B). These results indicate, that PHYA is epistatic to AHK2 and AHK3 in very low fluence light-induced germination. Concluding from that, CK receptors require light perception via phyA for the induction of germination in very low fluence light conditions. To verify the requirement of phyA for the germination of seeds with an altered CK content, also germination rates of *phyA ipt3,5,7* were analyzed. After induction of germination by a WL pulse, both wild-type and *phyA* seeds displayed unexpectedly low germination rates, while *ipt3,5,7* and *phyA ipt3,5,7* germinated well (Figure 22 C). This resulted in significantly increased germination rates of *phyA ipt3,5,7* seeds compared to *phyA* as well as compared to Col-0 in WL. In FR light, *phyA ipt3,5,7* mutant seeds germinated comparable to *phyA* seeds (Figure 22 D), confirming the results obtain from *phyA ahk2 ahk3* seeds. Based on this it may be concluded that seeds impaired in CK signal transduction or CK biosynthesis do not harbor an alternative, phyA-independent pathway for very low fluence light perception. The induction of germination clearly relies on phyA, therefore an alternative light perception pathway may be excluded as possible reason for the increased FR light sensitivity of CK mutant seeds in germination.

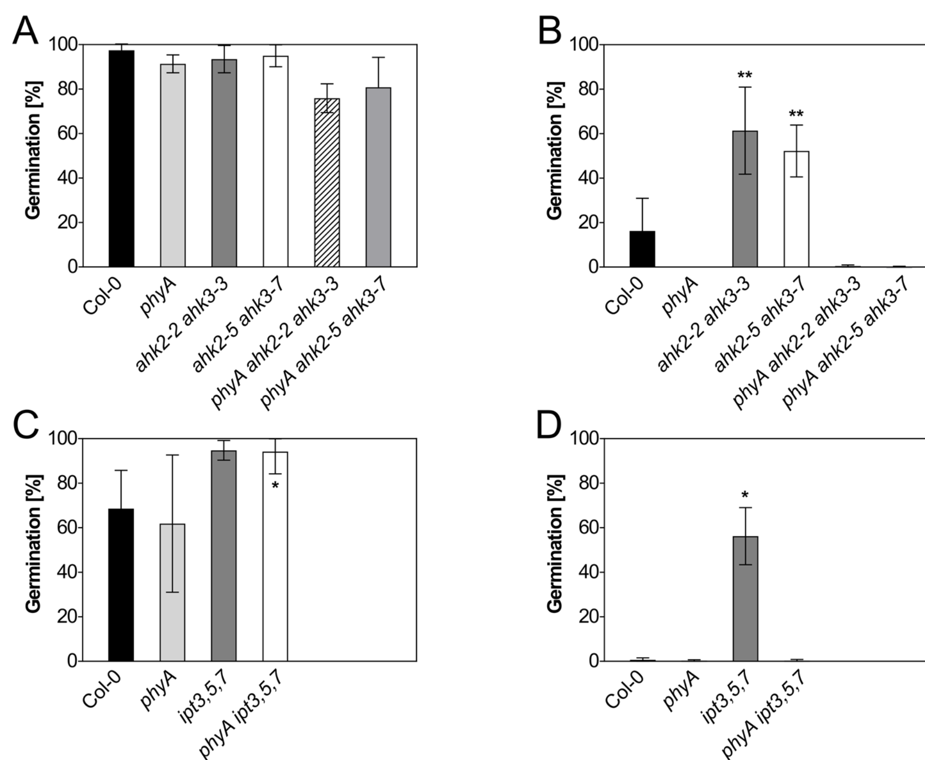

Figure 22. PhyA is required for the induction of germination by FR light, but not by WL, in seeds impaired in CK signaling or CK biosynthesis.
Figure 22 continued.
Germination rates were scored 96 h after the germination-inducing WL (A and C) or FR light pulse (B and D). Germination rates were determined for *phyA ahk2-2 ahk3-3* and *phyA ahk2-5 ahk3-7* (A and B) as well as for *phyA ipt3,5,7* seeds (C and D) and compared to the respective controls. Error bars indicate standard deviations. Asterisks represent statistically significant differences to phyA as calculated by Kruskal-Wallis-Test, post-hoc Dunn's test. * $p < 0.05$; ** $p < 0.01$.

3.2.3 phyA protein levels

As demonstrated in the previous section, phyA is essential in CK-deficient mutant seeds for VLFR germination. A plausible explanation for the increased germination rates of *ahk2 ahk3* seeds in very low fluence light conditions could be enhanced phyA protein levels in these mutants. The following experiments aimed to elucidate the kinetics of phyA protein accumulation over time during the imbibition process and to compare protein levels between wild-type and *ahk2 ahk3* mutant seeds.

The phyA levels in seeds were assessed by western blot combined with chemiluminescence detection and fluorescence-based protein detection technique. The timescale to quantify and compare phyA levels was chosen according to the experimental setup in seed germination assays. *phyA* mutant seeds imbibed for 48 h were analyzed as negative controls, no phyA protein was detected (Figure 23). The choice of an appropriate housekeeping protein to normalise the fluorescence signals was challenging, due to the specific type of tissue, the highly variable nature of protein levels in seeds and the limited availability of antibodies described for seed extracts. Actin, which is often used as a housekeeping protein was excluded due to unspecific bands in the western blot between 40- 70 kDa, that prevented a clear allocation of signal on the blot.

Instead, UGPase (51-53 kDa) was chosen as a housekeeping protein (Meng *et al.*, 2009) because UGPase is constantly translated in the course of seed imbibition in darkness and showed only a single band in western blots (Figure 23 A-D).

Since phyA protein is not present in dry seeds (Shinomura *et al.*, 1996), control samples were harvested after imbibing seeds for 1 h in darkness. It is known that phyA protein accumulates in the course of imbibition (Lee *et al.*, 2012b), reaching high levels in imbibed seeds after a 12 – 48 h dark period (see introduction section 1.4.1). Seed imbibition for 1 h and 6 h was not sufficient for the seeds to accumulate detectable levels of phyA protein (Figure 23). Both in wild-type and in *ahk2 ahk3* seeds, phyA levels became apparent 12 h after the onset of imbibition and show highest abundance after 24 and 48 h (Figure 23). In wild-type seeds, phyA levels reproducibly increased after a 48 h imbibition period compared to 24 h imbibed seeds (Figure 23 A, B, C, D). The gradual increase of phyA protein levels in *ahk2 ahk3* mutant seeds was clearly detectable in some blots (Figure 23 A), other blots showed no increase in phyA levels after 24 h (Figure 23 B and C) or only marginal phyA levels in all samples (Figure 23 D).

Because protein level quantifications were highly variable across biological replicates and experiments, the comparison of phyA protein accumulation patterns over time between CK double receptor mutant and wild-type seeds was difficult, independent of the method used for protein detection (Figure 23 A and B). To provide an approximation, all blots of one experiment, comprising seeds from five individual plants tested in different western blots were quantified. These quantifications revealed higher phyA levels in wild-type seeds after 48 h compared to *ahk2 ahk3* seeds (Figure 23 E).

Based on the experimental data, there was no evidence for increased phyA protein levels in *ahk2 ahk3* seeds. Nevertheless, taking into account the experimental difficulties, an increase in phyA protein levels could not be entirely ruled out as an explanation for the increased germination phenotype of *ahk2 ahk3* seeds in FR light conditions. A final evaluation and comparison of phyA protein levels in seeds would require additional experimentation.

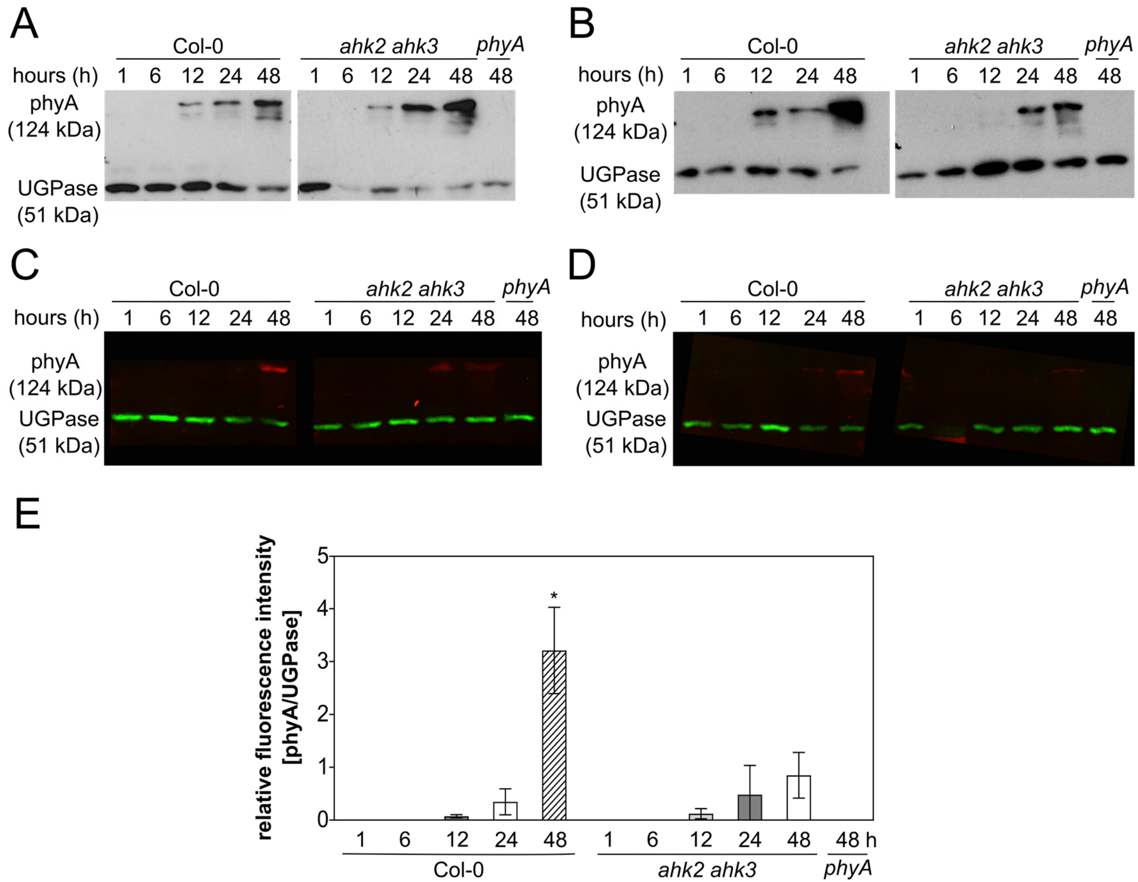

Figure 23. PhyA protein level in the course of seed imbibition in wild-type and *ahk2 ahk3* seeds.
PhyA protein level in the course of seed imbibition in wild-type and *ahk2 ahk3* seeds. Exemplary, proteins isolated from the seed population of two biological replicates (A and C/ B and D) were detected using western blot, followed by chemiluminescence immunodetection (A and B) or fluorescence immunodetection (C and D). UGPase protein was included as a housekeeping protein. (E) Quantification of phyA levels in the course of seed imbibition in darkness based on fluorescence protein quantification of seeds derived from five different biological replicates (for Col-0 and *ahk2 ahk3*) or 3 biological replicates (for *phyA*) per genotype. Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0, 1 h as calculated by Two-Way ANOVA, post-hoc Tukey's test. * $p < 0.05$. Pictures were taken and analyzed by Schmidt (2018).

3.3 The CK status in seed tissue with a higher maternal genome dosage influences seed germination in very low fluence FR light

3.3.1 Reduction of CK perception or CK levels in seed tissue with a higher maternal genome dosage revealed a crucial role of the tissue-specific CK status for germination

In the following section the impact of the maternal CK status on seed germination of the progeny in VLFR FR light conditions was investigated. For this purpose, germination of the F1 offspring derived from reciprocal crosses between wild-type and CK-deficient plants (*ahk2 ahk3* and *35S:CKX2*) was tested in FR light.

As control, crosses between wild type and wild type or mutant and mutant were performed. As expected, seeds derived fromcrosses between *ahk2 ahk3* showed significantly higher germination rates compared to seeds derived from wild type crossed to wild type in FR light (Figure 24 A).

The germination rate of the progeny of reciprocal crosses revealed an increased germination rate when seeds derived from *ahk2 ahk3* maternal plants pollinated with wild-type were compared to seeds derived from wild-type maternal plants pollinated with *ahk2 ahk3* pollen (Figure 24 A). The seeds derived from reciprocal crosses showed germination responses comparable to the respective maternal parent, this indicates a maternal effect shaping seed germination in FR light. For *CKX2* overexpressing plants, control crosses revealed a significantly higher germination rate of homozygous *35S:CKX2* seeds compared to the wild-type seeds (Figure 24 B). Further results showed that seeds originated from crosses between *35S:CKX2* maternal plants pollinated with wild-type pollen germinated slightly, but not significantly better than seeds originated from wild-type maternal plants pollinated with *35S:CKX2* pollen (Figure 24 B). This supports the hypothesis of maternal effects shaping the germination response of the progeny in FR light.

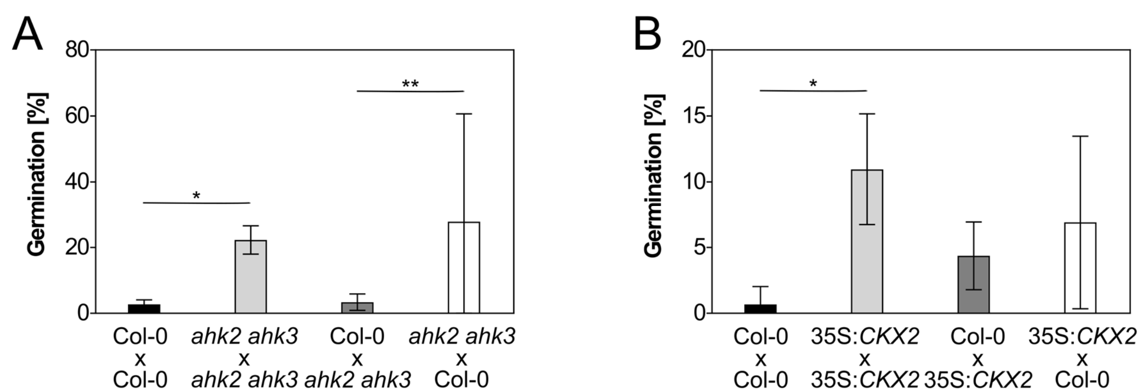

Figure 24. The CK status in maternal plants shapes germination of the offspring in FR light. Germination rates in FR light were examined (A and B). The genotype of the maternal parent is indicated in the upper, the paternal parent in the lower row of the label. As a control, wild-type crosses (Col-0 x Col-0) and *ahk2 ahk3* x *ahk2 ahk3* (A) or *35S:CKX2* x *35S:CKX2* (B) seeds were analyzed. For the graphs, results obtained from 3 independent experiments (A) or only one experiment (B) were depicted. Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0. Two-Way ANOVA, post-hoc Tukey´s test (A) or Kruskal-Wallis-Test, post-hoc Dunn´s test (B). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.3.2 Enhanced CK breakdown specifically in testa- or endosperm seed tissue does not affect germination in FR light

Because the CK status in seed tissue with a higher maternal genome dosage strongly impacts seed germination, the question arose if testa or endosperm is most prominent here.

To reduce the CK status in a specific tissue type, *CKX1,* encoding a CK catabolizing enzyme, was expressed under different seed tissue specific promoters. To ensure tissue specificity of the respective promoters, GUS-lines were analyzed as well. The respective promoter:*CKX1* lines and corresponding promoter:GUS lines used in this work were generated by Harder (2009), under supervision of Stefanie Zintl. Expression of the promoter:*CKX1* construct was verified for the lines used in this work by Harder (2009) (see material and method section 2.4.3). As a control for the biological relevance of an enhanced *CKX1* expression for seed germination, germination of constitutive *CKX1* overexpression lines were studied in FR light. Overexpression of *CKX1* resulted in significantly enhanced seed germination rates, even exceeding *ahk2 ahk3* seeds (Figure 25 A).

To analyze the effect of tissue-specific reduction of the CK status on germination rates, two representative lines were presented here, *ARR21:CKX1*, expressing *CKX1* in the endosperm and *GILT:CKX1*, expressing *CKX1* in the testa. According to eFP browser data (Winter *et al.*, 2007), *ARR21* is specifically expressed in the chalazal endosperm of developing seeds, already detectable in the pre-globular seed stage. In mature seeds, *ARR21* expression expands to the chalazal seed coat, where expression remains weaker than in the chalazal endosperm region in mature seeds (Figure 25 B). *GILT* (*GAMMA-INTERFERON-RESPONSIVE LYSOSOMAL THIOL REDUCTASE*, AT4G12960) was published to be strongly expressed in the outer seed coat layers, from the pre-globular to the linear cotyledon stage. The outer seed coat layer consists of inner and outer integument layers that will form the testa in mature dry seeds. Additionally, *GILT* is expressed in the chalazal seed coat until the linear cotyledon stage, albeit lower than in the outer seed coat (Figure 25 B).

First, eFP browser data were experimentally validated. Developing seeds expressing *ARR21:GUS* showed indigo blue GUS staining both in the chalazal and in the micropylar endosperm region (Figure 25 C). In the *GILT:GUS* line specifically the seed covering layers were stained (Figure 25 C). Since both eFP gene expression data and analysis of promoter:GUS lines confirmed tissue-specific activity of the promoters, the FR light germination of seeds expressing *ARR21:CKX1* and *GILT:CKX1* lines was analyzed. Germination did not significantly differ between wild-type seeds and those expressing *CKX1* in a tissue specific manner, both lines showed a slight, but not significant increase in germination compared to wild-type seeds (Figure 25 D). Based on these results, an increased CK catabolism in only one of the seed tissues does not seem to be sufficient to reach germination rates as in CK receptor double mutant seeds.

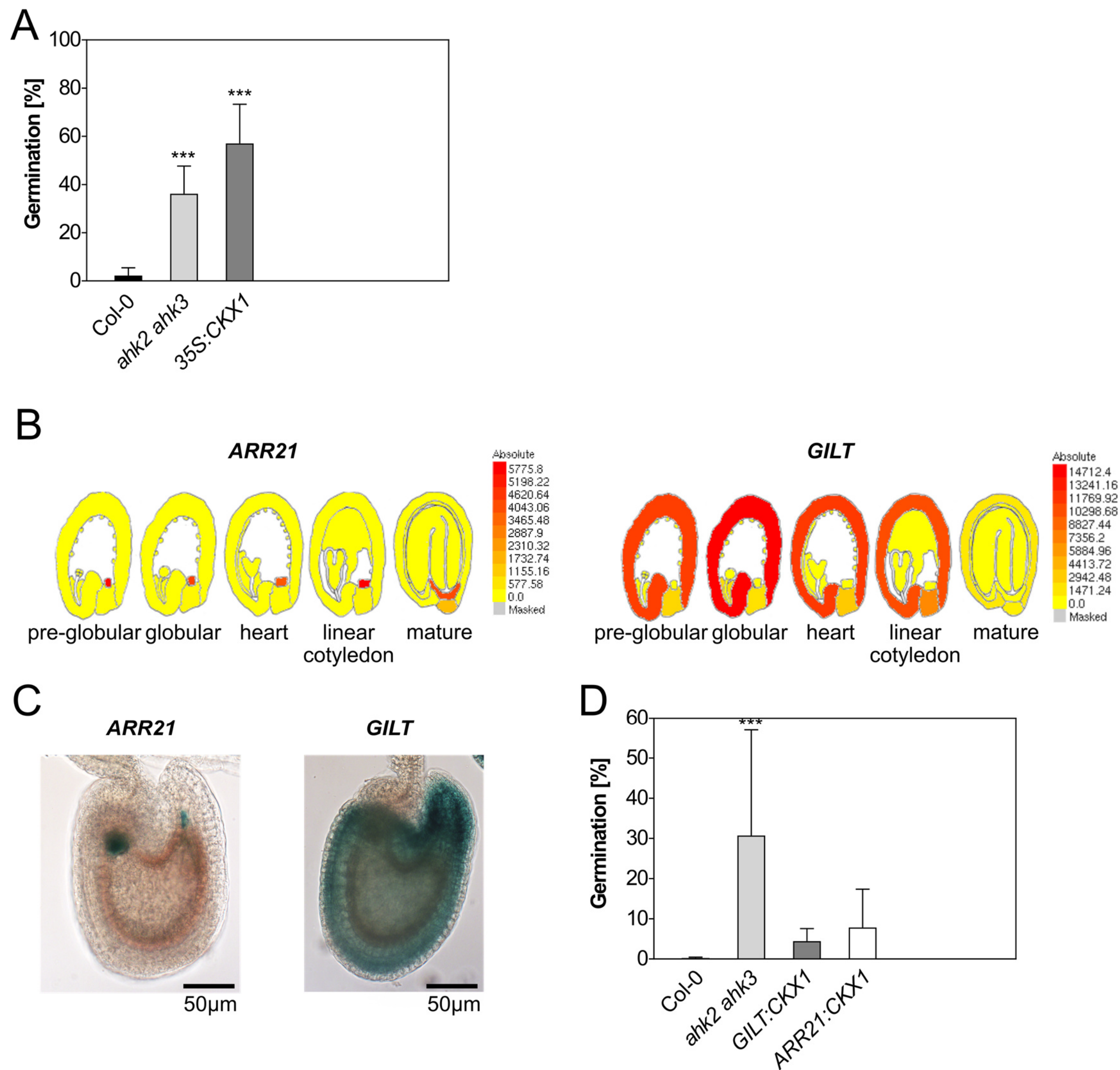

Figure 25. Tissue-specific reduction of the CK status in developing seeds does not allow to discriminate which maternally-derived tissue is of specific importance for germination in FR light.

(A) Germination rates of seeds overexpressing *CKX1* compared to wild type and *ahk2 ahk3*. (B) Expression data (developmental map) from *Arabidopsis* eFP browser at BAR (Schmid *et al.*, 2005, Winter *et al.*, 2007), visualizing *ARR21* and *GILT* expression patterns in developing seeds. (C) Visualization of *ARR21* and *GILT* expression in seeds by GUS staining of *ARR21:GUS* and *GILT:GUS* plants. (D) Germination rates in FR light of seeds with tissue-specific *CKX1* expression. Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0. Kruskal-Wallis-Test, post-hoc Dunn´s test (A) and Two-Way ANOVA, post-hoc Tukey´s test (D). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.4 Impaired CK perception causes no alterations in hormone level, but affects hormone sensitivity during germination

3.4.1 ABA and GA hormone levels are not functionally relevant for increased germination rates of CK receptor mutant seeds

Two plant hormones, GA and ABA, are known to act as master regulators of seed germination. The balance between GA and ABA levels and the sensitivity of their signaling pathways is known to regulate dormancy and the onset of germination (Finkelstein *et al.*, 2008; Yan and Chen, 2017). To determine whether an altered hormone level during seed imbibition may cause higher germination rates of CK mutant seeds in FR light, GA, ABA and CK hormone levels in imbibed seeds were measured and compared between wild-type and *ahk2 ahk3* seeds. Using the standard experimental protocol for germination assays as a basis (depicted in Figure 10), hormone levels were analyzed in dry seeds, in the midst (24 h after the start of imbibition) and the end of the imbibition period (48 h), just before the second, germination-inducing light pulse would be applied (see scheme in Figure 26 A). According to Weitbrecht *et al.* (2011) this time frame covers the water uptake phases II and III in the germination process.

3.4.1.1 GA

The most important bioactive GAs in Arabidopsis are GA_1, GA_3, GA_4 and GA_7. For seeds and the regulation of seed germination GA_4 is of outstanding importance (Yamaguchi, 2008). While GA_1 and GA_3 are classified as 13-hydroxylated GAs, GA_4 and GA_7 belong to the 13-non hydroxylated GAs (Bradford and Nonogaki, 2007).

In the course of imbibition, GA_1 was most abundant in dry seeds, with similar levels measured both in wild type and *ahk2 ahk3*. GA_1 levels significantly decreased after 24 h imbibition in both genotypes (Figure 26 B). Interestingly, in *ahk2 ahk3* seeds GA_1 levels were significantly higher compared to wild type after 24 h, but this difference was lost after 48 h of imbibition. Comparable to GA_1, highest GA_3 levels were present in dry seeds, then decreased non-significantly in wild-type and significantly in *ahk2 ahk3* seeds after 24 h of imbibition (Figure 26 C). Low GA_3 levels were maintained until the last measurements at 48 h. GA_4 was, in terms of absolute hormone level, the second most abundant GA in dry seeds after GA_{12} (Figure 26 D). GA_4 levels did not significantly differ between wild type and *ahk2 ahk3* at any tested point in time. The decrease during imbibition was significant over time in *ahk2 ahk3*, imbibed wild-type seeds displayed a non-significant tendency. GA_5 hormone levels were unaltered over time and genotype (Figure 26 E).

In plants, GA_{12} serves as precursor for both 13-hydroxylated and 13-non hydroxylated GAs (Yamauchi *et al.*, 2007; Hedden and Thomas, 2012) (Figure 7). In the presented data, GA_{12} was the most abundant GA regarding absolute hormone levels. In dry seeds GA_{12} levels were significantly higher in wild-type compared to *ahk2 ahk3* mutant seeds (Figure 26 I). In both genotypes a significant drop in GA_{12} levels 24 h after the onset of imbibition was measured. Low GA_{12} levels were maintained up to 48 h.

Via several biosynthesis intermediates GA_{20} is synthesized from GA_{53}, which is derived from hydroxylation of GA_{12} and serves as a precursor for 13-hydroxylated GA metabolites. GA_{20} levels were highest in dry seeds in both genotypes. Comparison of hormone levels between genotypes revealed a significantly increased GA_{20} abundance in wild-type dry as well as in 24 h imbibed seeds.

Over time GA_{20} levels significantly decreased in wild-type, but not in *ahk2 ahk3* seeds (Figure 26 J). Interestingly, in *ahk2 ahk3* a small, but significant increase in GA_{20} level from 24 to 48 h was measured.

Using GA_{20} as a substrate, GA3-oxidases biosynthesize GA_1 and GA_5 (Yamaguchi, 2008). GA_5 itself serves as precursor for the biosynthesis of GA_6 and the bioactive GA_3. As shown for GA_5, GA_6 levels were stable over time and between the genotypes (Figure 26 F). Remarkably, absolute GA_3 levels were more than ten times lower than GA_5 or GA_6 levels. Comparable to 13-hydroxylated GAs, also non-hydroxylated GAs are synthesized from GA_{12}. For example, GA_9 is produced from GA_{12} via several metabolic intermediates. Highest GA_9 levels were present in dry seeds and not significantly altered between wild-type and *ahk2 ahk3* seeds (Figure 26 H). In both genotypes, a consistent, but non-significant decrease in GA_9 levels in the course of imbibition was obvious.

GA_9 serves as a precursor for GA_7 and the biologically active and most important GA in germination, GA_4. GA_7 levels significantly decreased in the wild-type after 24 h, but not in *ahk2 ahk3*, where only a slightly decrease over time was measurable. Comparable to GA_9, equal levels of GA_7 were measured between the genotypes (Figure 26 G).

These results do not support the hypothesis that enhanced levels of active GAs, in *ahk2 ahk3* seeds (especially GA_4) cause the increased germination rates of *ahk2 ahk3* mutant seeds in FR light. In addition, there is no evidence that *ahk2 ahk3* accumulates GA precursors or biosynthesis intermediates more efficiently than wild-type seeds during imbibition.

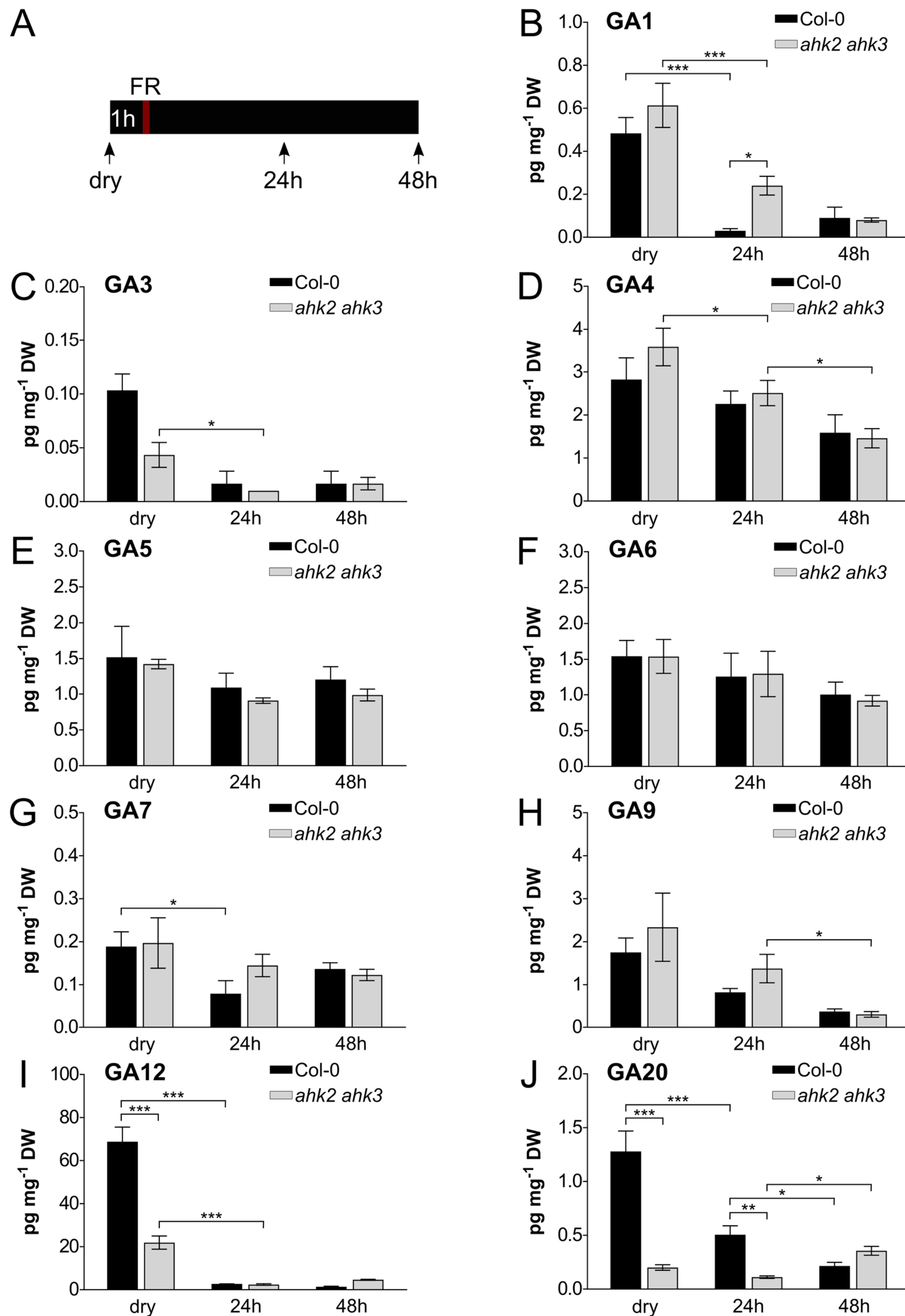

Figure 26. Endogenous GA levels in Col-0 and *ahk2 ahk3* mutant seeds.
Diagram depicting the experimental setup (A). Arrows indicate time of sampling. FR, far-red light pulse for 3 min with 90.6 µmol m^{-2}. After the FR light pulse, seeds were imbibed for 24 h or 48 h in darkness and GA metabolites were measured (B-J). Error bars indicate standard deviations. Asterisks represent statistically significant differences. Two-Way ANOVA, post-hoc Tukey´s test (B, D, E, F, G, H, I, J) and (C) Kruskal-Wallis-Test, post-hoc Dunn´s test (comparison over time) combined with Mann-Whitney test (comparison between genotypes). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. n = 3 biological replicates á 100 mg DW, dry weight.

3.4.1.2 ABA

The early seed imbibition phase is characterized by a rapid decrease of endogenous levels of ABA. Low levels are maintained in non-dormant seeds while in dormant seeds ABA levels increase again, due to ABA *de novo* synthesis (Ali-Rachedi *et al.*, 2004). A lower ABA level could therefore explain the higher germination rates of CK mutant seeds compared to wild type in the VLFR. In dry seeds, ABA was abundant in *ahk2 ahk3* and in wild-type seeds (Figure 27). ABA levels significantly decreased in both genotypes around 70 % when seeds were imbibed for 24 h and remained low thereafter (Figure 27). In sum, the comparison of endogenous ABA levels between CK receptor mutant and wild-type seeds revealed no significant differences between the genotypes at any tested point in time (Figure 27).

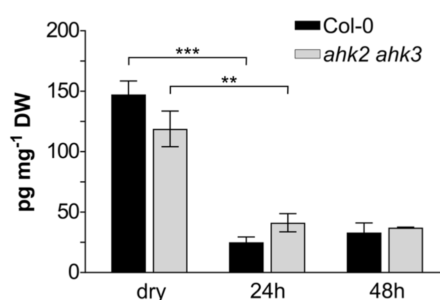

Figure 27. Endogenous ABA levels in Col-0 and *ahk2 ahk3* mutant seeds.
After the FR light pulse, seeds were imbibed for 24 h or 48 h in darkness and ABA metabolites were measured. Error bars indicate standard deviations. Asterisks represent statistically significant differences. Two-Way ANOVA, post-hoc Tukey´s test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. n = 3 biological replicates á 100 mg DW, dry weight.

3.4.1.3 CK

In order to connect CK levels to the seed germination process, the kinetics of different CK metabolites were evaluated in imbibed seeds. The focus of this analysis was set to free CK bases and CK ribosides. Measurements of other CK metabolites (including DHZ, O-glucosides, N-glucosides) did not reveal any intriguing differences (Table A3).

Regarding the free CK bases, both tZ and cZ levels in dry seeds were significantly increased in *ahk2 ahk3* (0.7 pg mg^{-1} dry weight (DW) and 0.9 pg mg^{-1} DW respectively) compared to wild-type seeds (0.3 pg mg^{-1} DW and 0.5 pg mg^{-1} DW) (Figure 28 A and C), while iP-type CK remained unchanged on a low absolute level (0.1 pg mg^{-1} DW in both genotypes) (Figure 28 E). tZ levels significantly decreased in both genotypes after 24 h of imbibition compared to dry seeds (Figure 28 A). In the course of imbibition, tZR levels were not significantly different between the genotypes (Figure 28 B). Highest tZR levels were measured in dry seeds, a significant decrease of 85% in the wild type and 98% in *ahk2 ahk3* was obvious after 24 h of imbibition. After that, levels stayed low in both genotypes. cZR levels were not significantly altered over time or between the genotypes (Figure 28 D). Opposite to other measured CKs, iPR levels increased significantly around 88% in *ahk2 ahk3* and non-significantly around 67% in wild type from dry seeds in the course of imbibition (Figure 28 F). After 24 h and 48 h *ahk2 ahk3* seeds had significantly higher iPR levels (47 % at 24 h and 22 % at 48 h) than wild-type seeds.

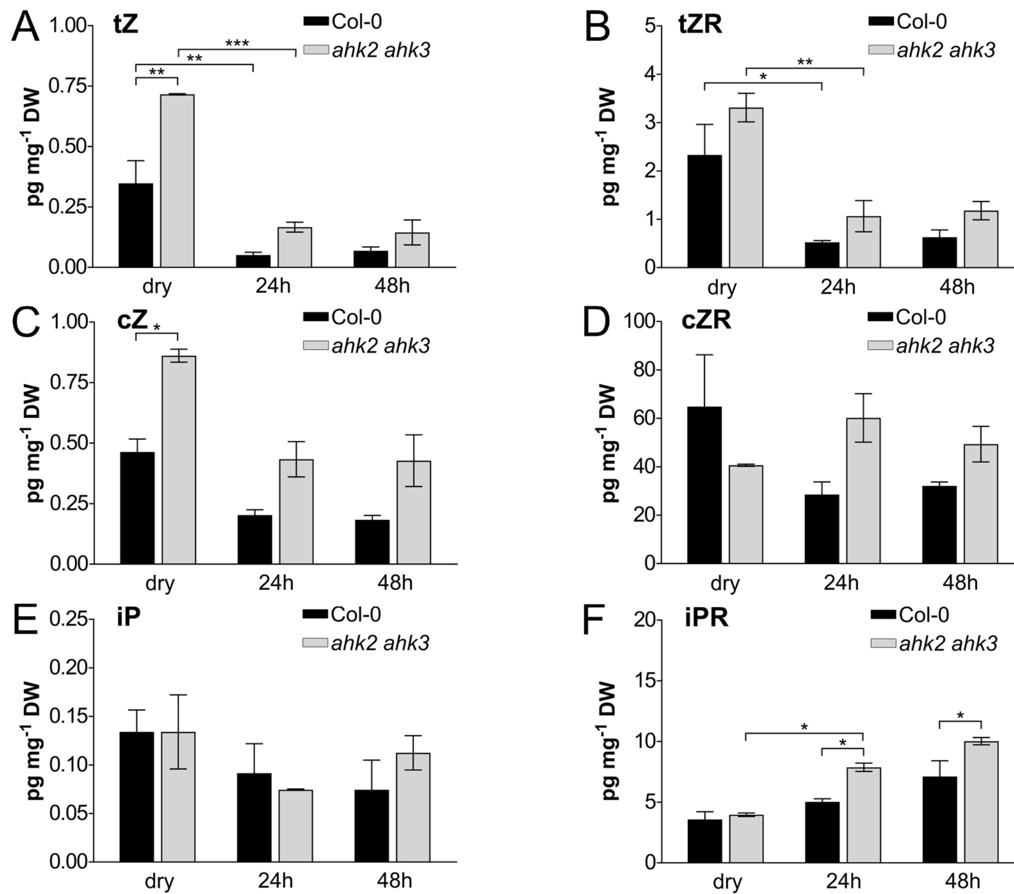

Figure 28. CK levels in Col-0 and *ahk2 ahk3* mutant seeds.
After the FR light pulse, seeds were imbibed for 24 h or 48 h in darkness and CK metabolites were measured. Error bars indicate standard deviations. Asterisks represent statistically significant differences. Two-Way ANOVA, post-hoc Tukey´s test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. n = 3 biological replicates á 100 mg DW, dry weight.

3.4.2 Hormone sensitivity

As shown in the previous section, no major differences between ABA and GA hormone levels were detectable in imbibed *ahk2 ahk3* receptor mutant and wild-type seeds. Nevertheless, an altered sensitivity towards ABA or GA may be causal for the observed differences in germination in very low fluence light conditions. Therefore, the sensitivity of seeds towards exogenously applied ABA or GA both in WL and FR light conditions was tested.

3.4.2.1 ABA sensitivity of CK mutants in germination is comparable to wild type both in WL and FR light conditions

In WL, a significant reduction in germination rates both in wild-type and *ahk2 ahk3* mutant seeds after was measured application of ABA (Figure 29 A). Application of 5 µM ABA suppressed germination almost completely in both genotypes.

A drastic reduction of germination levels in both genotypes was already detectable after application of 0.5 µM ABA, therefore ABA concentrations between 0 and 0.25 µM ABA were chosen for the analysis of germination. In FR light, *ahk2 ahk3* mutant seeds showed significantly higher germination rates of around 40 % compared to 1 % - 3 % in wild-type

seeds (Figure 29 B). With increasing ABA concentrations, reduced germination rates in FR light in *ahk2 ahk3* mutant seeds were observed (Figure 29 B). Wild-type germination rates did not exceed five percent in in FR light, even without ABA application, therefore a ABA-induced reduction of germination was not evident (Figure 29 B).

In conclusion, ABA application suppressed seed germination both in wild-type and in *ahk2 ahk3* mutant seeds in WL, in FR light this effect was measured for *ahk2 ahk3* mutant seeds. Based on these results, a comparison of ABA sensitivities in FR light between the genotypes was not undoubtedly possible.

