Characterization of mediators of the cytokinin transcriptional response

The characterization of ARR14 and the identification of novel modulators of the cytokinin response

Dissertation

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Disputation am:_____

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1. Introduction

1.1. The plant hormone cytokinin

In the late 19th - early 20th century Wiesner, Haberlandt, van Overbeek, Jablonski and Skoog postulated an unknown substance to promote cell division in plant tissue (Wiesner, 1892; Haberlandt, 1913; Jablonski and Skoog, 1954). Testing chemicals for their potential to induce cell division, Miller and colleagues finally isolated the first cytokinin in 1955 from autoclaved hering sperm DNA (Miller *et al*., 1955). Zeatin from maize was the first cytokinin to be isolated from plant tissue and being described chemically (Letham, 1965). In the following years several additional cytokinins were identified (Mok and Mok, 2001) and proved to be active as phytohormones (Schmitz and Skoog, 1972; Mok *et al*., 2005).

1.1.1. Cytokinin functions

Besides cell division, the first function assigned to cytokinin was to promote cell differentiation e.g. the formation of shoots from callus (Skoog and Miller, 1957; Skoog *et al*., 1965), but cytokinin is involved in various other processes (Hwang *et al*., 2012 ; Mok and Mok, 2001; Werner and Schmülling, 2009). I will focus on those functions relevant for the results I obtained in this study.

In roots cytokinin promotes the transition from the phase of cell division towards cell differentiation in cooperation with auxin by indirectly regulating auxin transport (Dello Ioio *et al*., 2008; Moubayidin *et al*., 2010; Perilli *et al*., 2010). In concert with auxin cytokinin plays a role in lateral root initiation and development (Kitomi *et al*., 2011; Kushwah *et al*., 2011; Zheng *et al*., 2011; Bielach *et al*., 2012; Hao and Cui, 2012). It inhibits lateral root formation (Böttger, 1974) and mediates cell differentiation in the root meristem via at least three distinct pathways (Dello Ioio *et al*., 2007). The first pathway is the direct transcriptional and posttranscriptional regulation of PIN-FORMED (PIN) proteins that regulate auxin efflux (Ruzicka *et al*., 2009; Marhavy *et al*., 2011; Zhang *et al*., 2011). The second pathway is an indirect regulation by prompting the expression of *ABSCISIC ACID INSENSITIVE 4 (ABI4)*, an abscisic acid regulated gene. *ABI4* codes for an APETALA 2 (AP2)-domain containing protein regulating *PIN*-expression (Shkolnik-Inbar and Bar-Zvi, 2010). The third pathway is a regulation via AHK3/ARR1-activated transcription of *SHORT HYPOCOTYL 2 (SHY2)*, an AUX/IAA transcriptional repressor regulating *PIN* expression (Dello Ioio *et al*., 2008; Moubayidin *et al*., 2010). Cytokinin acts also in the elongation zone of the root via induction of ethylene production (Stenlid, 1982; Cary *et al*., 1995)

stimulating auxin biosynthesis and auxin basipetal transport (Ruzicka *et al*., 2007). In embryo development, cytokinin and auxin crossregulate each other to establish the pattern needed for a functional root cell system (Müller and Sheen, 2008).

Cytokinin influences the apical dominance of shoots and roots. The auxin-dependent bud inhibition is reversed by cytokinin application (Wickson and Thimann, 1958). In bud inhibition auxin represses cytokinin biosynthesis (Tanaka *et al*., 2006). This, in concert with the action of strigolactones leads to an inhibition of bud outgrowth. By decapitation of the main stem the dormant axilary bud can become sensitive towards cytokinin derived from the main stem and grow out (Ferguson and Beveridge, 2009; Domagalska and Leyser, 2011).

Vascular patterning is affected by cytokinin (Mähönen *et al*., 2006; Bishopp *et al*., 2011a; Bishopp *et al*., 2011b). Cytokinin mutants showed severe defects in vasculature structure (see chapter 1.1.5.1) (Mähönen *et al*., 2000; Yokoyama *et al*., 2007; Argyros *et al*., 2008; Ishida *et al*., 2008b).

In the shoot apical meristem (SAM), cytokinin regulates cell identity via STIMPY (STIP) (Skylar *et al*., 2010) and size via cytokinin signaling components (Lee *et al*., 2009). The cytokinin signaling system acts on regulators of cell identity as *WUSCHEL (WUS)* and *CLAVATA (CLV)* and vice versa and is involved in maintenance of stem cell identity(Leibfried *et al*., 2005; Buechel *et al*., 2009; Gordon *et al*., 2009; Chickarmane *et al*., 2012).

Cytokinin influences also photosynthesis. It induces photosynthetic genes (Treharne *et al*., 1970), stimulates plastid ripening (Stetler, 1965) and alters thylakoid structure (Cortleven *et al*., 2011; Cortleven and Valcke, 2012). Cytokinin is able to delay leaf senescence (Richmond and Lang, 1957). Already in 1977 Wittenbach discovered the reversion of dark–induced senescence in *Triticum* by cytokinin (Wittenbach, 1977). The delay depends on the cytokinin signaling components (Kim *et al*., 2006). Senescence-specific endogenous cytokinin overproduction delays senescence (Gan and Amasino, 1995; Zhang *et al*., 2010; Merewitz *et al*., 2011a, b; Merewitz *et al*., 2012). Possibly this is at least partially realized through the induction of a hexokinase connecting cytokinin to sugar signaling to senescence phenomena (Swartzberg *et al*., 2011).

Cytokinin functions also as integrator of environmental signals (reviewed in Argueso *et al*., 2009; Choi *et al*., 2011). It is involved in rhizobiobial symbiosis. Badenoch-Jones discovered that root nodules accumulate cytokinin (Badenoch-Jones *et al*., 1984). Signaling components as well as the cytokinin-regulated CLAVATA3/ENDOSPERM SURROUNDING REGION (CLE) peptides are involved

in nodulation (see chapter 1.1.5.1) (Gonzalez-Rizzo *et al*., 2006; Murray *et al*., 2007; Tirichine *et al*., 2007; Mortier *et al*., 2012).

Recently also a function in pathogen response was shown for cytokinin. A transcription factor of the cytokinin signaling component was shown to directly interact with TGA3 and bind to the *PATHENOGENESIS-RELATED 1 (PR1)* promoter and a changed cytokinin level or sensitivity caused changes in the response towards *Pseudomonas syringae* (Choi *et al*., 2010). Independent of salicylic acid signaling, cytokinin upregulates phytoalexines in tobacco, leading to an increased resistance against *Pseudomonas syringae* (Grosskinsky *et al*., 2011). In high cytokinin concentrations the immunity is increased in a salicylic acid-dependent manner (Argueso *et al*., 2012).

Also abiotic stresses like salt stress and drought stress were connected to cytokinin. Itai and colleagues observed that cytokinin levels are higher in salt-stressed plants (Itai and Vaadia, 1971). Plants with a lowered cytokinin status due to increased cytokinin degradation (Werner *et al*., 2008; Werner *et al*., 2010; Nishiyama *et al*., 2012) or increased cytokinin biosynthesis (Merewitz *et al*., 2011a; Peleg *et al*., 2011; Qin *et al*., 2011) were more tolerant towards salt stress and drought stress.

Cytokinin is involved in sensing and regulating the nutrient status. It was shown to repress sodium transporter genes (Mason *et al*., 2010), and sulfur transporter genes (Maruyama-Nakashita *et al*., 2004b; Maruyama-Nakashita *et al*., 2004a) and is involved in gene regulation under phosphate starvation conditions (Franco-Zorrilla *et al*., 2002; Franco-Zorrilla *et al*., 2005). Genes involved in cytokinin signaling are regulated by nitrogen availability (Sakakibara *et al*., 1998; Taniguchi *et al*., 1998; Takei *et al*., 2001b; Sakakibara, 2003; Sakakibara *et al*., 2006).

1.1.2. Cytokinin as chemical

Chemically cytokinins are N^6-substituted adenine derivates (Skoog, 1967; Mok and Mok, 2001). Based on the side chain structure they are divided into two classes, the isoprenoid and the aromatic cytokinins (reviewed by Mok and Mok, 2001; Schmülling, 2004; Sakakibara, 2006; Hirose *et al*., 2008). Most of the biologically active natural cytokinins are of the isoprenoid type, e.g. iP (isopentenyl)-type and zeatin type cytokinins (reviewed by Schmülling, 2004). iP-type cytokinins can be converted into zeatin-type cytokinins by the P450 monooxygenases CYP735A1 and CYP735A2 (Takei *et al*., 2004a). *Trans*-zeatin is the most active zeatin in *Arabidopsis thaliana* and most other plants (Schmitz and Skoog, 1972) but in some plant species, e.g. maize, also the

cis-forms showed biological activity (Schmitz and Skoog, 1972; Emery *et al*., 1998; discussed in Mok and Mok, 2001; Veach *et al*., 2003). *Cis*-zeatin can be bound by *Arabidopisis* cytokinin receptors (Spichal *et al*., 2004; Yonekura-Sakakibara *et al*., 2004; Romanov *et al*., 2006). Aromatic cytokinins seem to be restricted to some plant species e.g. N-(o-hydroxybenzylamino)purine in *Populus* (Strnad *et al*., 1992). Receptor binding assays were performed to estimate the binding affinities towards different cytokinins in *Arabidopsis thaliana* (Romanov *et al*., 2006). The natural cytokinin levels *in planta* are about 0,7-2,5 nM (as reviewed by Werner and Schmülling, 2009), e.g. 5-125ng/ml in xylem exudate in bean (Bangerth, 1994).

1.1.3. Cytokinin metabolism

There are several ways to regulate the level of active cytokinin in the cell. The level can be regulated via biosynthesis, interconversion/conjugation and degradation (Figure 1.1).

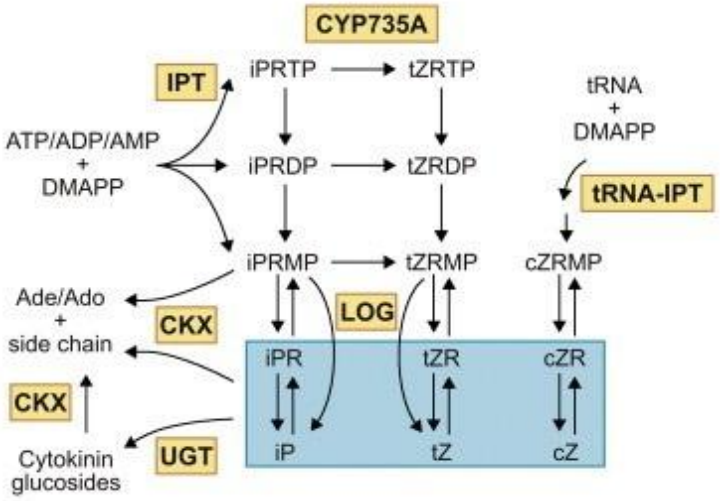


Figure 1.1: Cytokinin metabolism and interconversion. Cytokinin can be synthetized by IPT and tRNA-IPT enzymes. IPT enzymes use DMAPP and ATP/ADP/AMP as substrates and release the corresponding iP-adenosinephosphate. This can be converted to *t*Z-adenosinephosphates by CYP735A. LOG leads to the formation of free iP and *t*Z. CKX degrades iPRMP, iPR and iP to adenine/adenosine and the side chain. Glycosyltranferases synthesize cytokinin glucosides that can partially be degraded by CKX. tRNA-IPTs use DMAPP and tRNA as substrate to release *c*ZRMP. This can be converted to *c*ZR and *c*Z. CYP735A, P450 monooxygenase; IPT, isopentenyltransferase; LOG, LONELY GUY; UGT, glycosyltransferase; CKX, cytokinindehydrogenase/-oxidase; ATP/ADP/AMP, adenosine-tri/di/mono-phosphate; Ade, adenosine; iPRTP/iPRDP/iPRMP, isopentenyladenosine-tri/di/mono-phosphate; *t*ZRTP/*t*ZRDP/*t*ZRMP, *trans*-zeatinadenosine-tri/di/mono-phosphate; DMAPP, dimethylallyldiphosphate; *c*ZRMP, *cis*-zeatinadenosinemonophosphate; iPR, isopentenylriboside; iP, isopentenyl; *t*ZR, *trans*-zeatinriboside; *t*Z, *trans*-zeatin; *c*ZR, *cis*-zeatinriboside; *c*Z, *cis*-zeatin (modified from Werner and Schmülling, 2009)