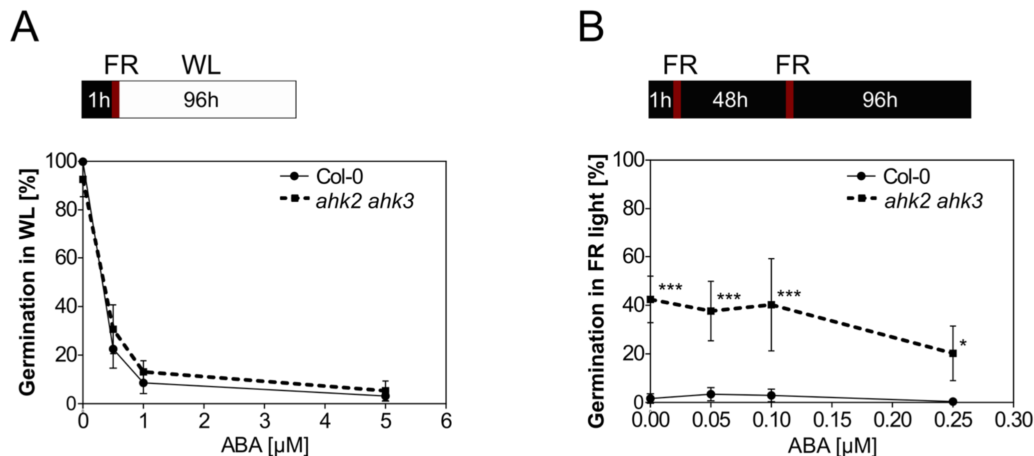

Figure 29. Germination of *ahk2 ahk3* mutant seeds is suppressed after ABA application in WL and in FR light.
Germination percentages in white light (WL) were examined in wild-type (Col-0) seeds as well as in seeds defective in CK perception (*ahk2 ahk3*) after exogenous application of 0 – 5 µM ABA (A) or 0 – 0.25 µM ABA (B). Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0, as calculated by Two-Way ANOVA, post-hoc Tukey test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. n= 4 biological replicates and 2 technical replicates per experiment and condition. FR, far-red light pulse, first pulse FR for 3 min with 90.6 µmol m^{-2} s^{-1}, second pulse FR 18 µmol $m^{-2}s^{-1}$ or constant white light (WL) 127 µmol $m^{-2}s^{-1}$.

3.4.2.2 GA sensitivity is increased in CK mutant seeds compared to the wild type in all tested light conditions

Next, the sensitivity towards externally applied GA was tested. To accentuate the effect of externally applied GA_{4+7}, the seeds were additionally supplied with a GA biosynthesis inhibitor, Paclobutrazol (Pac) (Nambara *et al.*, 1991; Jacobsen and Olszewski, 1993). Exogenous application of 10 µM Pac without additional supply of GA_{4+7} effectively repressed WL induced germination in wild type as well as in CK mutant seeds (Figure A2). In FR light, Pac significantly decreased germination in the *ahk2 ahk3* and *ipt3,5,7* mutants from 72 % and 94 % to 0.3 % and 0.8 % respectively (Figure A2). Germination was also reduced in wild-type seed from 2 % to 0 %. Although this effect was not significant due to the low baseline germination rate of only 2 %, these results suggest that all tested genotypes were responsive towards suppression of GA biosynthesis by Pac. Alterations in the sensitivity towards Pac between the genotypes were not tested in this work.

Since application of GA may induce the light-independent onset of germination (Debeaujon and Koornneef, 2000) dark- germination rates were determined as well. No seed germination was observed, when seeds were incubated in darkness without GA

supply (Figure A2). After application of 1 µM GA_{4+7} *ipt3,5,7* showed significantly increased germination rates (9.5 %) compared to wild-type seeds (2.5 %). Similarly, treatment with 10 µM GA significantly enhanced germination in darkness of *ahk2 ahk3* (70 %) and *ipt3,5,7* (69 %) seeds compared to the wild type (17 %) (Figure 30 A).

After induction of germination with a WL pulse, a higher GA sensitivity of CK mutant seeds was already measurable after application of 0.1 and 1 µM GA. CK receptor mutants and CK biosynthesis mutants germinated 72 % and 76 % respectively after application of 1 µM GA, while wild-type seeds germinated only 17 % (Figure 30 B). Nevertheless, application of 10 µM GA induced 100 % germination in wild-type seeds (Figure 30 B).

In FR light conditions, increased germination rates of *ahk2 ahk3* compared to wild type were already present as response to 1 µM GA treatment (Figure 30 C). When treated with 10 µM GA, both mutants germinated significantly better (*ahk2 ahk3* 82 % and *ipt3,5,7* 86 %) than wild-type seeds (46 %). After application of 30 µM GA, seeds of the tested genotypes germinated uniformly in WL and FR light (Figure 30 B and C).

In summary, a lower CK status or lower CK signal transduction results in increased GA sensitivity in darkness, in WL and in FR light. At higher GA concentrations, germination rates were comparable between the genotypes.

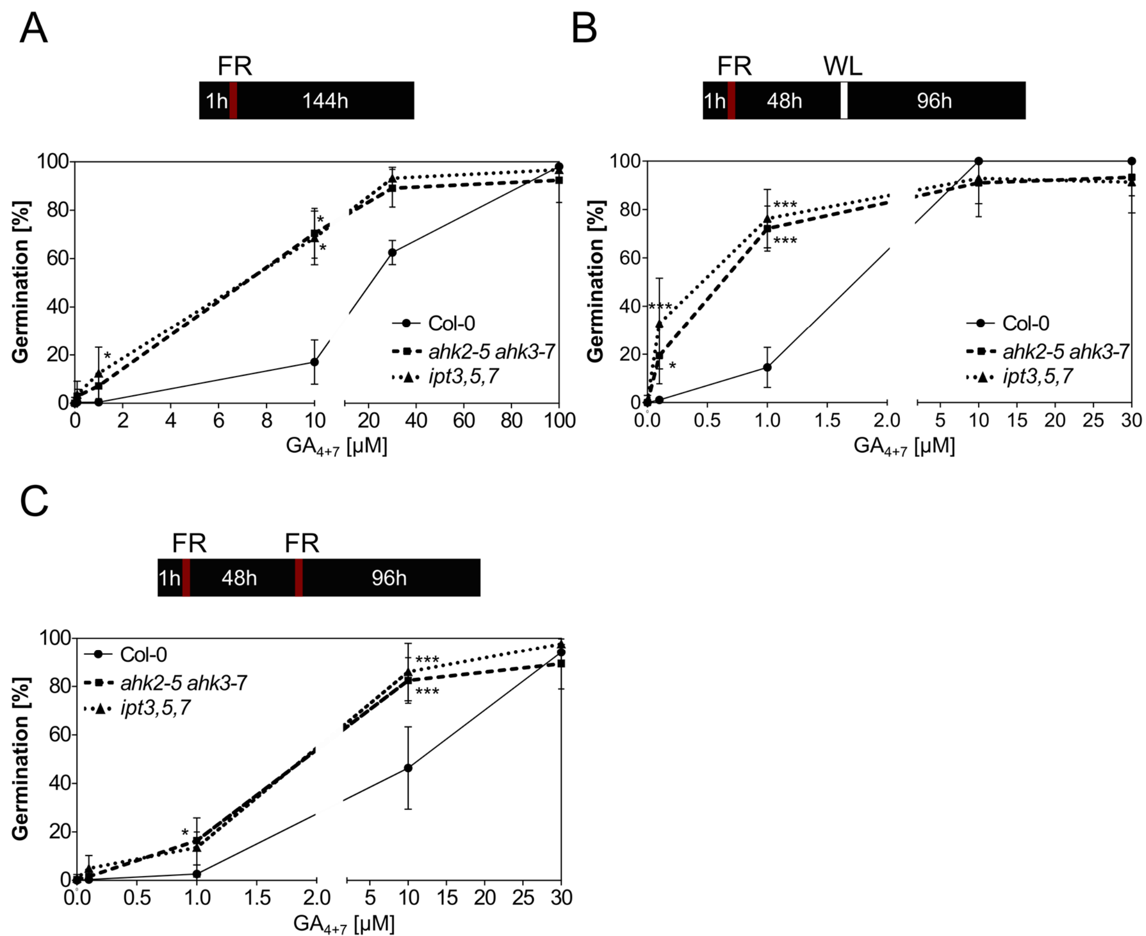

Figure 30. GA sensitivity is increased in darkness, WL and in FR light in seeds with a lower CK status.

Figure 30 continued
Germination percentages in darkness (A), white light (WL) (B) and FR light (C) were examined in wild-type (Col-0) seeds as well as in seeds defective in CK perception (*ahk2 ahk3*) or biosynthesis (*ipt3,5,7*) after exogenous application of 0.1 – 30 µM GA and 5 µM Paclobutrazol (Pac) (A) or 10 µM Pac (B and C). Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0, as calculated by Kruskal-Wallis-Test, post-hoc Dunn's test (A) or Two-Way ANOVA, post-hoc Tukey test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. FR, FR light pulse, first pulse FR for 3 min with 90.6 µmol m^2 s^{-1}, second pulse WL 127 µmol $m^{-2}s^{-1}$ or FR 18 µmol $m^{-2}s^{-1}$.

3.5 Analysis of the gene regulatory network underlying the negative impact of CK on seed germination

3.5.1 *In silico* identification of marker genes for germination in WL and FR light

3.5.1.1 Analysis of microarray data and identification of putative marker genes

The process of seed germination is accompanied by a major reprogramming of the seeds' genome (Nakabayashi *et al.*, 2005; Howell *et al.*, 2008; Narsai *et al.*, 2017). However, the gene regulatory network underlying the negative effect of CK on the germination process in FR light remains elusive.

In order to study gene expression in germinating seeds, an analysis of microarray data combined with gene expression studies by qPCR and RNA-Seq was performed.The aim of the *in silico* study was to identify a set of marker genes for seed germination, using publicly available microarray data. These set of marker genes should include genes specifically regulated at defined points in time during germination.

The Genevestigator database (Hruz *et al.*, 2008) was screened for datasets with an experimental setup resembling the experimental design used for germination assays in this work. For the induction of germination by a very low fluence FR light pulse after a dark imbibition phase, no microarray dataset covering the relevant timeframe was discovered in Genevestigator.

However, a dataset investigating changes in gene expression of cold-stratified seeds during WL germination over time was identified to fulfil most of the pre-defined criteria for selection of a dataset. These include the experimental setup, the time frame, the tissue used for analysis, and the ecotype (for details see material and methods section 2.10.3). The microarray study selected for analysis in this work was conducted and published by Narsai *et al.* (2011). Imbibed seeds were sampled over time, starting shortly (1 h) after the transfer to light, covering 12 h and 24 h, up to 48 h of WL illumination (Figure 31 A). Earlier sampling points analyzed in the microarray data set, namely during cold stratification, were not considered in this work.

After normalization of the raw data using the RMA method (Irizarry *et al.*, 2003), DEGs were identified. Two different methods were used in this work, the Cyber-T method combined with the PPDE method for false discovery rate calculation (Kayala and Baldi, 2012) and the $\log_2$ fold change analysis (Brenner *et al.*, 2005). A high proportion of all identified DEGs, namely 1857, which equals 13.7 % of all DEGs, were shared between the different time points, indicating that these genes were generally associated with germination (Cyber-T method, Figure 31 B).

In order to identify marker genes, genes specifically regulated at a certain time during the germination process were examined in more detail. 348 DEGs were identified as uniquely regulated 6 h after the seeds were transferred to WL compared to 1 h WL

incubation (Figure 31 B). The genes identified as differentially regulated specifically 6 h after transfer to light could not be classified into specific GO terms. 453 genes were differentially regulated specifically 12 h after the transfer to light (Figure 31 B) and again these genes could not be classified into specific GO terms.

After 24 h of light incubation, 2509 DEGs were identified, presenting the largest number of uniquely regulated DEGs at a certain time point (Figure 31 B). The GO terms enriched in this set of DEGs was related to “responses to CK” and two metabolic processes, the cellular amide and carboxyl acid metabolic processes (Figure A3). The GO term “responses to CK” is defined as any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production and gene expression) as a result of a CK stimulus (Carbon *et al.*, 2009; Mi *et al.*, 2019) and includes 592 genes in total.

48 h after the transfer to light, 644 genes were differentially expressed in seeds (Figure 31 B). Classification of DEGs at the time point 48 h revealed that these DEGs were associated with rRNA processing and metabolic processes as well as ncRNA processing (Figure A4). Incubation of seeds for 48 h in WL resulted most likely in a high proportion of already germinated seeds in the analyzed samples.

In order to identify genes potentially qualified as marker genes out of this pool, the $\log_2$-fold change differences in gene expression from 1 h illuminated seeds to the expression at the respective point in time was calculated. The number of genes identified (labeled as frequency) was plotted against the calculated $\log_2$-fold expression in a histogram (Figure 31 C). The expression of genes that were at least two-fold increased or decreased compared to 1 h imbibed seeds was plotted in the graph at the dashed lines at a $\log_2$-fold change of 1, representing a two-fold increase or $\log_2$-fold change of -1, labeling a two-fold decrease in expression (Figure 31 C).

At 6 h of WL illumination, the majority of 4895 genes was slightly upregulated, only a limited number of genes were at least two-fold up (33 genes)- or downregulated (7 genes). After 48 h of WL illumination, DEGs that were at least two-fold up (356 genes) - or downregulated (58 genes) were more abundant compared to 6 h (Figure 31 C).

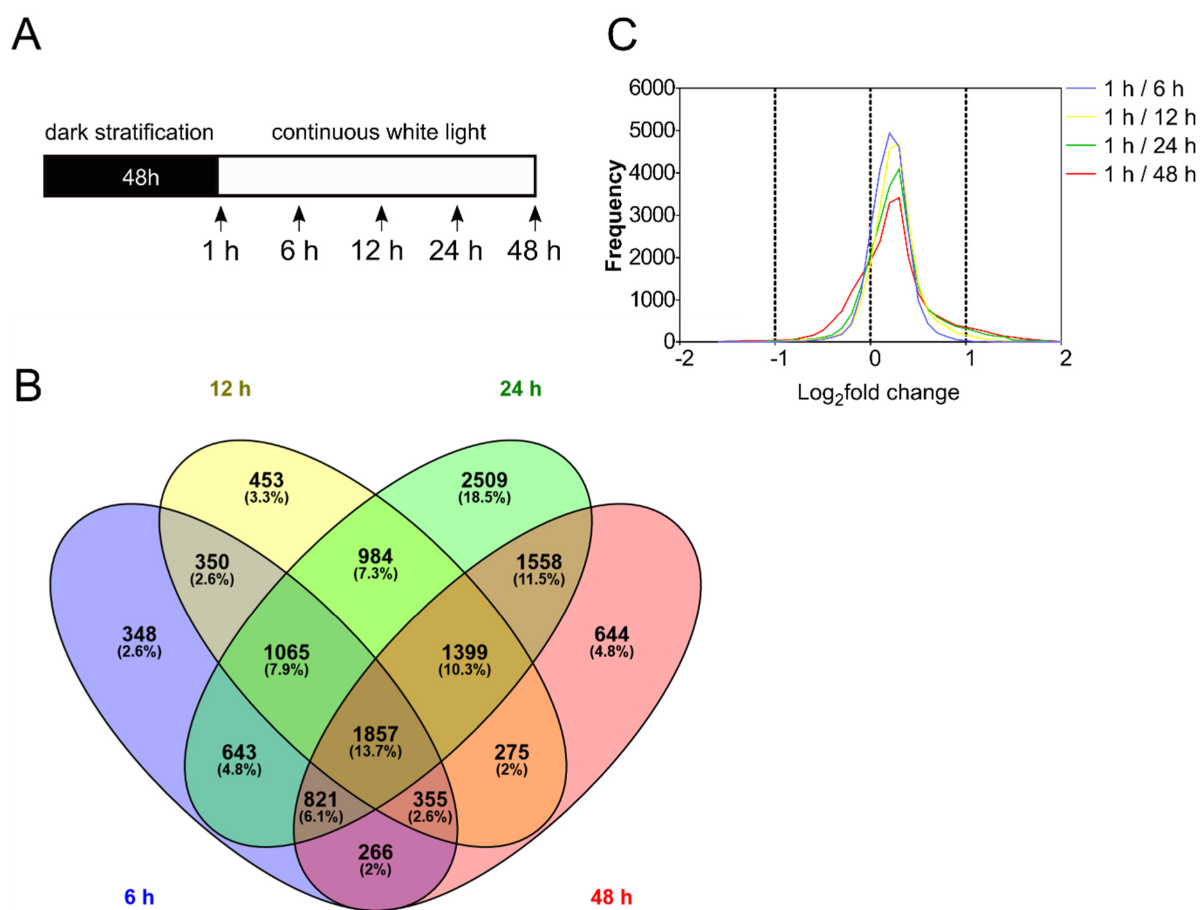

Figure 31. Analysis of microarray data GSE30223, published by Narsai *et al.* (2011). Schematic overview of the experimental design (A). Differentially expressed genes (DEGs) identified with the Cyber-T method ($p < 0.05$, PPDE > 0.96) and plotted in a Venn diagram for each time point (B). (C) Frequency distribution of DEGs identified with the log_2-fold change method. The y-axis represents the number of genes, on the x-axis the log_2-fold change expression of the genes is plotted for each time point relative to the expression levels in 1 h imbibed seeds. Dashed line at a log_2-fold change of 1 indicates a two-fold increase, the dashed line at -1 indicates a two-fold decrease in expression compared to 1 h.

For the selection of suitable marker genes, the log_2-fold change method was used. DEGs were filtered by highest log_2-fold change value at 6 h, 12 h, 24 h or 48 h compared to 1 h imbibed seeds. To further reduce the number of possible candidate genes, 15 DEGs with highest expression at the respective point in time were selected (Table A4). Downregulated genes were not considered further. If several genes were upregulated uniquely at one specific time point, the gene displaying highest expression levels was considered. All selected marker genes were also identified at the respective time point if the Cyber-T method was used for calculation (data not shown).

PYK10, encoding a glycosyl hydrolase and *XTH31*, a gene involved in cell wall biogenesis, were among the highest regulated DEGs at all time points. Following gene expression over time, both *PYK10* and *XTH31* expression in germination raised early and strongly upon illumination, reaching highest expression levels 12 h after the onset of illumination, the expression remaining constant thereafter (Figure 32 A and B).

A GDSL-motif esterase was identified as marker gene for early stages of germination. *GDSL* was identified among the 15 highest upregulated genes 6 h after the transfer to WL but was not among the highest upregulated genes at other time points, so *GDSL* might be qualified as a marker gene for this early stage of germination. *GDSL* expression peaked at 12 h of WL illumination and decreased thereafter (Figure 32 C).

Two marker genes were identified in seeds exposed to WL for 12 h. *CYP81F4*, belonging to the cytochrome P450 family, was identified among the highest upregulated genes in the seeds 12 h after the start of WL illumination (Figure 32 D). Following expression over time, *CYP81F4* expression levels peaked after 12 h, stayed stable at 24 h and decreased after 48 h of illumination. *JAL34*, encoding a mannose-binding lectin, represents a suitable marker gene for the time frame covering 12 h, 24 h and 48 h, where *JAL34* was among the Top 15 DEGs in germinating seeds. In the course of time, expression of *JAL34* was most pronounced after 24 h of WL illumination (Figure 32 E).

The arabinogalactan-encoding *FLA9* was identified among the highest upregulated genes 24 h after the onset of illumination. During germination, *FLA9* expression increased from 6 to 12 h, maximum expression levels were observed after 24 h, so *FLA9* might be a potential marker gene for the seed transcriptome after 24 . Thereafter, the expression level slightly decreased (Figure 32 F).

48 h after the transfer of seed to WL, a high proportion of seeds may already have completed germination. Therefore, it was not surprising to identify *LHB1B1*, a gene encoding a light harvesting complex subunit as a marker gene for this time point. *LHB1B1* expression steadily increased over time in light incubated seeds, reaching highest expression levels after 48 h in this dataset (Figure 32 G).

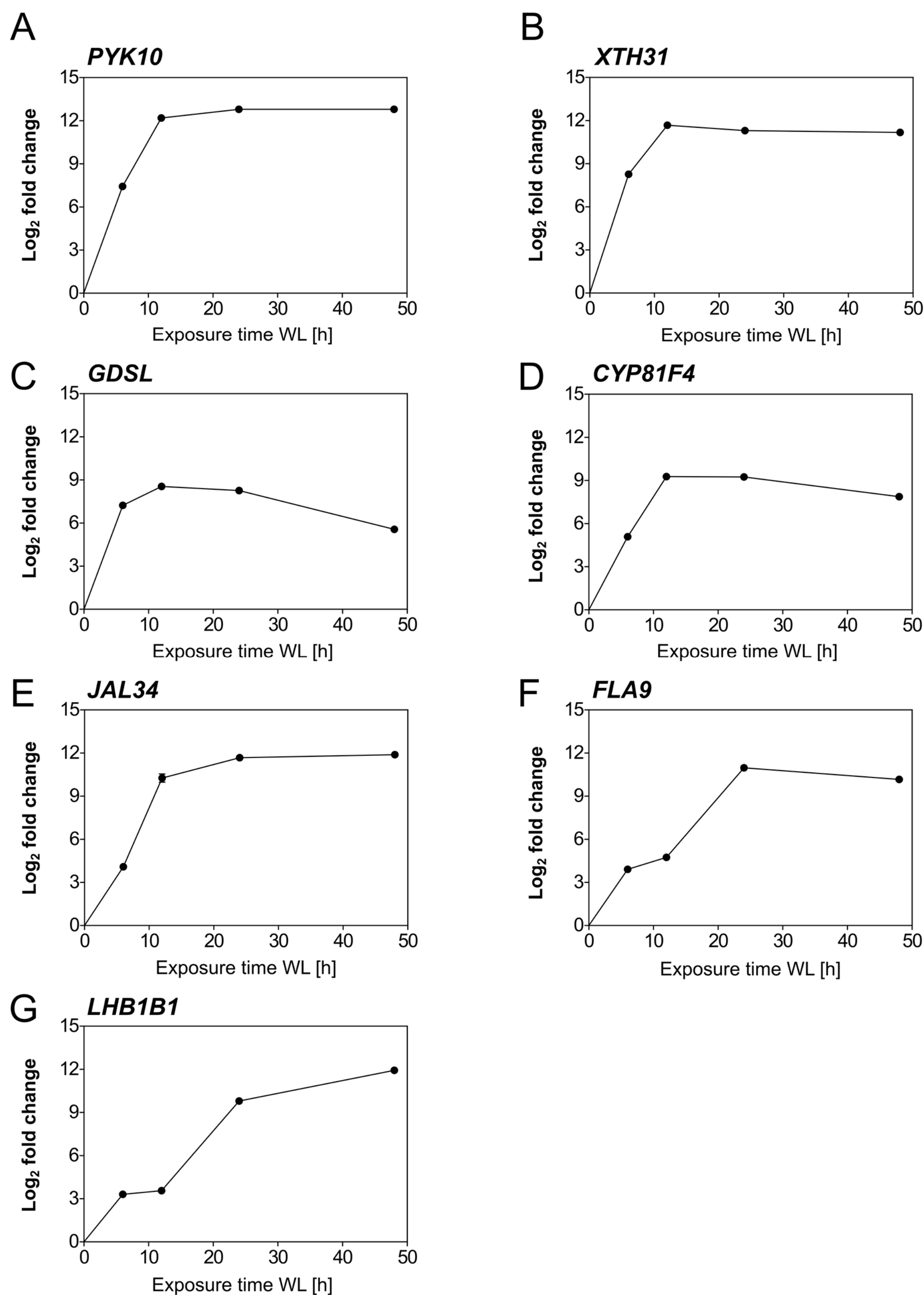

Figure 32. Gene expression analysis over time of identified marker genes from *in silico* microarray data.
Log_2-fold change of gene expression was calculated for every time point relative to the expression of the respective gene after 1 h of illumination. For the calculation, RMA-normalized microarray data were used (n = 3). WL, white light. Microarray data from Narsai *et al.* (2011).

3.5.1.2 *In silico* identified marker genes reveal an imbibition-related expression pattern in very low fluence light conditions

Following the identification of marker genes by *in silico* data analysis, the expression levels and kinetics of the candidate genes for germination were tested via qPCR in samples collected according to the protocols used in this work (schemes for example in Figure 33 A and B). In contrast to the experimental setup in the analyzed microarray, where seeds were constantly illuminated with WL, in this work the germination-inducing WL pulse was temporary limited to three hours. Additionally, in the dark imbibition period prior to the light pulse, the seeds were imbibed at room temperature and not subjected to stratification.

Gene expression was analyzed 12 h after the application of a germination-inducing WL pulse (see scheme Figure 33 A). Compared to 1 h imbibed seeds, the expression of the glycosyl hydrolase *PYK10* was twofold increased in wild type and sixfold in *ahk2 ahk3* seeds after a WL pulse was applied (Figure 33 B). Regarding *XTH31* expression, in wild-type seeds no increase in expression was measurable, but *ahk2 ahk3* seeds displayed a twofold increase in *XTH31* expression compared to 1 h imbibed seeds (Figure 33 B). The expression patterns of the remaining marker genes revealed no increase compared to 1 h imbibed seeds after the application of a germination-inducing WL pulse (Figure 33 11 B). To sum this up, for the *in silico* identified marker genes no light-dependent induction of expression could be confirmed via qPCR.

As mentioned before, no data set was available in the literature using a very low fluence FR light pulse for the induction of germination. Therefore, the *in silico* identified marker genes were tested under VLFR light conditions. Gene expression was analyzed after 28 h of dark imbibition, as well as early (1 h) and late (6 h) after a very low fluence FR light pulse (see scheme Figure 33 C). Gene expression relative to 1 h imbibed seeds of the respective genotype were plotted in a heatmap (Figure 33 D).

In wild-type seeds, the expression of all tested marker genes, except *PYK10*, increased less than twofold compared to 1 h imbibed seed (Figure 33 B), while in the published dataset analyzed before expression increased 500-fold (Narsai *et al.*, 2011). In *phyA* mutant seeds, the increase in expression was even more pronounced compared to wild-type seeds (Figure 33 D). So the increase in marker gene expression appears to be independent of the second light pulse, but related to the imbibition procedure. In *ahk2 ahk3* seeds, a gene expression pattern similar to wild type was observed, *GDSL* and *LHB1B1* expression was more pronounced compared to the wild type (Figure 33 D). The gene expression data underlying the heatmap may be found in Table A5.

In sum, the changes in gene expression observed here, especially in *phyA* mutant seeds, indicate an imbibition-regulated pattern of gene expression rather than an effect triggered by the germination-inducing FR light pulse.

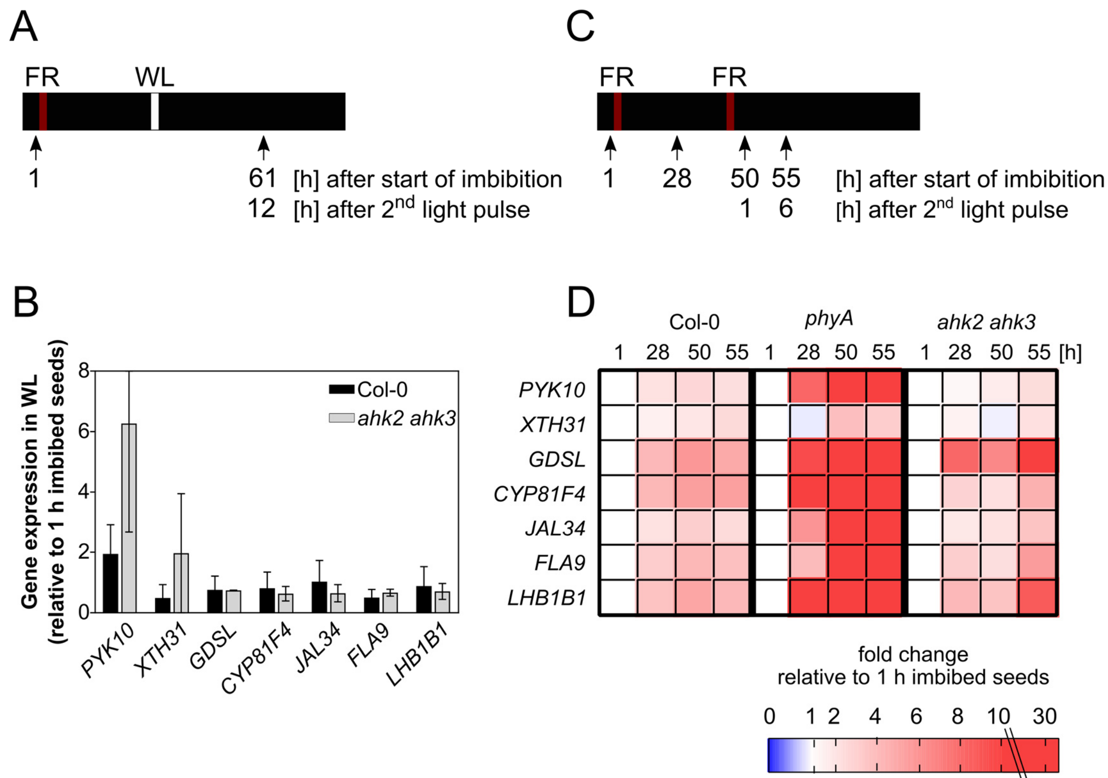

Figure 33. Gene expression analysis via qPCR of *in silico* identified marker genes reveal imbibition-related expression patterns.
Schematic overview indicating the experimental setup for gene expression analysis after a white light (WL) pulse (A). (B) Gene expression 12 h after a WL pulse, as indicated in scheme (A) relative to 1 h imbibed seeds in wild-type and in *ahk2 ahk3* receptor mutant seeds. (C) Schematic overview of the experimental setup for gene expression analysis in very low fluence light conditions. (D) Heat map of transcript levels analyzed by qPCR. The transcript level of 1 h imbibed seeds of every genotype was set to 1 (outer left column in every section). *PP2A* was used as a reference gene. Blue color indicates downregulation, red color upregulation relative to 1 h imbibed seeds.

3.5.2 The influence of CK on germination in FR light is not correlated with differential expression of classical germination-associated genes

As the identification of suitable marker genes for germination after a very low fluence FR light pulse using *in silico* microarray data was not expedient, a different approach to assess the gene regulatory network underlying the altered germination response in CK-deficient seeds was used.

In this section, the expression of a set of genes, which were already described in the literature to be associated with the regulation of germination (Ogawa *et al.*, 2003; Seo *et al.*, 2006; Oh *et al.*, 2009; Yao *et al.*, 2015), was analyzed via qPCR in wild-type and in CK receptor double mutant seeds. Seeds derived from the *ipt3,5,7* mutant were only included in the analysis of later stages of germination (see scheme Figure 34 B). Marker genes identified from the *in silico* data analysis were not included in this set, since their expression was shown to be regulated solely by imbibition. The set of genes for analysis was selected based on their previous description to be relevant for germination and the availability primers with a high efficiency in seed material.

Here, gene expression was analyzed early (one and six hours, Figure 34 A) and late (twelve and 24 hours, Figure 34 B) after application of a FR light pulse and plotted as

relative expression difference to 1 h imbibed wild-type seeds (outer left column). The gene expression analysis was conducted to monitor gene expression patterns of transcripts associated with ABA -and GA metabolism, ABA signaling components and light signaling, that might affect seed germination.

In the literature, ABA biosynthesis-associated genes like *ABA1*, *NCED6* and *NCED9* were described to be decreased by red light treatment (Seo *et al.*, 2006; Oh *et al.*, 2007; Seo *et al.*, 2009), but not by FR light. *NCED3* was upregulated fourfold in Col-0 seeds as response to a second, germination-inducing FR light pulse while expression was unaltered in *ahk2 ahk3* mutant seeds (Figure 34 A). Since *phyA* mutant seeds displayed *NCED3* expression patterns resembling wild type, while being unable to respond to the applied FR light pulse, a functional relevance of altered *NCED3* expression was not supported here. In the wild type, the ABA biosynthesis associated genes *NCED6* and *NCED9* were not regulated in late stages of germination (Figure 34 B). In contrast, in *ahk2 ahk3* and *ipt3,5,7* these genes were downregulated twofold (Figure 34 B). *ABA1* and *ABI5* expression showed similar downregulation over time both in wild-type and in *phyA* mutant seeds (Figure 34 A), while *ABI3* was not regulated at all among all genotypes, therefore these genes were not analyzed more into detail.

RRL1 (*RETARDED ROOT GROWTH-LIKE*) was described to cause an increased response to ABA in seed germination (Yao *et al.*, 2015). After testing *RRL1* expression in germinating seeds, a two- to threefold increased expression was measurable six and twelve hours after FR light application in the wild-type, whereas in *ahk2 ahk3* seeds *RRL1* was downregulated (Figure 34 A). After an initial downregulation, *RRL1* expression in *phyA* was slightly upregulated.

CYP707A1, encoding an ABA catabolizing enzyme and *GA20ox1* (*GA20 OXIDASE 1*), which is involved in GA biosynthesis, displayed an expression pattern similar to *RRL1* (Figure 34 A). Expression was upregulated in wild-type seeds, but downregulated in *ahk2 ahk3* seeds. In *phyA* both genes were initially downregulated compared to one hour imbibed wild-type seeds, but expression increased after 50 h and 55 h of imbibition. Neither *ahk2 ahk3* nor *phyA* reached wild type-like levels of *CYP707A1* or *GA20ox1* expression. Monitoring *GA20ox1* expression at later stages of germination (Figure 34 B), revealed that expression was rather downregulated in wild-type as well as in *ahk2 ahk3* and *ipt3,5,7* seeds. Expression of *GA2ox3*, which encodes a GA catabolizing enzyme, was upregulated two- to threefold in wild-type seeds and threefold in *ahk2 ahk3* mutant seeds (Figure 34 B). For the induction of germination, the release of DELLA-mediated inhibition by GA is of crucial importance (Peng *et al.*, 1997; Ogawa *et al.*, 2003), so the gene expression patterns of *GAI* and *RGL2* were monitored. *GAI* and *RGL2* were slightly upregulated in the wild type over time (Figure 34 A). In *phyA* mutant seeds, *GAI* and *RGL2* levels were initially lower in *phyA* seeds compared to 1 h wild-type seeds, but also upregulated over time. In *ahk2 ahk3* seeds, *RGL2* was fourfold higher expressed relative to 1 h imbibed wild-type seeds after 28 h of imbibition. Other *DELLA* genes were not tested, due to the lack of qualified qPCR primers.

To sum this up, genes potentially interesting here were characterized by expression pattern that differ in wild-type and in *phyA* mutant seeds, since an FR light-associated effect on gene expression level would not be expected in seeds unable to perceive FR light. This was the case for *RRL1*, *CYP707A1* and *GA20ox*. Also interesting were genes regulated at later stages of germination, for example *NCED6* and *NCED9*.

Next, the gene expression of light-associated genes was analyzed in more detail. *PHYA*, encoding the primary light sensor for FR light, plays an outstanding role in germination-induction via FR light (refer to introduction section 1.4.1). Although phyA protein level were not obviously altered (refer results section 3.2.3), the *PHYA* gene expression exceeded the expression of any other gene analyzed in this qPCR study, which was the reason for separate plotting of *PHYA* expression in Figure 34. *PHYA* expression increased in wild-type seeds during imbibition eightfold and was stable thereafter (1 h and 6 h after the germination-inducing light pulse) (Figure 34 A). In *ahk2 ahk3* mutant seeds, basal *PHYA* expression was comparable to wild-type seeds, but expression strongly increased up to 20-fold 28 h after the onset of imbibition and remained high thereafter (Figure 34 A).

Gene expression of *PHYB*, which encodes a photoreceptor activated majorly by red light, and *HY5*, a gene encoding a phytochrome-interacting factor regulating light-associated signal transduction downstream the photoreceptors, were not regulated at all in very low fluence FR light germination (Figure 34 A). *OWL1*, a gene associated specifically with the VLFR (Kneissl *et al.*, 2009), was not regulated in the wild type and also showed no substantial alterations in expression over time in *ahk2 ahk3* and *phyA* seeds. *SOM* and *SLY* are both associated with signal integration between light and hormone metabolism (refer to introduction section 1.4.2). While *SLY1* was not regulated in wild-type seeds, *SOM* was downregulated (Figure 34 A). *phyA* mutant seeds showed a similar expression pattern, in *ahk2 ahk3* seeds both genes were not regulated compared to 1 h wild-type seeds (Figure 34 A).

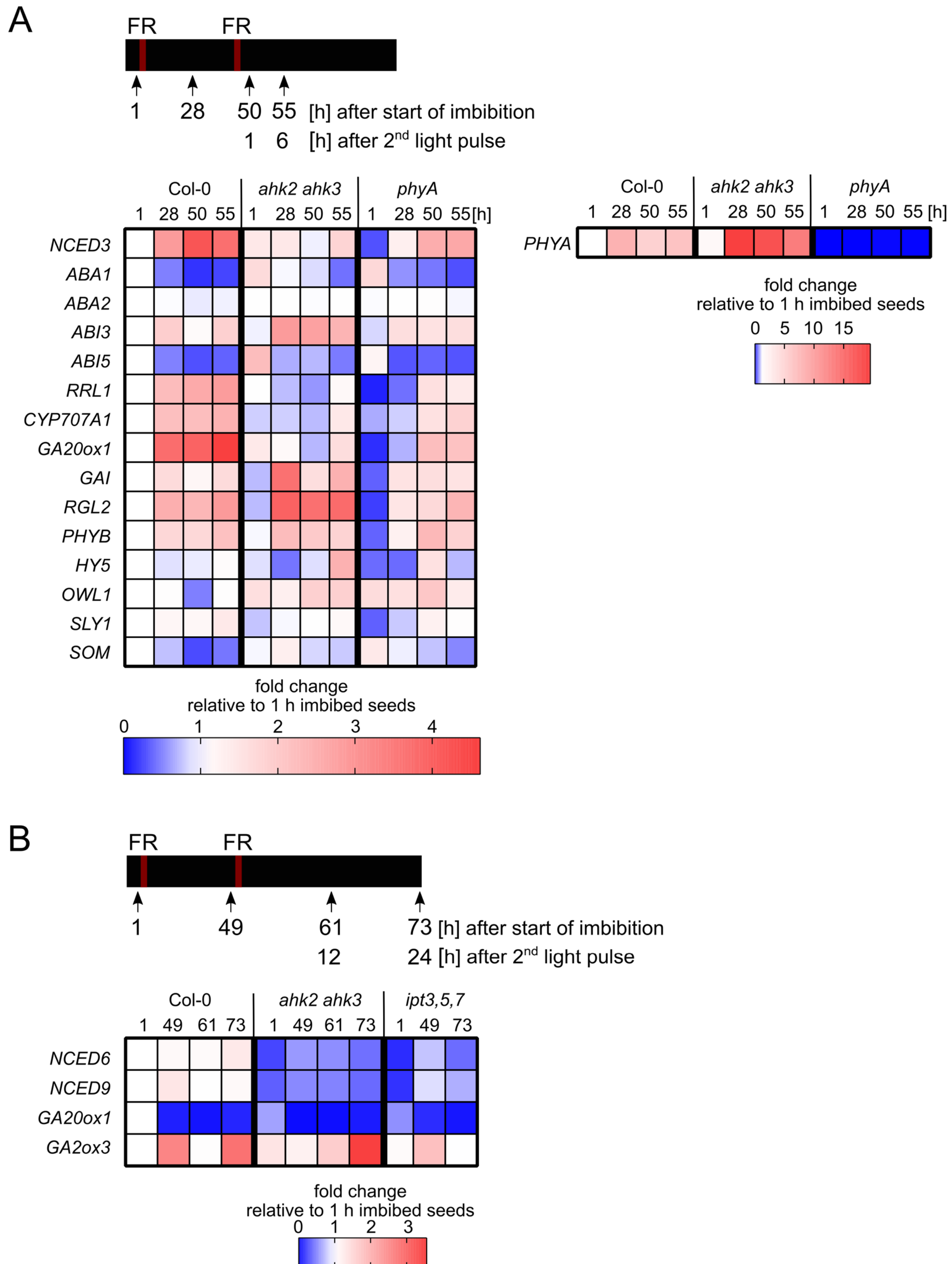

Figure 34. Gene expression analysis of ABA- GA- and light-associated genes in the course of FR light induced seed germination.
Gene expression analysis of ABA- GA- and light-associated genes in the course of FR light induced seed germination. The experimental setup of the respective analysis is indicated in the upper part of each panel. Gene expression was analyzed in the imbibition phase of germination prior to the second, germination-inducing FR light pulse in Col-0, *ahk2 ahk3* and *phyA* mutant seeds (A). After induction of germination, gene expression was assessed early (1 h and 6 h after the second FR light pulse) (B) and late (12 h and 24 h after the second FR light pulse) (C).

Figure 34 continued.
PHYA transcript level were plotted separately (in A and B). The transcript level of 1 h imbibed wild-type seeds was set to 1 (outer left column, n = 4). *PP2A* was used as a reference gene. Blue color indicates downregulation, red color upregulation relative to 1 h imbibed wild-type seeds. FR, FR light pulse, first pulse FR for 3 min with 90.6 µmol m^{-2} s^{-1}, second pulse with 18 µmol $m^{-2}s^{-1}$ FR.

3.5.3 RNA-Seq analysis

3.5.3.1 Pre-requirements for RNA-Seq analysis

The seed transcriptome during germination in VLFR light conditions has been assessed in this work by testing a set of genes, which were published to play a crucial role in the germination process. Nevertheless, no distinct group of genes was identified which could explain the increased germination rates of seeds with a reduced CK status or signal transduction. To uncover the effect of CK on the transcriptional level, RNA-Seq analysis was performed.

As a quality control, the germination rates of the seed set intended for analysis was tested both in WL and FR light, to ensure seed viability and the absence of dormancy. Induction of germination with a WL pulse resulted in more than 96 % germination in all tested genotypes (Figure 35 A), so seed dormancy or lack of seed viability was excluded. In FR light conditions, both *ahk2 ahk3* and *ipt3,5,7* seeds showed significantly higher germination rates (64 % and 80 % respectively) compared to wild type (36.5 %) (Figure 35 B). These results are in accordance with the germination response observed earlier in this work. For RNA-Seq, only wild-type and *ahk2 ahk3* samples were analyzed.

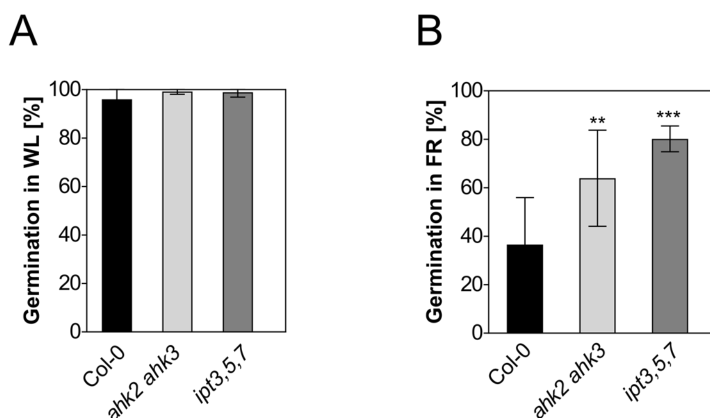

Figure 35. Germination analysis of seed set designated for RNA-Seq analysis.
Germination rates in WL (A) and in FR light (B) in the wild type and in seeds defective in CK perception (*ahk2 ahk3*) or CK biosynthesis (*ipt3,5,7*). Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0 as calculated by (A) Kruskal-Wallis-Test, post-hoc Dunn's test or (B) One-Way ANOVA, post-hoc Dunnett's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. n = 4 biological replicates comprising seeds of 8 plants.

For RNA-Seq sample preparation, wild-type and *ahk2 ahk3* mutant seeds were harvested in dim green safe light after one hour of imbibition, prior to the application of a phyB inactivating FR light pulse.

To monitor imbibition, samples were harvested at the end of a two days dark imbibition phase (49 h after sowing). In order to compare transcriptomic changes dependent on the light quality of the second, germination-inducing light pulse, imbibed seeds were either illuminated with a WL or with a FR light pulse. Samples were harvested 6 h after the second light pulse was applied (55 h after the onset of imbibition), or 12 h after the germination-inducing light pulse (61 h after the onset of imbibition) (Figure 36).

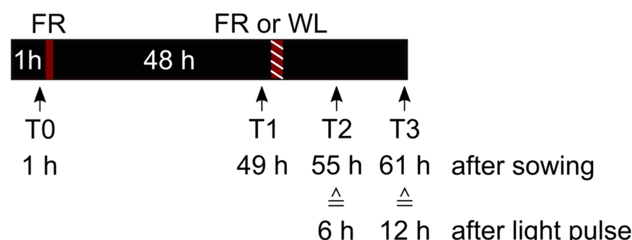

Figure 36. Schematic overview indicating the experimental setup for the RNA-Seq analysis. Schematic overview indicating the experimental setup for the RNA-Seq analysis. T indicates "timepoint"; FR, far-red light pulse; WL, white light pulse. First pulse of FR was applied for 3 min with 90.6 µmol m^{-2} s^{-1}, the second pulse applied at the end of the imbibition phase either FR light 5 min 18 µmol $m^{-2}s^{-1}$ or WL 75 min. 150 µmol $m^{-2}s^{-1}$. Three biological replicates were harvested, each containing 5 mg seed material (dry weight) consisting of pools of 8 plants.

3.5.3.2 Exploratory data analysis

In this work, a PCA and a sample clustering heatmap were performed to ensure similarity between the biological replicates which are expected to cluster together (Conesa *et al.*, 2016; Li and Li, 2018). The PCA analysis was conducted in R, as input the raw, non-normalized data was used. Principal component 1 (PC1, x-axis) represents 90 % and PC2 (y-axis) represents 6 % of the total variation in the data (Figure 37). Together, PC1 and PC2 cover 96 % of data variation, therefore no further principal components were analyzed. In the PCA, samples clustered into four major clusters (Figure 37; red circles). Biological replicates cluster closely together, indicating a high biological reproducibility (Figure 37). In the following, the clusters were described into more details, from left to right in the PCA plot (Figure 37). WT_T0 samples cluster together with *ahk2 ahk3*_T0 samples (Figure 37; Cluster 1). Cluster 2 comprises WT_T1 samples and *ahk2 ahk3*_T1 samples (Figure 37; Cluster 2). 6 h after application of a WL pulse (T2), *ahk2 ahk3* samples cluster with wild-type samples (Figure 37; Cluster 3). Also 12 h after a WL pulse, wild-type samples cluster together with *ahk2 ahk3* samples (Figure 37; Cluster 4).

After induction of germination with a FR light pulse no separate clusters were identified. *ahk2 ahk3* biological replicates accumulated in two distinct areas, according to the timepoint of sampling (Figure 37; brown and orange), while the biological replicates in the wild type displayed a higher degree of variation (Figure 37; light green and green).

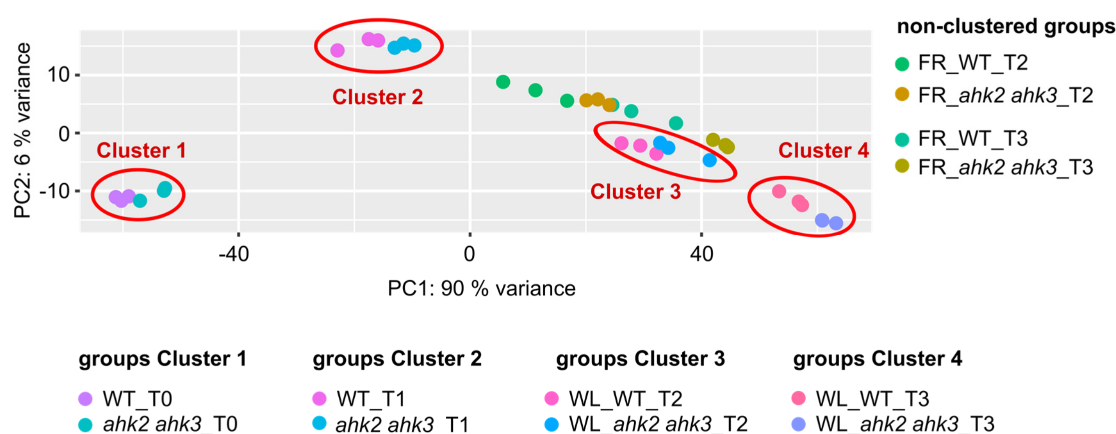

Figure 37. Principal component analysis of RNA-Seq data showing clustering of biological replicates.
Four clusters can be clearly distinguished. The transcriptome of seeds imbibed for 1 h is similar in both genotypes, WT_T0 biological replicates cluster together with *ahk2 ahk3*_T0 (Cluster 1). T1 refers to seed samples harvested after 49 h of imbibition, WT_T1 samples cluster together with *ahk2 ahk3*_T1 samples (Cluster 2). Samples harvested 6 h after a germination-inducing WL pulse cluster together, irrespective of the seeds´ genotype (WL_WT_T2 and WL_*ahk2 ahk3*_T2, Cluster 3). Also 12 h after induction of germination by a WL pulse, WL_WT_T3 biological replicates cluster together with WL_*ahk2 ahk3*_T3 samples (Cluster 4). In samples subjected to FR light treatment for the induction of germination, the transcriptome of *ahk2 ahk3* seeds differs from wild-type seeds, so no cluster could be identified (non-clustered groups).

The results obtained from PCA analysis were confirmed by a color-coded hierarchical clustering heatmap displaying Euclidian distance (Figure A5). Overall, the heatmap revealed a clustering of the samples in four major clusters. This is in line with the findings of the PCA, which also identified four major sample clusters.

To sum this up, reproducibility of the biological replicates was demonstrated both by PCA and a color-coded heatmap, therefore the RNA-Seq dataset was regarded as qualified for further analysis. The next step in the analysis of the RNA-Seq dataset was the identification of differentially expressed genes, based on negative binomial distribution using DESeq2 (Love *et al.*, 2014).

3.5.3.3 Transcriptomic changes during WL-induced germination in the wild type

In order to investigate transcriptomic changes in wild-type seed germination, differentially expressed genes in wild-type seeds were analyzed over time by comparing transcriptomes of one hour imbibed seeds to samples either harvested at the end of the imbibition phase (49 h), six hours (55 h) or twelve hours after a germination-inducing WL treatment (61 h) (see scheme Figure 36). Genes that were either significantly up - or downregulated (as calculated with the Bonferroni-method, $p \leq 0.05$) at least twofold compared to one hour imbibed wild-type seeds were selected and analyzed.

A large proportion of genes were commonly differentially regulated in all tested conditions, independent of illumination (3966 genes, Figure 38). As response to a WL stimulus, a set of 6352 DEGs was specifically regulated after 55 h or 61 h after the onset of imbibition in comparison to 1 h imbibed seeds (Figure 38).

For further analysis, transcripts differentially regulated in the wild type solely by imbibition (1009 genes, Figure 38) were excluded.

To sum this up, in accordance to other publications analyzing seed germination, a major reprogramming of the transcriptome occurs, both prior and after the induction of germination by a light pulse.

To gain a deeper insight into the biological processes in which the WL-regulated genes in wild type are involved, GO term analysis was performed. Genes differentially regulated after a WL pulse were enriched in GO terms involved in rRNA processing and metabolism, replication, ribosomes or ncRNA (Figure 38 B). But also GO terms related to mitochondria and chloroplasts, as well as peptide metabolism, gene expression and mRNA processing were identified, although less pronounced than the previously mentioned ones (Figure 38 B).

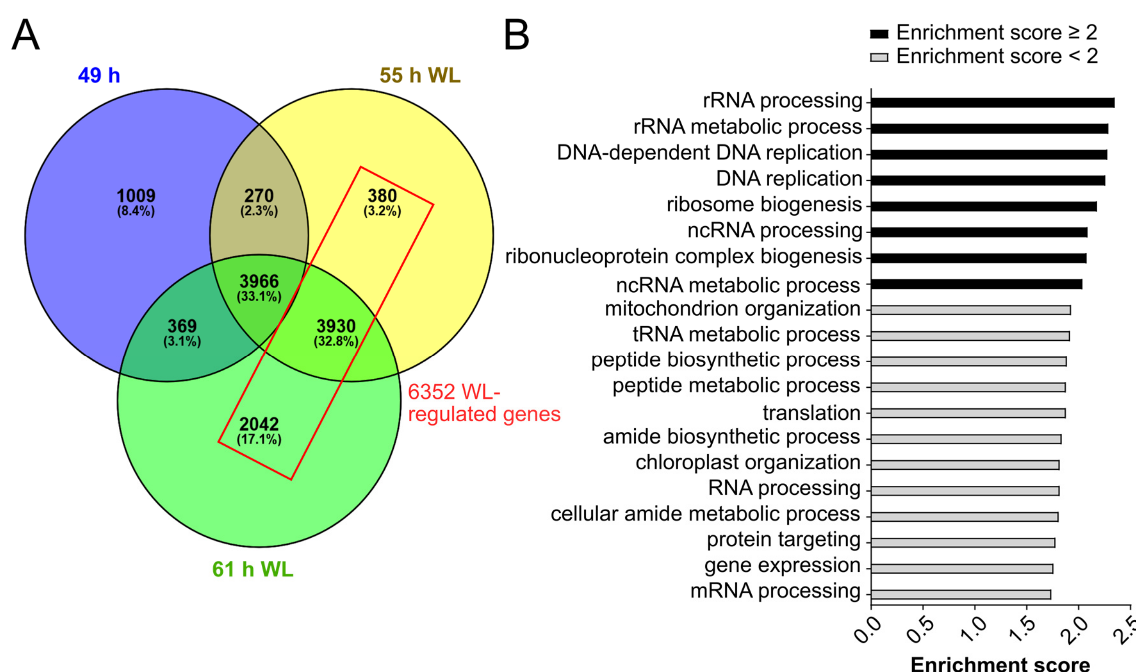

Figure 38. Venn diagram and GO term analysis of differentially expressed genes in wild-type seeds during imbibition and germination.
(A) Venn diagram showing the number of differentially expressed genes overlapping after imbibition (49 h), six hours (55 h WL) and twelve hours (61 h WL) after WL illumination, always compared to 1 h imbibed seeds. Only significantly regulated genes (Bonferroni; p-value ≤ 0.05) are depicted in the Venn diagram. Red box indicates genes specifically regulated after induction of germination by a WL pulse in wild-type seeds. (B) Gene Ontology analysis of WL-regulated genes in wild-type seeds. Gene lists included in the GO term analysis were indicated as red box in (A). Enrichment score abbreviates gene enrichment score.

In order to analyze and compare the wild type germination in WL conditions to the literature, the following part will focus on genes significantly upregulated in response to a WL pulse, downregulated genes were not considered further.

Overrepresented GO categories in the set of genes upregulated after a WL pulse were comparable to previous studies, analyzing *A. thaliana* seed germination in WL either with stratification prior to light treatment (Narsai *et al.*, 2011) or without stratification (Nakabayeshi *et al.*, 2005) (Figure 39).

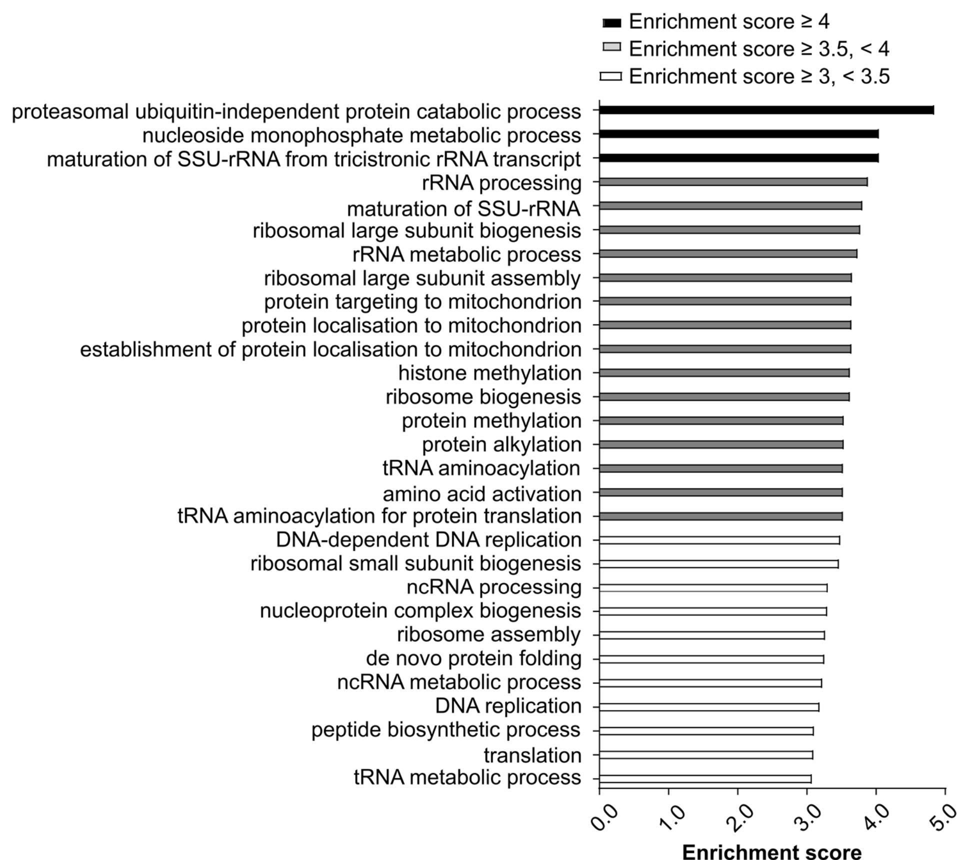

Figure 39. Gene Ontology analysis of genes upregulated in wild-type seeds after exposure to WL.
Only GO categories with the highest gene enrichment scores are included. Enrichment score abbreviates gene enrichment score.

In order to resolve the temporal aspect of gene regulation after a WL pulse in wild-type seeds, genes specifically upregulated early or late after a WL pulse were examined into more detail. Out of 380 DEGs specifically regulated six hours (early) after a WL pulse (55 h; Figure 38). The 15 strongest upregulated genes were examined closer (Table 18). Among them, three genes were functionally related to reproduction. *CUC1* has been described to be relevant for embryo development (Kamiuchi *et al.*, 2014), AT1G13230 for stimulation of seed production after infection with the symbiotic fungi *Piriformospora indica* (Shahollari *et al.*, 2007) and *MIR824A* is functionally relevant for the repression of flowering (Hu *et al.*, 2014). Neither these genes nor the other genes identified were described to be functionally relevant for seed germination.

Table 18. Highest upregulated genes in wild-type seeds, six hours after induction of germination by a WL pulse compared to seeds imbibed for 1 h.
Log_2 FC indicates log_2-fold change. Significant regulated genes were selected based on p-values ≤ 0.05 according to Bonferroni. Only the 15 highest upregulated genes were considered here. ATG numbers of genes mentioned in the text were indicated in bold.

ATG	Name	log_2 FC	p-value	short description
AT5G22580		6.63	0.0205	Stress responsive A/B Barrel Domain-containing protein
AT1G72416		6.58	0.0016	Chaperone DNA J-domain superfamily protein
AT2G40200		6.44	0.0030	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AT4G09500		6.21	0.0065	UDP-Glycosyltransferase superfamily protein
AT1G69160		6.06	0.0186	Suppressor
AT5G37500	*GORK*	6.01	0.0142	Gated outwardly-rectifying K channel
AT5G08185	*MIR162A*	5.91	0.0277	MicroRNA162A
AT3G52561		5.91	0.027	Hypothetical protein
AT3G15170	***CUC1***	**5.89**	**0.0482**	**NAC (No Apical Meristem) domain transcriptional regulator superfamily protein**
AT1G12070		5.81	0.043	Immunoglobulin E-set superfamily protein
AT5G12050		5.77	0.0473	Rho GTPase-activating protein
AT1G13230		**5.74**	**0.0455**	**Leucine-rich repeat (LRR) family protein**
AT1G31240		4.62	0.0034	Bromodomain transcription factor
AT4G24415	***MIR824A***	**4.44**	**0.0055**	**MicroRNA824A**
AT1G73850		4.38	0.0143	DNA ligase (DUF1666)

Twelve hours after induction of germination, a massive reprogramming of the transcriptome was measurable in wild-type seeds. In total, 2042 genes were differentially regulated twelve hours after the WL pulse was applied (61 h; Figure 38). Two genes, AT4G31330 and AT3G13760 were highly upregulated in WL germination and described to be downregulated after CK application (Rashotte *et al.*, 2003; Lee *et al.*, 2007). Also seed development associated genes were identified (Table 19), *BRCA2*(IV) and AT5G47330 have been described earlier to be involved in early embryo development (Pagnussat *et al.*, 2005; Yu, 2005).

Table 19. Highest upregulated genes in wild-type seeds, twelve hours after induction of germination by a WL pulse compared to seeds imbibed for 1 h.
Log2 FC indicates log2-fold change. Significant regulated genes were selected based on p-values ≤ 0.05 according to Bonferroni. Only the 15 highest upregulated genes were considered here. ATG numbers of genes mentioned in the text were indicated in bold.

ATG	Name	log_2 FC	p-value	short description
AT1G29080		9.52	2.9114E-10	Papain family cysteine protease
AT4G31330		**8.95**	**1.2983E-09**	**Transmembrane protein, putative**
AT5G55340		8.27	3.6748E-07	MBOAT (membrane bound O-acyl transferase) family protein
AT2G41475	*ATS3*	8.13	7.94E-07	Embryo-specific protein 3
AT4G12870		8.05	8.5792E-07	Gamma interferon responsive lysosomal thiol (GILT) reductase family protein
AT4G00020	***BRCA2(IV)***	**7.83**	**1.2882E-06**	**BREAST CANCER 2 like 2A**
AT4G08685	*SAH7*	7.77	2.588E-05	Pollen Ole e 1 allergen and extensin family protein
AT2G23010	*SCPL9*	7.75	4.1108E-06	Serine carboxypeptidase-like 9
AT5G03760	*ATCSLA09*	7.71	1.5001E-05	Nucleotide-diphospho-sugar transferases superfamily protein
AT3G62060		7.65	4.6395E-06	Pectinacetylesterase family protein
AT4G02330	*AtPMEPCRB*	7.61	7.1199E-06	Plant invertase/pectin methylesterase inhibitor superfamily
AT1G04020	*BARD1*	7.61	4.932E-06	Breast cancer associated RING 1
AT3G54560	*HTA11*	7.59	6.2786E-06	Histone H2A 11
AT2G23630	*SKS16*	7.58	7.698E-06	SKU5 similar 16
AT5G47330		**7.57**	**1.4249E-08**	**Alpha/beta-Hydrolases superfamily protein**
AT3G13760		**7.55**	**1.49E-05**	**Cysteine/Histidine-rich C1 domain family protein**

To summarize, analysis of transcripts regulated either shortly or later after a WL pulse, which is capable to induce germination of almost the entire wild-type seed population, revealed pronounced expression of seed development associated transcripts, but also genes linked to CK or flowering time were identified to be differentially regulated in wild-type seed germination induced by WL.

3.5.3.4 Imbibition induces only minor transcriptional alterations between wild-type and CK receptor mutant seeds

This part of the RNA-Seq analysis aimed to elucidate, if transcriptomic changes during imbibition between *ahk2 ahk3* and wild-type seeds are causal for the increased germination of *ahk2 ahk3* seeds in FR light. To do so, two different aspects were analyzed. On the one hand, differential gene expression between the genotypes after one hour and 49 h were analyzed (Figure 40 A). On the other hand, dynamics of transcriptomic changes in the imbibition phase were compared between the genotypes (Figure 40 B), setting the focus on transcriptomic changes induced by and functionally related to the imbibition process.

Interestingly, only 68 genes were differentially expressed after one hour and 49 h of imbibition between the genotypes (Figure 40 A). Due to this low number of DEGs, significantly overrepresented GO terms could not be identified. In one hour imbibed seeds, 33 genes were differentially regulated in the *ahk2 ahk3* mutant compared to wild-type, 20 of these genes were up- , while 13 genes were downregulated (Figure 40 , Table 20).

Eight DEGs were regulated both after one hour and 49 h of imbibition, four of them were upregulated (Table 21). In this set, none of the upregulated genes was related to known germination-, development- or imbibition pathways. Considering downregulated DEGs, *AHK3* and a gene of yet unknown function (AT4G04223) were identified as the strongest downregulated genes (Figure 40 A, Table 21) in *ahk2 ahk3* mutant seeds. At the end of the imbibition period, prior to the application of a germination-inducing light pulse, 26 genes were differentially regulated in the *ahk2 ahk3* mutant compared to wild-type seeds (Figure 40 A, Table 22). *FTM1/AAD6*, encoding a protein involved in fatty acid metabolism (Jin *et al.*, 2017), was the highest upregulated transcript in *ahk2 ahk3* seeds at the end of the imbibition phase. Other genes upregulated at the end of the imbibition period in *ahk2 ahk3* seeds were not associated directly with seed development or germination pathways. The major proportion of DEGs at the end of the imbibition period was upregulated, while only five genes were downregulated in CK receptor double mutant seeds (Table 22). Among the downregulated genes, *AHK2* was identified.

Next, DEGs regulated during the process of germination in both genotypes was monitored. In total, 4819 DEGs were regulated both in wild-type and in *ahk2 ahk3* mutant seeds (Figure 40 B), equaling 72 % of all DEGs identified here. This supports the notion that imbibition is similarly regulated in both genotypes.

In sum, imbibition in both wild-type and *ahk2 ahk3* mutant seeds seems to be regulated similarly on transcriptional level. Based on the presented data, it seems unlikely that transcriptional changes evident during the imbibition process are causal for the observed differences in FR light induced germination between the genotypes.

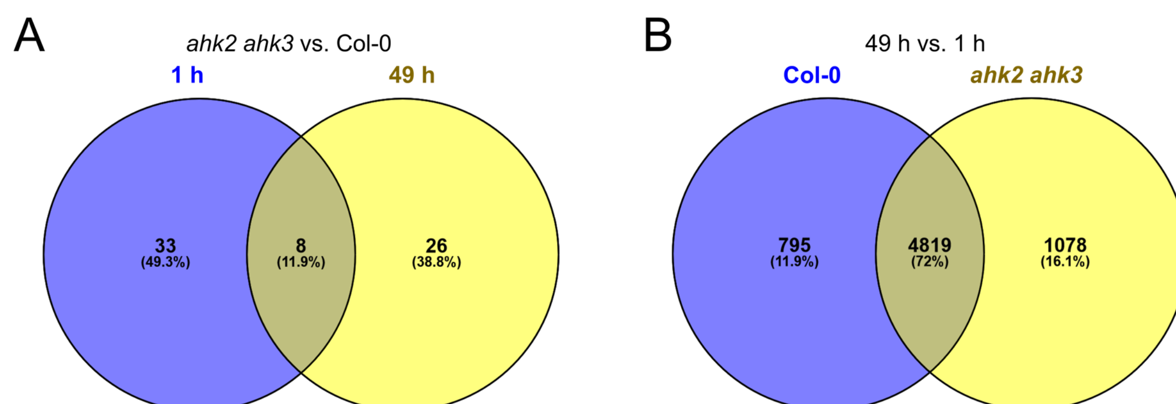

Figure 40. Venn diagram of differentially expressed genes during imbibition.
Depicted in (A) are DEGs exclusively regulated in *ahk2 ahk3* compared to wild type after 1 h, DEGs expressed after 1 h and 49 h (overlap) and DEGs exclusively regulated in *ahk2 ahk3* compared to wild type after 49 h of imbibition. In (B) differentially regulated genes in the course of imbibition in wild-type and *ahk2 ahk3* mutant seeds are plotted. Only significantly regulated genes (Bonferroni; p-value ≤ 0.05) are depicted in the Venn diagrams.

Table 20. Differentially expressed genes in *ahk2 ahk3* seeds compared to wild-type seeds after 1 h of imbibition.
Log_2 FC indicates log_2-fold change. Significant regulated genes were selected based on $p \leq 0.05$ according to Bonferroni. ATG numbers of genes mentioned in the text were indicated in bold.

ATG	Name	log_2 FC	p-value	short description
AT3G25290		2.13	0.01600861	Auxin-responsive family protein
AT2G28550	*RAP2.7*	1.77	0.02524783	Related to AP2.7
AT5G25190	*ESE3*	1.56	0.00012024	Integrase-type DNA-binding superfamily protein
AT5G19520	*MSL9*	1.52	0.00033216	Mechanosensitive channel of small conductance-like 9
AT2G20300	*ALE2*	1.45	0.00093391	Protein kinase superfamily protein
AT1G68240		1.43	0.00857911	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AT1G48600	*PMEAMT*	1.42	0.04908354	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
AT3G19820	*DWF1*	1.40	2.5201E-08	Cell elongation protein / DWARF1 / DIMINUTO (DIM)
AT3G21090	*ABCG15*	1.35	0.00307811	ABC-2 type transporter family protein
AT2G19800	*MIOX2*	1.34	0.00131217	Myo-inositol oxygenase 2
AT2G17230	*EXL5*	1.23	7.746E-06	EXORDIUM like 5
AT2G41370	*BOP2*	1.21	0.00104048	Ankyrin repeat family protein / BTB/POZ domain-containing protein
AT1G77760	*NIA1*	1.20	1.6046E-05	Nitrate reductase 1
AT2G41560	*ACA4*	1.19	0.00020235	Autoinhibited Ca (2) -ATPase, isoform 4
AT3G14220		1.18	0.00295686	GDSL-like Lipase/Acylhydrolase family protein
AT2G39420		1.17	0.00012484	Alpha/beta-Hydrolases superfamily protein
AT2G06925	*PLA2-ALPHA*	1.08	0.00846844	Phospholipase A2 family protein
AT5G25460	*DGR2*	1.04	0.0206882	Transmembrane protein, putative (Protein of unknown function, DUF642)
AT5G56860	*GNC*	1.04	4.9236E-07	GATA type zinc finger transcription factor family protein
AT2G26330	*ER*	1.02	0.03239308	Leucine-rich receptor-like protein kinase family protein
AT3G07650	*COL9*	-1.02	0.00253076	CONSTANS-like 9
AT3G05165		-1.09	0.00288542	Major facilitator superfamily protein
AT1G36370	*SHM7*	-1.11	1.433E-05	Serine hydroxymethyltransferase 7
AT1G77450	*NAC032*	-1.13	0.00179691	NAC domain containing protein 32
AT5G01820	*SR1*	-1.15	0.00059486	Serine/threonine protein kinase 1
AT5G67080	*MAPKKK19*	-1.17	0.00025487	Mitogen-activated protein kinase kinase kinase 19
AT2G01280	*MEE65*	-1.22	2.0185E-07	Cyclin/Brf1-like TBP-binding protein
AT4G21990	*APR3*	-1.22	1.1926E-10	APS reductase 3
AT1G11440		-1.29	0.01325506	Hypothetical protein
ATCG00020	*PSBA*	-1.30	5.3758E-08	Photosystem II reaction center protein A
AT1G62170		-1.40	0.04659797	Serine protease inhibitor (SERPIN) family protein
AT5G40670		-1.43	9.082E-06	PQ-loop repeat family protein / transmembrane family protein
AT1G62180	*APR2*	-1.46	1.8929E-13	5' Adenylylphosphosulfate reductase 2

Table 21. Differentially expressed genes in *ahk2 ahk3* compared to wild-type seeds both after 1 h and 49 h of imbibition.
Log_2 FC (49 h) indicates log_2-fold change after 49 h of imbibition, 1 h timepoints were not included. Significant regulated genes were selected based on p-values ≤ 0.05 according to Bonferroni. ATG numbers of genes mentioned in the text were indicated in bold.

ATG	Name	log_2 FC (49 h)	p-value	short description
AT2G01422		4.22	2.8021E-93	
AT1G53480	*MRD1*	2.79	1.7876E-14	Mto 1 responding down 1
AT1G53490	*HEI10*	2.08	2.993E-22	RING/U-box superfamily protein
AT2G25980		1.09	4.5796E-09	Mannose-binding lectin superfamily protein
AT1G67105		-1.78	8.6171E-09	
AT5G33393		-2.87	1.2098E-22	Hypothetical protein
AT4G04223		**-3.69**	**2.3801E-10**	
AT1G27320	***HK3***	**-4.95**	**7.968E-185**	**Histidine kinase 3**

Table 22. Differentially expressed genes in *ahk2 ahk3* seeds compared to wild-type seeds after 49 h of imbibition.
Log_2 FC indicates log_2-fold change. Significant regulated genes were selected based on p-values ≤ 0.05 according to Bonferroni. ATG numbers of genes mentioned in the text were indicated in bold.

ATG	Name	log_2 FC	p-value	short description
AT1G43800	***FTM1***	**4.60**	**0.00016877**	**Plant stearoyl-acyl-carrier-protein desaturase family protein**
AT5G12020	*HSP17.6II*	4.09	0.0065882	17.6 kDa class II heat shock protein
AT2G16060	*HB1*	2.71	0.04868672	Hemoglobin 1
AT3G20110	*CYP705A20*	2.22	0.0091623	Cytochrome P450, family 705, subfamily A, Polypeptide 20
AT1G23410		2.02	0.0145733	Ribosomal protein S27a / Ubiquitin family protein
AT2G40970	*MYBC1*	1.93	0.02905427	Homeodomain-like superfamily protein
AT4G27170	*SESA4*	1.62	0.00031934	Seed storage albumin 4
AT2G19590	*ACO1*	1.61	0.02414891	ACC oxidase 1
AT3G48440		1.50	0.03825789	Zinc finger C-x8-C-x5-C-x3-H type family protein
AT5G46280	*MCM3*	1.46	0.03113616	Minichromosome maintenance family protein
AT1G12805		1.39	0.02251179	Nucleotide binding protein
AT1G65370		1.35	0.01017564	TRAF-like family protein
AT1G54000	*GLL22*	1.34	0.00431314	GDSL-like Lipase/Acylhydrolase superfamily protein
AT1G28100		1.34	4.3332E-12	Hypothetical protein
AT1G64970	*G-TMT*	1.30	0.02037684	Gamma-tocopherol methyltransferase
AT1G11580	*PMEPCRA*	1.29	0.04310371	Methylesterase PCR A
AT5G14920		1.23	0.00276414	Gibberellin-regulated family protein
AT3G23800	*SBP3*	1.14	0.0001704	Selenium-binding protein 3
AT5G51750	*SBT1.3*	1.10	1.5394E-05	Subtilase 1.3
AT3G52290	*IQD3*	1.07	0.04179501	IQ-domain 3
AT1G28110	*SCPL45*	1.06	0.00199902	Serine carboxypeptidase-like 45

Table 22 continued.

AT4G39800	*MIPS1*	-1.15	0.00037807	Myo-inositol-1-phosphate synthase 1
AT1G52560		-1.18	0.0001752	HSP20-like chaperones superfamily protein
AT5G35750	***HK2***	**-1.34**	**3.7903E-05**	**Histidine kinase 2**
AT5G33390		-3.73	1.4274E-09	Glycine-rich protein
AT2G24940	*MAPR2*	-23.37	0.03367275	Membrane-associated progesterone binding protein 2

3.5.3.5 Transcriptomic changes during seed germination in response to FR light depend on the seeds' CK status

After evaluation and comparison of transcriptomic changes between *ahk2 ahk3* and wild-type seeds during the imbibition process, the next part of the analysis focused on FR light induced changes in gene expression over time. It was hypothesized that transcriptional changes caused by the reduced CK perception in *ahk2 ahk3* mutants occur after FR light perception.

First of all, DEGs significantly up- or downregulated in *ahk2 ahk3* seeds compared to wild type at different time points, both prior (1 h and 49 h) and after (55 h and 61 h) the application of a FR light pulse, were analyzed (Figure 41). Seven genes were commonly differentially regulated between the genotypes in all conditions, independent of an inducing FR light pulse (Figure 41). Every single of these seven genes, including *AHK3*, were already differentially regulated between the genotypes during imbibition (Table 21). This indicates that the differential regulation of the respective transcripts is related to the genotype rather than the treatment, so these genes were not analyzed further because the treatment effect cannot be determined with certainty.

Furthermore, three genes were commonly differentially regulated after imbibition as well as six and twelve hours after FR light application (Figure 41). In contrast to the abovementioned seven genes, the respective genes were not differentially expressed in one hour imbibed seeds. Again, all three genes, including *AHK2*, were already included in the list of genes differentially regulated after 49 h of imbibition (Table 22) and not analyzed further here.

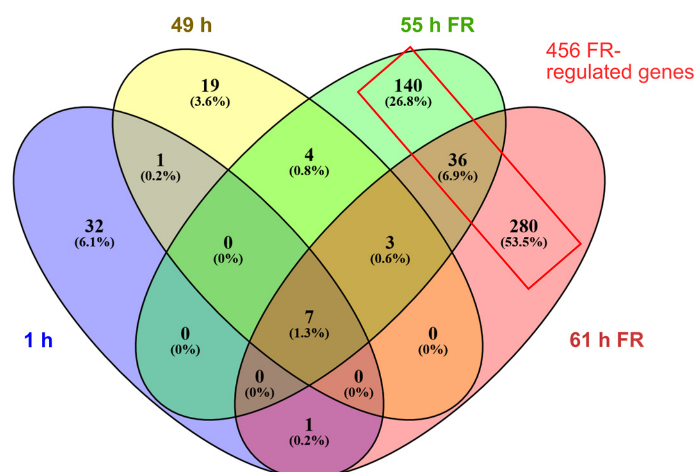

Figure 41. Transcripts differentially regulated in *ahk2 ahk3* seeds compared to wild type at different timepoints prior (1 h and 49 h) and after induction of germination with a FR light pulse (55 h and 61 h).

Figure 41 continued.
Only significantly regulated genes (Bonferroni; p-value ≤ 0.05) are depicted in the Venn diagram. Red box indicates genes differentially regulated in *ahk2 ahk3* seeds after FR light application, irrespective of the timepoint.

In the following, DEGs deregulated as a consequence of FR light application were studied more closely. In total, 456 genes were shown to be differentially expressed after a germination-inducing FR light pulse (red box, Figure 41). Among them, 140 genes were exclusively up- or downregulated in *ahk2 ahk3* seeds six hours after FR light application (Figure 41). The number of DEGs exclusively regulated in *ahk2 ahk3* seeds in response to a FR light pulse doubled twelve hours after light application, 280 DEGs were identified (Figure 41). 36 genes were identified in the overlap, indicating differential expression both six and twelve hours after FR light application (Figure 41).

Analysis of the entire FR light regulated set of genes revealed an enrichment in GO terms involved in seed oilbody biogenesis, lipid storage, seed maturation and maintenance of location (Figure 42). Less pronounced, but still clearly overrepresented were transcripts classified into functional groups related to water deprivation and ABA, but also related to development, lipids and response to external stimuli, coldness in this case (Figure 42). These results provide valuable information by stressing pathways relevant for germination, which were up to now not directly related to light or seed germination in a broader sense.