1.1.3.1. Biosynthesis

The first step of cytokinin biosynthesis is performed by Isopentenyl transferases (IPTs). IPT proteins synthesize isopentenyladenosine 5′-monophosphate (iPMP) from dimethylallylpyrophosphate (DMAPP) and AMP. They were discovered in the T-region of the bacterial Ti-plasmid (Buchmann *et al*., 1985) and later described also in *Arabidopsis* (Takei *et al*., 2001a). The first IPTs used to increase the cytokinin level in plants were derived from agrobacteria (Smigocki, 1991; Gan and Amasino, 1995; Rupp *et al*., 1999). ATP/ADP-dependent IPT enzymes produce iP and *trans*-zeatin-type cytokinins whereas tRNA-dependent IPT enzymes are responsible for the production of *cis*-zeatin-type cytokinins. Two *Arabidopsis* tRNA-*IPT* genes; *IPT2* and *IPT9*, were identified and analyzed in *knockout* approaches (Miyawaki *et al*., 2006). ATP/ADP-dependent *IPT* genes are members of a small multigene family with nine members in *Arabidopsis thaliana.* They show differential expression domains and levels and can regulate the cytokinin abundance and spatial distribution (Kakimoto, 2001; Takei *et al*., 2001a; Miyawaki *et al*., 2004; Takei *et al*., 2004b; Miyawaki *et al*., 2006). IPT enzymes are involved in the integration of environmental signals and development. *IPT3* expression in *Arabidopsis thaliana* is regulated by nitrate availability (Miyawaki *et al*., 2004; Takei *et al*., 2004b), *IPT5*- and *IPT7*-transcription are upregulated by auxin treatment (Miyawaki *et al*., 2004). IPT enzymes have been used as a tool to increase the cytokinin content in a specific spatial and temporal manner by utilizing specific promoters. These experiments linked cytokinin to various functions. A bacterial *IPT* driven by the *Hsp70* promoter increased the expression of the meristem regulators *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA* (*KNAT)* and *SHOOTMERISTEMLESS* (*STM)* (Rupp *et al*., 1999). *IPTs* were used to increase the cytokinin content of plants in a senescence-specific manner by combining them with the *SENESCENCE-ASSOCIATED GENE 12* (*SAG12)* promoter (Gan and Amasino, 1995; Merewitz *et al*., 2011a, b; Merewitz *et al*., 2012) Using this tool, Gan and Amasino observed a delay in senescence in tobacco leaves (Gan and Amasino, 1995). In *SAG12::IPT* creeping bentgrass the photosynthesis, water use efficiency, and root viability were increased under water stress conditions. The *IPT* expression resulted in protein and metabolite accumulation and increased the drought resistance of the plants (Merewitz *et al*., 2011a, b; Merewitz *et al*., 2012). *HSP18.2::IPT* in peanut increased the drought tolerance as well (Qin *et al*., 2011). *IPT* expressed under the control of a *SENESCENCE ASSOCIATED RECEPTOR PROTEIN KINASE (SARK)* promoter in rice changed the sink-source-relations in drought-stressed rice towards stronger sinks (Peleg *et al*., 2011).

Active cytokinins can be released by the cytokinin hydroxylases CYP735A1 and CYP735A2 (Takei *et al*., 2004a).

1.1.3.2. Interconversion and conjugation

The most active forms of cytokinins are the free nucleobases (Schmitz and Skoog, 1972; Spiess, 1975; Åstot *et al*., 2000), but also ribosides seem to be active (Spichal *et al*., 2004). Nucleobases can be obtained by dephosphorylation and deribosylation of nucleotides. In 1977 and 1981 adenosine nucleosidases were discovered in barley and wheat germ (Guranowski and Schneider, 1977; Chen and Kristopeit, 1981a). A nucleotidase was also discovered in wheat germ cells (Chen and Kristopeit, 1981b). Free bases can also be directly released in a single step reaction by a cytokinin nucleoside 5'-monophosphate phosphoribohydrolase named LONLEY GUY (LOG) (Kurakawa *et al*., 2007). Expression patterns and single *knockouts* as well as multiple *knockouts* were analyzed by Kuroha and Tokunaga and colleagues (Kuroha *et al*., 2009; Tokunaga *et al*., 2012). Cytokinin ribosides can be converted to inactive nucleotides by an adenosine kinase (Kwade *et al*., 2005; Schoor *et al*., 2011).

The level of active cytokinin is also regulated by interconversion or conjugation of the free bases. A N-glycosylation can occur in the N^3, N^7 or N^9 position and inactivate the cytokinin irreversible whereas O-glycosylations are reversible and may play a role as storage form (Munoz *et al*., 1990). In 1975 Letham and colleagues demonstrated that N^7- and N^9-glucosides are less active than the free bases in de-rooted radish seedlings (Letham *et al*., 1975). Together with Etsch, Letham observed N-glycosylation of cytokinins . In 1979 Entsch and colleagues identified a N^7-glycosyltransferase (Entsch, 1979). Recently Wang and colleagues analyzed a mutant of *UGT76C2*, a N-glycosyltransferase (Wang *et al*., 2011). In 1999 a zeatin-O-glycosidase was discovered and proved to be active in *Phaseolus* (Martin *et al*., 1999).

1.1.3.3. Degradation

Degradation of cytokinins is performed by CYTOKININ OXIDASEs (CKXs). They were first discovered in tobacco (Paces *et al*., 1971) and isolated from maize crude extract that showed cytokinin degrading enzyme activity (Whitty and Hall, 1974; Brownlee *et al*., 1975; Burch, 1989). CKX enzymes oxidate selectively the unsaturated N^6-isoprenoid side chain of the cytokinin to release the adenine or adenosine (McGaw, 1983). They can degrade free cytokinin bases and nucleosides and show a certain substrate-specificity (Galuszka *et al*., 2007; Kowalska *et al*.,

2010). Cyclic or saturated side chains are mostly resistant towards CKX degradation (McGaw, 1983; Hare, 1994). Aromatic cytokinins are degraded by CKX enzymes with low efficiency (Kowalska *et al*., 2010). Phenylurea-type cytokinins were found to be strong inhibitors of CKX activity (Chatfield and Armstrong, 1986; Laloue and Fox, 1989). *CKX* genes are expressed in a specific spatial and temporal manner as described in Werner et al (2003) (Werner *et al*., 2003). Overexpression of *CKX* genes causes the so-called "cytokinin-deficiency syndrome". Tobacco and *Arabidopsis* plants with the "cytokinin-deficiency syndrome" display stunted shoots, a smaller and less active shoot apical meristem (SAM), less reproductive organs and enhanced root systems (Werner *et al*., 2001; Werner *et al*., 2003). Overexpression of *CXK* genes in *Arabidopsis* as well as in tobacco, led to a shift in source-sink relations (Werner *et al*., 2008). *CKX* expression correlated with e.g. grain weight in *Triticum arvense* (Zhang *et al*., 2012) and hydrogen peroxide accumulation (Cueno *et al*., 2012). A root-specific overexpression increased specifically the root biomass, resulting in enhanced drought resistance, better performance in sulfur- or magnesium deficiency and led to the accumulation of S, P, Mo, Mg, Zn and Cd in the shoots (Werner et al., 2010). *CKX* genes have been studied in several plant species e.g. tobacco (Werner *et al*., 2001), *Arabidopisis thaliana* (Werner *et al*., 2003), maize (Lohar *et al*., 2004; Smehilova *et al*., 2009), tomato (Cueno *et al*., 2012), wheat (Galuszka *et al*., 2004; Mameaux *et al*., 2012) and barley (Schluter *et al*., ; Galuszka *et al*., 2004; Mameaux *et al*., 2012).