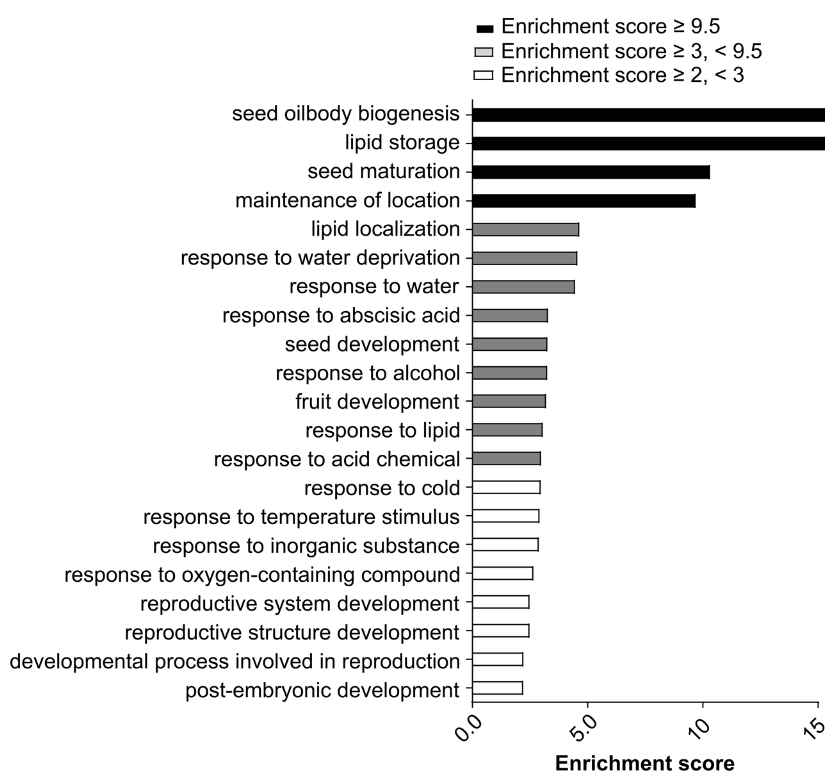

Figure 42. Gene Ontology analysis of FR-associated transcripts differentially regulated in *ahk2 ahk3* mutant seeds compared to wild type.
Only GO categories with the highest gene enrichment scores are included.

To refine the analysis and investigate differentially abundant transcripts into more detail, the lists of FR light associated transcripts, which may still contain genes differentially regulated in *ahk2 ahk3* mutant seeds solely during imbibition, were re-analyzed. In order to exclude transcripts already deregulated in *ahk2 ahk3* seeds during imbibition, these genes were subtracted from the set of FR light-regulated genes in *ahk2 ahk3* mutant seeds (Figure 43).

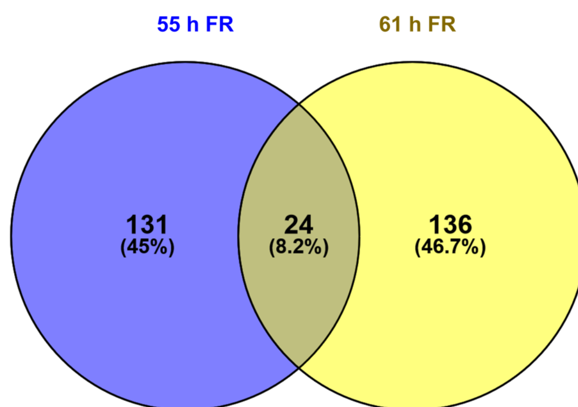

Figure 43. Refined analysis of FR light-regulated DEGs.
Out of 456 transcripts differentially regulated in FR light, transcripts already deregulated in *ahk2 ahk3* seeds during imbibition were subtracted, and the remaining transcripts plotted. Only significantly regulated genes (Bonferroni; p-value ≤ 0.05) are depicted in the Venn diagram.

Among the FR light regulated set of transcripts, 131 genes were differentially regulated exclusively six hours after perceiving FR light (Figure 43).

Out of 131 transcripts the 15 highest up- or downregulated genes six hours after FR light application in *ahk2 ahk3* seeds were examined closer. It is interesting to note that among the highest upregulated genes (Table 23), environmental stress-associated genes were present like *HSP18.2*, *RCI2B* and AT2G37130, which are associated with heat-, cold- or pathogen stress, respectively (Medina *et al.*, 2001; Chassot *et al.*, 2007; Liu *et al.*, 2013). Furthermore, genes encoding proline-rich proteins were found (*PRP2, PRP4* and AT1G23720) (Table 23). Downregulated genes were associated with phosphate transport (*PHT5.2*) (Liu *et al.*, 2016), auxin- and CK (*JLO* and *NAC067*, respectively), whereby only *JLO* has been described to be functionally relevant for embryo development (Kim *et al.*, 2006; Hsieh *et al.*, 2011) (Table 24).

Table 23. Highest upregulated genes six hours after application of FR-light in *ahk2 ahk3* seeds compared to wild-type.
Log_2 FC indicates log_2-fold change. Significant regulated genes were selected based on $p \leq 0.05$ according to Bonferroni. Only the 15 highest upregulated genes were considered here. ATG numbers of genes mentioned in the text were indicated in bold.

ATG	Name	log_2 FC	p-value	short description
AT3G16410	*NSP4*	6.41	0.00965	Nitrile specifier protein 4
AT2G21140	***PRP2***	**6.20**	**0.00115**	**Proline-rich protein 2**
AT5G46900		4.58	0.02409	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT1G12080		4.56	0.02182	Vacuolar calcium-binding protein-like protein
AT4G30170		4.34	0.00033	Peroxidase family protein
AT3G16240	*DELTA-TIP*	4.29	0.02431	Delta tonoplast integral protein
AT1G67328		4.27	0.00009	Natural antisense transcript overlaps with AT1G67330
AT3G05890	***RCI2B***	**4.21**	**0.01234**	**Low temperature and salt responsive protein family**
AT3G23050	*IAA7*	4.18	0.01416	Indole-3-acetic acid 7
AT4G38770	***PRP4***	**4.16**	**0.00035**	**Proline-rich protein 4**
AT2G37130		**4.13**	**0.00786**	**Peroxidase superfamily protein**
AT5G59720	***HSP18.2***	**4.13**	**0.02714**	**Heat shock protein 18.2**
AT1G23720		**4.11**	**0.00166**	**Proline-rich extensin-like family protein**
AT3G23810	*SAHH2*	3.91	0.01592	S-adenosyl-l-homocysteine hydrolase 2
AT5G44020		3.89	0.00267	HAD superfamily, subfamily IIIB acid phosphatase

Table 24. Downregulated genes six hours after application of FR-light in *ahk2 ahk3* seeds compared to wild-type.
Log_2 FC indicates log_2-fold change. Significant regulated genes were selected based on $p \leq 0.05$ according to Bonferroni. ATG numbers of genes mentioned in the text were indicated in bold.

ATG	Name	log_2 FC	p-value	short description
AT4G11810	***PHT5.2***	**-1.69**	**0.0224818**	**Major Facilitator Superfamily with SPX (SYG1/Pho81/XPR1) domain-containing protein**
AT4G01520	***NAC067***	**-1.54**	**0.0000047**	**NAC domain containing protein 67**
AT4G28790		-1.42	0.0010188	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AT5G24380	*YSL2*	-1.31	0.0041653	YELLOW STRIPE like 2
AT1G02405		-1.27	0.0010915	Proline-rich family protein
AT2G19310		-1.13	0.0000384	HSP20-like chaperones superfamily protein
AT1G12064		-1.13	0.0000153	Transmembrane protein
AT3G54366		-1.12	0.0000001	
AT4G00220	***JLO***	**-1.09**	**0.0028351**	**Lateral organ boundaries (LOB) domain family protein**
AT3G11600		-1.08	0.0114261	E3 ubiquitin-protein ligase
AT5G65590		-1.06	0.0002667	Dof-type zinc finger DNA-binding family protein
AT5G06270		-1.02	0.0002945	Hypothetical protein

Transcripts deregulated both six and twelve hours after application of a germination-inducing FR light pulse were particularly interesting, since differential expression of these genes might influence the germination response both in early and later stages of germination in response to FR light in CK receptor mutant seeds. 24 genes were identified to be specifically regulated both at the 55 h and 61 h timepoint (Figure 43). The FR light-induced regulation of the genes identified here was consistent after six or twelve hours, meaning that genes downregulated early after the FR light pulse were downregulated after twelve hours as well (data not shown).

Notably, seven genes were upregulated, but 17 genes were downregulated both short and long after FR light application (Table 25). Upregulated genes were associated with cell wall organization and release of mucilage from the seed coat (*EXT4*, *BGAL4* and *BXL1*). Among the downregulated genes, *SOM* and *NCED9* were identified, two genes well described in germination (Seo *et al.*, 2006; Kim *et al.*, 2008). SOM acts as a key negative regulator of germination downstream of PIF1 (Kim 2008) and *NCED9* encodes a key enzyme in ABA biosynthesis and is transcriptionally repressed in the seed germination process (Seo *et al.*, 2006; Oh *et al.*, 2007). The two strongest downregulated genes in *ahk2 ahk3* compared to wild-type were *MEE8,* a maternal effect embryo arrest factor (Cho *et al.*, 2011) and an ABA-importer encoding gene called *AIT3*, which also transports GA_3 (Kanno *et al.*, 2012) (Table 25).

Table 25. Differentially expressed genes six hours (55 h) and twelve hours (61 h) after FR light application in *ahk2 ahk3* seeds compared to wild type.
Log_2 FC indicates log_2-fold change. Significant regulated genes were selected based on $p \leq 0.05$ according to Bonferroni. ATG numbers of genes mentioned in the text were indicated in bold.

ATG	Name	log_2 FC (55 h)	p-value	short description
AT1G76930	***EXT4***	**3.58**	**7.0705E-10**	**Extensin 4**
AT5G47330	*PPT1*	3.07	0.01785689	Alpha/beta-hydrolases superfamily protein
AT5G56870	***BGAL4***	**2.54**	**0.00366691**	**Beta-galactosidase 4**
AT5G35630	*GS2*	2.40	0.04798331	Glutamine synthetase 2
AT5G49360	***BXL1***	**1.75**	**0.00086415**	**Beta-xylosidase 1**
AT2G44500		1.42	2.3987E-07	O-fucosyltransferase family protein
AT4G35770	*SEN1*	1.23	0.03021743	Rhodanese/Cell cycle control phosphatase superfamily protein
AT1G15330		-1.00	0.00052121	Cystathionine beta-synthase (CBS) protein
AT5G61820		-1.01	0.00022283	Stress up-regulated Nod 19 protein
AT1G48990		-1.02	0.00014044	Oleosin family protein
AT1G03790	***SOM***	**-1.03**	**8.1132E-06**	**Zinc finger C-x8-C-x5-C-x3-H type family protein**
AT1G78390	***NCED9***	**-1.05**	**0.0362821**	**Nine-cis-epoxycarotenoid dioxygenase 9**
AT3G56350		-1.07	0.0426192	Iron/manganese superoxide dismutase family protein
AT1G17810	*BETA-TIP*	-1.13	0.02390822	Beta-tonoplast intrinsic protein
AT4G22390		-1.14	0.00950765	F-box associated ubiquitination effector family protein
AT1G22500	*ATL15*	-1.17	0.00202221	RING/U-box superfamily protein

Table 25 continued.

AT1G67100	*LBD40*	-1.18	2.4772E-07	LOB domain-containing protein 40
AT4G37390	*BRU6*	-1.24	6.7363E-06	Auxin-responsive GH3 family protein
AT2G31980	*CYS2*	-1.25	5.7844E-08	PHYTOCYSTATIN 2
AT5G55370		-1.25	1.6272E-07	MBOAT (membrane bound O-acyl transferase) family protein
AT1G71250		-1.26	3.8505E-10	GDSL-like Lipase/Acylhydrolase superfamily protein
AT1G70840	*MLP31*	-1.27	0.04672118	MLP-like protein 31
AT1G25310	***MEE8***	**-1.46**	**6.4503E-05**	**Basic helix-loop-helix (bHLH) DNA-binding family protein**
AT3G25260	***AIT3***	**-1.65**	**1.7268E-05**	**Major facilitator superfamily protein**

Twelve hours after FR light application, a set of 136 regulated genes was identified (Figure 9). In this set, genes specifically upregulated in *ahk2 ahk3* seeds (Table 26) were associated amongst others with the cell wall. For example, the most highly upregulated gene, *CASP5* is functionally relevant in the context of casparian stripe formation in roots (Roppolo *et al.*, 2011). But also *GATL3*, *EXPA6* and *AGAL2* were described in association with cell wall modifications (Iglesias *et al.*, 2006; Chrost *et al.*, 2007; Kong *et al.*, 2011). Moderately upregulated genes were described in embryo development and consequently expressed in developing embryos (AT5G01870) (Spencer *et al.*, 2007) (Table 26).

Table 26. Transcripts upregulated twelve hours after application of FR-light in *ahk2 ahk3* seeds compared to wild-type.
Log_2 FC indicates log_2-fold change. Significant regulated genes were selected based on p-values ≤ 0.05 according to Bonferroni. ATG numbers of genes mentioned in the text were indicated in bold.

ATG	Name	log_2 FC	p-value	short description
AT5G15290	***CASP5***	**7.22**	**0.01308201**	**Uncharacterized protein family (UPF0497)**
AT3G48260	*WNK3*	2.62	3.4065E-05	With no lysine (K) kinase 3
AT1G35420		2.02	0.0072556	Alpha/beta-Hydrolases superfamily protein
AT1G13250	***GATL3***	**2.00**	**0.00691995**	**Galacturonosyltransferase-like 3**
AT1G50630		1.75	0.00731293	Extracellular ligand-gated ion channel protein
AT5G01870		**1.74**	**0.00152994**	**Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein**
AT4G12870		1.73	0.00399019	Gamma interferon responsive lysosomal thiol (GILT) reductase family protein
AT5G45680	*FKBP13*	1.62	0.00442363	FK506-binding protein 13
AT4G21870		1.56	5.9529E-07	HSP20-like chaperones superfamily protein
AT4G01700		1.56	2.3771E-07	Chitinase family protein
AT2G28950	***EXPA6***	**1.53**	**0.01818972**	**Expansin A6**
AT2G39350	*ABCG1*	1.50	0.0011139	ABC-2 type transporter family protein
AT2G27385		1.49	0.00051544	Pollen Ole e 1 allergen and extensin family protein
AT5G08370	***AGAL2***	**1.49**	**0.00382021**	**Alpha-galactosidase 2**
AT3G07010		1.37	0.00098028	Pectin lyase-like superfamily protein

Among the strongest downregulated genes twelve hours after FR light, again cell wall associated genes (AT5G44360 and AT4G36700) (Irshad *et al.*, 2008), but also a negative regulator of seed germination (*DOG1-like 3*, AT4G18690) were identified (Table 27) (Bentsink *et al.*, 2006). Furthermore, also transcripts of late embryogenesis-related genes as *ECP63* and *EM1* were downregulated in *ahk2 ahk3* seeds. In addition, *EM1* was shown to be ABA inducible and its expression was repressed in the course of germination (Manfre, 2005).

Table 27. Transcripts downregulated twelve hours after application of FR-light in *ahk2 ahk3* seeds compared to wild-type.
Log_2 FC indicates log_2-fold change. Significant regulated genes were selected based on p-values ≤ 0.05 according to Bonferroni. ATG numbers of genes mentioned in the text were indicated in bold.

ATG	Name	log_2 FC	p-value	short description
AT4G28800		-3.29	0.00962552	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AT5G44360		**-2.08**	**2.1683E-05**	**Oxido-reductase**
AT2G36640	***ECP63***	**-2.07**	**6.8665E-24**	**Embryonic cell protein 63, Late embryogenesis-related**
AT4G18690		**-2.04**	**0.00123545**	**DOG1-like 3**
AT4G09590	*NHL22*	-2.00	1.972E-09	NDR1/HIN1-like 22
AT3G51810	***EM1***	**-1.98**	**2.5336E-17**	**Stress induced protein**
AT2G02930	*GSTF3*	-1.98	5.1036E-09	Glutathione S-transferase F3
AT4G36700		**-1.92**	**2.1897E-13**	**RmlC-like cupins superfamily protein**
AT1G13340		-1.87	7.3376E-09	Regulator of Vps4 activity in the MVB pathway protein
AT2G25890		-1.83	2.6461E-09	Oleosin family protein
AT5G25180	*CYP71B14*	-1.82	1.483E-12	Cytochrome P450, family 71, subfamily B, polypeptide 14
AT4G01970	*STS*	-1.81	8.5344E-12	Stachyose synthase
AT5G18450		-1.76	1.7346E-06	Integrase-type DNA-binding superfamily protein
AT5G38780		-1.75	8.2225E-05	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
AT4G10490		-1.73	9.2793E-07	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein

3.5.3.6 Analysis of transcriptional changes associated with WL or FR light induced germination

This part of the RNA-Seq analysis aims to track and compare the transcriptional responses of wild-type and *ahk2 ahk3* mutant seeds which were able to complete germination in the respective light environment. So data from ahk2 ahk3 seeds treated with WL or FR light and wild-type seeds treated with a WL pulse were analyzed here. Wild-type seeds treated with FR light were not considered. The focus of the analysis was set to transcriptomic changes early (six hours) after the application of the second, germination-inducing light pulse (see scheme Figure 36).

In order to exclude transcriptional changes occurring as a consequence of imbibition, the transcriptome of 55 h imbibed seeds treated with either FR or WL were compared to 49 h imbibed seeds. Identified DEGs were plotted into a Venn diagram (Figure 44).

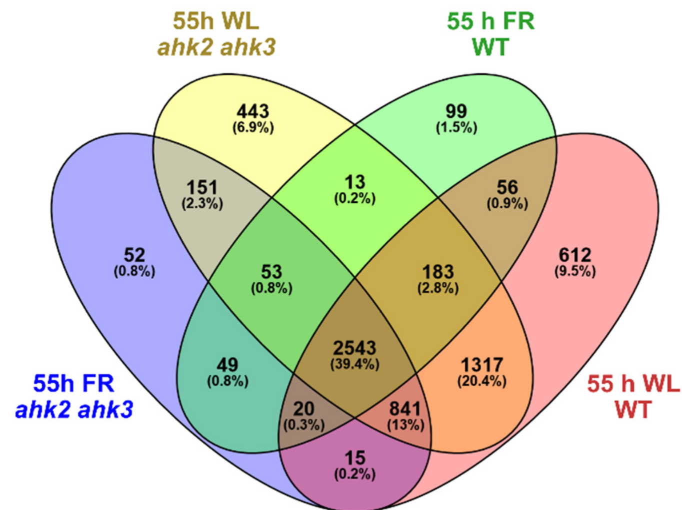

Figure 44. Venn diagram of transcripts differentially regulated in 55 h imbibed seeds. Differentially expressed genes in wild-type and *ahk2 ahk3* mutant seeds six hours after a WL or a FR light pulse, relative to 49 h imbibed seeds of the respective genotype were plotted. Only significantly regulated genes (Bonferroni; p-value ≤ 0.05) are depicted in the Venn diagram.

The largest proportion of transcripts (2543, equaling 39.4 % of all DEGs in this set) were commonly differentially regulated in all conditions and genotypes tested here, pointing either to a light quality-independent regulation, or a late imbibition-related regulation. Therefore, these genes were not analyzed into more detail.

Genes regulated in samples where high germination rates in both genotypes were expected (based on the germination assay conducted previously) were analyzed into more detail. The comparison of DEGs in *ahk2 ahk3* seeds treated with a FR light or WL pulse and in wild-type seeds treated with WL revealed an overlap of 841 genes (Figure 44). GO term analysis of the 841 gene overlap between these samples revealed an overrepresentation of transcripts associated with responses to abiotic stimuli, especially stress stimuli (Figure 45).

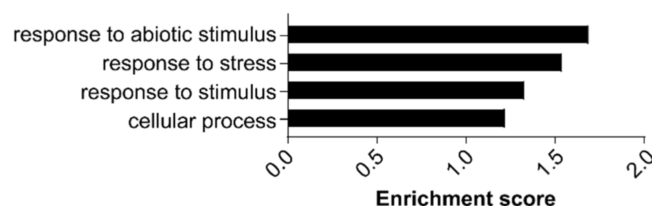

Figure 45. Gene Ontology analysis of germination-related genes six hours after illumination. Classification of 841 DEGs overlapping in *ahk2 ahk3* mutant seeds in FR and in WL and in wild-type seeds subjected to WL treatment.

Taking a closer look at the highest upregulated genes among the 841 DEGs identified in germinating wild-type and *ahk2 ahk3* mutant seeds, transcripts associated with CK and ABA were most prominent. *AHP6*, encoding a negative regulator of CK signaling, was identified among the strongest upregulated transcripts in seeds able to complete germination (Table 28). Also *GIS*, which was functionally characterized in the context of CK and GA-dependent trichome formation (Yan *et al.*, 2012), and two SAUR-like auxin responsive transcript were strongly upregulated in seeds likely to complete germination. In addition, *NCED3*, which is associated with ABA biosynthesis in water-stressed plants (Tan *et al.*, 2003), was upregulated (Table 28). In contrast to other members of the NCED family, which were more prominent in the regulation of seed germination, *NCED3* expression levels remained low during seed maturation and development (Frey *et al.*, 2012). Furthermore, *LPP2* was upregulated in seeds likely to germinate. As an integrator of the ABA signal, LPP2 has been described to act upstream of ABI4 in ABA signaling (Katagiri *et al.*, 2005). To summarize this part, both CK and ABA-related pathways seem to be differentially expressed in seeds able to complete germination.

Table 28. Transcripts upregulated in *ahk2 ahk3* seeds after application of FR- or WL pulse and in wild-type seeds treated with WL.
Log_2 FC indicates log_2-fold change compared to imbibed seeds of the respective genotype (49 h). Significant regulated genes were selected based on p-values ≤ 0.05 according to Bonferroni. ATG numbers of genes mentioned in the text were indicated in bold.

ATG	Name	log_2 FC *ahk2 ahk3* FR 55 h	p-value *ahk2 ahk3* FR 55 h	log_2 FC *ahk2 ahk3* WL 55 h	p-value *ahk2 ahk3* WL 55 h	log_2 FC wild type WL 55 h	p-value wild type WL 55 h	short description
AT5G01881		6.79	7.50E-04	7.42	3.34E-05	6.49	0.00312476	Transmembrane protein
AT3G58070	***GIS***	**4.24**	**1.38E-03**	**5.14**	**9.17E-07**	**3.28**	**0.00019186**	**C2H2 and C2HC zinc fingers superfamily protein**
AT1G75580		4.16	4.39E-02	4.98	2.32E-04	3.34	0.00084999	SAUR-like auxin-responsive protein family
AT1G15080	***LPP2***	**3.74**	**5.94E-06**	**4.31**	**5.50E-09**	**4.35**	**4.4309E-06**	**Lipid phosphate phosphatase 2**
AT1G80100	***HP6***	**3.54**	**2.22E-05**	**3.48**	**4.23E-05**	**3.22**	**0.04403927**	**Histidine phosphotransfer protein 6**
AT5G05490	*SYN1*	3.48	1.61E-02	3.71	2.62E-03	3.98	0.02415645	Rad21/Rec8-like family protein
AT4G31320		**3.40**	**2.98E-04**	**3.76**	**5.94E-06**	**3.38**	**0.00035848**	**SAUR-like auxin-responsive protein family**
AT3G22100		3.39	4.15E-02	3.93	5.61E-04	3.48	0.01952599	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AT3G14440	***NCED3***	**3.34**	**1.34E-02**	**3.97**	**5.12E-05**	**3.79**	**0.00269707**	**Nine-cis-epoxycarotenoid dioxygenase 3**
AT3G48280	*CYP71A25*	3.07	3.00E-02	3.48	8.04E-04	4.50	0.00063869	Cytochrome P450, family 71, subfamily A, polypeptide 25
AT2G37560	*ORC2*	3.00	2.23E-07	3.20	7.47E-09	3.85	0.00959591	Origin recognition complex second largest subunit 2
AT5G37500	*GORK*	2.98	6.81E-06	3.48	2.93E-09	3.05	7.0577E-05	Gated outwardly-rectifying K channel
AT5G24660	*LSU2*	2.90	1.09E-04	4.09	1.50E-12	3.82	8.5384E-15	Response to low sulfur 2
AT3G19850		2.86	7.70E-03	3.50	7.64E-06	3.92	0.00011633	Phototropic-responsive NPH3 family protein
AT2G40150	*TBL28*	2.73	2.12E-04	3.08	1.53E-06	2.66	0.00114565	TRICHOME BIREFRINGENCE-LIKE 28
AT4G34790		**2.50**	**1.02E-02**	**3.96**	**1.06E-11**	**4.58**	**1.9531E-11**	**SAUR-like auxin-responsive protein family**
AT5G56320	EXPA14	2.45	1.02E-04	2.41	1.79E-04	2.41	0.02428981	Expansin A14

Aside from the above described set of genes, the Venn diagram (Figure 44) revealed a set of 52 genes exclusively deregulated in *ahk2 ahk3* mutant seeds six hours after a germination-inducing FR light pulse. These genes may be decisive for enhanced germination of CK receptor mutant seeds in response to a FR light pulse, therefore these set was examined into more detail. Due to the low number of DEGs, GO term analysis revealed no significantly enriched groups, therefore, the highest upregulated genes were analyzed.

The strongest upregulated gene, AT2G34010, encodes a protein of unknown function. It was described previously as a homologue of a TOPLESS (TPL) corepressor in yeast (Chen *et al.*, 2014). A development-associated gene, *CDKD1;1* was upregulated specifically after FR light treatment in CK receptor mutant seeds. *cdkd1;1* mutants were described as defective in post-embryonic development (Shimotohno *et al.*, 2006; Takatsuka *et al.*, 2015) Furthermore, the hormone transporter *AZG1* was significantly upregulated in *ahk2 ahk3* seeds six hours after FR light application. It encodes a plant adenine-guanine transporter (Mansfield *et al.*, 2009). Noteworthy, two FLA genes were among the highest upregulated genes in *ahk2 ahk3* seeds after FR light application, *FLA10* and *FLA4*/*SOS5*. Both encoded proteins belong to different groups within the FLA protein family (MacMillan *et al.*, 2010).

To sum this up, upregulation of genes related to seed development, hormone transporter and genes encoding FLA proteins associated to the cell wall and the regulation of seed mucilage seem to be specifically important for germination of CK receptor double mutant seeds in germination-inducing light conditions.

Table 29. Transcripts upregulated in *ahk2 ahk3* seeds six hours after a FR light pulse.
Log$_2$ FC indicates log$_2$-fold change compared to *ahk2 ahk3* imbibed seeds (49 h). Significant regulated genes were selected based on p-values ≤ 0.05 according to Bonferroni. Only the 15 highest upregulated genes were considered here. ATG numbers of genes mentioned in the text were indicated in bold.

ATG	Name	log$_2$ FC	p-value	short description
AT2G34010		**2.79**	**0.00816577**	**Verprolin**
AT5G14890		2.67	0.01034746	Potassium transporter
AT4G13710		2.21	0.03579657	Pectin lyase-like superfamily protein
AT3G10960	***AZG1***	**2.08**	**0.00028601**	**AZA-guanine resistant 1**
AT3G60900	***FLA10***	**2.04**	**0.01625987**	**Fasciclin -like arabinogalactan-protein 10**
AT5G54970		1.97	0.01090025	Hypothetical protein
AT5G02520	*KNL2*	1.93	4.9095E-07	Dentin sialophosphoprotein-like protein
AT2G39675	*TAS1C*	1.90	0.00074581	Trans-acting siRNA1c primary transcript
AT4G39970		1.89	0.00755852	Haloacid dehalogenase-like hydrolase (HAD) superfamily protein
AT3G46550	***FLA4/SOS5***	**1.86**	**1.4773E-05**	**Fasciclin-like arabinogalactan family protein**
AT4G19130		1.74	0.02270556	Replication factor-A protein 1-like protein
AT2G19170	*SLP3*	1.56	0.0313313	Subtilisin-like serine protease 3
AT1G19950	*HVA22H*	1.52	0.00728424	HVA22-like protein H (ATHVA22H)
AT1G73690	***CDKD1;1***	**1.50**	**0.01045452**	**Cyclin-dependent kinase D1;1**
AT2G38210	*PDX1L4*	1.42	0.03739672	Putative PDX1-like protein 4

After detailed analysis of transcriptomic changes six hours after the germination-inducing second light pulse, this part is focused on later stages of germination. In the following part, transcriptomic changes twelve hours after light application were analyzed. Twelve hours after application of a germination-inducing light pulse the overall number of DEGs clearly increased (Figure 46). For example, the number of DEGs deregulated in all conditions tested doubled twelve hours after a light pulse, from 2543 six hours after light application to 5585 genes (Figure 44 and Figure 46). This again supports the notion, that in the course of imbibition and germination major transcriptomic changes occur.

Twelve hours after a germination-inducing light pulse, the Venn diagram revealed 1003 genes, representing 11 % of all DEGs, deregulated in seeds likely to complete germination, namely in *ahk2 ahk3* seeds treated with a FR or WL pulse and in wild-type seeds treated with WL (Figure 46).

Classification of these deregulated transcripts revealed a substantially different set of GO terms late after light application as compared early after light application. While six hours after light treatment in seeds likely to germinate, GO terms related to abiotic stimuli and stress were prominent, twelve hours after perceiving a light pulse metabolic processes were overrepresented (Figure 47).

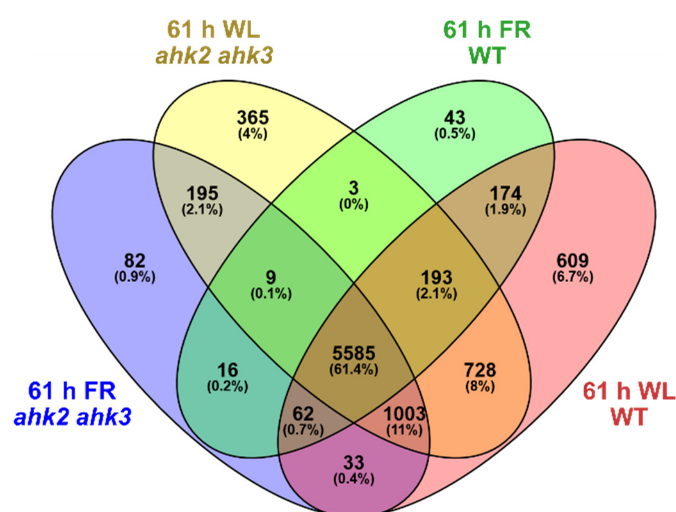

Figure 46. Venn diagram depicting the number of transcripts differentially regulated in 61 h imbibed seeds.

Differentially expressed genes in wild-type and *ahk2 ahk3* mutant seeds twelve hours after a WL or a FR light pulse, always relative to 49 h imbibed seeds of the respective genotype were plotted. Only significantly regulated genes (Bonferroni; p-value ≤ 0.05) are depicted in the Venn diagram.

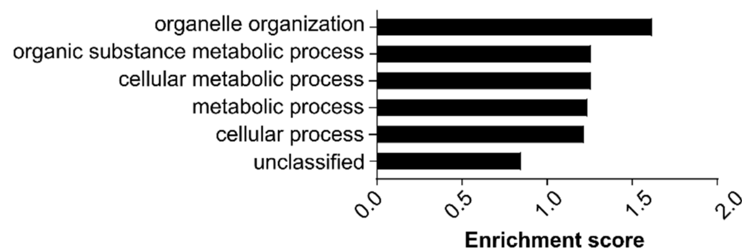

Figure 47. Gene Ontology analysis of germination-related genes twelve hours after illumination.
Classification of 1003 DEGs overlapping in *ahk2 ahk3* mutant seeds in FR and in WL and in wild-type seeds subjected to WL treatment.

The highest upregulated genes in the set of 1003 DEGs in germinating seeds twelve hours after application of a germination-inducing FR or WL pulse belong to various biological processes (Table 30). *FAF1* expression was drastically increased compared to imbibed seeds 49 h after the onset of imbibition. *FAF1* was previously described as being highly expressed in developing embryos (Wahl *et al.*, 2010). AT5G60520 encodes a LEA protein and occurred previously in a screen for CK regulated genes (Lee *et al.*, 2007). Interestingly, also *ADAP* was identified among the highest upregulated transcripts in germinating seeds. *ADAP* was also named *WRI3* and described as a transcriptional activator of fatty acid biosynthesis and rescued the wrinkled seed phenotype of *wri1-4* seeds (To *et al.*, 2012). Furthermore, AT2G34010, encoding a protein of unknown function, was in the list of highest upregulated genes in germination seeds at late timepoints. Interestingly, this transcript was the strongest upregulated transcript in the list of genes deregulated in *ahk2 ahk3* seeds in 55 h imbibed seeds (Table 29). It encodes a homologue of a TPL corepressor in yeast (Chen *et al.*, 2014), an involvement in seed germination or a connection to CK has not been described in the literature.

Table 30. Transcripts upregulated twelve hours after induction of germination in *ahk2 ahk3* seeds by FR or WL and wild-type seeds pulsed with WL.

Log_2 FC indicate log_2-fold change compared to 49 h imbibed seeds. Significant regulated genes were selected based on p-values ≤ 0.05 according to Bonferroni. Only the 15 highest upregulated genes were considered. ATG numbers of genes mentioned in the text were indicated in bold.

ATG	Name	log_2 FC *ahk2 ahk3* FR 61 h	p-value *ahk2 ahk3* FR 61 h	log_2 FC *ahk2 ahk3* WL 61 h	p-value *ahk2 ahk3* WL 61 h	log_2 FC wild type WL 61 h	p-value wild type WL 61 h	short description
AT3G45320		6.81	0.000565859	7.98	1.42114E-06	5.62	0.0034	Transmembrane protein
AT4G16220		6.25	0.007760209	6.78	0.000720244	4.74	0.0037	GDSL-like Lipase/Acylhydrolase superfamily protein
AT1G14800		6.22	0.026345222	6.83	0.002095796	6.17	0.02987869	Nucleic acid-binding, OB-fold-like protein
AT4G02810	***FAF1***	**5.44**	**0.005780422**	**5.30**	**0.012403168**	**5.05**	**0.04305493**	**FANTASTIC four-like protein (DUF3049)**
AT4G03292		5.38	1.53876E-05	5.67	1.6812E-06	6.46	0.00186829	Polynucleotidyl transferase, ribonuclease H-like superfamily protein
AT5G60520		**4.21**	**0.011634178**	**4.65**	**0.000654577**	**4.60**	**0.02134718**	**Late embryogenesis abundant (LEA) protein-like protein**
AT1G16060	***ADAP***	**4.10**	**0.002495775**	**4.47**	**0.000150961**	**3.15**	**0.0003521**	**ARIA-interacting double AP2 domain protein**
AT5G22810		3.91	1.74983E-09	4.82	4.97638E-16	3.64	3.9375E-11	GDSL-like lipase/acylhydrolase superfamily protein
AT1G80690		3.86	0.000366171	4.34	4.80919E-06	3.45	4.0139E-05	PPPDE putative thiol peptidase family protein
AT5G11540	*GulLO3*	3.86	0.027744566	4.49	0.000325199	5.69	0.00227182	D-arabinono-1,4-lactone oxidase family protein
AT5G36120	*CCB3*	3.60	2.72494E-05	4.35	3.57501E-09	4.67	1.0821E-06	Cofactor assembly, complex C (B6F)
AT4G17380	*MSH4*	3.45	0.014595237	3.33	0.037656585	3.98	0.00099544	MUTS-like protein 4
AT2G34010		**3.38**	**1.20627E-05**	**3.64**	**4.79644E-07**	**3.33**	**0.02166959**	**Verprolin**
AT4G11320		3.32	1.45022E-06	4.80	3.22789E-17	4.26	2.2909E-14	Papain family cysteine protease
AT1G17285		3.18	8.36457E-07	3.22	5.08144E-07	2.15	0.02782986	transmembrane protein

3.5.3.7 Expression pattern of CK-related transcripts in the germination process

The objective of this study was to monitor transcriptional changes in CK-associated genes in the wild-type and in *ahk2 ahk3* mutant seeds. A set of well-known CK-related genes were chosen according to genes analyzed in (Cortleven *et al.*, 2019b).

First, genes associated with CK synthesis were investigated. In wild-type seeds, *IPT*s were rather downregulated, except *IPT9*, which was slightly, but significantly upregulated twelve hours after application of a light pulse, irrespective of light quality (Figure 48).

CK biosynthesis genes were similarly regulated in *ahk2 ahk3* mutant seeds (Figure 48). *IPT* genes were rather downregulated in germination, as in wild-type seeds *IPT9* was slightly but significantly upregulated at the later stage of germination (Figure 48). Members of the *LOG* family, except *LOG9*, tended to be not- or slightly upregulated in germination in wild-type seeds, both in WL and in FR light conditions (Figure 48). *LOG* transcripts in *ahk2 ahk3* seeds tended to be upregulated in imbibition and the germination phase, similar to wild-type seeds (Figure 48). In wild-type seeds, genes encoding CK catabolizing *CKX1*, *CKX2* and *CKX4* showed a trend towards downregulation, only *CKX3* was significantly downregulated in imbibed seeds, albeit not thereafter. Only *CKX5* was significantly upregulated after FR light illumination as well as six hours after WL application (Figure 48). In CK receptor mutant seeds, *CKX1*, *CKX2* and *CKX3* tended to be downregulated, again comparable to wild-type seeds during germination or in the imbibition phase, respectively. *CKX5* was significantly upregulated after FR light illumination as well as after WL application and *CKX7* expression was significantly increased in the imbibition phase (Figure 48).

Notably, genes encoding CK receptors significantly regulated in the course of seed germination in wild type. *AHK3* was significantly downregulated in wild-type seeds, both after WL and FR light illumination (Figure 49). In *ahk2 ahk3* mutant seeds the transcript levels of *AHK2* and *AHK3* genes were very low, as expected, both in control samples as well as in the samples for comparison. Nevertheless, also *AHK4* is downregulated in *ahk2 ahk3* mutant seeds, representing the same tendency as in wild-type seeds (Figure 49).

Regarding genes encoding phosphotransmitter proteins, *AHP5* was downregulated late after a FR or WL pulse in both genotypes, *AHP2* and *AHP3* were upregulated in WL conditions and late after a FR light pulse. *AHP6* has been identified previously (Table 28) among the strongest upregulated genes in WL-treated wild-type seeds. The heatmap clearly demonstrated pronounced *AHP6* expression in WL-treated wild-type seeds as well as in *ahk2 ahk3* seeds (Figure 49).

None of the A-type ARRs was significantly regulated in WL or FR light-induced germination in the wild-type. This was also true for A-type ARR regulation in *ahk2 ahk3* mutant seeds, except *ARR4* which was upregulated significantly after imbibition (Figure 49).

Similarly, the majority of B-type *ARR* genes was not differentially regulated in germination in wild-type seeds. While all B-type ARRs tended to be downregulated during imbibition, responding to a light pulse induced differences in transcript abundance. While *ARR1* and *ARR2* were significantly downregulated after light application in both genotypes, *ARR10* and *ARR12* were slightly upregulated in response to light and similarly in both genotypes (Figure 49). Lastly, C-type *ARR*s were not regulated at all in both genotypes

(Figure 49). Strikingly, based on the expression pattern of CK-associated transcripts, it was not possible to distinguish non-germination inducing FR illumination from germination-inducing WL illumination on a transcriptional level in wild-type seeds.

To sum this up, in CK-associated transcript regulation during imbibition and germination in *ahk2 ahk3* mutant seeds was very similar to the regulation in wild-type seeds, no mutant-specific deregulation patterns were found here.