Another possibility to degrade cytokinins is the deamination. Goble and colleagues identified a cytokinin deaminase that deaminates N^6-isopentenyladenine to isopentenylamine and hypoxanthine (Goble *et al*., 2011).

1.1.4. Transport of cytokinin

Another level of regulation of the cytokinin level is compartmentalization or transport from cell to cell. For the vasculature it is known, that the cytokinin type levels differ in xylem and phloem. *Trans*-zeatin is enriched in the xylem, iP-type cytokinins are enriched in the phloem sap (reviewed by Hirose *et al*., 2008). Cytokinins are transported short distance via PURINE PERMEASEs (PUPs) and EQUILIBRATIVE NUCLEOSIDE TRANSPORTERs (ENTs). The PUP transporters were discovered in 2000 by Gillissen and colleagues and displayed differential cytokinin affinities (Gillissen *et al*., 2000). PUP1 and PUP2 were shown to transport adenine energy-dependent but with high affinity (Bürkle *et al*., 2003). ENT2 in rice exhibited strong substrate specificity and transported iPR (Hirose *et al*., 2006). Cedzich and colleagues stated the existence of a high-affinity and a low affinity transport system for cytokinin uptake in

Arabidopsis. Hereby the PUP transporters would cover the low affinity transport (Cedzich *et al*., 2008). Intracellular transporters specific for cytokinin have not been identified up to now (Hirose *et al*., 2008). Long distance transport seems also to be important as shown by grafting experiments using cytokinin biosynthesis mutants and wild type (WT) plants (Matsumoto-Kitano *et al*., 2008).

1.1.5. Cytokinin signaling

The elucidation of the cytokinin signaling pathway started with the discovery of *CYTOKININ INSENSITIVE 1 (CKI1)* in an activation tagging screen (Kakimoto, 1996). The mutant exhibited constitutive cytokinin responses like rapid cell division and shoot induction in a callus assay in the absence of cytokinin. When *CKI1* was compared to known genes, the similarity to the ethylene receptor *ETHYLENE RESISTANT 1 (ETR1)* became obvious (Chang *et al*., 1993). *ETR1* and *CKI1* showed similarity to the receptors of the bacterial two-component system. In bacteria, a simple two-component system is composed of a histidine kinase and response regulator. The histidine kinase senses a signal and translocates a phosphoryl group from a conserved histidine residue in the transmitter domain to a conserved aspartate residue of the receiver domain of the response regulator (reviewed by West and Stock, 2001 and Chang and Stewart, 1998) (Figure 1.2A). Because of the sequence similarity, a two component system was proposed for the cytokinin signal transduction in plants and later confirmed by various groups (Kakimoto, 1996; D'Agostino and Kieber, 1999; Hwang and Sheen, 2001).

A hybrid histidine kinase (HK) first transfers the phosphoryl group intramolecularly to an aspartate of its own receiver domain. Subsequently a histidine phosphor transfer protein (HPT) is phosphorylated at a canonical histidine residue. When it shuttles to the nucleus it phosphorylates a response regulator (RR) at the conserved aspartate in the receiver domain (D'Agostino and Kieber, 1999) (Figure 1. 2B).

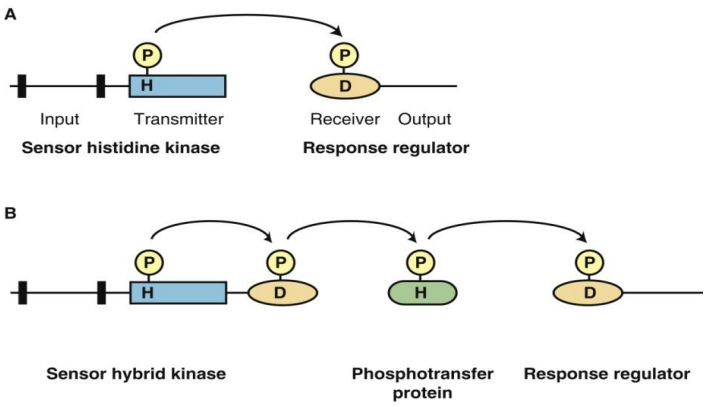


Figure 1.2: Two component systems. (A) Bacterial two component system and (B) multistep two-component situation in plants. In the bacterial system, the phosphate is transferred from a sensor histidine kinase to a response regulator. In the more complex situation in of the multistep two component system in plants, a response regulator domain of the sensor kinase and phosphotransfer protein are interposed. (Schaller *et al*., 2011)

Response regulators in *Arabidopsis* were isolated by several groups, e.g. *INDUCED BY CYTOKININ 6* and *7 (IBC6* and *ICB7)* (Brandstatter and Kieber, 1998), *ARBIDOPSIS RESPONSE REGULATORs 3-7* (*ARR3-7*) (Imamura *et al*., 1998). Expression analyses revealed that some response regulators are induced by cytokinin (Kiba *et al*., 1999) and by resupply of nitrate to nitrate-starved plants (Taniguchi *et al*., 1998). In maize Sakakibara and colleagues identified the response regulator *CYTOKININ-INDUCIBLE PROTEIN 1* (*ZmCip1)*, which was upregulated in its expression by cytokinin and nitrogen (Sakakibara *et al*., 1998). Later *ZmRR1* and *ZmRR2* were identified and characterized (Deji *et al*., 2000; Deji *et al*., 2002). By screening EST databases for histidine phosphotransfer proteins Miyata and Suzuki and colleagues identified *AHP1-3* (Miyata *et al*., 1998; Suzuki *et al*., 1998) and a phosphate transfer from AHPs to ARRs was shown (Imamura *et al*., 1998; Miyata *et al*., 1998; Suzuki *et al*., 1998). *AHPs* were able to complement a yeast strain deficient in the orthologue *YPD1* (Miyata *et al*., 1998; Suzuki *et al*., 1998). Nakamura and colleagues showed that AHP1 is able to phosphorylate CKI1 as well (Nakamura *et al*., 1999). In 2003, Asakura and colleagues proved the interactions between histidine phosphotransfer proteins and response regulators in maize (Asakura *et al*., 2003). To gain more insight into the mechanism of the *Arabidopsis* cytokinin signaling system and get hints for specific functions of its components Dortay and colleagues performed interaction studies by using the yeast two-hybrid system. An interaction matrix within the two-component system and screens to identify new interactors were presented and showed e.g. interactions between AHPs and receptors and AHPs and response regulators (Dortay *et al*., 2006; Dortay *et al*., 2008).