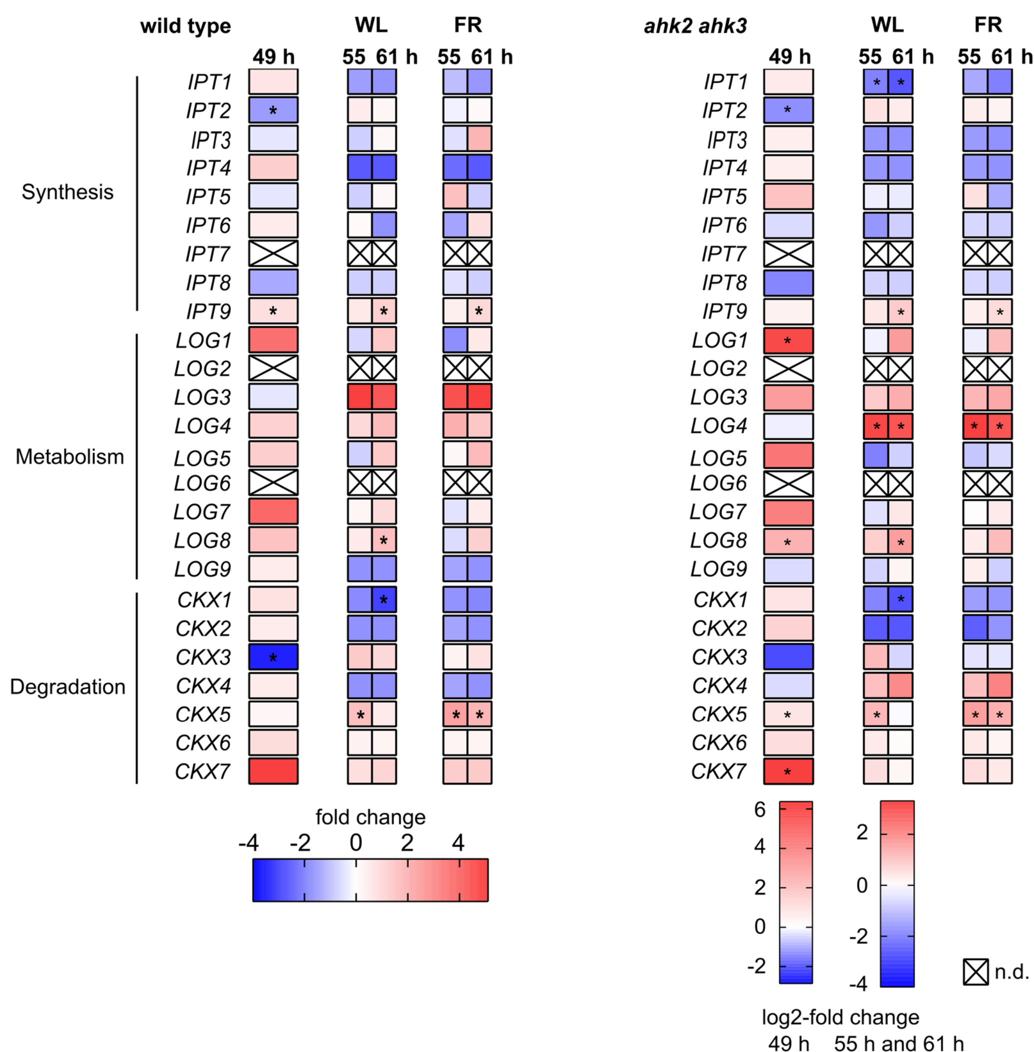

Figure 48. Expression of CK associated biosynthesis-, conversion- and catabolism- genes during imbibition and after application of a FR or WL pulse in wild-type and *ahk2 ahk3* mutant seeds.

Asterisks indicate statistically significant differences (*, $p < 0.05$) as compared to the respective control samples (49 h compared to 1 h imbibed seeds; 55 h and 61 h compared to 49 h imbibed seeds). Blue color indicates downregulation, red color upregulation, n.d., not defined.

Figure 49. Expression of CK signaling-associated genes during imbibition and after application of a FR or WL pulse in wild-type and *ahk2 ahk3* mutant seeds.
Asterisks indicate statistically significant differences (*, $p < 0.05$) as compared to the respective control samples (49 h compared to 1 h imbibed seeds; 55 h and 61 h compared to 49 h imbibed seeds). Blue color indicates downregulation, red color upregulation, n.d., not defined.

3.5.3.8 Expression pattern of ABA, GA and other germination-related transcripts during germination

To understand the dynamics of gene expression in the germination process, in this section expression of genes associated with ABA, GA or for germination in general were analyzed in WL and FR light in wild-type and *ahk2 ahk3* mutant seeds.

Genes encoding proteins catalyzing ABA biosynthesis were downregulated during imbibition in both genotypes, with exception of *NCED6* and *NCED9* which were slightly upregulated, *NCED6* in *ahk2 ahk3* seeds was even significantly upregulated during imbibition (Figure 50). After a light pulse, irrespective of the light quality, genes related to ABA biosynthesis were downregulated (Figure 50). *NCED3* did not fit this pattern in both genotypes, since expression was downregulated during imbibition and tended to be upregulated after a light pulse (Figure 50). Regarding ABA catabolism, two of three ABA catabolism-related transcripts (*CYP707A*s) were slightly upregulated after light application in wild-type seeds, but not regulated or downregulated during imbibition (Figure 50). In contrast, in *ahk2 ahk3* mutant seeds *CYP707A1* and *CYP707A3* were upregulated during imbibition (Figure 50), expression after a light pulse was equivalent to wild-type expression (Figure 50). Since no data were available for *ABI5*, only *ABI3* was analyzed as a representative of ABA-downstream signaling. *ABI3* was slightly, but significantly downregulated after FR and WL illumination in both genotypes (Figure 50). To sum this up, ABA-related transcripts were regulated similarly in wild-type and *ahk2 ahk3* mutant seeds after a light pulse, independent of light quality. In the imbibition phase, two ABA-catabolism related genes were upregulated in *ahk2 ahk3* seeds, suggesting an earlier reduction of ABA levels in the mutant seeds.

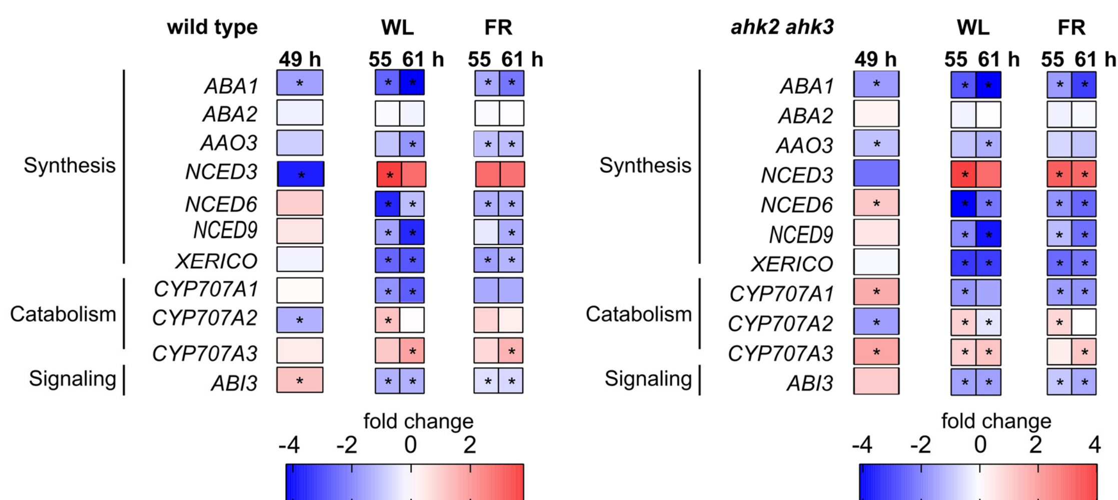

Figure 50. Expression of ABA-related transcripts during imbibition and after application of a FR or WL pulse in wild-type seeds and in *ahk2 ahk3* mutant seeds.
Asterisks indicate statistically significant differences (*, $p < 0.05$) as compared to the respective control samples (49 h compared to 1 h imbibed seeds; 55 h and 61 h compared to 49 h imbibed seeds).

Next GA-related transcripts were analyzed. GA- biosynthesis related transcripts were upregulated both in wild-type and in *ahk2 ahk3* mutant seeds during imbibition (Figure 51). Among them, *GA3ox* transcripts were upregulated and *GA20ox3* was downregulated both after WL and after FR-light treatment (Figure 51) in both genotypes. Additionally, in *ahk2 ahk3* seeds, *GA20ox2* was downregulated significantly in response to a WL pulse, after FR-light treatment downregulation was not significant (Figure 51). Transcript levels of the GA-degrading *GA2ox2* gene were significantly increased in both genotypes during imbibition but decreased thereafter (Figure 51). All analyzed GA-receptors were downregulated in wild-type seeds both after FR and WL illumination (Figure 51). Solely *GID1B* was slightly upregulated in imbibed seeds (Figure 51), this pattern was consistent between wild-type and *ahk2 ahk3* mutants. Regarding *DELLA* transcript expression, encoding negative regulators of germination, it could be shown that three of four DELLAs included in the analysis were upregulated during imbibition, but downregulated thereafter (Figure 51) in wild-type and in *ahk2 ahk3* mutant seeds. Opposite to that, *RGL1* expression was upregulated in later stages of the germination process, irrespective of the light condition or genotype (Figure 51).

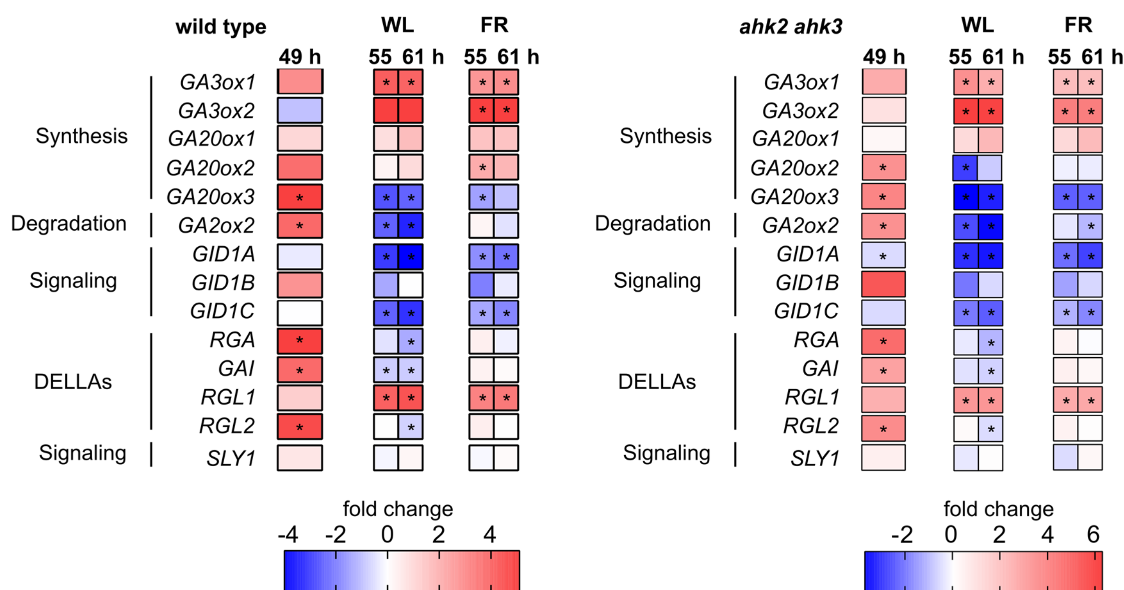

Figure 51. Expression of GA-related transcripts during imbibition and after application of a FR or WL pulse in wild-type and *ahk2 ahk3* mutant seeds.
Asterisks indicate statistically significant differences (*, $p < 0.05$) as compared to the respective control samples (49 h compared to 1 h imbibed seeds; 55 h and 61 h compared to 49 h imbibed seeds).

Apart from the regulation of germination by ABA- or GA-related processes, many other factors are known to be crucial for the regulation of the highly complex germination process. Transcripts encoding the photoreceptors PHYA and PHYB were upregulated during imbibition, both in wild-type and in *ahk2 ahk3* mutant seeds but downregulated thereafter (Figure 52). Other phytochrome-encoding genes were downregulated throughout imbibition and germination in both genotypes (Figure 52). Regarding phytochrome-interacting factors, transcript abundance of different members of the PIF family was comparable in wild-type and in *ahk2 ahk3* seeds. *PIF1* and *PIF6* were upregulated during

imbibition and downregulated after a light pulse was applied (Figure 52). *PIF8* may be regulated differently in wild-type and in *ahk2 ahk3* mutant seeds, although differential expression was not significant. While *PIF8* transcript level were slightly upregulated in wild-type imbibition and downregulated during germination, in *ahk2 ahk3* mutant seed *PIF8* regulation was invers, displaying downregulation during imbibition and upregulation during germination (Figure 52).

PIF-related transcripts were upregulated in imbibition and germination, both in wild-type and in *ahk2 ahk3* mutant seeds, while all SUPPRESSOR OF PHYA-related transcripts included in this analysis were downregulated (Figure 52). In *ahk2 ahk3* mutant seeds, only *SPA1* and *SPA3* were upregulated after induction of germination by WL. *COP/DET/FUS*-related transcripts were downregulated in both genotypes (Figure 52). Regarding light signaling components directly downstream of the photoreceptors, *HY5* was 32-fold downregulated after imbibition and 32-fold upregulated in wild-type seeds exclusively after a WL pulse (Figure 52). In *ahk2 ahk3* seeds, the *HY5* regulation pattern was similar to wild-type seeds (Figure 18). *FHY1* and *FHL*, important for nuclear import of phyA, were upregulated during imbibition, but downregulated both after FR as well as after WL treatment in both genotypes (Figure 52).

Transcripts related to seed germination and dormancy were also regulated similarly between the genotypes. *BBX19* and *BBX21* transcript level were increased after imbibition, but reduced after a light pulse, independent of light quality. Differential expression of *OWL1*, *SOM* and *DOG1*, prominent regulators of seed germination, were shown to be downregulated in both genotypes and light conditions (Figure 52).

To sum this up, differential regulation of transcripts related to germination or light did not differ between wild-type and CK receptor mutant seeds, indicating that regulation of gene expression is not coupled to CK signal transduction via AHK2 or AHK3. So a relevance for shaping the VLFR seed germination phenotype of CK receptor mutants is not assumed.

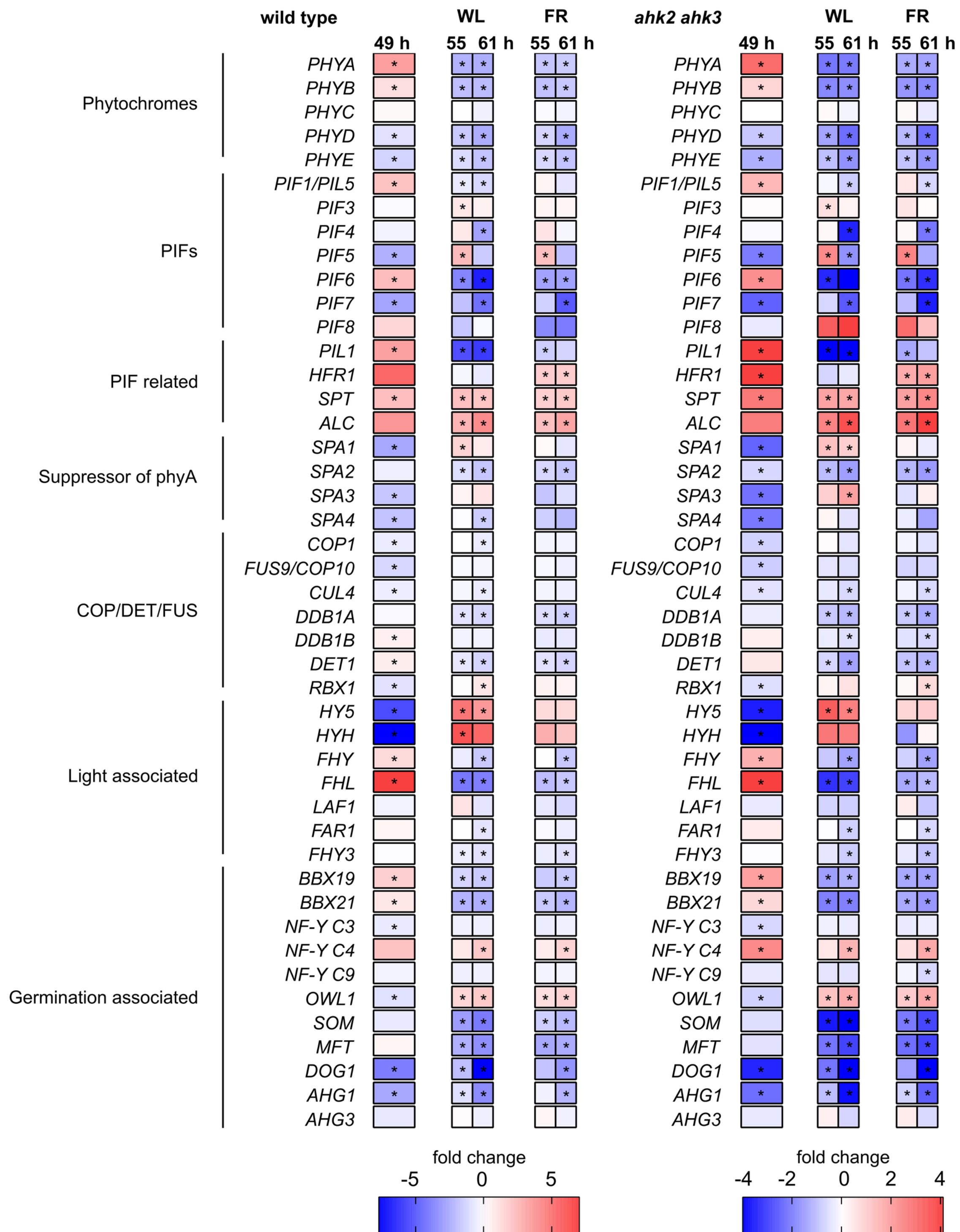

Figure 52. Expression of germination and light-related transcripts during imbibition and after application of a FR or WL pulse in wild-type and in *ahk2 ahk3* mutant seeds.
Asterisks indicate statistically significant differences (*, $p < 0.05$) as compared to the respective control samples (49 h compared to 1 h imbibed seeds; 55 h and 61 h compared to 49 h imbibed seeds).

3.6 The impact of light and nutrient availability on seed germination

In priming research the biological role of a long-term memory in *r*-strategists, such as *Arabidopsis thaliana* are discussed (Ganguly *et al.*, 2017; Lämke and Bäurle, 2017). In the field of seed germination research, establishment and inheritance of a long-term memory, for example via seed dormancy is widely accepted. It was recently shown that maternal day length and maternal light/shade environment affect seed germination characteristics (Imaizumi *et al.*, 2017; Vayda *et al.*, 2018).

3.6.1 Seed priming by parental light conditions is advantageous for germination in the second offspring generation

Environmental factors such as the light environment of parental plants during seed development determine or “prime” the germination phenotype of their offspring (McCullough and Shropshire Jr, 1970; Hayes and Klein, 1974). It seems that information on the parental growth environment is passed to the next generation, which is advantageous for the offspring that is likely to germinate in an environment similar to one the parents experienced (Galloway and Etterson, 2007). The following section aimed to elucidate if very low fluence FR light conditions experienced by parental plants may affect the germination phenotype of seeds and if CK may play a role in this process.

In order to analyze intergenerational effects of the light environment on seed germination, parental plants were grown under shade-enriched or non-shade enriched light during their entire life including seed set and seed development. Shade light is characterized by a high proportion of FR light (R/FR ratio of 0.3) while, in contrast, non-shade light contains a lower FR proportion, resulting in a R/FR ratio of 1.1. Then germination rates of the seeds derived from parental plants grown under shaded or non-shaded conditions were determined and compared in FR light.

Growing parental plants with or without shade did not affect offspring germination in FR light in the wild-type or in *ahk2 ahk3* mutant seeds (Figure 53 A). In nature, shaded plants do not only face a shift in the R/FR light ratio, but also a reduction of the overall light quantity. To control for the reduced light quantity in shade, the light quantity applied to parental plants was reduced from 103 µmol m^{-2} s^{-1} to 62 µmol m^{-2} s^{-1}, referred to as weak light. Seeds derived from plants grown in weak light with shade or weak light without shade did not show alterations in germination induced by FR light (Figure 53 B), only the overall germination rates were reduced when parental plants were grown in weak light conditions (Figure 53 A and B). These results demonstrate that parental exposure to shade does not seem to alter the ability of F1 generation seeds to germinate in VLFR conditions, irrespective of the overall light quantity. Seeds derived from plants grown in shade light for two subsequent generations showed higher germination rates in FR light compared to seeds derived from plants grown under non-shaded conditions (Figure 53 C). This increase in FR light-induced germination was significant only in wild-type seeds (67 % in shade light and 31 % in non-shade light) as *ahk2 ahk3* seeds derived from non-shaded parental plants already germinated at high rates of 80 % (Figure 53 C). These results suggest, that the parental light environment affects offspring germination, when the conditions were stable for more than one generation. To clarify a probable role for CK in this process, further experimentation would be necessary.

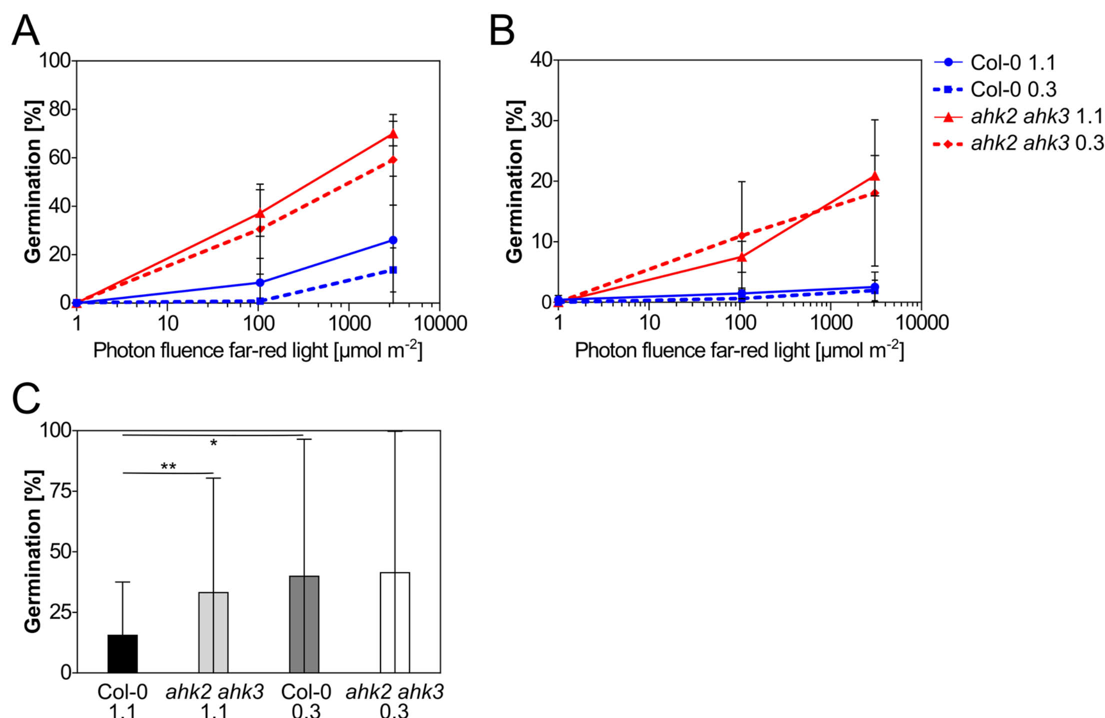

Figure 53. Parental light environment affects germination of offspring after germination induction with a FR light pulse.
Parental plants were grown either in 103 µmol m^{-2} s^{-1} WL conditions (A and C) or in 62 µmol m^{-2} s^{-1} weak WL conditions (B) in different red to far-red (R/FR) ratios for one generation (A and B) or two generations (C). 1.1 refers to a R/FR ratio of 1.1, which equals non-shade conditions. 0.3 refers to a R/FR ratio of 0.3, regarded as shaded conditions. Germination was induced by a FR light pulse of 105 µmol m^{-2} or 3066 µmol m^{-2} (A and B) or 5400 µmol m^{-2} (C). Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0, as calculated by One-Way ANOVA, post-hoc Tukey´s test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.6.2 Nitrogen fertilization increases germination rates in FR light

Seed dormancy and seed germination are directly linked to the nitrogen availability at the site of plant growth and seed dispersal. Nitrogen availability affects both of these traits in *A. thaliana* (Alboresi *et al.*, 2005; Vidal *et al.*, 2014). Fertilization of parental plants positively affected both wild-type and *ahk2 ahk3* germination. *ahk2 ahk3* seeds germinated significantly higher in FR light when the parental plants were fertilized (22 %) compared to non-fertilized parental plants (3 %) (Figure 54). This difference was also measurable in wild-type seeds (Figure 54), indicating that the nitrogen supply affects germination in FR light independent of the CK perception in seeds.

The exogenous application of nitrogen during imbibition further increased germination rates in FR light in a dose-dependent manner, both in wild-type and in CK receptor double mutant seeds. In wild-type seeds, addition of 0.1 mM KNO_3 during imbibition resulted in increased germination rates in seeds derived from non-fertilized parental plants (2 % compared to 0.7 % without nitrogen application during imbibition) or from fertilized parental plants (43 % compared to 16 % without nitrogen application during imbibition) (Figure 54). Highest wild-type germination rates were measured after feeding of 1 mM KNO_3 during imbibition, wild-type seeds from non-fertilized parents germinated at a frequency of 8 %, while seeds from fertilized parental plants germinated at a frequency of 67 % (Figure 54).

The effect of exogenous nitrogen application was also detectable in *ahk2 ahk3* mutant seeds. Application of 0.1 mM KNO_3 resulted in 43 % and 10 % germination in FR light in seed derived from fertilized and non-fertilized parental plants, respectively. Application of 1 mM KNO_3 further increased germination rates in seed derived from fertilized parental plants to 47 % and to 21 % in seeds from non-fertilized parents (Figure 54). Interestingly, wild-type seeds even exceeded *ahk2 ahk3* germination rates in FR light in seeds subjected to fertilization of the parental plants and fertilization during imbibition (Figure 54).

To sum this up, these results suggest that seeds are sensitive to externally applied fertilization treatment with nitrogen, which positively influences germination in FR light. Furthermore, information on the parental fertilization status affected offspring germination in very low fluence germination conditions. Both effects seem to be independent of CK perception via AHK2 and AHK3.

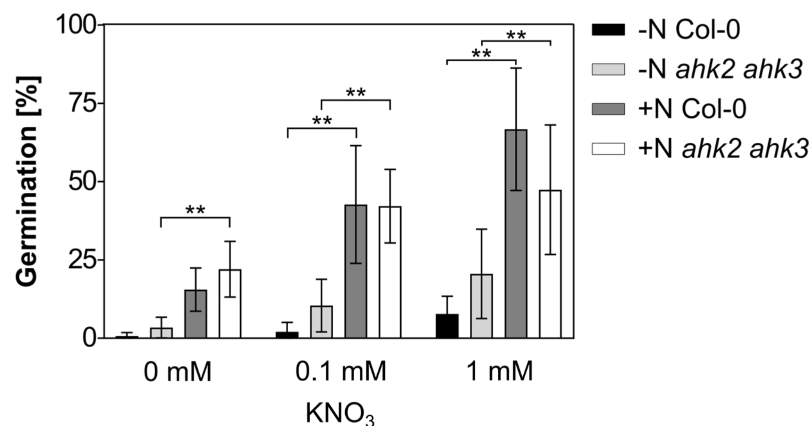

Figure 54. Nitrogen supply increases germination rates in FR light both after exogenous nitrogen supply and after fertilization of parental plants.
Germination rates were determined in the wild type (Col-0) and in CK receptor double mutant seeds (*ahk2 ahk3*) derived from either fertilized (+N) or non-fertilized (-N) parental plants. Additionally, exogenous nitrogen fertilization with 0.1 or 1 mM KNO_3 was applied during germination. Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0, as calculated by Two-Way ANOVA, post-hoc Tukey test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. n = 6 biological replicates á minimum 140 seeds.

3.7 Seed characteristics shape the seed germination rates in very low fluence FR light

3.7.1 Seed coat removal revealed a negative influence of the seed coat on germination

As has been described earlier in this work, the expression of CK-catabolizing *CKX1* in maternal seed tissue affects the seed germination response in very low fluence FR light (see results section 3.3.2).

In this part, the contribution of the seed coat to germination in FR light was investigated by removal of the seed coat from imbibed seeds. The experimental procedure of seed coat dissection has been described previously (Lee *et al.*, 2010). Whole seeds do not germinate in darkness after an inhibiting FR light pulse (Figure 55 A), but removal of the seed coat resulted in germination rates of 17 % both in wild-type and in *ahk2 ahk3* coatless embryos (Figure 55 A).

After pre-treatment with WL (see scheme Figure 55 B), coatless wild-type embryos were published to germinate well (Lee *et al.*, 2012). In this work, coatless wild-type embryos subjected to the published protocol failed to germinate (Figure 55 B). *ahk2 ahk3* coatless embryos germinated, but germination rates did not exceed 22 % (Figure 15 B). Non-dissected wild-type seeds exhibited residual germination rates of 5 % after WL treatment (Figure 55 B), indicating that the FR light pulse applied after the WL treatment was not sufficient to entirely block germination. For *ahk2 ahk3* seed, no germination was measurable under these conditions (Figure 55 B).

When non-dissected seeds were tested for their ability to germinate after application of a very low fluence FR light pulse, *ahk2 ahk3* seeds showed higher germination rates (63%) compared to wild-type seeds (26%) as expected (Figure 55 C). Removal of the seed coat increased germination rates in FR light in both genotypes to 100 % (Figure 55 C).

So, removal of the seed coat positively affects germination in very low fluence FR light, independent of the seeds' genotype. Nevertheless, future experimentation is required. Especially other seed batches and different CK mutant should be tested to clarify the role of CK in the seed coat, and the relevance of this tissue in dark or WL conditions.

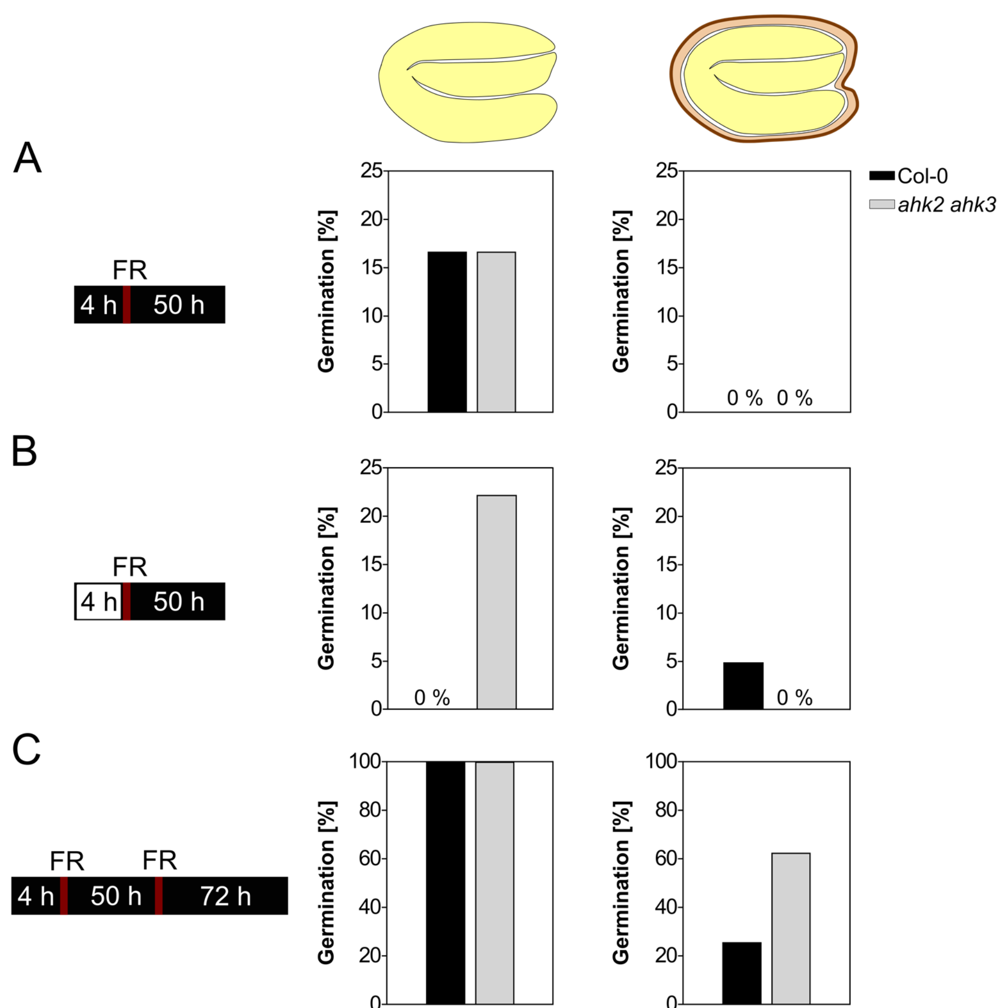

Figure 55. Removal of the seed coat affects seed germination in different light conditions. Seed without (graphs on the left) or with (graphs on the right) seed coat were tested for their germination rates (A) in darkness, (B) after white light (WL) pre-treatment or (C) in the standard very low fluence FR light protocol. Light treatments are indicated in the schemes on the left. Seeds of two plants per genotype were pooled, of this pool 10-15 seeds per genotype and treatment were dissected and the testa was removed. Germination rates in non-dissected seeds were analyzed using n ≥ 30 seeds per genotype and treatment.

3.7.2 Small seed size is advantageous for germination in FR light

CK has been shown to play a role in the regulation of seed size in *A. thaliana* (see introduction section 1.5). In order to investigate a possible functional relationship between seed size and the ability to complete germination in very low fluence FR light conditions, seed sizes (1000 corn weight and seed size class) and germination rates of different mutants in the CK pathway were compared.

The automated sorting of seeds into size classes did not reveal any differences in seed size between wild-type and the tested CK mutant seeds (Figure 56 A). By analyzing and comparing the 1000-seed weight between the genotypes, *ahk2-5 ahk3-7* mutant seeds displayed a significantly reduced seed weight of 13 mg, while *ipt3,5,7* seeds were significantly heavier (25 mg) compared to wild-type seeds (17 mg) (Figure 56 B). *35S:CKX2* seed weight was comparable to wild type (Figure 56 B).

In order to investigate, if the seed size is connected to the ability to complete germination under VLFR light conditions, germination rates of different sized wild-type and *ahk2 ahk3* receptor mutant seeds (manually sieved) were compared. The seeds classified into the largest size class (diameter > 300 µm) did not germinate after induction of germination by a FR light pulse, irrespective of their genotype (Figure 56 C). Seeds with a diameter of >250 to 300 µm germinated after a FR light pulse, as expected *ahk2 ahk3* seeds, showed higher germination rates (7 %) compared to wild-type seeds (0.5 %) (Figure 56 C). The smallest seeds tested here (diameter < 200 µm) showed highest overall germination rates (wild-type seeds germinated 2 %, *ahk2 ahk3* seeds 11 %). Thus, smaller seeds germinated at higher rates in FR light conditions as compared to larger seeds. This tendency was significant in *ahk2 ahk3* seeds, and visible but non-significant in wild-type seeds (Figure 56 C).

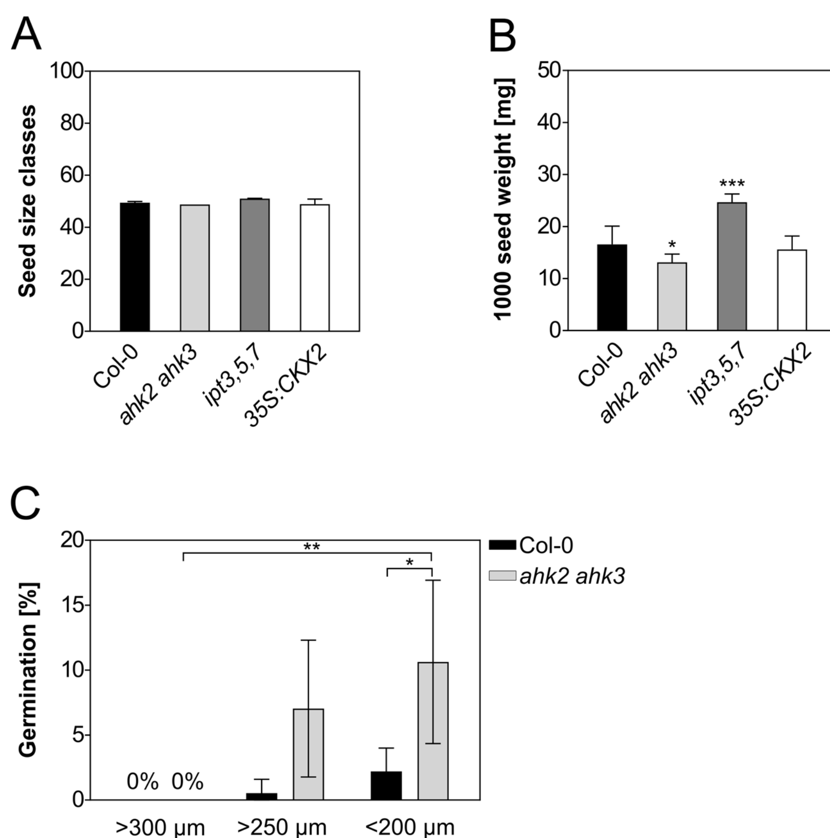

Figure 56. CK mutant seed sizes differ in their size and seed size affects germination in FR light. (A) Seeds were classified in arbitrary size classes. *A. thaliana* seeds cover size classes from 15 to 132. (B) 1000 seed weight of CK mutant and transgenic seeds and respective wild-type seeds. (C) Germination rates of large (diameter > 300 µm), medium (diameter > 250 µm) and small (diameter less than 200 µm) were scored 96 h after the FR light pulse in wild-type (Col-0) seeds as well as in seeds impaired in CK perception (*ahk*2 *ahk3*). Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0, as calculated by Kruskal-Wallis-Test, post-hoc Dunn's test (A) or One-Way ANOVA, post-hoc Dunnett's test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.7.3 The ability to germinate in FR light is retained in seeds subjected to long-term storage

Long-term storage of seeds results in a loss of seed viability due to protein- and non-enzymatic lipid oxidation by reactive oxygen species (Clerkx *et al.*, 2004). Besides environmental storage conditions also intrinsic, genetic factors affect seed longevity (Walters *et al.*, 2005; Nguyen *et al.*, 2015). Seed longevity is a multigenic trait (Rajjou *et al.*, 2008), as various aging-related QTLs have been identified in *A. thaliana* (Nguyen *et al.*, 2012). In this work, non-dormant seeds were tested for their germination rates in FR light after different dry storage periods. This study aimed to unravel if the enhanced germination phenotype of *ahk2 ahk3* seeds would persist over time. To control seed viability, germination rates were analyzed after induction of germination by a WL pulse first. For the FR light germination experiments, only seed batches were chosen that did not show decreased WL germination rates. WL germination rates of *ahk2 ahk3* seeds did not exceed 75 % any more after three years of storage (Figure 57 A), therefore three years old *ahk2 ahk3* seeds were excluded from the analysis of germination in FR light conditions. In WL, both genotypes showed high germination rates up to seed batches stored for two and a half years. In FR light, seeds with a reduced CK signal perception germinated significantly better in FR light (4–9 %) compared to the wild type (0.1-0.5 %) (Figure 57 B). These differences in germination rates were maintained up to 2.5 years after harvest.

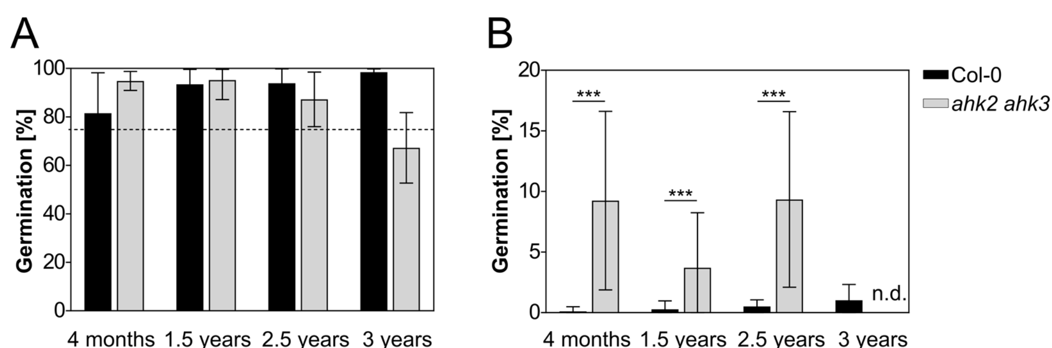

Figure 57. Differences in germination between genotypes in FR light persist over time. Germination rates were scored 96 h after the germination-inducing WL (A) or FR light pulse (B). Dashed line in (A) labels a germination rate of 75 %, which is the minimum WL germination rate required for a seed set to be considered for FR light germination tests. Error bars indicate standard deviations. Asterisks represent statistically significant differences to Col-0 as calculated by Two-Way ANOVA, post-hoc Tukey's test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. n = 6 biological replicates á minimum 140 seeds.