Homologs of the two component system have been bioinformatically identified and analysis of expression patterns was performed e.g. in maize (Chu *et al*., 2011), rice (Ito and Kurata, 2006; Pareek *et al*., 2006; Tsai *et al*., 2012), *Lotus* (Ishida *et al*., 2009), *Physcomitrella* (Ishida *et al*., 2010) and grapevine (Fernandes *et al*., 2009). For a comprehensive overview about the cytokinin two component system in plants other than *Arabidopsis thaliana* see Hellmann *et al*. (2010). A scheme of the current model of cytokinin signal transduction is depicted in Figure 1.3.

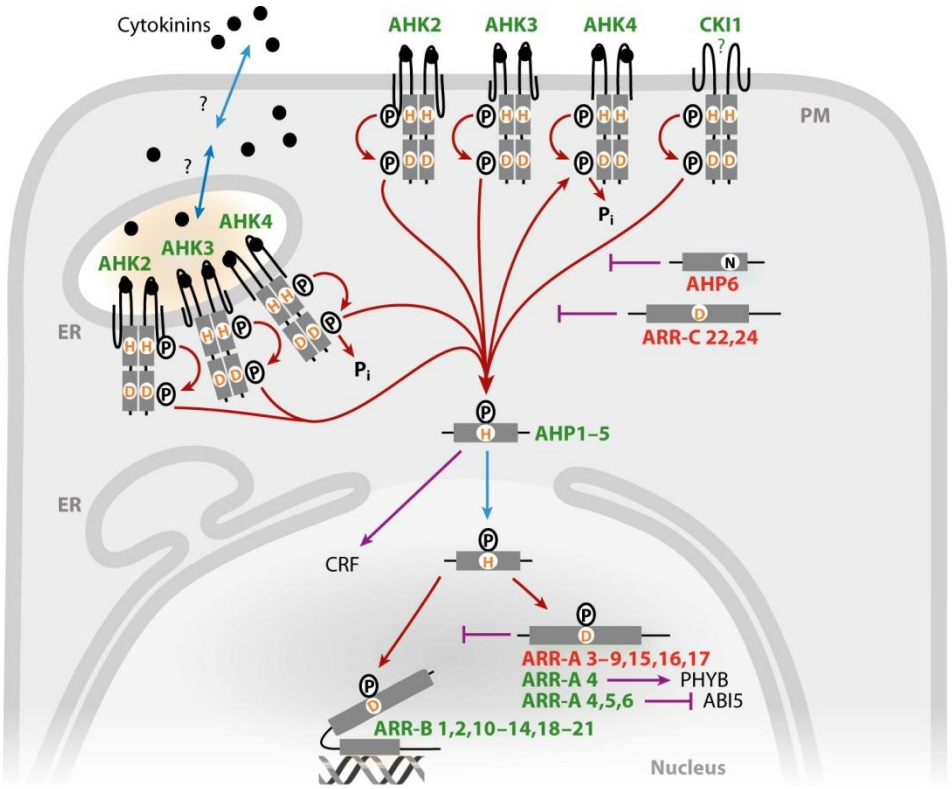


Figure 1.3: Model of cytokinin signaling. Cytokinin is perceived by the membrane-bound receptors ARABIDOPSIS HISTIDINE KINASE 1 (AHK1), AHK2 and AHK3 and a phosphate signal is transferred to the nucleus by ARABIDOPSIS HISTIDINE TRANSFER PROTEINs (AHPs). ARABIDOPSIS RESPONSE REGULATORs of type-A or type-B (ARR-As or ARR-Bs) are phosphorylated. The receptor CYTOKININ INSENSITIVE 1 (CKI1) integrates signals into the cytokinin signal transduction pathway, CYTOKININ RESPONSE FACTORs (CRFs) are phosphorylated by the AHPs. AHPs and type-c ARRs (ARR-C) inhibit cytokinin signaling. ARR-As inhibit ARR-B phosphorylation. *PHYTOCHROME B* (*PHYB*) and *ABA INSENSITIVE 5 (ABI5)* are regulated by ARR-As (modified from Hwang *et al*., 2012).

1.1.5.1. The cytokinin receptors

The first real cytokinin receptor to be identified was CYTOKININ RESISTANT 1/*ARABIDOPSIS* HISTIDIN KINASE 4/WOODEN LEG (CRE1/AHK4/WOL). *wol* was a *gain-of-function* mutant defective in vasculature development. It exhibited a reduced cell number, no asymmetric cell divisions and therefore just protoxylem structures (Mähönen *et al*., 2000). *cre1* was another mutant allele of the *CRE1/AHK4/WOL* cytokinin receptor. It was identified due to its reduced cytokinin responsiveness (Inoue *et al*., 2001). Yamada and colleagues discovered that the *wol* mutation abolishes cytokinin binding (Yamada *et al*., 2001). CRE1/AHK4/WOL showed cytokinin-dependent kinase activity when expressed in yeast (Ueguchi *et al*., 2001a).

In *Arabidopsis thaliana* there are three cytokinin receptors serving as a start of the signaling cascade, CRE1/AHK4/WOL, AHK2 and AHK3. They share the feature of a CHASE (Cyclases/Histidine kinases Associated Sensory Extracellular) domain (Anantharaman and Aravind, 2001; Mougel and Zhulin, 2001), a varying number of transmembrane domains, a histidine kinase domain and a receiver domain (Mähönen *et al*., 2000; Inoue *et al*., 2001; Suzuki *et al*., 2001b; Ueguchi *et al*., 2001b; Ueguchi *et al*., 2001a; Yamada *et al*., 2001) (Figure 1.4).

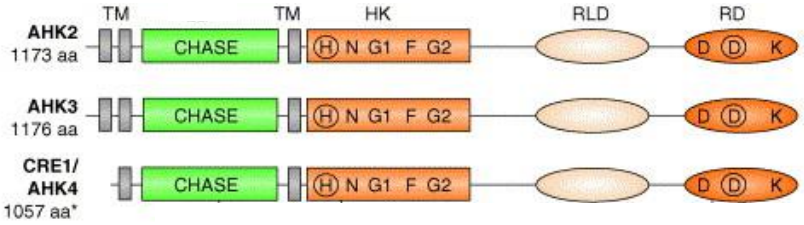


Figure 1.4: Structure of the cytokinin receptors. The CHASE domain is flanked by transmembrane domains (TM). A histidine kinase domain (HK), a receiver-like domain (RLD) and a response regulator domain (RD) with a DDK phosphorylation motif are located at the predicted cytoplasmatic side (modified from Heyl and Schmülling, 2003).

Suzuki and colleagues showed an interaction of AHK4 with HPTs (Suzuki *et al*., 2001b). Sakai and colleagues presented a signal transduction cascade from CRE1/AHK4 to ARR1 to ARR6 (Sakai *et al*., 2001). CRE1/AHK4 is able to act as a kinase or a phosphatase to the HPTs (Mähönen *et al*., 2006).

Loss-of-function analyses revealed the roles of the other two cytokinin receptors in *Arabidopsis* in cytokinin signaling and showed, that they act partially redundant to each other (Higuchi *et al*., 2004; Riefler *et al*., 2006; Stolz *et al*., 2011).

Expression analyses revealed, that *CRE1/AHK4* was stronger expressed in the roots whereas the other two receptors showed higher and ubiquitous expression in the shoot (Higuchi *et al*., 2004; Nishimura *et al*., 2004). In *Arabidopsis* the receptors were localized at least partially to the endoplasmatic reticulum where also cytokinin binding activity was discovered (Caesar *et al*., 2011; Wulfetange *et al*., 2011). Similar results were observed in maize (Lomin *et al*., 2011).

CRE1/AHK4 was not activated by cytokinin ribotides and ribosides in a bacterial binding assay whereas AHK3 was (Spichal *et al*., 2004). In a live cell binding assay Romanov and colleagues observed that AHK3 bound also dihydro-zeatin, iP and ribosides (Romanov *et al*., 2005; Romanov *et al*., 2006; Romanov and Lomin, 2009). Promoter swap experiments revealed that CRE1/AHK4 can replace AHK2 but not AHK3. This could be due to the fact that binding preferences for iP are higher for both CRE1/AHK4 and AHK2 and lower for *trans*-zeatin. So there is no complete redundancy among the receptors (Stolz *et al*., 2011). In maize, ZmHK1 responded stronger to iP whereas ZmHK2 was more sensitive towards *trans*-zeatin. Both analyzed maize receptors showed activity after *cis*-zeatin application (Yonekura-Sakakibara *et al*., 2004). OsHK6 from rice preferred iP, whereas OsHK4 had a higher affinity towards *trans*-zeatin (Choi *et al*., 2012).

The identification of residues involved in cytokinin binding and the structural resolution of the CHASE domain can facilitate the design of improved synthetic cytokinins or cytokinin inhibitors. For this, different experiments were performed. To identify conserved residues responsible for cytokinin binding, an evolutionary proteomics approach was conducted. By bioinformatic comparison, the conserved residues were identified and then proven to be important for receptor activation in a binding assay (Heyl *et al*., 2007).

In 2011, the crystal structure of the CHASE domain of CRE1/AHK4 was published. It consists of a long stalk helix and two PAS domains, that are connected by a helical linker. The last β-strand of the membrane proximal PAS domain is linked to the stalk helix by a disulphide bridge. The central β-sheets of the two PAS domains and two more β-strands form a binding pocket for cytokinin. The structure showed similarity to bacterial histidine kinase sensor domains (Hothorn *et al*., 2011).

Surprisingly, in rice, one of the potential receptors identified contained a CHASE domain coupled to a serine/threonine kinase domain instead of a histidine kinase domain and was named CHARK/OsCRL4 (CHASE domain Receptor-like serine/threonine Kinase) (Han *et al*., 2004; Ito and Kurata, 2006; Pareek *et al*., 2006). *CHARK/OsCRL4* expression complemented the *cre1* mutant but the mechanism still needs to be elucidated (Han *et al*., 2004).

The double mutant *ahk2 ahk3* showed a semi-dwarfed phenotype (Nishimura *et al*., 2004). The leaf cell number was decreased as well as the chlorophyll content but the root system was increased (Riefler *et al*., 2006). Single *knockouts* or double *knockout* combinations other than *ahk2 ahk3* exhibited no obvious phenotype (Higuchi *et al*., 2004; Nishimura *et al*., 2004).

To examine the importance of cytokinin sensing for the plant, triple receptor *loss-of-function* mutants were generated. Triple receptor *knockouts* exhibited reduced cytokinin sensitivity and a smaller and less active SAM. The plants lacked induction of cytokinin-inducible response regulators (Higuchi *et al*., 2004; Nishimura *et al*., 2004). In triple receptor *knockouts* the germination time was shortened, a lower sensitivity towards far red light was observed and plants required less light. The seeds were twice the size of WT seeds (Riefler *et al*., 2006). AHKs influence the sensitivity to red light. The modulation of the PHYTOCHROME B (*PHYB)* activity state by cytokinin is absent in the receptor triple *knockout* plant (Mira-Rodado *et al*., 2007). Although the receptor triple *knockout* was impaired in growth and did not grow without sugar supplement as reported by Skylar (Skylar *et al*., 2010), it was still able to germinate, grow and set seeds (Higuchi *et al*., 2004). Obviously for the basic morphology and development, the receptors are not essential.

Although the receptors act quite redundantly, researchers were able to assign specific functions to the receptors. Analysis of *loss-of-function* mutants related their functions to germination in different light regimes. Double mutants showed an increased germination rate in dark, white light, red light and far red light compared to WT (Riefler *et al*., 2006). AHK3 is involved in senescence. In the *ore12-1*, an *AHK3 gain-of-function* mutant, the senescence was delayed and the regulation occurred via ARR2 (Kim *et al*., 2006). AHK4 mediated cytokinin-induced programmed cell death. Additionally the receptors regulated the steady state level of endogenous cytokinin. AHK2 and AHK3 regulated the level of *cis*-zeatin, all receptors are involved in regulation of *trans*-zeatin and iP (Riefler *et al*., 2006; Vescovi *et al*., 2012). AHK2 and AHK3 seem to play a role in secondary growth. The *loss-of-function* single mutants showed less layers of procambium. The defects in cambium proliferation were more pronounced in the *ahk2 ahk3* double mutant (Hejatko *et al*., 2009).

The receptors play a pronounced role in symbiotic interaction. RNAi-induced silencing of *Medicago CRE1* resulted in lower nodule induction by *Sinorhizobium meliloti*. The regulation of *NODULE INCEPTION* (*NIN)* by cytokinin seems to be dependent on CRE1 (Gonzalez-Rizzo *et al*., 2006). In *Lotus*, the *HK1 loss-of-function* mutant *hit1* (*Hyperinfected1*) showed an increase in infection threads and exhibited no *NIN* activation. *hit1* failed in cortical cell divisions during

nodulation (Murray *et al*., 2007). A *gain-of-function* mutant of *HK1* on the other hand showed spontaneous nodulation (Tirichine *et al*., 2007).