4 Discussion

4.1 CK negatively regulates seed germination in various light conditions

4.1.1 Seed dormancy is affected by the seeds' CK status but is not causal for the repression of germination

In CK mutant seeds as well as in the wild type, primary dormancy was lost after 30 to 49 days of dry storage (Figure 14). This is consistent with the time span of around 30 days the loss of primary dormancy requires, as described for Col-0 seeds (Piskurewicz *et al.*, 2016). In order to exclude the influence of dormancy on the outcome of germination experiments, in this work all seed sets were after-ripened for six months prior to usage. Shorter after-ripening periods revealed reduced dormancy of *35S:CKX2* and higher dormancy of *35S:CKX4* compared to the wild type (Figure 14). This is in line with the germination pattern in FR light, which will be discussed later. Neither wild-type nor CK receptor mutant seeds developed secondary dormancy which would result in reduced WL germination rates.

4.1.2 CK represses R light-induced, phyB-dependent seed germination

The phyB-mediated induction of germination by R light is the most prominent pathway to induce seed germination. Seeds are highly sensitive towards low fluences of R light as it effectively promoted the onset of germination in the LFR (Neff *et al.*, 2000; Bae and Choi, 2008). The first objective of this work was to investigate the connection between R light-induced seed germination and CK. In low fluence R light, CK receptor double mutant seeds showed higher germination rates compared to the wild type (Figure 8) which is consistent with the findings published by Riefler *et al.* (2006). This suggests that CK signal perception hampers phyB-mediated seed germination. However, this effect was not visible when germination was induced by higher R light fluences (Figure 8), indicating that the repression of germination by CK is especially relevant under low light conditions. Consequently, in WL conditions CK mutant seeds germinate indistinguishable from wild-type seeds (Riefler *et al.*, 2006 and Figure A1).

Among the CK receptor double mutant seeds, the increased germination in response to low fluent R light was only significant in *ahk2-5 ahk3-7* seeds (Figure 8 B), the *ahk2-2 ahk3-3* allele combination showed a non-significant tendency to germinate at higher rates compared to wild-type (Figure 8 C). While *ahk2-5 ahk3-7* represents a weak allele combination, *ahk2-2 ahk3-3* is regarded as strong allele combination. This definition origins in the finding, that *ahk* triple mutants carrying the *ahk2-2 ahk3-3* alleles additional to *cre1* were unable to form viable seeds (Nishimura, 2004; Higuchi *et al.*, 2004), while triple mutants containing the *ahk2-5 ahk3-7* alleles developed viable seeds (Riefler *et al.* 2006). In both allele combinations the T-DNA insertion resulted in null or strong hypomorphic alleles (Nishimura, 2004; Higuchi *et al.*, 2004; Riefler *et al.*, 2006), so in both cases a loss of function is assumed. Nevertheless, alterations in phenotype between the different *AHK* allele combinations were also evident in female gametophyte development (Cheng and Kieber, 2013). These authors attributed the observed differences in residual levels of *AHK3* expression in *ahk3-3* allele containing combinations, although *ahk3-3* is regarded as the stronger allele compared to *ahk3-7*. This may also explain the higher germination of *ahk2-5 ahk3-7* seeds compared to *ahk2-2 ahk3-3* seeds in this work.

The AHK4 receptor plays only a minor role in the phyB-dependent LFR, as double mutant combinations including *ahk4/cre1* (*ahk2-5 cre1-2* and *ahk3-7 cre1-2*) did not differ in their germination rates from wild-type seeds in R light (Figure 8 B).

Mutations of single AHK receptors were not sufficient to significantly increase seed germination rates (Figure 8 C), what argues against a prominent role of single AHK receptors in this process and is presumably attributed to the high functional redundancy among AHKs (Ueguchi *et al.*, 2001b; Nishimura, 2004).

As *ahk2 ahk3 cre1* mutant plants and the resulting seeds show strong phenotypical defects, as dwarfed growth, a limited number of seeds that show increased seed sizes (Riefler *et al.*, 2006), offspring of these lines was not included in germination experiments in this work. Phenotypical alterations of the seed morphology might affect germination, seed size for example influenced timing of germination (Elwell *et al.*, 2011). The effect of seed size on germination will be discussed later (refer to discussion section 4.7). Reduction of CK level by increased CK catabolism was not sufficient to confer increased germination rates in R light (Figure 8), suggesting that CK signal transduction is more effective to repress germination compared to reduced CK levels. In conclusion, CK negatively regulates germination in low fluence R light conditions.

4.1.3 CK represses the reversibility of phyB-dependent germination after R/FR illumination

The reversion of phyB to the inactive form prevented phyB-mediated germination in wild-type seeds but had a milder effect on the inhibition of germination in *ahk2 ahk3*, *ahk3* single mutants and *35S:CKX2* seeds (Figure 9). Interestingly, loss of an individual receptor, namely AHK3 also resulted in enhanced germination rates after R/FR light treatment, suggesting a more pronounced role of AHK3 in germination.

A connection between phyB and proteins associated with the CK signal transduction pathway has been demonstrated by Sweere *et al.* (2001) and Mira-Rodado *et al.* (2007). The authors showed that ARR4 stabilizes the active Pfr form of phyB in R light (Sweere *et al.*, 2001). Consequently, *ARR4* overexpressors accumulated more active phyB (Mira-Rodado *et al.*, 2007). On the other hand, ARR4 is a negative regulator of CK signaling, so overexpression of *ARR4* may result in lower activity of the CK signaling pathway, which in turn would further promote germination. In this work, the role of ARR4 in phyB-dependent germination was not analyzed in detail. To figure out, whether the interaction between ARR4 and phyB affects R light induced germination, the germination response of *ahk2 ahk3 arr4* mutants may be analyzed in R light. If the loss of *ARR4* in *ahk2 ahk3* seeds results in additive effects, measurable by increased germination rates compared to *ahk2 ahk3* in R light, this may indicate that ARR4 and AHK2/AHK3 alter germination via distinct pathways. Also phosphorylation of ARR4 is required for phyB interaction (Mira-Rodado *et al.*, 2007), so seeds expressing of a non-phosphorylable *ARR4* version may be tested for their germination response in R light.

It has to be taken into account, that the Sweere *et al.* (2001) and Mira-Rodado *et al.* (2007) analyzed seedlings and measured hypocotyl elongation, while in this work seed germination was investigated. In plants, the processes of hypocotyl elongation and germination partially use the same molecular mechanisms, but the role of the components might vary greatly. For example, COP1 is a negative regulator of both seed germination and hypocotyl elongation (Chen *et al.*, 2004; Yu *et al.*, 2016), while HY5, a prominent

positive regulator of photomorphogenesis, has a negative impact on the onset of seed germination by promoting ABA signaling (Chen *et al.*, 2008).

The second possibility to explain the germination phenotype of CK mutants after R/FR light treatment, is the induction of germination by phyA. In the course of dark imbibition of seeds, phyA accumulates (Lee *et al.*, 2012a). After illumination with R light, both phytochromes were activated and the effect of phyA was masked by the strong phyB effect (Shinomura *et al.*, 1996; Botto *et al.*, 1996). Consequently, the increased germination rates after a phyB-inactivating light pulse were due to germination induction by phyA. Another strong hint for a prominent role of phyA is the fact, that germination is triggered in *ahk2 ahk3* seeds more effectively when low amounts of R light were applied (Figure 8), since phyA is activated also by very low amounts of light compared to phyB (Shinomura *et al.*, 1996).

To sum this up, CK negatively regulates phyB-dependent LFR germination, however a putative role of ARR4 in this process remains to be studied.

4.1.4 The repression of phyA-mediated germination by CK requires a functional CK metabolism and signaling pathway

Apart from the negative regulation of phyB-dependent LFR by CK, germination assays revealed that CK also negatively affected the phyA-mediated VLFR. In order to specifically induce germination via phyA, the germination inducing R light pulse was replaced by a FR light pulse. Due to variations in their activation spectra of the five phytochromes in *A. thaliana*, only phyA is induced by FR light (Shinomura *et al.*, 1996).

In FR light-induced germination assays, both *ahk2-2 ahk3-3* and *ahk2-5 ahk3-7* mutants showed drastically increased germination rates compared to the wild-type (Figure 10), so both strong and weak allele combinations had a similar impact on germination. Both in phyA- and in phyB-mediated germination, double mutant combinations including *ahk3* (*ahk2-2 ahk3-3*, *ahk2-5 ahk3-7* and *ahk3-7 cre1-2*) tended to germinate at higher rates as compared to allele combinations not containing *ahk3* (Figure 10). Interestingly, the loss of AHK3 in *ahk3* single mutants was not sufficient to increase germination rates. Nevertheless, AHK3 played a more pronounced role in the repression of germination in FR light compared to AHK2 or AHK4/CRE1. RNA-Seq data revealed a significant downregulation of *AHK3* transcript levels in seeds after WL and FR light illumination (Figure 49), supporting the idea of a more pronounced role of AHK3 in the seed germination process. A reason for that may be found in the ligand preferences of AHK receptors. AHK3 has a high affinity towards tZ and a lower affinity towards iP, so tZ might be the most important CK repressing germination (Spíchal *et al.*, 2004; Romanov *et al.*, 2006; Stolz *et al.*, 2011).

During development, tZ levels in seeds were high, but drastically decreased about twelve days after flowering (Kanno *et al.*, 2010). CK measurements in dry seeds conducted in this work showed increased tZ level in *ahk2 ahk3* compared to wild-type seeds (Figure 28), thus confirming results obtained by Riefler *et al.* (2006). Albeit, measuring CK profiles during imbibition revealed that tZ levels significantly decreased in both wild-type and *ahk2 ahk3* seeds after 24 h of imbibition compared to dry seeds, and stayed low until 48 h of imbibition, so a reduction of CK level during imbibition might support the onset of germination.

Ipt2 ipt9 mutants, which are characterized by a reduced production of cZ, are not affected in phyA-dependent germination thus supporting the hypothesis that tZ might be

the most prominent CK repressing germination. AHK receptors have a sevenfold lower affinity towards cZ compared to tZ (Romanov *et al.*, 2006; Stolz *et al.*, 2011). Additionally, cZ -type CK showed similar accumulation characteristics compared to tZ (Figure 28), so a general reduction of CK level during imbibition occurred.

To sum this up, not only the overall CK level is decisive for the induction of germination, also downstream CK signaling is required to mediate the repressive effect of CK on seed germination in VLFR light conditions. This was illustrated in *ahk2 ahk3* seeds, where all measured CK isoforms over-accumulated compared to wild-type seeds (Figure 28), but the seed germination pattern showed a reduced CK-dependent repression of germination.

4.1.4.1 Repression of phyA-dependent germination requires CK biosynthesis and is negatively affected by CK degradation

To refine the involvement of individual components of the CK signaling pathway, the germination phenotype of CK mutants and transgenic lines defective in CK biosynthesis, with enhanced CK catabolism, impaired CK signal transduction or signal output was investigated in VLFR light conditions. Seeds impaired in CK biosynthesis showed increased germination rates compared to wild type in FR light, even exceeding germination of *ahk2 ahk3* mutant seeds (Figure 15). Impaired iP and tZ production in *ipt3,5,7* and *ipt1,3,5,7* mutants drastically increased germination, what implies that the repression of germination by CK is mediated by iP- and/or tZ type CKs. The available *ipt4 ipt8* mutants had a WS background, both mutants and wild-type seeds did not germinate after a FR light pulse (data not shown). So if *IPT4* and *IPT8* participate in the regulation of phyA-dependent germination remained unclear.

Despite the role of CK biosynthesis by IPTs, gain-of-function of one individual IPT was not sufficient to lower germination rates in FR light. This was shown in germination assays using *rock4* seeds, which have a missense mutation in the CK synthesis gene *IPT3*, resulting in a gain-of-function version of IPT3 (Figure 16; Jensen, 2013). RNA-Seq analysis revealed only minor changes in *IPT* expression in the germination process both in wild-type and in *ahk2 ahk3* mutant seeds (Figure 49). Probably, *IPT* expression level in mature seeds are not decisive for the repression of germination by CK. Presumably, the physiological settings in the seeds, resulting in increased germination rates in FR in mature seeds, were already established during seed development.

Ectopic expression of a bacterial *IPT* under control of a seed-specific lectin promoter in tobacco revealed that *IPT* expression is effective to increase CK accumulation in seeds, promotes cell division in the embryo and increases embryo diameter (Ma *et al.*, 2008). The effects were most striking in later stages of development (16-21 DAF), what may imply that CK affects embryo development more in later developmental phases. In *B. napus* has been shown that *BnIPT1*, *BnIPT3*, *BnIPT5*, *BnIPT7* and *BnIPT8* are expressed early in seed development (from fertilization up to the heart stage, Figure 3) and expression was decreased in mature seeds (Song *et al.*, 2015). So in higher order *ipt* mutants, the lack of IPT activity throughout seed development may affect germination characteristics in mature seeds. To test, in which developmental phase the lack of CK biosynthesis affects the seed germination phenotype in VLFR conditions, an inducible, seed specific repression of *IPT*s at different stages of seed development may be appropriate to resolve this question.

Reduction of the CK status by overexpression of *CKX2* increased phyA-dependent germination, overexpression of *CKX4* did not (Figure 10). For germination experiments,

35S:CKX lines with a less strong phenotype were chosen according to the phenotypic descriptions in Werner *et al.* (2003). Interestingly, after illumination with R/FR light, seeds overexpressing *CKX2* responded stronger than *CKX4* overexpressing ones. The higher germination phenotype of seeds derived from *CKX* transgenic lines was confirmed also in *35S:CKX1* (Figure 25), suggesting similar effects in both phenotypically strongly affected and less affected lines.

One indication for different functions of individual *CKX* genes may be speculated based on different expression patterns in seed development. In wild-type seeds, *CKX2* is highly expressed in the micropylar endosperm at the pre-globular stage of development, *CKX4* is not expressed in developing seeds (eFP browser, data from Le *et al.* 2010). Overexpression of *CKX* genes under control of a 35S promoter resulted in systemic expression of the respective genes. The products of *CKX2* and *CKX4* are predicted to have a similar subcellular localization, the apoplast (Werner *et al.*, 2003) but differ in slightly in their substrate specificity. CKX4 has a higher affinity for tZ at pH 6.5 compared to CKX2, this difference was even more pronounced at pH 8 (Galuszka *et al.*, 2007). Still it is surprising that the overexpression of these genes had different consequences for germination in FR light.

Opposite to an enhanced CK degradation in mutants lacking CK catabolism via CKX, lower germination rates in FR light compared to wild-type seeds were expected. *ckx3 ckx5* loss-of-function mutants over-accumulate tZ and have a higher activity of placenta tissue, resulting in increased seed production (Bartrina *et al.*, 2011). But even quintuple *ckx* mutants showed only subtle differences in germination rates after a FR light pulse compared to wild-type seeds (Figure 15). This may be due to the experimental setup, which was not optimal to test the effect of higher CK levels in seeds. Because of the low wild-type germination rates in VLFR FR light, a reduction of germination was difficult to detect.

4.1.4.2 Repression of phyA-dependent germination requires CK signaling

Mutant lines harbouring constitutive active gain-of-function variants of *AHK2* and *AHK3* showed alterations in reproductive development. *rock3* mutant lines displayed an increased seed yield and a higher number of siliques, while *rock2* plants had more siliques but no increase in seed yield (Bartrina *et al.*, 2017). Counterintuitively, *ahk2 ahk3* seeds germinated at high frequencies in FR light, but *rock2 rock3* seeds showed no significant alteration in germination in FR light, and rather tended to germinate to higher rates than wild-type seeds as well (Figure 16). Interestingly, expression analysis of the well-established CK reporter system *ARR5:GUS* demonstrated previously the increased *ARR5:GUS* expression in *rock2* and *rock3* single mutants in *A. thaliana* seedlings (Bartrina *et al.*, 2017). Since the downstream effects of constitutive signaling at the level of the CK receptors are unknown, a deregulation of downstream components of the CK signaling pathway, which might affect germination, remains elusive.

Impaired CK signaling downstream of the CK receptors was sufficient to lift the repression of germination by CK. Loss of AHPs downstream of the receptors (*ahp2,3,5* mutant seeds) conferred high germination rates in FR light (Figure 16). *AHP2* and *AHP5* were highly expressed in the endosperm compared to other silique tissues (Day *et al.*, 2008), but the loss of function of *AHP2* and *AHP5* was not sufficient to alter germination in FR light (Figure 16). AHPs show a high functional redundancy in the shoot and root

(Hutchison *et al.*, 2006; Deng *et al.*, 2010), what might explain the wild type-like germination, if only two AHPs are lacking (Figure 16).

AHP6 acts as a negative regulator of the CK signaling pathway, since it does not propagate the phosphate signal to downstream response regulators (Mähönen *et al.*, 2006). *AHP6* was expressed in developing embryos (Mähönen *et al.*, 2006). In this work was demonstrated that a loss of the negative regulation of the CK signaling system by *AHP6* did not affect germination (Figure 16) leading to the conclusion that AHP6 is not a central regulator of phyA-mediated VLFR.

B-type *ARR*s, which showed a highly specific expression profile in seeds (Day *et al.*, 2008), may be particularly interesting for seed germination, since the response of one specific seed tissue or part of a seed tissue can be sufficient to alter germination characteristics of the entire seed. One example is the micropylar endosperm region, where cell elongation of the tissue is correlated with testa rupture (De Giorgi *et al.*, 2015). In *A. thaliana*, *ARR19* and *ARR21* were specifically expressed in the chalazal endosperm of developing seeds (Tiwari *et al.*, 2006; Day *et al.*, 2008). Additionally, overexpression of *ARR21* resulted in increased *IPT4* expression (Kiba *et al.*, 2005; Day *et al.*, 2008) so loss of *ARR21* expression might decrease CK biosynthesis and consequently increase germination in FR light. To analyze the consequence of a site-specific loss of CK downstream signaling, *arr19* and *arr21* seeds were tested for their germination response in FR light. The loss of function of *ARR19* and *ARR21* either in single or double mutant combination neither resulted in higher germination rates in FR light nor in abortion of seeds in the siliques (Figure 17, Figure 18). That argues against a prominent role of *ARR19* and *ARR21* in seed development and in phyA-dependent seed germination. The hypothesis of a site-specific CK action in the chalazal endosperm that might repress germination was not supported in this work.

Apart from ARR19 and ARR21, the most prominent and the most studied B-type ARRs in *A. thaliana* are ARR1, ARR10 and ARR12 (Mason *et al.*, 2004; Mason *et al.*, 2005; Ishida *et al.*, 2008). In phyA-mediated germination, double mutant combinations of *ARR1*, *ARR10* and *ARR12* showed no differences to wild-type seeds (Figure 18). To further analyze the role of ARR1, ARR10 and ARR12 transcription factors in germination, FR light-induced germination should be analyzed in *arr1 arr10 arr12* seeds. Noteworthy, RNA-Seq data showed no differences in B-type *ARR*s expression, B-type *ARR*s were regulated similarly between wild-type and *ahk2 ahk3* seeds in germination (Figure 49). Nevertheless, RNA-Seq data can only provide hints, but may not provide definite evidence for the importance of B-type ARRs in germination. Loss-of-function of other B-type ARR family members, such as *arr2* and *arr14* did not affect germination in VLFR FR light (Figure 18).

Based on these results, loss of one or two functional B-type ARRs does not affect seed germination in FR light, what may be explained by a general high functional redundancy of B-type ARRs (Nguyen *et al.*, 2016), presumably also in the regulation of germination. Thus, higher-order mutants should be tested in FR light germination assays. To sum this up, the results obtained in this work do not provide a clear picture about the relevance of B-type ARRs in the CK-mediated repression of germination in VLFR light conditions.

A-type ARRs act redundantly as negative feedback regulators of the CK signaling circuit (To *et al.*, 2004). The lack of feedback regulation is expected to result in a more active CK signal transduction and thereby a further repression of FR light-induced germination. Strikingly, *arr3,4,5,6,8,9* seeds lacking six of ten A-type *ARR*s germinated

comparable to wild-type seeds (Figure 19). Transcriptomic analysis revealed no changes in the expression profile of A-type *ARR* genes during the germination process (Figure 49). As mentioned above, one of these ARRs, namely ARR4, had already been connected to phyB in the literature (Sweere *et al.*, 2001). In this work, the role of ARR4 in phyA-dependent germination was tested. Neither *ARR4* overexpression nor *ARR4* loss-of-function were affected in germination after a FR light pulse (Figure 19). This is congruent with the results of Mira-Rodado *et al.* (2007), in their work a direct interaction between phyA and ARR4 was not evident. So, a similar regulation of phyB and phyA by ARR4 in FR light seems unlikely.

Apart from the tested ARRs, other A-type ARRs might be more relevant for germination. Among the A-type ARRs, ARR7 and ARR15 are presumably the most important regulators of germination as they integrate CK and auxin signals in embryo development (Müller and Sheen, 2008). In FR light, *arr7 arr15* mutant seeds exhibited repeatedly, but not significantly lower germination rates compared to wild-type seeds (Figure 19). The potential role as germination-promoting actors is further supported by the notion, that ABI4 was able to bind to their promoters and repressed *ARR7* and *ARR15* expression in germinating seeds (Huang *et al.*, 2017).

Collectively, a decreased CK status as well as an impaired CK signal transduction positively influences seed germination, strengthening the role of CK as a negative regulator of the phyA-dependent VLFR. Nevertheless, the role of CK signaling components such as A- and B-type ARRs needs further clarification.

4.1.5 CK alters the kinetics of germination and effectively represses it in very low light conditions

Induction of germination via phyA requires only very low light fluences and is not specific for certain light qualities. Already a photon fluence of 0.5 – 10 µmol m^{-2} FR light is sufficient to induce germination (Shinomura *et al.*, 1996). Apart from FR light, a wide variety of irradiations from UV to visible light induces phyA action and triggers VLFR germination (Shinomura *et al.*, 1996; Botto *et al.*, 1996). Consistently, in CK receptor double mutant seeds, germination was effectively induced by blue light and by FR light illumination with 10-18 µmol m^{-2} (Figure 11, Figure 12).

Variation in the seeds' physiological status including phytohormones may account for differential timing of germination. In this work was shown, that seeds with a reduced CK perception germinated earlier in WL conditions compared to wild-type seeds, this effect was even more pronounced in FR light (Riefler *et al.*, 2006; Figure 13). The repression of germination by CK seems to be relevant especially in unfavorable light conditions.

As has been previously demonstrated, hormone sensitivity can be light-dependent. This was studied by applying a GA2ox2 non-cleavable GA_4 analogue to seeds, in FR light the seeds were more sensitive towards the non-cleavable GA than in R light (Yamauchi *et al.*, 2007). This indicates that not only the hormone status or signal transduction, but also the light environment shapes the seed germination response via an altered hormone sensitivity. A similar model might be suggested for CK, where the repression of germination by CK is more effective in FR light than in WL. Ecologically, with the help of alterations in the CK status or light-dependent CK sensitivity in individual seeds a different timing of germination may be achieved, lower CK levels might facilitate early germination in FR light conditions. Ecologically, different timing of seed germination is advantageous for the seed

population to avoid competition among the siblings and minimize the risk of extinction of all individuals due to unfavourable environmental conditions (Nonogaki, 2014).

4.1.6 Externally applied CK does not repress germination

Because CK negatively affects seed germination, in this work the implications of external CK application should be clarified. A recent study provided evidence that CK application accelerates seed germination in *Medicago* (Araújo *et al.*, 2019). In contrast, even though external BA application rescued the ABA-mediated block of cotyledon greening in *A. thaliana* seedlings, CK treatment did not affect germination in WL conditions (Guan *et al.*, 2014). Regarding the CK concentrations used in the study showing positive effects of BA on germination, a 1000-fold higher BA concentration was used (Araújo *et al.*, 2019) compared to the study in *A. thaliana* where CK treatment had no effects (Guan *et al.*, 2014). In this work, even 5000-fold lower concentrations of CK were capable to induced mild effects in wild-type seeds, CK application seemed to enhance germination capacity in FR light rather than repressing it (Figure 20). This indicated, that seed were able to perceive even low BA concentrations applied externally. Seeds also responded to external application of GA, ABA or nitrogen fertilizer which shaped their germination phenotype (Batak *et al.*, 2002; Finkelstein *et al.*, 2008). CK was, as well as other phytohormones, able to penetrate the seed-covering layers and alter the germination response of treated seeds. Basically, external CK application to mature seeds had the opposite effect as internal alterations of the CK status or CK signaling. This may indicate that the negative impact of CK on VLFR germination was established already during seed development. Thus, the external application of CK to mature, dry seeds does not have the same effect as a reduced CK status during seed development.

4.2 phyA is required for FR light-induced germination but CK does not affect absolute phyA levels

The onset of light-dependent germination strongly depends on phyA and phyB in FR and R light respectively. But also phyD and phyE were described to play a role in seed germination (Hennig *et al.*, 2002; Martel *et al.*, 2018). PhyD is required for seed germination when seeds were in a secondary dormancy state induced by high temperature, referred to as thermoinhibition of germination (Martel *et al.*, 2018). phyE contributes to germination in continuous blue and FR light, acting in concert with phyA (Hennig *et al.*, 2002).

Nevertheless, for the induction of germination by FR light, phyA is essential and the most prominent photoreceptor, thus a negative effect of CK on phyA either in a direct or indirect way may be assumed. Since the repression of germination by CK was most pronounced in the phyA-dependent VLFR, a negative regulatory function of CK on phyA was assumed. Analyses of seeds lacking phyA and either CK biosynthesis or CK perception revealed germination rates mimicking the *phyA* single mutant phenotype (Figure 22). These results indicate that *PHYA* is epistatic to *AHK2* and *AHK3* in very low fluence light-induced germination. Based on these results, seeds impaired in CK signal transduction or CK biosynthesis do not harbor an alternative, phyA-independent pathway for very low fluence light perception. These results also do not support the participation of other photoreceptors, such as phyD or phyE in the regulation of germination in FR light.

The increased light sensitivity of CK mutants led to the speculation that CK negatively regulates phyA protein abundance or protein accumulation. In this work, the accumulation

pattern described by Lee *et al.* (2012b), where phyA protein level accumulated in the first 12 h of dark imbibition, reached a plateau phase after 12 h with no further increase after 24 h and 48 h, could not be confirmed (Figure 23). In contrast, wild-type phyA levels reproducibly increased after 48 h imbibition compared to 24 h imbibed seeds (Figure 23). A reason for that might the experimental conditions. In contrast to the work of Lee *et al.* (2012b), in this work seeds were not exposed to WL during sowing and imbibition. Pre-treatment with WL prior to the application of the first phyB-reverting FR light pulse induced higher germination rates as compared to seeds which were strictly kept in darkness prior to FR light treatment (data not shown), indicating that in WL pre-treated seeds signaling cascades are activated, which could not be inhibited by a phyB-inactivating light pulse any more. So the delayed accumulation of phyA in this work, as compared to Lee *et al.*, 2012b, may be a result of the lacking WL pre-treatment. In *ahk2 ahk3* mutant seeds the effect of phyA accumulation between 24 h and 48 h was less reproducible (Figure 23).

Quantification of absolute phyA levels revealed no evidence for increased phyA levels in *ahk2 ahk3* seeds, so a repressive effect of CK on phyA abundance seems unlikely but could not be excluded in this work, due to the experimental setup. The reasons for that were the highly variable results obtained from the western blot protein quantification, the unstable UGPase housekeeping gene expression patterns and the high intraspecific biological variance between the tested seeds derived from in parallel grown and harvested parental plants. Since CK did not seem to alter phyA levels directly, however, CK might impact phyA indirectly, for example by affecting its subcellular distribution or activity. Active phyA was imported in the nucleus in a light-dependent manner (Li *et al.*, 2011). Active phyA physically interacted with FHY1 and FHL in the cytoplasm (Desnos *et al.*, 2001; Hiltbrunner *et al.*, 2005; Genoud *et al.*, 2008). With the help of the NLS sequence of its signaling partners, phyA translocated to the nucleus (Hiltbrunner *et al.*, 2005; Zhou *et al.*, 2005). Analysis of the RNA-Seq dataset in this work revealed no correlation between CK and *FHY1/FHL*. Both genes were upregulated during imbibition but downregulated after a light pulse both in *ahk2 ahk3* and wild-type seeds (Figure 52). To study if CK negatively affects protein-protein interaction between phyA and FHY1 or FHL, an *in planta* bimolecular fluorescence complementation assays (BiFC) in an *ahk2 ahk3* background could be used. Furthermore, phyA uptake assays, using fluorescence-labelled versions of phyA would help to clarify if CK hinders phyA nuclear import.

CK might also induce phosphorylation of phyA to decrease its stability without affecting absolute protein levels. The phosphorylation of phyB triggered dark reversion, in phyA phosphorylation triggered rapid degradation (Medzihradszky *et al.*, 2013). Phosphorylation of phyB was independent of the interaction with ARR4, indicating that accelerated dark reversion by phosphorylation is mediated by an alternative pathway (Medzihradszky *et al.*, 2013). A possible connection between phyA and ARR4 with regard to phosphorylation remains elusive.

Absolute phyA protein levels at the analyzed timepoints did not seem to be decisive for increased germination rates of CK mutants, but probably the phyA-dependent regulation of the transcriptome might affect germination. After induction of germination by a FR light pulse, the expression of 11% of the genome was regulated by phyA (Ibarra *et al.*, 2013). Several links between CK and phyA signaling on transcript level had been described. Genome-wide transcript profiling found deregulation of light-signaling components in CK-deficient plants. *PHYA* transcripts were downregulated, while *COP1/SPA* transcripts, which are associated with phyA degradation, were upregulated in CK-deficient plants in the shoot

and in the root (Brenner and Schmülling, 2012). This is in line with the notion, that CK treatment upregulated *PHYA* (Brenner *et al.*, 2005). Additionally, phyA upregulated the CK-related signaling components *CRF1*, *CRF2* and *CRF3* (Ibarra *et al.*, 2013). In this work, *PHYA* expression was 20-fold increased in CK receptor mutant seeds during dark imbibition as compared to wild-type seeds, suggesting a negative effect of CK on *PHYA* transcript abundance (Figure 34). The contradictory findings in Brenner *et al.* (2012) and this work may be based on the different test systems (seeds or seedlings) used. While in WL-treated seedlings, light perception is primarily regulated via phyB, dark-imbibed seeds strongly rely on light perception via phyA. So the impact of CK on *PHYA* expression in WL may be less informative compared to the expression pattern specifically in FR-light induced seed germination. However, a negative feedback mechanism might be plausible as well, since phyA upregulated *CKX5* expression, which catabolizes CK (Ibarra *et al.*, 2013).

At the end of the imbibition period, global transcript analysis via RNA-Seq revealed high *PHYA* transcript levels both in wild-type and in CK receptor mutants, which decreased after a WL or FR light pulse (Figure 52). This is congruent with the notion, that *PHYA* expression was negatively regulated by light (Somers and Quail, 1995), gene expression rapidly decreased after five hours of illumination with FR light (Cantón and Quail, 1999). Recapitulatory, the negative effect of CK on seed germination could not be easily explained by altered phyA protein levels. To clarify the role of *PHYA* gene expression and potential consequences for phyA downstream targets, further experimentation is required.

4.3 The negative impact of CK on FR light-induced germination seems to be largely independent of ABA and GA

4.3.1 GA

GA is a long known and intensively studied phytohormone in the context of seed dormancy and germination, but is also involved in numerous other plant traits, such as root and shoot elongation, flowering and seed development (Achard and Genschik, 2009; Daviere and Achard, 2013; Hedden and Sponsel, 2015).

4.3.1.1 GA hormone level are not regulated by CK during imbibition

Plants carefully balance hormonal levels in seed development, especially GA levels are tightly coupled to ABA. GA_{12}, together with the 13-non hydroxylated GA_{15} and GA_4 are the dominant GA isoforms in germinating seeds (Ogawa *et al.*, 2003).

In dry seeds GA_{12} and GA_4 were identified as most abundant GA isoforms (Figure 26). This was expected since GA_4 is the most effective isoform to induce germination (Yang *et al.*, 1995; Ogawa *et al.*, 2003). In this work could be shown that the seeds' CK status did not alter GA_4 level in dry seeds but seemed to hinder GA_1 accumulation in dry seeds (Figure 26). GA_1 is regarded as positive regulator of germination as well but is less prominent compared to GA_4. In developing siliques of *COGWHEEL* (*cog*) mutants, which germinated to higher percentages than wild-type seeds after deterioration treatment, higher GA_1 levels were evident (Bueso *et al.*, 2016).

As compared to GA level in dry seeds, level in the course of imbibition and germination are of specific interest. GA level in dry seeds are only partly suitable as indicator for the seed germination phenotype, GA level during and at the end of the imbibition phase allow more precise predictions (Yamauchi *et al.*, 2007). Since CK receptor mutants showed higher germination rates in FR light, an increased accumulation of

germination-promoting GA might be reasonable. This was not confirmed experimentally in this work, both GA_1 and GA_4 level were drastically decreased during imbibition (Figure 26). This decrease was evident in wild-type as well as in *ahk2 ahk3* seeds, suggesting a CK-independent regulation of bioactive GA levels in imbibed seeds. One plausible explanation for these findings might be, that GA level were analyzed in dark imbibed seeds in this work, but the dynamics of GA level in germinating seeds is strongly light dependent (Yamaguchi *et al.*, 1998; Oh *et al.*, 2007). In continuous light, GA_4 accumulated in germinating seeds, reaching highest levels 40 h after exposure to light (Ogawa *et al.*, 2003). So it would make sense to measure GA level after FR light exposure and compare them between wild-type and *ahk2 ahk3* seeds.

Comparable to GA_1 and GA_4, a decrease of all other measured GA isoforms during seed imbibition was evident in this work (Figure 26), albeit previous research found no strong regulation of these GA isoforms in the course of seed germination (Ogawa *et al.*, 2003). This decrease of GA isoforms may be explained by an initial leakage of solutes from imbibing seeds in the course of germination. This mechanism reduces the overall hormone content, including the concentration of germination-inhibitors such as ABA and is a sign for membrane damage (Matilla *et al.*, 2005; Weitbrecht *et al.*, 2011).

To sum this up, elevated GA level in CK-deficient seeds are not causal for the increased germination rates in FR light, suggesting that the seeds CK status is not relevant for GA accumulation during imbibition.

4.3.1.2 Transcriptional regulation of the GA metabolism genes is independent of CK in FR light-induced germination

On a transcriptional level, insights into the regulation of GA metabolism-associated genes in FR light are rare. First, GA biosynthesis genes were analyzed. In this work, the GA biosynthesis-associated genes *GA3ox* and *GA20ox* were slightly upregulated early in the imbibition process (Figure 51), but in later stages of germination *GA20ox1,* another GA biosynthesis-associated gene, was downregulated (Figure 34). It is known that GA biosynthesis genes displayed a low activity in darkness after a first FR light pulse (Yamaguchi *et al.*, 2002), what causes a lack of GA *de novo* production. GA biosynthesis genes were upregulated in a light-dependent manner via phytochromes (Yamaguchi *et al.*, 1998; Ogawa *et al.*, 2003; Oh *et al.*, 2007), but obviously short WL or FR light pulses as applied in this work, did not induce a strong upregulation of GA biosynthesis genes. Interestingly, application of WL and FR light to induce germination seemed to affect GA biosynthesis gene expression in a similar way (Figure 51), suggesting that phyA- and phyA/phyB-mediated induction of germination feed into similar pathways in the regulation of GA-associated genes.

The GA catabolism-associated *GA2ox3* was upregulated during imbibition (Figure 51), mirroring expression patterns of *GA2ox2*, the most prominent GA catabolism-associated gene in germination, which was described as well as upregulated during of imbibition (Seo *et al.*, 2006). *GA2ox2* was repressed in a light-dependent manner (Yamaguchi 1998, Ogawa 2003, Oh 2007), but again short light pulses seem to be less effective.

Considering the role of CK in the regulation of GA-associated genes, CK repressed *GA20ox* and *GA3ox* expression in seedlings (Brenner *et al.*, 2005). In this work, no connection between CK and the regulation of GA-associated genes was evident (Figure 34). GA biosynthesis genes as well as GA catabolism-associated genes were similarly

regulated in wild-type and *ahk2 ahk3* receptor mutant seeds (Figure 51). Summarizing this, GA metabolism was not affected by CK on a transcriptional level in germinating seeds.

4.3.1.3 CK negatively influences GA sensitivity in FR light-induced germination

To understand the role of GA in the seed germination process, its viable to note that not only absolute hormone level regulate germination, but hormone sensitivity has a major impact on the germination response. The seeds' GA sensitivity depends on environmental conditions such as light and temperature. Cold treatment for example increased the sensitivity of seed germination towards externally applied GA (Derkx and Karssen, 1993; Debeaujon and Koornneef, 2000; Yamauchi, 2004).

In this work CK negatively regulated the sensitivity towards externally applied GA_4, since both a lower CK biosynthesis and lower CK signal transduction resulted in increased GA sensitivity (Figure 30). It is important to note that these results were highly reproducible for *ipt3,5,7* seeds, but less reproducible for *ahk2 ahk3* seeds. The increased GA sensitivity was independent of the light conditions used to induce germination, similar effects were observed in darkness, in WL and in FR light (Figure 30). These results indicate that the negative regulation of GA sensitivity by CK in seeds may be an additional mechanism for CK to suppress germination. But these findings do not explain the observed light-specificity of the CK -mediated repression of germination, so additional mechanisms presumably coupled to phytochromes are necessary to confer light-specificity.

Crossings of CK mutants with mutants of the biosynthesis GA pathway, for example *ga3ox* or *ga20ox* mutants, might allow a first idea if the regulation of germination by GA is upstream of the regulation by CK. Testing the seeds' sensitivity towards the GA biosynthesis inhibitor Pac might reveal if CK decelerates GA biosynthesis. Assuming a negative regulatory effect of CK on GA biosynthesis, CK mutant seeds should be less sensitive towards applied Pac compared to wild-type seeds. This has already been shown for *arr1 arr12* mutants, which were insensitive towards externally applied Pac in the DELLA-mediated cotyledon opening (Marín-de la Rosa *et al.*, 2015), indicating that an altered Pac sensitivity due to the CK status might be a regulatory mechanism to control GA biosynthesis in germination.

4.3.1.4 Expression profiles of genes encoding GA-associated signaling components were not dependent on CK or FR light in germination

Multiple GA downstream components regulate and fine-tune GA signaling throughout the plants' life. In this work, the analysis of GA downstream components and their potential role in the regulation of FR light germination by CK and in conferring an increased GA sensitivity in CK receptor mutant seeds was analyzed based on RNA-Seq data, which may provide only a limited significance.

The GA signal is perceived by GID1 receptors, which seem to function as positive regulators of seed germination in the light (Griffiths *et al.*, 2006; Willige *et al.*, 2007; Voegele *et al.*, 2011). Analysis of transcriptomic data showed that transcript level of *GID1a* and *GID1c* were regulated irrespective of light quality or CK in germination (Figure 51), thus a role of GA receptors in the repression of VLFR germination by CK was not supported. Following the idea, that CK interfered with GA downstream signaling components to regulate germination, DELLA genes were analyzed in more detail. During imbibition *DELLA* expression was slightly upregulated (Figure 51) and downregulated after induction of germination, independent of the light quality (Figure 51). Only *RGL1* was upregulated in

later stages of germination, what was not expected since *RGL2* was described as most important regulator of germination (Lee *et al.*, 2002). Since no alterations between the genotypes were measured, *DELLA* gene expression during germination seems to be independent of CK. A CK-mediated upregulation of *RGA* and *GAI* as in seedlings (Brenner *et al.*, 2005) was not evident in the germination process. The analysis was carried out on transcript level only, although information on protein level would have been more informative, since DELLAs are prone to fast degradation after induction of germination (Tyler *et al.*, 2004).

Another convergence point between DELLAs and CK are the physical interactions of RGA and GAI with the B-type ARRs ARR1, ARR2 and ARR14 (Marín-de la Rosa *et al.*, 2015). But in VLFR conditions ARR1, ARR2 and ARR14 were not identified as major regulators of the CK-mediated suppression of germination (Figure 18), although *arr1 arr2 arr14* mutant should be generated and tested for their germination phenotype in FR light. Concluding from this, the action of the ARR-DELLA module does not seem to be a major regulatory mechanism for phyA-dependent germination.

Thus, the increased sensitivity of CK receptor mutant seeds towards GA, as discussed previously (discussion section 4.3.1.3), did not seem to be coupled to known GA signaling-associated components, at least on a transcriptional level.

4.3.2 ABA

The regulation of ABA is of crucial importance for the onset of seed germination. ABA is important to prevent premature germination and delays the onset of germination when environmental conditions are not optimal for the emerging seedling (Holdsworth *et al.*, 2008b).

4.3.2.1 ABA hormone level are not directly regulated by CK during imbibition

Additional to the analysis of the impact of CK on the transcriptional regulation of ABA-associated transcripts, ABA level were measured. ABA level dropped in the course of imbibition both in CK-deficient and in wild-type seeds (Figure 27). This was expected, since a reduction of ABA level prior to germination is vital to allow germination. In non-dormant seeds of the *A. thaliana* ecotype Cape Verde Island the ABA content decreased rapidly during the first 24 h of imbibition before it reached a stable state (Ali-Rachedi *et al.*, 2004). Interestingly, the overall ABA content in seeds was not affected by the seeds' CK status (Figure 27). This has already been demonstrated in seedlings of seven-day-old *ahk2 ahk3* receptor mutants (Kumar and Verslues, 2015). Consequently, also the strong reduction of ABA level in imbibed seeds seems to be independent of CK.

One plausible explanation for the reduction of ABA level, besides by leakage of solutes out of the seeds (Matilla *et al.*, 2005; Weitbrecht *et al.*, 2011) are internal regulatory mechanisms, such as the catabolic degradation of active ABA in imbibed seeds (Ali-Rachedi *et al.*, 2004). To summarize this part, this work revealed no indications that CK affects ABA level in imbibed or germinating seeds.

4.3.2.2 Transcriptional regulation of ABA metabolism genes is independent of CK in FR light-induced germination

For the onset of germination, ABA-dependent processes are regulated in a light dependent manner. R light downregulated ABA biosynthesis-associated genes like *ABA1*, *NCED6* and *NCED9* (Seo *et al.*, 2006; Oh *et al.*, 2007; Seo *et al.*, 2009). But this downregulation was

not observed in FR light conditions in qPCR analysis conducted in the present thesis (Figure 34). These data suggest that the regulation of ABA biosynthesis by FR light strongly differs from the regulation induced by R light illumination. Furthermore, there was no evidence for a CK-induced regulation of ABA biosynthesis genes in germinating seeds, so a reduced expression of ABA biosynthesis genes in FR light germination does not seem to be decisive for the higher germination rates in seeds lacking CK perception.

But not only biosynthesis of ABA is regulated in seeds, also a coordinated ABA degradation is pivotal for the regulation of ABA level. R light induced the ABA catabolic gene *CYP707A2* (Oh *et al.*, 2007). Analysis of *CYP707A* genes in germination via qPCR and RNA-Seq showed upregulation in wild-type seeds (Figure 34, Figure 50), in *ahk2 ahk3* seeds analyzed via qPCR a downregulation of ABA-catabolizing genes was found, while in the RNA-Seq dataset upregulation of *CYP707A* genes was evident (Figure 50). In sum, to resolve the question if CK alters the expression of ABA degradation-associated transcripts, a more detailed expression analysis of *CYP707A* genes in FR light conditions should be conducted.

4.3.2.3 The influence of CK on ABA sensitivity in FR light-induced germination is still unclear

As in the case of GA, not only ABA content but also ABA sensitivity is crucial for the seeds' germination phenotype (Kawakami *et al.*, 1997). Since no evidence was found arguing in favor of a CK-induced increase in ABA level during imbibition, the impact of CK on the sensitivity towards externally applied ABA was tested. It was found that ABA treatment suppressed germination in WL independent of the seeds' CK status (Figure 29). In FR light, CK receptor mutants were sensitive to ABA application, but comparison to the sensitivity of wild-type seeds was difficult, due to the low wild-type germination rates. To overcome this problem and test if CK alters sensitivity towards exogenous ABA, additional experiments should be conducted. For example, endogenous ABA biosynthesis may be suppressed in the seeds prior to ABA application. One ABA biosynthesis inhibitor widely used in germination assays is norflurazon, which suppresses carotenoid biosynthesis and effectively prevents endogenous ABA accumulation (Chamovitz *et al.*, 1991). Besides comparing the consequences of reduced ABA biosynthesis for FR light germination between CK-deficient and wild-type seeds, also tests with balanced application of exogenous ABA and norflurazon may provide helpful insights in ABA dynamics dependent on CK.

4.3.2.4 ABA downstream signaling components are regulated independently of CK in FR light-mediated germination

CK and ABA downstream signaling components are linked to each other in germination (Wang *et al.*, 2011; Huang *et al.*, 2017). For instance, in *IPT8* overexpressing seeds the A-type ARRs ARR4, ARR5 and ARR6 suppressed *ABI5* expression and physically interacted with ABI5 (Wang *et al.*, 2011). In phyA-dependent germination, *ABI5* was downregulated over time in wild-type, in *ahk2 ahk3* and in *phyA* seeds (Figure 34 A), what indicates a FR light and CK status- independent regulation.

Also ABI4 is coupled to CK in WL germination. ABI4 was capable to bind the promoters of the A-type ARRs *ARR6*, *ARR7* and *ARR15* to repress germination (Huang *et al.*, 2017). The growth phenotype of *abi4 ahk2 ahk3* plants suggested that ABI4 acts downstream of AHK2 and AHK3 in mediating CK responses during plant growth (Huang *et*

al., 2017). Unfortunately, *ABI4* transcript level were not analyzed due to technical difficulties in the present thesis, therefore no statement about its possible involvement in FR light induced germination is possible in this work.

ABI3 was also analyzed on transcript level in germination. ABI3 is transcription factor repressing germination by the coordinated action with PIF1, which induced *SOM* expression in imbibed seeds (Park *et al.*, 2011). *ABI3* expression in qPCR measurements was similar between the genotypes (Figure 34 A), while in the RNA-Seq dataset *ABI3* showed a slight downregulation after WL and FR light illumination (Figure 50), suggesting a light-quality independent regulation. Also, a regulation by CK seems implausible, since *ABI3* regulation was similar in wild-type and CK receptor mutant seeds (Figure 34 A).

Another, less well described ABA signaling component was analyzed in this work. RRL1 confers an increased response to ABA in *A. thaliana* seed germination (Yao *et al.*, 2015). *RRL1* expression seemed to be reduced in *ahk2 ahk3* seeds compared to wild-type and *phyA* seeds, what may indicate a lower ABA sensitivity of *ahk2 ahk3* seeds in FR light germination (Figure 34 A). To follow this idea, crossings between *ahk2 ahk3* mutants and *RRL1* overexpressing lines and subsequent germination assays with exogenously applied ABA may clarify if loss of CK signaling can restore ABA sensitivity in *RRL1* overexpressors. To sum this up, there is only limited experimental evidence that ABA downstream signaling components are regulated by CK in the VLFR germination in seeds.

4.4 The CK status of the maternal parent is important for seed germination in FR light

Mature seeds consist of an embryo, the testa and the endosperm. The genotype of the testa is identical to the mother plant, while the endosperm tissue contains two copies of the maternal and one copy of the paternal genome resulting in an increased maternal genome dosage. In *A. thaliana*, the endosperm is a single cell-layer tissue, which is metabolically active (Olsen, 2004). The endosperm is also capable to respond to a light stimulus by releasing ABA towards the embryo to repress seed germination (Lee *et al.*, 2010; Lee *et al.*, 2012b). Presumably also CK plays a role in maternal seed tissues. tZ was more abundant in the maternal silique envelope compared to developing seeds at the end of the late maturation phase (Kanno *et al.*, 2010).

In the framework of this thesis was hypothesized that the CK status in testa or endosperm may alter the seed germination response in FR light. To test that, the germination phenotype of F1 seeds derived from reciprocal crosses between wild-type and plants with a lower CK status or signal transduction were analyzed. Seeds with a reduced CK signal transduction or increased CK catabolism in maternal seed tissues displayed higher germination rates compared to seeds with a higher wild-type genome dosage in the maternal tissue (Figure 24). This indicates that the CK status in maternal tissue is important for phyA-dependent germination.

To refine in which of the maternal tissues the CK status plays a role, plant lines were analyzed which express *CKX1* under control of either a testa- or an endosperm-specific promoter. It was found that a reduction of the CK status specifically in one maternal tissue was not sufficient to increase germination rates significantly in FR light (Figure 25). These findings may indicate that the CK status in both seed tissues with a higher maternal genome dosage is instructive for the higher germination rates in FR light. To confirm site-specific promoter activity, GUS staining was conducted and a site-specific activity of the

respective promoters was confirmed (Figure 25). Nevertheless, the actual transcript abundance of *CKX1* and CKX1 activity in the testa and the endosperm should be measured in these lines.

A multitude of maternal effects is known to alter seed germination (Galloway, 2001; He *et al.*, 2014) but the identification of the molecular mechanism how CK is involved here, lies beyond the scope of this work. In the following, some ideas and potential roles of CK in maternally-derived seed tissue are collected and may provide starting points for further research.

4.4.1 Testa characteristics may be altered by CK

Since both the CK status in the testa and in the endosperm appear to be important for germination, the role of the testa tissue will be discussed in the following section.

The dead testa tissue regulates the light spectrum and intensity that reaches the embryo by its pigmentation (Yamauchi *et al.*, 2007; Donohue, 2009). Testa pigmentation is based on oxidation of colorless proanthocyanidins, they confer a brown color to mature seeds after their oxidation (Debeaujon *et al.*, 2000). There are indications that CK might play a role in testa pigmentation, but experimental data in the literature is scarce. In oilseed rape, CK measurements revealed a reduced CK content in darker seeds compared to lighter seeds (Wan *et al.*, 2013). According to the results in this work, rather the opposite effect would be expected, less pigmentation in seeds with a reduced CK status might increase light permeability and thus confer higher germination rates. Measurements of anthocyanidines in seeds with a reduced CK status or an altered CK signal transduction could reveal if CK impacts testa pigmentation. Interestingly, in *A. thaliana* seed coat color is associated with testa permeability, as demonstrated by staining of transparent testa mutants (Debeaujon *et al.*, 2000) although the mechanism is still not fully understood. To gain deeper insight, if CK affects testa permeability or testa pigmentation, further experimentation is required. Therefore, a tetrazolium staining assays should be conducted using different mutants with an increased or decreased CK status (Debeaujon *et al.*, 2000). On the basis of the staining intensity of the embryo, the permeability of the testa may be evaluated.

4.4.2 CK action in the endosperm may regulate phyA-dependent germination

Endosperm growth is tightly coupled to seed size. A functional relevance of CK affecting seed size has been described earlier (Werner *et al.*, 2003). Presumably, the reduction of CK content or signaling in maternal seed tissues is due to an increased cell size in the endosperm or testa. This would result in reducing the mechanical constrains which hinder germination. In germinating seeds, the micropylar endosperm cell size was positively correlated with testa rupture, an increased cell size thus favors germination (De Giorgi *et al.*, 2015). Only recently has been demonstrated that cell expansion in the micropylar endosperm and in micropylar-endosperm adjacent region, which is coupled to *EXPANSIN2* (*EXPA2*) expression, reduced the mechanical constrains of the seed coat and was a prerequisite for the completion of germination (Sánchez-Montesino *et al.*, 2019). To test if altered cell sizes are causal for the germination phenotype of CK mutant seed, micropylar endosperm cell size in *35:CKX1*, *35S:CKX2* and in *ahk2 ahk3* seeds should be measured.

On a molecular level, the CK status in seed tissues with a higher maternal genome dosage may also be coupled to other phytohormone signaling pathways. So CK might act in concert with ABA to regulate germination in seed tissues with a higher maternal genome

dosage, multiple convergence points connect ABA and CK in the regulation of germination. As described above, ABA level and ABA sensitivity were not affected by CK on a whole seed level. But for fine-tuning of hormone signals, also the release of hormones from the seed coat affected the germination response, as shown in seed coat bedding assays (Lee *et al.*, 2012b). It may be speculated that CK may specifically affect ABA level or sensitivity in the endosperm or in the embryo, what would presumably not alter overall ABA content or ABA sensitivity measured in the present thesis. To evaluate if CK signaling in the endosperm promotes ABA accumulation or ABA release towards the embryo, ABA level in the seed coat and in dissected embryos should be measured.

In this work could be shown that the presence of a seed coat negatively influences germination in all tested light conditions (Figure 55) what is in accordance to the described negative impact of seed coats on germination (Lee *et al.*, 2010). To elucidate, if seed coats also release CK and to assess a potential light specificity, seed coat bedding assays as described in Lee *et al.* (2010) should be conducted using CK mutant seed coats, which are expected to lack a repressing effect on dissected embryos.

4.4.3 Epigenetic mechanisms may confer the repression of phyA-dependent germination by CK

Maternal effects may be established via epigenetic modifications as well. Seed size and seed germination were controlled by epigenetic mechanisms (Li *et al.*, 2013; Narsai *et al.*, 2017). Crossings between plants with different ploidy revealed that an excess of maternal genome dosage resulted in a reduced endosperm growth and thus smaller seeds, an excess of paternal genome dosage had opposite effects (Adams *et al.*, 2000). This parent-of-origin effect relied on CG methylation patterns, suppression of paternal CG methylation had similar effects as an excess of maternal genome dosage (Adams *et al.*, 2000). Epigenetic mechanisms were also involved in the regulation of germination. For example, at the end of germination a large-scale demethylation of the genome marked the transition from seed to seedling (Narsai *et al.*, 2017).

Up to today, it is not known whether CK alters DNA methylation or demethylation patterns, but there are hints that link CK to epigenetic modifications. The effect of exogenously applied CK on reproductive traits in *B. napus* such as number of ovules and seeds was transmitted to the next generation presumably by stable epigenetic modifications (Zuñiga-Mayo *et al.*, 2018). Epigenetic mechanisms were also capable to alter the CK status. The expression of the CK catabolizing *CKX2* in the endosperm was activated by the IKU pathway and promotes endosperm growth (Garcia *et al.*, 2005; Li *et al.*, 2013). A demethylated paternal genome and a reduction of the repressive H3K27me3 marks altered *CKX2* expression (Li *et al.*, 2013).

Combining this information with the results of the higher germination rates of seeds with a maternally reduced CK status or signal transduction (Figure 24), a connection between CK and epigenetic effects in the regulation of germination may be proposed but experimental evidence is lacking. Since DNA methylation positively affects seed germination and *CKX2* expression, the methylation status of CK-related genes in maternal seed tissues should be analyzed, optimally during seed germination in FR light.

To sum this up, the connection between CK and the regulation of phyA-dependent seed germination may involve epigenetic mechanisms, but this needs further experimental clarification.

4.5 Dissecting transcriptomic alterations underlying the impact of CK on seed germination

The gene regulatory network underlying the negative effect of CK in the germination process in FR light has not been studied before. In order to examine this process more closely and to eventually generate hypotheses based on transcriptomic changes potentially interesting for the regulation of FR-dependent germination by CK, the transcriptome of germinating seeds was investigated in the present thesis. To do so, an *in silico* analysis of a published dataset was conducted, gene expression was assessed via qPCR and via RNA-Seq analysis.

1) First of all, putative marker genes for different time points of seed germination were identified *in silico* using a publicly available microarray data set (Narsai *et al.*, 2011). The dataset provides transcriptomic data from seeds germinated in continuous WL (Narsai *et al.*, 2011). This dataset was chosen, because no RNA-Seq or microarray data set was available where FR light-treated seeds were studied. Also a dataset where only a temporally limited WL pulse was applied to induce germination, as in the present thesis, was not available in the literature. 2) The marker genes identified *in silico* were tested via qPCR for their expression after induction of germination by a temporally limited WL pulse and 3) for their expression after a FR light pulse. Additionally, 4) the *in silico* data set was utilized to gain more general insights into the regulation of seed germination, what will be shortly discussed here as well.

Next, the transcriptome of germinating seeds was investigated by RNA-Seq analysis. Thereby, RNA-Seq analysis was used in the present work as a tools to identify transcriptional regulation at different time points of the seed germination process and the consequences of an altered CK status on transcriptional regulation both in WL and in FR light (Yazdanpanah *et al.*, 2017; Tognacca *et al.*, 2019).

5) To ensure significance and comparability of the RNA-Seq dataset generated in the present thesis to published studies analyzing transcriptomic changes in germinating seeds, the results of the RNA-Seq analysis conducted in this work were compared to the literature. Since no datasets for FR light-induced germination are available in the literature, differentially expressed genes in WL treated wild-type seeds were used for these comparisons. Additionally, transcriptomic changes over time in wild-type seeds are discussed shortly. Next, 6) the transcriptome of seeds able to complete germination in WL or FR light was analyzed in order to develop hypothesis about transcriptomic changes associated with the onset of germination in general.

To take a closer look at transcriptional alterations due to the seeds CK status in different phases of germination, 7) the transcriptome of imbibed wild-type and *ahk2 ahk3* seeds was analyzed. One of the most important analysis was conducted by 8) comparing transcriptomic changes in response to FR light between wild-type and *ahk2 ahk3* seeds. Nevertheless, to assess and evaluate the functional relevance of transcriptional changes for the seed germination process, further experimentation would be required using classical genetic assays, such as mutant or transgenic lines of the identified differentially regulated genes. Lastly, 9) the transcriptional regulation of CK metabolism -or signaling at different timepoints of WL and FR light-induced germination will be discussed shortly.

4.5.1 *In silico* identification of marker genes for WL germination and their expression in WL and FR light

1) First, the *in silico* study targeted to identify putative marker genes for different time points of seed germination using publicly available microarray data will be discussed. Out of the available microarray data set (Narsai *et al.*, 2011), a set of putative WL regulated marker genes was extracted (Figure 31). These genes were regulated at distinct time points in continuous WL during germination (Figure 32).

Next, 2) the expression of the putative marker genes was tested by qPCR in seeds treated with a temporally limited WL pulse, as was used throughout the present thesis. All putative marker genes showed only a slight induction twelve hours after a germination-inducing WL pulse (Figure 33). This indicates that the strong induction of expression apparent in continuous WL cannot be reached by illumination of seeds with a temporally limited WL pulse.

So the transcriptional regulation in seeds in response to continuous light versus a temporally limited light pulse seems to differ, although the outcome which is the effective induction of germination is similar. Not only the duration of illumination, also the pre-treatment seeds were subjected to might cause differences in gene expression. For example, Narsai *et al.* (2011) conducted a cold pre-treatment prior to imbibition, whereas in this work no cold treatment was applied.

3) To test, whether the set of putative marker genes identified *in silico* might be used as marker genes for FR light-induced germination, the expression of the putative marker genes in FR light was studied. In phyA-dependent FR light conditions, the strongest induction of the *in silico* identified genes was measured in *phyA* mutant seeds unable to perceive FR light (Figure 33). This indicates that the induction of gene expression is independent of light perception and solely related to the imbibition process. Because of these results, none of the putative in silico identified marker genes was qualified to serve as a marker gene for transcriptome analysis in WL- or FR light-dependent germination.

4) Apart from the search for marker genes, *in silico* data were also utilized to gain more general insights into the regulation of seed germination. The microarray dataset revealed that a large proportion of differentially expressed genes was regulated across all analyzed timepoints (Figure 31). This was expected since the onset of germination is accompanied by a reprogramming of the seeds' transcriptome (Nakabayashi *et al.*, 2005; Howell *et al.*, 2008; Narsai *et al.*, 2017).

Interestingly, a large number of DEGs was identified after 24 h of WL incubation (Figure 31) compared to 1 h imbibed seeds. This is in line with the timeframe when seeds in continuous WL start germinating and a major transcriptome reprogramming is expected. The closer examination of these differentially regulated transcripts after 24 h of continuous WL incubation revealed an enrichment of genes belonging to the GO term “responses to CK” (Figure A3). Up to today, the onset of germination or the protrusion of the radicle was not associated with the regulation of CK-associated transcripts (Narsai *et al.*, 2017; Tognacca *et al.*, 2019). Additionally, germination experiments conducted in the frame of the present thesis showed that WL-induced germination was independent of the seeds' CK status (Figure A1). On a transcriptional level, a connection between WL-induced germination and CK may be drawn via the *CYP81F4* gene. *CYP81F4*, which was listed as a potential marker gene for germination in the present thesis (Figure 32) encodes cytochrome P450 family protein and is regulated by CK as shown in a meta-analysis of microarray data (Brenner and Schmülling, 2015). In order to further investigate the impact

of CK on the seeds' transcriptome in WL, an in-depth study of transcriptomic changes in WL in *ahk2 ahk3* seeds over time would be a promising approach.

4.5.2 RNA-Seq analysis of WL-induced germination in wild-type seeds

5) In order to compare the transcriptomic data generated in the frame of the present work to previously published seed transcriptomic studies, DEGs identified in WL treated wild-type seeds were analyzed into more detail. In wild-type seeds, genes upregulated in WL belonged to the GO terms DNA or RNA metabolism, RNA processing, translation, mitochondrial proteins and histone-associated GO terms (Figure 39). The study published by Narsai *et al.* (2011), which was also used for the *in silico* analysis discussed in the previous section, identified in two out of four DEG clusters a significant overrepresentation of genes related to mitochondria and DNA or RNA metabolism. This corresponds to the GO terms identified in the RNA-Seq analysis conducted in this work. Additionally, Nakabayashi *et al.* (2005) and Tognacca *et al.* (2019) identified similar GO terms in germination. Remarkably, genes tightly associated with the regulation of germination (as for example *PIF*s, *DELLA*s, GA-and ABA-associated transcription factors, *DOG1*, *SLY*) were not identified as strongly regulated transcripts in the present thesis (Figure 50, Figure 51, Figure 52). This is in line with the findings in other studies where no- or only few of the “classically” germination-associated genes were identified as significantly differentially regulated (Nakabayashi *et al.*, 2005; Narsai *et al.*, 2011; Narsai *et al.*, 2017). To summarize this part, the comparison of GO terms identified in the present thesis and their comparison to previous research confirm the reliability and quality of the RNA-Seq analysis conducted in this work. Interestingly, commonly known signaling hubs regulating germination do not belong to the most strongly regulated transcripts in WL.

Next, transcriptomic changes at different time points of the germination process are discussed. Closer examination of transcripts regulated six hours after WL illumination identified genes (Table 18) were associated with developmental processes, such as embryo development and the repression of flowering (Hu *et al.*, 2014; Kamiuchi *et al.*, 2014).

Twelve hours after illumination two genes, namely AT4G31330, encoding a putative transmembrane protein and AT3G13760, encoding a Cysteine/Histidine-rich C1 domain family protein, which have been described as negatively regulated by CK application were identified among the strongest upregulated transcripts in germinating wild-type seeds (Rashotte *et al.*, 2003; Lee *et al.*, 2007) (Table 19). Despite their regulation on transcriptional level, their functional relevance in the germination process remains open. In order to examine the regulation of germination in a timely resoluted manner into more detail the RNA-Seq dataset might provide a good starting point for further analyzes.

To summarize this, the most strongly regulated transcripts in WL were associated with seed developmental processes, flowering and CK. To study the role of CK in WL induced germination, the analysis of DEGs in *ahk2 ahk3* seeds after a WL pulse might provide helpful insights to investigate the consequences of an altered CK status on the transcriptome in WL- induced germination.

4.5.3 RNA-Seq analysis of transcriptional changes in seeds capable to complete germination in WL and/or FR light

6) This part of the RNA-Seq analysis aimed at tracking and comparing the transcriptional responses of wild-type and *ahk2 ahk3* mutant seeds, which are expected to germinate

based on germination assays conducted in the present work. High germination rates are expected in wild-type seeds treated with a WL pulse and *ahk2 ahk3* seeds treated with a WL or FR light pulse, so genes differentially regulated in these samples were analyzed. This set of transcripts may be useful to develop hypothesis about transcriptomic changes associated with the onset of germination in general.

To analyze transcriptomic alterations on a temporal level, transcripts differentially regulated early after the germination-inducing light pulse (6 h) and later after the germination-inducing light pulse (12 h) were investigated.

Six hours after application of the germination-inducing light pulse transcripts associated with responses to abiotic stimuli, especially stress stimuli were identified (Figure 45). Among the strongest upregulated transcripts *AHP6* was found (Table 28), indicating that potentially the negative regulation of CK signaling by AHP6 might favor germination. However, analysis of *ahp6* loss-of-function mutants (Figure 16) provided no hint, that AHP6 is relevant for the regulation of germination in FR light. It might thus be possible, that AHP6 supports light-dependent germination by inhibiting the CK signaling pathway but is not the major component in this process. Other DEGs identified as being upregulated during germination were related to seed development, hormone transport, cell-wall and seed mucilage. Their functional relevance for the regulation of germination remains to be tested.

Remarkably, overrepresented GO terms varied in the course of germination. Instead of abiotic stress-related transcripts, genes associated with metabolic processes were overrepresented at later stages of germination (Figure 47). This might be due to the major changes on a transcriptional and metabolic level from seed to seedling metabolism.

Interestingly, a connection between the fatty acid metabolism in seeds and the onset of germination was evident. *ADAP*, also named *WRI3*, a transcriptional activator of fatty acid biosynthesis was identified among the upregulated DEGs (Table 30), which is also able to rescue the wrinkled seeds phenotype *wri1-4* seeds (To *et al.*, 2012). A direct connection to germination was found in Lee *et al.* (2009), they described better germination and partial insensitivity to ABA of *adap* knock-out seeds, suggesting a negative regulatory role of *ADAP* in seed germination via the ABA pathway (Lee *et al.*, 2009). To uncover a potential connection between the fatty acid metabolism and light-dependent germination, analyses of the germination response of lipid metabolism mutants or lipid signaling mutants in FR light might provide additional information.

4.5.4 The seeds' CK status has only a minor effect on transcriptomic changes during imbibition

During imbibition the seeds' pre-germinative metabolism is induced, conferring sensitivity towards environmental cues. This fact is used for example in a process called seed priming, where seeds are dried again after imbibition before they lose desiccation tolerance, in order to improve germination performance and accelerate the onset and the uniformity of germination (Paparella *et al.*, 2015). Thus, it was hypothesized that the higher germination rates of CK deficient seeds in FR light are based on transcriptional alterations occurring in the imbibition phase, prior to the perception of a germination-inducing light pulse.

So in the next part, 7) differentially expressed genes in *ahk2 ahk3* and wild-type seeds are discussed. Upon imbibition, a reprogramming of the transcriptome was evident, more than 5000 genes were differentially regulated both in wild-type and in *ahk2 ahk3* seeds (Figure 40). Remarkably, the genotypes shared a major proportion of these DEGs, supporting that transcriptional changes during imbibition are independent of CK perception. This is corroborated by the low number of DEGs identified when comparing transcriptional changes between wild-type and *ahk2 ahk3* seeds after one and 49 h of imbibition (Figure 40). An explanation for the largely similar regulation might be the high proportion of transcripts reflecting the embryonic maturation program expressed in early phases of imbibition (Galland *et al.*, 2014).

To uncover potential interesting genes deregulated during the imbibition process and related to CK, uniquely regulated DEGs in *ahk2 ahk3* seeds were examined closer. At the end of the imbibition period *FTM1/AAD6*, encoding a protein involved in fatty acid metabolism, was the highest upregulated transcript in *ahk2 ahk3* (Figure 40 A, Table 22). A close relative of *AAD6*, *AAD5* has been shown to be highly expressed in developing embryos during seed development (Jin *et al.*, 2017). Apparently, also a gene of yet unknown function (AT4G04223) was strongly downregulated in *ahk2 ahk3* seeds, and it may be worth to analyze its function as potential germination repressor (Table 21).

In sum, imbibition in both wild-type and *ahk2 ahk3* mutant seeds appear to be regulated similarly on transcriptional level. Based on the presented findings major differences of transcriptional regulation during imbibition do not seem causal for the increased germination rates of seed with a reduced CK signal transduction.

4.5.5 The seeds' CK status alters transcriptional regulation during FR light-induced germination

In this section 8) differentially regulated genes in *ahk2 ahk3* seeds as a response to FR light illumination are discussed. It was hypothesized that the onset of germination in *ahk2 ahk3* seeds in FR light is based on transcriptional alterations that differ from wild-type seeds illuminated with FR light, of which only a small proportion of seeds complete germination.

Seven genes were differentially regulated between the genotypes in one hour imbibed seeds, during imbibition and after application of the germination-inducing FR light pulse (Figure 41). These genes were not analyzed further here, because their regulation is not solely dependent on FR light, the observed differential expression is presumably due to the seeds genotype only. Nevertheless, they may still be relevant for the regulation of germination in wild-type and CK receptor mutant seeds.

In the following, only DEGs deregulated as a consequence of FR light application in *ahk2 ahk3* are discussed into more detail. Regarding the quantity of deregulated genes,

after subtraction of imbibition-regulated genes the number of DEGs exclusively regulated in *ahk2 ahk3* seeds in response to a FR light pulse was similar six and twelve hours after FR light illumination (131 and 136 respectively, Figure 43). This indicates that the onset of germination by FR light is not associated with an increase in DEGs in later phases of the germination process compared to the wild type. To evaluate the significance of these findings, the gene regulation in *ahk2 ahk3* seeds in FR light might be assessed at different timepoints, using *ahk2 ahk3* seeds imbibed for one hour as reference and compared the gene regulation over time in wild-type seeds subjected to a WL pulse.

In order to investigate which pathways were deregulated on a transcriptional level dependent on the seeds CK status in FR light, GO terms were analyzed into more detail. Overrepresented GO categories revealed that seed lipid-associated transcripts (GO terms seed oilbody biogenesis, lipid storage and maintenance of location) and seed maturation-associated transcripts were differentially regulated in *ahk2 ahk3* seeds in response to FR light (Figure 42). The regulation of lipid metabolism has been coupled to seed germination responses previously (Manz *et al.*, 2005), but not in the context of either very low fluence light or CK. Lipid mobilization in seeds is coupled to ABA, as it represses mobilization predominantly in the embryo, not in the endosperm (Manz *et al.*, 2005; Penfield *et al.*, 2006). So presumably in *ahk2 ahk3* seeds lipids are better mobilized compared to the wild type. CK might negatively affect this process, but the functional relevance of this process remains elusive.

Transcripts deregulated both six and twelve hours after application of a germination-inducing FR light pulse were particularly interesting, since differential expression of these genes might influence the germination response both in early and later stages of germination in response to FR light in CK receptor mutant seeds. Upregulated genes were associated with cell wall organization and release of mucilage from the seed coat (Table 25). This may provide hints to differences in the cell wall organization in *ahk2 ahk3*, which are important for germination (Steinbrecher and Leubner-Metzger, 2018). The rigidity of the cell wall sets mechanical constrains to germination, especially in the region of the micropylar endosperm (Steinbrecher and Leubner-Metzger, 2018). To confirm a biological significance, further experimentation would be required. By measuring the rigidity of the cell wall and endosperm weakening puncture-force measurements may be conducted (Finch-Savage and Leubner-Metzger, 2006).

Transcripts downregulated in *ahk2 ahk3* after a FR light pulse included *NCED9*, an ABA biosynthesis gene and *SOM*, a negative regulator of germination downstream of *PIF1* (Kim 2008). The repression of *NCED9* was confirmed in this work via qPCR in *ipt3,5,7* and *ahk2 ahk3* seeds (Figure 34). ABA levels are not altered in *ahk2 ahk3* seeds, however a tissue-specific repression of *NCED9* might be relevant for phyA-dependent germination. Analysis of *SOM* expression by qPCR revealed the strongest downregulation in wild-type seeds after FR light (Figure 34), so a specific role in the regulation of germination by CK seems unlikely.

4.5.6 Genes encoding components of CK metabolism and signaling are not strongly deregulated in CK receptor double mutant seeds

9) In order to monitor transcriptional changes in CK-associated genes during germination and compare their regulation in wild-type and *ahk2 ahk3* seeds, a set of well-known CK-associated genes was analyzed for their transcriptional regulation. Interestingly, the analyzed transcripts were regulated very similarly during imbibition and germination in *ahk2 ahk3* and wild-type seeds (Figure 49). The lack of a mutant-specific deregulation of CK-associated genes may indicate, that the CK status in mature seeds rather than the expression of CK-related genes during imbibition or germination is instructive for the onset of germination. Based on this assumption, expression of CK-associated transcripts might be more relevant during seed development rather than in mature seeds.

In germination CK biosynthesis-associated genes were rather downregulated, while *LOG* genes tendent to show upregulation in germination (Figure 48). So rather the conversion of CK than the *de novo* production might be regulated during germination. If CK plays a negative role in the regulation of germination, a more pronounced upregulation of *CKX* genes might be expected but is not seen here. The majority of A- B- and C-type *ARR* genes were not significantly regulated during germination (Figure 49), thus a role in the regulation of germination by differential expression is not supported by the presented data.

4.6 The role of CK as a priming agent

In this part of the discussion, the role of CK in intergenerational memory establishment (´priming´) will be discussed. The term ´priming´ in this context does not refer to the technique of seed priming, as described before (refer to introduction section 1.7). The definition for priming by environmental stress (Hilker *et al.*, 2016), which defines the priming stimulus as temporally limited stress event that prepares plants for future stress incidents (´triggering´), does not adequately match the analyses conducted in the present thesis. Rather, priming is defined here as a seed status which enhances the germination response of seed in response to a FR light triggering stimulus.

In seed germination research, establishment and inheritance of long-term effects transferred to the next generation, for example via seed dormancy, is commonly known (Ganguly *et al.*, 2017). Long-term memory can be established either by inter- or by transgenerational effects. Intergenerational memory is defined as a memory detected in the first offspring generation of primed plants, and presumably includes cues introduced by the mother plant directly (Lämke and Bäurle, 2017). If the memory of the priming persists for at least two generations generations, it is defined as transgenerational priming (Lämke and Bäurle, 2017).

The mechanisms for long-term memory establishment in *r*-strategists, such as *Arabidopsis thaliana,* are under debate (Ganguly *et al.*, 2017; Lämke and Bäurle, 2017). Transgenerational priming probably involves epigenetic mechanisms for memory establishment (Lämke and Bäurle, 2017) but evidence in the literature is inconclusive. For example, repeated priming by drought stress does not result in variations of the DNA methylome which is inherited transgenerationally (Ganguly *et al.*, 2017). On the other hand, the intergenerational memory of osmotic stress is established via DNA methylation (Wibowo *et al.*, 2016).

The role of CK as a priming agent in response to biotic stress has been described previously (Choi *et al.*, 2010; Cortleven *et al.*, 2019a). Also a recent study found that

information about exogenously applied CK on reproductive traits in *B. napus* seeds were transmitted to the next generation presumably by stable epigenetic modifications (Zuñiga-Mayo *et al.*, 2018). In the plant generation following the CK treated one, germination in soil and vegetative growth was comparable to wild-type plants, but in the reproductive phase flower morphology was similar to CK-treated plants, indicating that CK induced a long-term memory that is activated in the reproductive phase (Zuñiga-Mayo *et al.*, 2018). So, the transfer of information about environmental stimuli, such as light and nutrients, from parental plants to the offspring and the connection to CK are discussed.

4.6.1 Priming by parental light conditions is advantageous for germination in the second offspring generation but independent of CK

The light conditions experienced by parental plants are of specific importance, since *A. thaliana* seeds are dispersed in close vicinity to their parental plants. So the light conditions the parents face are similar to the light conditions the emerging seedlings have to cope with after completion of germination (Roach and Wulff, 1987). If parental plants are shaded during vegetative growth or reproduction, this indicates the presence of neighboring vegetation. Interestingly, the perception of a canopy by maternal plants has a stronger effect on seed germination than the presence of a canopy during seed imbibition (Leverett *et al.*, 2016). This indicates that parental light conditions directly alter germination of the offspring.

It was hypothesized that for offspring of shaded plants the ability to germinate in shade may be advantageous. But in this thesis, parental exposure to shade during vegetative and reproductive growth did not alter the phyA-dependent germination of the first offspring generation, irrespective of the overall light quantity applied (Figure 53). Interestingly, if the environmental conditions the plants were grown were kept stable for two generations, phyA-dependent germination of the offspring was altered. The second offspring generation of shade-grown wild-type plants germinated almost as well as *ahk2 ahk3* seeds in FR light (Figure 53). These findings do not support the hypothesis of a direct information transfer from parental plants to their offspring about the surrounding light environment which alters germination in VLFR light conditions. For the establishment of a memory about parental light environments, two subsequent generations perceiving the shade light seem to be required. This may indicate that the established memory is too weak to alter offspring germination after only one generation but may accumulate after two generations, thus affecting germination.

Studies analyzing WL germination found that maternal exposure to shade during seed maturation even decreases germination rates of the offspring (Vayda *et al.*, 2018), what was not the case in this work. These discrepancies may be explained by the different light regimes used to induce germination. If for example phyB-dependent germination would be less efficient, this would explain the germination phenotype described by Vayda *et al.* (2018) but would not affect FR light germination rates measured in this work.

Next, it was investigated if plants with a reduced CK status are capable to inform their offspring about the environmental light conditions. *Ahk2 ahk3* seeds germinated to high percentages in FR light, independent of the light conditions experienced by their parents (Figure 53), suggesting no role of CK to establish a memory in seeds on parental light conditions. The experiments discussed here need to be repeated with different seed sets to ensure reliability and reproducibility of these findings.

4.6.2 Nitrogen supply affects phyA-dependent seed germination

Maternal seed provisioning during development with nutrients, hormones, proteins and transcripts impacts seed germination and seed dormancy (Holdsworth *et al.*, 2008b; Donohue, 2009). Earlier studies demonstrated that plant fertilized with nitrogen produced seeds that were less dormant compared to non-fertilized plants (Alboresi *et al.*, 2005). Interestingly, nitrogen supply resulted in the accumulation of high levels of CK in seedlings, arguing in favor of a functional connection between CK and nitrogen (Kiba *et al.*, 2011; Landrein *et al.*, 2018). In this work, nitrogen supply of parental plants increased phyA-dependent germination in a concentration-dependent manner in their offspring, both in wild-type and in *ahk2 ahk3* seeds (Figure 54). This indicates that the germination-promoting effect of N is independent of the seeds' CK status. But not only fertilization of the respective parental plants affects germination rates. Seed are also able to take up N from their environment during imbibition, positively affecting their germination rates (Alboresi *et al.*, 2005). This was also true for phyA-dependent germination, increasing nitrogen concentrations applied during seed imbibition resulted in increased germination rates in FR light (Figure 54).

To sum this up, nitrogen supply of parental plants as well as direct application of nitrogen to imbibing seeds increased germination rates in FR light, this effect was independent of the seeds' CK status.

4.7 Seed size and seed age affect germination rates in FR light

Seed development as well as seed characteristics influence the germination response. In the following, the impact of seed size and seed longevity for the onset of phyA-dependent germination will be discussed.