The receptors were also found to mediate abiotic and biotic stress responses. Mason and colleagues showed that a *knockout* of *AHK3* and *AHK4* led to a lower sodium accumulation in leaves (Mason *et al*., 2010). *AHK3* and *CRE1/AHK4* seem to play a role during phosphate starvation. *CRE1/AHK4* expression was downregulated by phosphate starvation (Franco-Zorrilla *et al*., 2002) and in the *ahk3* mutant, the cytokinin-induced repression of phosphate-starvation responsive genes was lowered and the sucrose sensitivity elevated. Additional mutation of *CRE1/AHK4* enhanced the phenotype (Franco-Zorrilla *et al*., 2005). The cytokinin-induced downregulation of sulfur transporters was dependent on CRE1/AHK4 (Maruyama-Nakashita *et al*., 2004a). Also iron transporters were repressed by cytokinin application dependent on AHK3 and CRE1/AHK4 (Seguela *et al*., 2008). In *Arabidopsis*, *ARR* expression was induced by cold temperatures. For this, AHK2 and AHK3 are required (Jeon *et al*., 2010). Loss of those two receptors increased also the drought and salt tolerance of plants (Tran *et al*., 2007; Kang *et al*., 2012). In *Medicago*, *HK1*, *HK2* and *CRE1/AHK4* expression was induced by salt stress (Merchan *et al*., 2007; Coba de la Pena *et al*., 2008). The cytokinin receptors act negatively on ABA signaling (Tran *et al*., 2007; Jeon *et al*., 2010; Mason *et al*., 2010). In biotic stress, specific combinations of receptors were involved. Symptoms of *Rhodococcus fascians* infection are dependent on AHK3 and CRE1/AHK4 (Pertry *et al*., 2009) whereas AHK2 and CRE1/AHK4 are needed for growth stimulation by *Piriformospora indica* infection (Vadassery *et al*., 2008).

For CKI1 the connection to cytokinin remains unclear. *CKI1* loss was lethal for *Arabidopsis thaliana*. It resulted in the inability to undergo a normal megagametogenesis and the female gametophyte was degraded. The mutation was not transmitted through the female germ line (Pischke *et al*., 2002; Hejatko *et al*., 2003). Deng and colleagues also observed the low transmittance of the *cki1 loss-of-function* mutation but could rescue plants by overexpression of the response regulator *ARR1* or an *IPT* under the control of the *CKI1* promoter (Deng *et al*., 2010). The *loss-of-function* mutant of *CKI1* exhibited defects in cambial cell maintenance and had a dysfunctional two-component system. Overexpression resulted in ectopic response of the two-component system (Kakimoto, 1996; Hejatko *et al*., 2009). Loss of the *CKI1* function pronounced the vascular defects in the *ahk2 ahk3* mutant, overexpression complemented the *ahk2 ahk3* vascular phenotype partially (Hejatko *et al*., 2009). The function of CKI1 in cytokinin signaling has to be elucidated, e.g. whether it is able to react to cytokinin without a cytokinin binding CHASE domain.

Hwang and colleagues discussed whether the *Arabidopsis* triple receptor *loss-of-function* mutant survived because of compensation through CKI1 or because some of the alleles in the triple receptor are no true *knockouts* (Hwang *et al*., 2012). Hints for *knockdowns* instead of *knockouts* in the analysis were found by Kinoshita-Tsujimura and colleagues (Kinoshita-Tsujimura and Kakimoto, 2011). Not all combinations of alleles were able to set seeds after flowering and embryo sacs were absent or the megagametophyte was not completely developed. Defects in anther dehiscence, pollen maturation and germination induction through the stigma and female gametophyte formation and maturation occurred (Kinoshita-Tsujimura and Kakimoto, 2011).

1.1.5.2. The histidine phosphotransfer proteins

The transfer of the phosphate from the receptors to the response regulators in plants is carried out by HISTIDINE PHOSPHOTRANSFER PROTEINs (HPTs) (D'Agostino and Kieber, 1999). They have been identified by HPT domain search (Miyata *et al*., 1998; Suzuki *et al*., 1998). In *Arabidopis*, there are five AHPs present with a conserved phospho-accepting histidine residue (Suzuki *et al*., 1998; reviewed by Heyl and Schmülling, 2003; Hutchison *et al*., 2006; Hutchison and Kieber, 2007)(Figure 1.5).

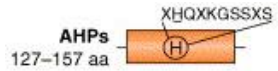


Figure 1.5: Model of a histidine phosphotransfer protein. The phospho-accepting histidine residue is marked (Heyl and Schmülling, 2003).

HPTs have first been described as cytosolic proteins that translocate to the nucleus after cytokinin treatment (Hwang and Sheen, 2001; Yamada *et al*., 2004) but Punwani and colleagues observed, that AHPs shuttle between cytoplasm and nucleus independent from cytokinin (Punwani *et al*., 2010).

AHP genes are expressed tissue specifically. *AHP1* is mainly expressed in roots, *AHP2* in flowers, *AHP3* in roots and leaves (Suzuki *et al*., 1998; Hradilova *et al*., 2007). *AHP4* is expressed at a low level (Suzuki *et al*., 2000). Its expression is downregulated by osmotic and salt stress. AHP4 was discussed to be a negative regulator of cytokinin signaling (Hutchison *et al*., 2006). The *AHP4* gene turned out to be expressed in inflorescences and *loss*- and *gain-of-function* analyses revealed that it is a negative regulator in secondary wall thickening of the endothecium (Jung *et al*., 2008). *AHP5* is expressed ubiquitously (Hradilova *et al*., 2007). For *AHP4* and *AHP5* different splicing variants were observed (Tanaka *et al*., 2004; Hradilova *et al*., 2007).

Microarrays revealed that the expression of *AHPs* is not regulated by cytokinin (Rashotte *et al*., 2003; Brenner *et al*., 2005), except for *AHP5. AHP5* was found to be cytokinin-regulated in an experiment overexpressing an *IPT* gene (Hoth *et al*., 2003).

The interaction between AHPs, AHKs and ARRs was shown by Dortay and colleagues and interaction- and phosphorylation studies were carried out also by Suzuki and colleges and Tanaka and colleagues with the ARRs (Suzuki *et al*., 1998; Suzuki *et al*., 2001a; Tanaka *et al*., 2004; Dortay, 2006). AHPs also interacted with CKI1 (Deng *et al*., 2010) and in a yeast two-hybrid screen, an interaction between AHP2 and TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR 10 (TCP10) was observed (Suzuki *et al*., 2001a).