4.7.1 A small seed size is advantageous for germination in FR light

The interconnection between seed size and the seeds' germination response is not yet fully understood. CK is a prominent regulator of seed size, higher order CK mutants impaired in CK signal transduction or transgenic seeds with an increased CK catabolism (expressing *35S:CKX*) have enlarged seeds (Werner *et al.*, 2003; Riefler *et al.*, 2006). In this work, comparison of size classes revealed no differences between CK mutant- and transgenic lines and wild-type seeds, indicating that the size distribution is similar across genotypes, but the seed weight differed (Figure 56). Seed biomass of *35S:CKX2* seeds resembled wild-type seeds (Figure 56), whereas *ipt3,5,7* seeds showed an increased seed weight (Figure 56). Reduced CK perception resulted in a reduced seed weight in *ahk2 ahk3* seeds (Figure 56). These results do not match earlier descriptions of the seed size phenotype of CK mutants (Werner *et al.*, 2003; Riefler *et al.*, 2006). The enlarged seed size of *ipt3,5,7* might be explained by a severe growth reduction of the respective plants, what might result in increased seed weight.

Regarding the connection between seed size and seed germination, previous studies demonstrated that in plants producing only a limited number of seeds the provisioning of these seeds was increased and they germinated faster in WL (Sills and Nienhuis, 1995). In *A. thaliana*, the onset of germination might be affected by seed size, but experimental evidence greatly varies between experiments, even in the same study (Elwell *et al.*, 2011). In the present thesis, small wild-type seeds tended to show higher germination rates in FR light compared to larger wild-type seeds (Figure 56). Alike, smaller

ahk2 ahk3 seeds germinated to higher percentages compared to larger *ahk2 ahk3* seeds in phyA-dependent germination conditions (Figure 56).

These results demonstrate, that positive correlation between seed size and seed germination in WL (Sills and Nienhuis, 1995) are not transferable to germination in FR light, where small seeds germinated to higher rates. One hypothesis to explain these results might be, that small seeds have to start germinating earlier after burial underneath a layer of soil where they only perceive the FR light spectrum (Scopel *et al.*, 1991) in order to survive. Larger seeds, which are better supplied to survive unfavorable environmental conditions, might persist in the soil for a longer time and induce the onset of germination if light conditions are more favorable.

To further study the connection between seed size and FR light germination in the wild type, experiments should be conducted where a significant number of siliques are removed from the plants. The seeds derived from the remaining siliques are expected to be larger and may be tested for their germination response in FR light.
Integrating the information about the different seed sizes in CK mutants and the effect of seeds size on phyA-dependent germination, it may be concluded that the CK status, not the seed size, is instructive for germination in FR light, since both large *ipt3,5,7* and small *ahk2 ahk3* seeds showed high germination rates (Figure 15).

4.7.2 The repressive effect of CK on phyA-mediated germination is retained in seeds during long-term storage

Seed longevity is a complex trait which involves both genetic factors and the environmental conditions the seeds perceive themselves during storage (Walters *et al.*, 2005). The loss of seed viability over time during storage is among others due to the oxidation of proteins and nucleic acids (Clerkx *et al.*, 2004). Only recently, phyA-dependent germination has been connected to seed aging. phyA-dependent germination in wild-type seeds was increased in old seed batches. This has been connected to an increase in oxidative stress in aged seeds and high endogenous polyamine levels (Kim *et al.*, 2019).

In the framework of this thesis was investigated, whether the negative effect of CK on germination in FR light would persist over time. In seeds subjected to long-term storage the repression of germination by CK in FR light was maintained up to 2.5 years after harvest (Figure 57). For these assays, only seed batches showing high WL germination rates were selected. Interestingly, an increase in phyA-dependent germination over time in wild-type seeds as published by Kim *et al.* (2019) was never observed in germination assays conducted in this work.

To sum this up, the negative effect of CK on phyA-dependent germination is not relieved over time by mechanisms related to seed aging.

4.8 Summary and future perspectives

In this work, the influence of CK on germination especially in very low fluence light was investigated. In the present thesis it was shown that CK negatively regulates germination in low fluence R light and in very low fluence FR and blue light conditions. However, dark germination rates and germination in WL were unaffected by the seeds CK status. The repressive effect of CK on seed germination is most pronounced in VLFR FR light, thus representing a novel way to regulate VLFR germination in *A. thaliana*.

The repressive effect of CK on phyA-mediated germination requires a functional CK metabolism and signaling pathway. In this work, seeds impaired in CK biosynthesis, with an increased CK catabolism, with a reduced signal perception at the level of AHK receptors and seeds impaired in CK signal transduction via AHPs showed higher germination rates compared to wild-type seeds. However, the role of A- and B-type ARRs in the VLFR germination is still unclear. Based on that, further research should be focused on the identification of components of the CK signal transduction pathway downstream of AHPs. Among the A- and B-type ARRs higher order mutants should be tested for their germination phenotype in FR light.

Interestingly, CK does not solely affect seed germination rates in FR light, but also the timing of germination is delayed by CK. This might provide a hint towards the ecological relevance of this regulation. Alterations in CK status in individual seed of a given seed population might separate the onset of germination on a temporal level, thus minimizing the risk of competition amongst the siblings.

The photoreceptor phyA is indispensable for the onset of germination in FR light for CK mutant seeds. Since phyA is the most important phytochrome regulating VLFR germination, a direct repressive effect of CK on phyA abundance was hypothesized but not confirmed in the present thesis. To exclude an increased phyA abundance in CK mutant seeds further studies measuring phyA protein level should be conducted, using different CK mutants. However, CK might impact phyA indirectly, which was not tested in the framework of the present thesis. To test if CK negatively affects subcellular distribution, phyA nuclear import or activity, phyA uptake assays utilizing labelled phyA protein might be conducted. Another possibility would be to study protein-protein interaction between phyA and FHY1 or FHL in CK mutants. To test if CK induces increased phyA phosphorylation and thereby decreasing phyA stability, photoreceptor phosphorylation assays should be conducted.

Because of their outstanding importance, the connection between CK, ABA and GA in the regulation of germination was analyzed more into detail in this work.

Hormone measurements revealed that GA level in CK deficient seeds are not elevated and therefore are not causal for the increased germination rates in FR light. During imbibition, GA levels decreased in seeds independent of their genotype, suggesting a CK independent regulation of bioactive GA levels in imbibed seeds. Since GA levels are regulated in a light-dependent manner in germinating seeds, GA measurements after an inducing FR light pulse might provide a more precise picture of GA levels in CK deficient seed when germination is induced by FR light. In contrast to GA levels, CK negatively influences Ga sensitivity. To investigate the connection between CK and GA into more detail, genetic crossings of *ahk* receptor mutants an GA biosynthesis -or signaling mutants should be conducted and the resulting seeds analyzed for their germination response in different light conditions.

Measurements of ABA level in imbibed seeds revealed no decrease of ABA in CK receptor mutant seeds, but the sensitivity towards externally applied ABA should be subject of further investigation. To do so, a balanced application of ABA and an ABA biosynthesis inhibitor should be used in order to reduce, but not completely inhibit seed germination in FR light also in wild-type seeds.

Using reciprocal crossing experiments, it was shown that the CK status in maternal tissue is important for phyA-dependent germination. Thereby, a reduction of the CK status specifically in testa or endosperm, was not sufficient to increase germination rates

significantly in FR light. These findings may indicate that the CK status in both maternally derived seed tissues is important for germination in FR light. In order to investigate the role of CK in maternal tissues more into detail, tetrazolium staining assays using different mutants with an increased or decreased CK status might clarify, if CK negatively affects testa permeability. Another interesting pathway, how CK in maternal seed tissues might regulate germination would be a tissue-specific alteration of ABA levels, ABA sensitivity or the ABA release towards the embryo during FR light induced germination. To test this, ABA level in the seed coat and in dissected embryos should be measured. Apart from hormonal regulation, also epigenetic mechanisms might shape the germination response of CK mutant seeds. Measuring the methylation status of CK-related genes in maternal seed tissues might provide hints whether or not a tissue-specific regulation of CK-associated genes is relevant for germination.

In the present thesis, also the gene regulatory network underlying the negative effect of CK on the germination process in FR light was studied. Interestingly, the seeds' CK status had only a minor effect on transcriptomic changes during imbibition. The transcriptome of seeds during FR light-induced germination was altered dependent on the seeds CK status. Overrepresented GO categories revealed that seed lipid-associated transcripts and seed maturation-associated transcripts were differentially regulated in *ahk2 ahk3* seeds in response to FR light. Additionally, cell-wall organization-associated transcripts were differentially regulated in *ahk2 ahk3* seeds. Up to present no functional connection between CK and the regulation of lipid metabolism or cell wall organization in germination is known. To identify a possible biological significance, crossings between CK mutants and lipid- or cell wall- associated mutants might be conducted. Another way to test the effect of CK on cell wall rigidity are puncture-force measurements, which might reveal an altered stability of the seed coat in CK mutant seeds.

Analyzing the impact of parental light conditions in this thesis provides evidence, that growth of parental plants in shade light conditions did not affect the germination response in FR light of their offspring. However, presumably growth of two plant generations in these conditions increased germination in FR light. To investigate this into more detail, further plant generations should be analyzed for their germination response in FR light. Additionally, by shifting offspring of shade-grown plants to non-shade conditions and vice versa might reveal, if the respective offspring would resemble the germination phenotype of their parental plants in response to FR light.

Furthermore, it has been shown that a small seed size is advantageous for germination in FR light, and that seeds with a reduced CK status showed higher germination rates in FR light independent of their seed size. Regarding seed age, the repressive effect of CK on germination in FR light was retained in aged seeds during long-term storage. To clarify the importance of seed size and seed age, different experiments, including artificial aging experiments and germination assays analyzing different sized seed of additional CK mutants may be conducted.

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Appendix

Table A1. Primer sequences for genotyping.

		Primer sequences (5´-> 3´)	
Mutant		**forward**	**reverse**
ahk2-5	Wild-type allel	GCAAGAGGCTTTAGCTCCAA	TTGCCCGTAAGATGTTTTCA
	Mutant allel	forward wild-type allel	SAIL
ahk2-2tk	Wild-type allel	GTCTATAACTTGTGAGCTCTTGAATC	GCTCGTGTCATAGACAGCAAAGGTC
	Mutant allel	forward wild-type allel	ATAACGCTGCGGACATCTAC
ahk3-3	Wild-type allel	CTTGTGATTGCGTTACTTGTTGCAC	GCAGGCCTATGGTCCACAACCACAG
	Mutant allel	TGGTTCACGTAGTGGGCCATCG	reverse wild-type allel
ahk3-7	Wild -type allel	CCTTGTTGCCTCTCGAACTC	CGCAAGCTATGGAGAAGAGG
	Mutant allel	forward wild-type allel	GABI Kat
ahp6-1	Wild-type allel	GAGCAGTTCTTGCAGCTTCAGCAG	AGGGTTTCGCTTCGGTAGC
	Mutant allel	GACGAGCAGTTCTTGCAGCTTCTG	CGTCACGAACCCTACGAGCACC
ahp6-3	Wild-type allel	GAGCAGTTCTTGCAGCTTCAGCAG	AGGGTTTCGCTTCGGTAGC
	Mutant allel	LbB1.3	reverse wild-type allel
arr10-5	Wild-type allel	CATTGGAGTTGTTGAGGGAGA	CGATGATGAGACTGGTTGGA
	Mutant allel	forward wild-type allel	Lba1
arr12-1	Wild-type allel	TAACAACGACGAACCAAGCA	TTGGCAGAGTCACAGAATGG
	Mutant allel	forward wild-type allel	Lba1
arr1-3	Wild-type allel	CTTCAAGCACTAGCCGTCACAGGTCAGTT	AATGTTATCGATGGAGTATGCGTCAAAGT
	Mutant allel	forward wild-type allel	Lba1
arr3	Wild-type allel	CCTGGAATGACTGGATACGA	AGTTCCTTCGTGAGCAAAGAG
	Mutant allel	LbB1.3	reverse wild-type allel
arr4	Wild-type allel	CTGAAACAGGAATCGTCCAA	CGGTGGTATCGTTTGTCAGT
	Mutant allel	LbB1.3	reverse wild-type allel
arr5	Wild-type allel	AAGCAAAGGCATGTGAGGTG	GGTTTTGCGTCCCGAGATGT
	Mutant allel	LbB1.3	reverse wild-type allel
arr6	Wild-type allel	TGAGCAGGAACTTTACTTTGTGA	ATGCTACCGAGGAAGATGGA
	Mutant allel	forward wild-type allel	LbB1.3
arr7	Wild-type allel	GGCGGTTTGCAGACTCACTTACCTGA	GGCAAAGGGGCTTCTAATC
	Mutant allel	forward wild-type allel	LbB1.3
arr8	Wild-type allel	TCTTTCTCTCGTTGTCTCCTTG	CATTTGGGTCGTTGTCATCT
	Mutant allel	LbB1.3	reverse wild-type allel
arr9	Wild-type allel	GATTGGTTGCTTTCAACAGG	ATCATATTCCCCTCTCTGCATT
	Mutant allel	LbB1.3	reverse wild-type allel
arr14	Wild-type allel	ATGCCGATCAACGATCAGTTTC	CTATCTTTGTCTTGAAGATCT
	Mutant allel	ATGCCGATCAACGATCAGTTTC	LbB1.3
arr15	Wild-type allel	TCCACATTGTGAAACCTTAGATTTCTT	AACGTCCGCAATGTGTTATTAAGTTGTC
	Mutant allel	forward wild-type allel	LbB1.3
arr19	Wild-type allel	AGAGAGTTGTTAGGGTTAGGGT	TCCACGTCATTCGCGGTTTA
	Mutant allel	Lba1	reverse wild-type allel
arr21	Wild-type allel	GTTTGATTCCGAGCCAACCG	CGCTTCTCCTTGTTGATGCTT
	Mutant	LbB1.3	reverse wild-type allel
ipt1	Wild-type allel	CAACGATTCGACCCAAAGTT	GCTCCAACACTTGCTCTTCC
	Mutant allel	forward wild-type allel	TGGTTCACGTAGTGGGCCATCG
ipt3	Wild-type allel	CCAACTTGTCGTATATCATTCGTACAGTG	TGGAGAGATTCGCCATGTGACAG
	Mutant allel	forward wild-type allel	CAACACGTGGGTTAATTAAGAATTCAGTAC
ipt5	Wild-type allel	TGCATGACGGCTCTAAGACA	TCGAGCTCTGGAACTCCAAT
	Mutant allel	TGGTTCACGTAGTGGGCCATCG	reverse wild-type allel
ipt7	Wild-type allel	CTACCGGATCGGGTAAGTCTC	GCTACAAGATTCTCCCAAGCC
	Mutant allel	forward wild-type allel	TGGTTCACGTAGTGGGCCATCG
phyA-211	Wild-type allel	TTATCCACAGGGTTACAGGG	GCATTCTCCTTGCATCATCC
	Mutant allel	GTCACAAGATCTGATCATGGC	AACAACCGAAGGGCTGAATC
T-DNA specific primer			
Lba1	TGGTTCACGTAGTGGGCCATCG		
LbB1.3	ATTTTGCCGATTTCGGAAC		
SAIL	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC		
GABI Kat	CCCATTTGGACGTGTAGACAC		

Table A2. Primer sequences for qPCR

Gene	ATG	Primer forward	Primer reverse
ABA1	AT5G67030	GGGCTTGGTCCTCTGTCTTT	GGTCATCGGCTTTGTCAGTG
ABA2	AT1G52340	ACTCGCTTTGGCTCATTTGC	CCGTCAGTTCCACCCCTTTT
ABI3	AT3G24650	CGGGAGGGACCTGGATGTATT	CCATCACTGGCGGTAATTGAG
ABI5	AT2G36270	GTGGTGGTGAGAATCATCCGT	CACCCTCGCCTCCATTGTTAT
CYP707A1	AT4G19230	CCGCCTTGTTTCTCACTCTCT	GGAGGGAGTGGGAGTTTGGAA
CYP81F4	AT4G37410	GTTGCCTCATCAGAGACCTCC	GTGGCACATCGTATCCTCCG
FLA9	AT1G03870	AAGGTATGACGGTGTTCGCA	GGTTTGTTTGGCCGGTGAAG
GA2ox3	AT5G07200	TCCACCTGACCCGTTTGGTTAC	TACTCAAGCCAGCCAAGGTCAC
GA20ox1	AT4G25420	CATTCGTCCCAACCCCAA	TGATGCTGTCCAAAAGCTCTCT
GAI	AT1G14920	AGCGTCATGAAACGTTGAGTCAGTG	TGCCAACCCAACATGAGACAGC
GDSL	AT4G26790	ACTTTGCCACTGGTGTCTGT	CACTTCCTTCCATAGCGGCA
HY5	AT5G11260	CGATGAGGAGATACGGCGAG	CGCTTGTTCTCTTTCTCCGC
JAL34	AT3G16460	TGGTGCTTTCGACGGTGTAA	ACAAACGAGACCGCTCCAAT
LHB1B1	AT2G3443	CTCTCTCCTCTCCTGCTTTGAC	GCCCATCTGCTGTGGATAAC
NCED3	AT3G14440	AGGTCGCAAGATTCGGGATT	GCGGATTTCAGACAGGACACTC
NCED6	AT3G24220	ACCGGGTCGGATATAAATTGGGTTG	CCCGGGTTGGTTCTCCTGATTC
NCED9	AT1G78390	AACCGCCGCTATGGTTTTAGACG	CCAGTCACCGGAAGGTTATGCAC
OWL1	AT2G35720	GTTTGTCGCTTCAACGGGTTT	GCTCCGAGTCCACCAGATTC
PHYA	AT1G09570	TGGCAGATTTCATGCTGATGGC	TCCGGTATGCGTTAACCTGATCTC
PHYB	AT2G18790	TTGGAGGCCACAGACTTGAACG	TCCCTCTTTAGCACAAATGAACCG
PP2A	At3g25800	GACAAAACCCGTACCGAG	CCATTAGATCTTGTCTCTCTGCT
PYK10	AT3G09260	TTTTCTCGCACGCTGGCTAT	TGATCGTCCGTCTTGGCATT
RGL2	AT3G03450	GACCCGAATCTGAAACCTTAGTG	GCTCAACCACCGTTACGATACT
RRL1	AT5G13610	CCGCCATGGGGCGGCTCTGTTGTTGAT	CCGCACGTGTCACAAGGATGCCCGAGAC
SLY1	AT4G24210	CGGTGATGCTCACAACGAGA	GCAGCCGATGTTAGTCCAGT
SOM	AT1G03790	AGCAATCAGCGTCTCCATCTCCAG	CCGTCATCGCCGTGAAATCG
XTH31	AT3G44990	GGGCTCTCAACACCAACGTA	ATGACATTTCGGTCGCCACT

Table A3. Various CK metabolites were measured in Col-0 and *ahk2 ahk3* mutant seeds. CK metabolites were measured in dry seeds and seeds which were imbibed for 24 h or 48 h in darkness. n.d., not detected. Unit pg/mg dry weight.

Genotype	sample	DHZ	DHZOG	DHZR	DHZROG	DHZ7G
Col-0	dry	n.d.	0.472	1.322	n.d.	n.d.
	dry	n.d.	0.786	1.657	n.d.	n.d.
	24 h imbibed	n.d.	n.d.	0.556	n.d.	n.d.
	24 h imbibed	n.d.	n.d.	0.640	n.d.	n.d.
	48 h imbibed	n.d.	n.d.	0.723	n.d.	n.d.
	48 h imbibed	n.d.	n.d.	0.722	n.d.	n.d.
ahk2 ahk3	dry	n.d.	1.016	2.610	n.d.	n.d.
	dry	n.d.	1.099	2.610	n.d.	n.d.
	24 h imbibed	n.d.	n.d.	1.368	n.d.	n.d.
	24 h imbibed	n.d.	n.d.	1.911	n.d.	n.d.
	48 h imbibed	n.d.	n.d.	1.240	n.d.	n.d.
	48 h imbibed	n.d.	n.d.	1.415	n.d.	n.d.
		tZOG	**tZR**	**tZROG**	**tZ7G**	
Col-0	dry	n.d.	1.884	0.206	3.084	
	dry	n.d.	2.778	0.271	3.534	
	24 h imbibed	n.d.	0.500	n.d.	0.842	
	24 h imbibed	n.d.	0.549	n.d.	0.995	
	48 h imbibed	n.d.	0.525	n.d.	0.684	
	48 h imbibed	n.d.	0.736	n.d.	0.704	
ahk2 ahk3	dry	1.016	3.102	0.402	9.984	
	dry	1.099	3.520	0.368	8.378	
	24 h imbibed	n.d.	0.837	0.112	2.494	
	24 h imbibed	n.d.	1.293	n.d.	2.310	
	48 h imbibed	n.d.	1.046	0.121	2.923	
	48 h imbibed	n.d.	1.314	n.d.	2.938	
		cZOG	**cZR**	**cZROG**	**cZ9G**	
Col-0	dry	n.d.	49.757	0.888	1.177	
	dry	n.d.	79.947	0.790	1.129	
	24 h imbibed	n.d.	24.791	0.350	0.212	
	24 h imbibed	n.d.	32.205	0.507	0.206	
	48 h imbibed	n.d.	33.195	0.369	0.219	
	48 h imbibed	n.d.	30.919	0.365	0.307	
ahk2 ahk3	dry	n.d.	40.294	1.216	1.241	
	dry	n.d.	40.931	1.165	1.099	
	24 h imbibed	n.d.	53.083	0.499	0.364	
	24 h imbibed	n.d.	67.251	0.550	0.452	
	48 h imbibed	n.d.	44.148	0.615	0.363	
	48 h imbibed	n.d.	54.528	0.547	0.366	
		iPR	**iP7G**	**iP9G**		
Col-0	dry	3.127	53.621	0.890		
	dry	4.027	83.359	1.281		
	24 h imbibed	5.207	40.036	n.d.		
	24 h imbibed	4.844	54.293	n.d.		
	48 h imbibed	8.034	32.800	n.d.		
	48 h imbibed	6.192	37.136	n.d.		
ahk2 ahk3	dry	4.071	73.909	1.615		
	dry	3.868	119.903	1.833		
	24 h imbibed	7.634	37.260	n.d.		
	24 h imbibed	8.115	49.332	n.d.		
	48 h imbibed	9.814	43.251	n.d.		
	48 h imbibed	10.236	48.885	n.d.		

Table A4. List of the 15 DEGs with highest expression at the respective point in time. DEGs were filtered by highest log_2-fold change value at 6 h, 12 h, 24 h or 48 h compared to 1 h imbibed seeds. Only significantly regulated genes ($p < 0.05$) were considered. The identified marker genes are depicted in bold.

Timepoint	Gene name	ATG	log2 fold change	p-value
6h	*PYK10*	**At3g09260**	1.7133729	2.257E-05
	XTH31	**At3g44990**	1.4941774	0.0002744
		At1g18250	1.4560552	8.572E-05
		At3g45010	1.4108984	8.482E-05
		At5g18660	1.3341945	0.000131
	GDSL	**At4g26790**	1.3039783	7.784E-06
		At5g57420	1.2480373	7.552E-06
		At4g22250	1.2451616	1.76E-07
		At4g22756	1.233184	6.365E-05
		At2g22170	1.229537	0.001005
		At4g01450	1.219135	1.327E-06
		At1g34245	1.2154337	3.089E-05
		At3g17680	1.2077115	6.059E-06
		At5g05290	1.2067794	1.063E-06
		At2g24270	1.1969328	0.0001005
12h		AT3G09260	2.4277706	1.555E-08
		AT3G44990	1.9930516	8.022E-06
	JAL34	**AT3G16460**	1.8938003	0.0004536
		AT3G45010	1.805616	4.323E-06
		AT1G18250	1.7849222	4.19E-05
		AT5G18660	1.7662536	5.663E-05
		AT2G22170	1.7128452	2.816E-05
		AT3G15950	1.7093846	2.443E-09
	CYP81F4	**AT4G37410**	1.6687029	0.0001029
		AT3G20370	1.6550307	0.0004159
		AT3G16390	1.6359292	3.215E-05
		AT1G69526	1.6345567	0.0001213
		AT5G15580	1.6253243	4.777E-07
		AT3G16450	1.6214491	0.0019619
		AT5G26280	1.6140028	3.415E-05
24h		At3g09260	2.497332	2.217E-05
		At1g66280	2.2522223	6.829E-08
		At5g26260	2.1026973	n.d.
		At3g16460	2.0800873	0.0001036
		At4g23670	2.0505674	7.683E-06
		At3g16450	2.0346177	7.331E-05
		At4g23680	1.9636915	1.257E-05
		At3g61470	1.9583729	3.959E-06
		At3g44990	1.9453589	6.833E-06
	FLA9	**At1g03870**	1.9409985	4.087E-08
		At2g22170	1.929264	2.671E-05
		At3g20370	1.9165039	3.175E-06
		At3g15950	1.9121849	6.044E-05
		At5g18660	1.8907423	4.453E-05
		At1g55670	1.888792	8.475E-06
48h		At3g09260	2.4975986	1.168E-05
		At4g23670	2.2962646	4.89E-06
		At3g16450	2.2105299	2.672E-05
		At1g66280	2.2076943	5.434E-05
		At4g23680	2.1914554	4.811E-06
		At3g61470	2.1839871	3.117E-09
	LHB1B1	**At2g34430**	2.1497866	8.05E-06
		At4g26010	2.1162504	2.036E-07
		At3g26520	2.1128942	2.887E-05
		At4g02270	2.1125078	5.267E-06
		At3g16460	2.1063659	4.596E-07
		At1g55670	2.069143	4.292E-05
		At5g26260	2.0647212	8.229E-05
		At5g67400	2.0611847	2.249E-32
		At4g25820	2.0544457	1.548E-07

Table A5. Gene expression analysis via qPCR of *in silico* identified marker genes.
Gene expression data underlying the heatmap in Figure 33. Given are the log2 fold change values relative to the transcript level of 1 h imbibed seeds of every genotype, which was set to 1 (outer left column in every section). *PP2A* was used as a reference gene.

	1 h Col-0		**28 h Col-0**		**50 h Col-0**		**55 h Col-0**	
Gene name	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PYK10	1	0	2.36	0.88	2.88	0.40	2.54	0.60
XTH31	1	0	1.67	1.33	2.14	0.99	2.72	1.46
GDSL	1	0	4.31	2.72	5.88	0.47	5.05	1.91
CYP81F4	1	0	4.38	2.88	5.56	0.69	5.52	1.40
JAL34	1	0	2.34	0.74	3.28	0.69	2.67	0.60
FLA9	1	0	3.34	2.11	4.38	0.76	3.93	1.45
LHB1B1	1	0	3.81	2.48	5.15	0.71	4.33	1.39
	1 h phyA		**28 h phyA**		**50 h phyA**		**55 h phyA**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PYK10	1	0	8.22	11.03	19.63	14.18	19.91	10.45
XTH31	1	0	0.92	0.55	4.06	3.88	3.32	2.31
GDSL	1	0	9.48	16.22	29.56	24.90	36.22	24.57
CYP81F4	1	0	9.99	15.33	36.90	29.75	33.79	22.34
JAL34	1	0	6.04	8.04	12.63	9.27	15.22	9.15
FLA9	1	0	4.18	6.71	16.18	15.60	13.37	8.69
LHB1B1	1	0	10.75	18.73	27.16	21.26	39.56	26.01
	1 h *ahk2 ahk3*		**28 h *ahk2 ahk3***		**50h *ahk2 ahk3***		**55h *ahk2 ahk3***	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PYK10	1	0	1.34	1.93	1.90	1.05	2.64	1.39
XTH31	1	0	1.59	1.46	0.94	0.72	2.46	1.88
GDSL	1	0	8.12	15.82	6.60	8.47	14.80	11.72
CYP81F4	1	0	3.10	6.17	2.44	3.06	4.67	3.42
JAL34	1	0	2.13	3.69	2.37	1.63	3.78	2.33
FLA9	1	0	3.27	6.20	2.55	3.12	5.62	3.86
LHB1B1	1	0	4.33	8.62	3.78	4.78	8.73	5.79

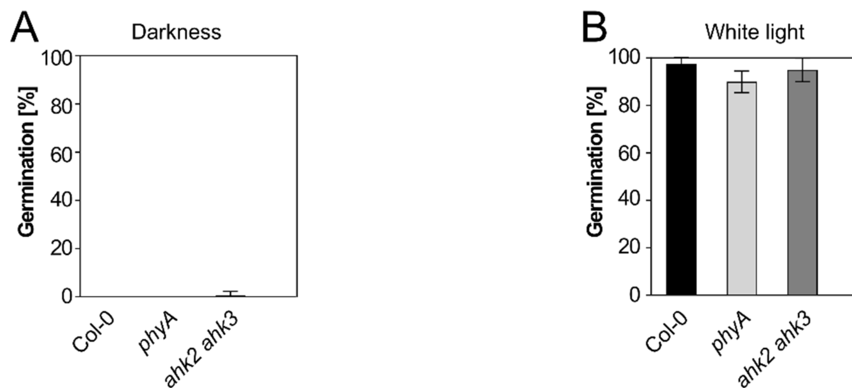

Figure A1. Germination rates in darkness and white light were similar between wild-type seeds, CK receptor mutant and *phyA* seeds.
Error bars indicate standard deviations.

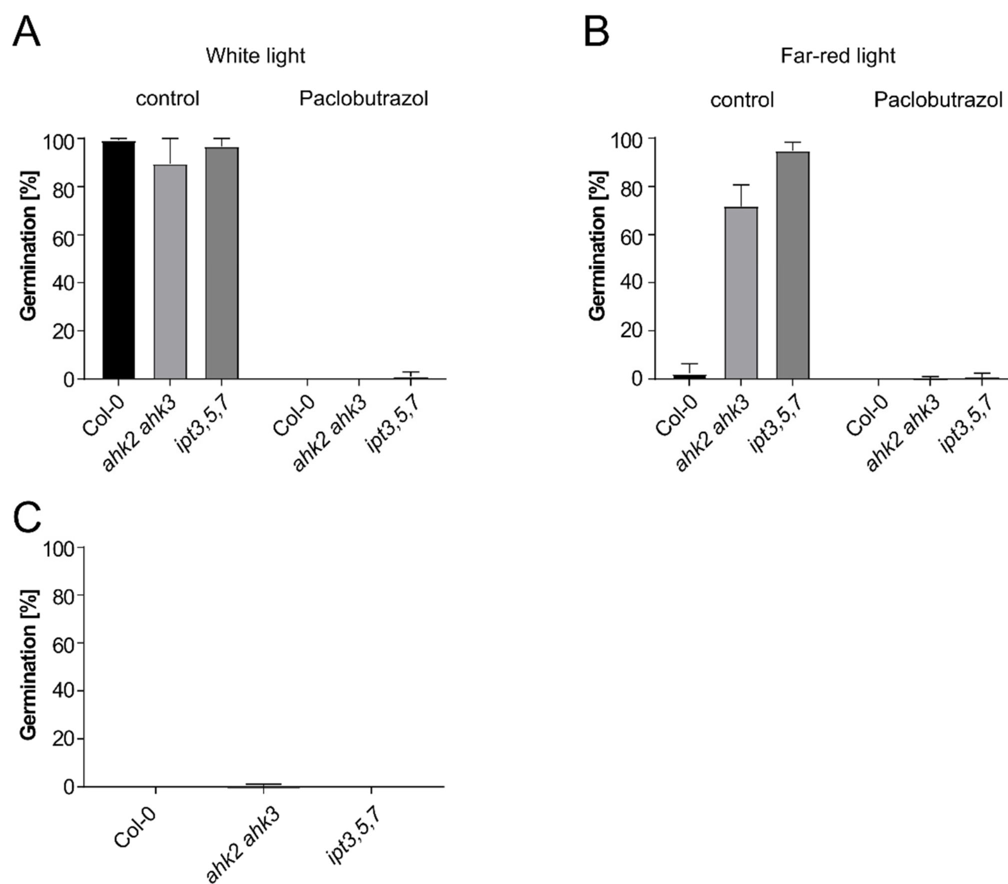

Figure A2. Reduction of germination rates after Paclobutrazol application.

A) Germination rates without (control) and with Paclobutrazol (10 µM) supply to the germination medium in white light (A) or far-red light (B). No seed germination was observed in the wild type or in CK mutant seeds (*ahk2 ahk3*, *ipt3,5,7*) when they were incubated in darkness without GA or Paclobutrazol supply (C).

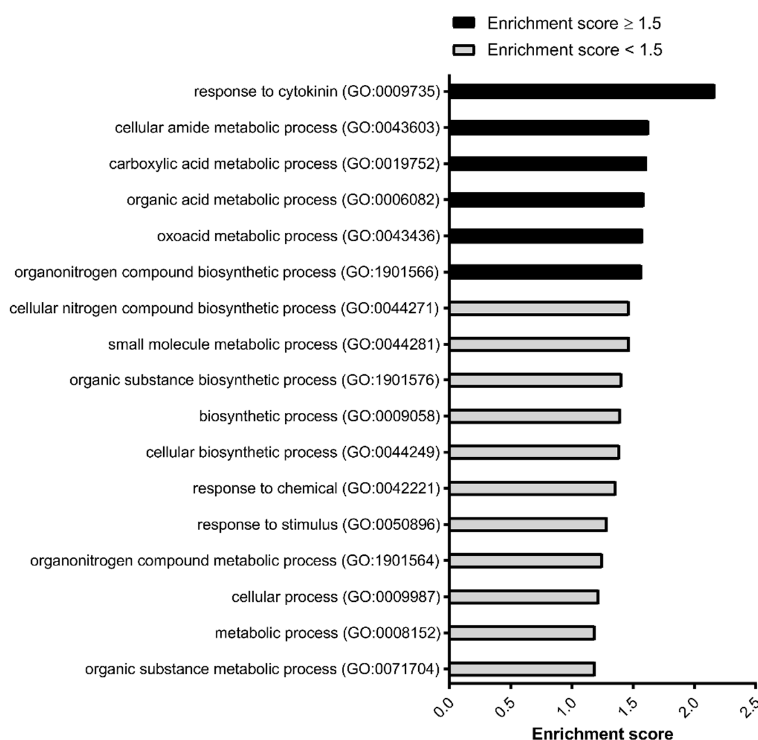

Figure A3. Gene Ontology analysis of genes uniquely regulated after 24 h of light incubation in wild-type seeds from microarray dataset published by Narsai *et al.* (2011). Enrichment score abbreviates gene enrichment score.

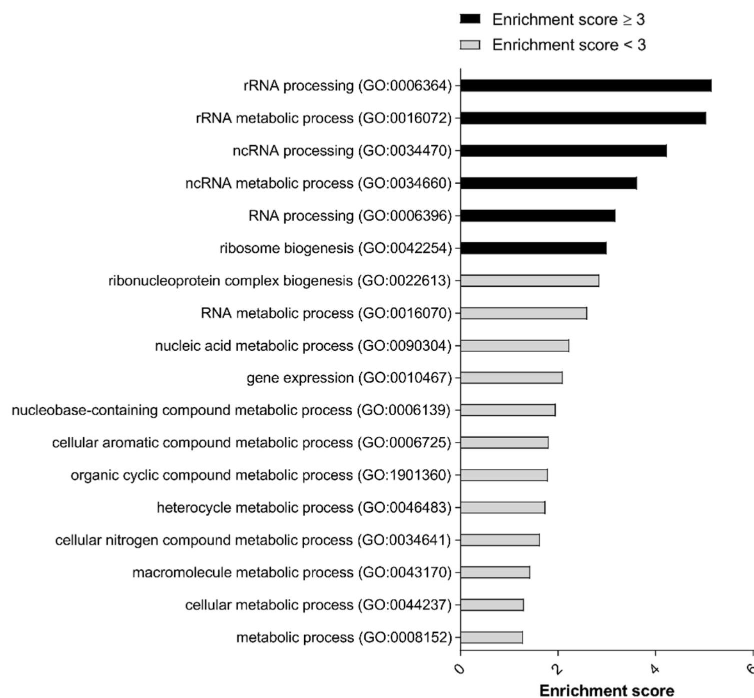

Figure A4. Gene Ontology analysis of genes uniquely regulated after 48 h of light incubation in wild-type seeds from microarray dataset published by Narsai *et al.* (2011). Enrichment score abbreviates gene enrichment score.

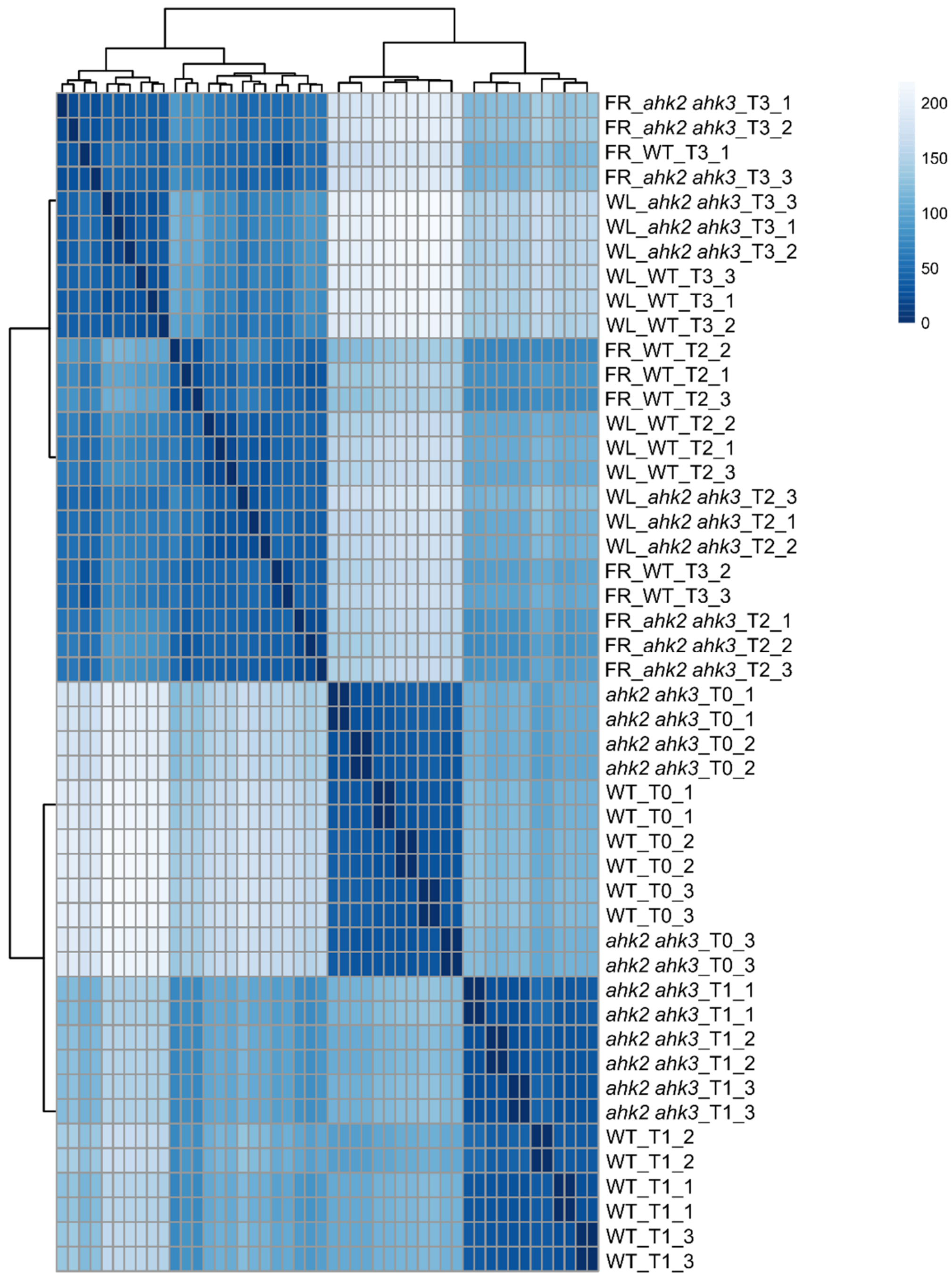

Figure A5. Color-coded hierarchical clustering heatmap displaying euclidian distance.

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