Mutants overexpressing *AHP2* were hypersensitive towards cytokinin (Suzuki *et al*., 2002). Hutchinson and colleagues observed no obvious phenotype for *AHP loss-of-function* single mutants, but multiple mutants exhibited a reduced sensitivity towards cytokinin. Multiple *knockout* mutants were also less fertile, showed a reduced vascular development and a shorter primary root (Hutchison *et al*., 2006). Deng and colleagues observed defects in megagametogenesis in the quintuple *AHP loss-of-function* mutant similar to the *cki1* mutant phenotype. They proposed that the AHPs act downstream of CKI1 (Deng *et al*., 2010). A loss of *AHP2*, *AHP3* and *AHP4* functions led to decreased sodium levels in shoots (Mason *et al*., 2010).

AHP6 does not contain the HPT-typical histidine residue. It lacks the ability to transfer the phosphate to the response regulators and was therefore classified as pseudo AHP. AHP6 acts as negative regulator of cytokinin signaling (Suzuki *et al*., 2000; Mähönen *et al*., 2006). It is expressed in developing protoxylem, the pericycle cells, in the shoot apex and young leafs (Tanaka *et al*., 2004; Mähönen *et al*., 2006). AHP6 is crucial for protoxylem formation. Cytokinin negatively regulated the spatial expression pattern of *AHP6* whereas AHP6 negatively regulates cytokinin signaling (Mähönen *et al*., 2006). A *loss-of-function* of *AHP6* in the *ckx3 ckx5* background caused larger and more active reproductive meristems due to a delay in SAM differentiation (Bartrina *et al*., 2011).

In 2005 the crystal structure of ZmHP2 was published. Conserved residues for interaction with receiver domains were identified (Sugawara *et al*., 2005). *AHPs* have also been analyzed in rice, maize and *Catharanthus* (Asakura *et al*., 2003; Papon *et al*., 2004; Ma and Tian, 2005). For an overview see Hellmann et al. (2010).

1.1.5.3. The response regulators

In *Arabidopsis thaliana* 23 response regulators were identified. According to their structure and the ability to be induced by cytokinin they were divided into three groups, type-A, type-B and type-C response regulators (Imamura *et al*., 1999; Kiba *et al*., 1999). The type-B response regulators, ARR1, ARR2, ARR10, ARR11, ARR12, ARR13, ARR14, ARR18, ARR19, ARR20 and ARR21, are *trans*-acting factors mediating the cytokinin response (Sakai *et al*., 2000; Hwang and Sheen, 2001; Sakai *et al*., 2001; Taniguchi *et al*., 2007). The type-A response regulators, *ARR3*, *ARR4*, *ARR5*, *ARR6*, *ARR7*, *ARR8*, *ARR9*, *ARR15*, *ARR16*, and *ARR17*, are primary cytokinin response genes as they are induced by cytokinin treatment (Brandstatter and Kieber, 1998; Sakakibara *et al*., 1998; D'Agostino *et al*., 2000; Rashotte *et al*., 2003; Brenner *et al*., 2005). The type-C response regulator family in *Arabidopsis* consists of *ARR22* and *ARR24* which share structural similarity to type-A response regulators but lack the upregulation upon cytokinin treatment (Kiba *et al*., 2004; Gattolin *et al*., 2006; Horák *et al*., 2008). All classes of response regulators have an N-terminal receiver domain to accept the phosphate from the HPTs. This is in contrast to pseudo response regulators that lack the conserved aspartate in the HPT domain. They cannot be directly activated via the cytokinin signaling system (Makino *et al*., 2000), although some of them seem to be regulated by cytokinin in their expression (Brenner *et al*., 2005). Pseudo response regulators are involved in the regulation of the circadian clock. A characterization of those in *Brassica* was published by Kim and colleagues (Kim *et al*., 2010; Kim *et al*., 2012a).

1.1.5.3.1. The type-B response regulators

Type-B response regulators are classified in three subgroups based on their phylogenetic relationship. The first subgroup contains the ubiquitously expressed *ARR1*, *ARR2*, *ARR10*, *ARR12*, *ARR11*, *ARR14* and *ARR18*. The two smaller groups are specifically expressed in reproductive organs and consist of *ARR13* and *ARR21* and of *ARR19* and *ARR20* (Mason *et al*., 2004; Tajima *et al*., 2004). All type-B response regulators have the N-terminal autoinhibitory receiver domain and a DNA-binding myb-like domain, the GARP (*Golden 2*, *ARR* and *Psr1*) domain (Lohrmann *et al*., 1999; Sakai *et al*., 2000; Hosoda *et al*., 2002) (Figure 1.6).

The first type-B response regulators to be discovered were ARR1 (Sakai et al., 1998), ARR2/ARABIDOPSIS RESONSE REGULATOR-LIKE PROTEIN 5 (ARR2/ARP5) (Sakai et al., 1998; Buchholz et al., 1998) and ARR11/ARP3 (Buchholz et al., 1998).

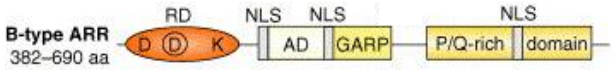


Figure 1.6: Model of a type-B response regulator. Type-B response regulators have a receiver domain (RD) with the DDK motif necessary for the phosphotransfer. Nuclear localization signals (NLS) lead those transcription factors to the nucleus. N-terminal of the DNA-binding GARP domain, there is an activation domain (AD) located. The C-terminus often consists of P/Q-rich domains (Heyl and Schmülling, 2003).

The aspartate residue in the receiver domain is important for the phosphorelay and function of the protein. It was shown that a non-phosphorylable mutated version of ARR2 cannot complement the *arr2 loss-of-function* mutant although phosphorylation is not required for DNA-binding (Hass *et al*., 2004).

The N-terminus seems to act autoinhibitory on the protein function. DNA binding of ARR1 and ARR2 was increased by removal of their N-terminus (Sakai *et al*., 2001). Removal of the first 45 basepairs of ARR18 created a constitutive active form of the protein (Liang *et al*., 2012) and plants overexpressing ARR14, ARR20 or ARR21 without N-terminus exhibited a growth phenotype in contrast to plants overexpressing the full length protein (Tajima *et al*., 2004). The C-terminus can be variable and often contains P/Q-rich acidic domains that seem to be important for the transactivation capacity (Imamura *et al*., 1998; Sakai *et al*., 2000; Hosoda *et al*., 2002; Imamura *et al*., 2003).

When the C-terminus of ARR1 or ARR2 was fused to the yeast GAL4, it was able to transactivate the GAL4 promoter (Sakai *et al*., 2000; Lohrmann *et al*., 2001).

ARR1 and ARR2 lacking the receiver domain bound to 5'(G/A)GAT(T/C)3' in electromobility shift assays. The motif was identified in a random oligonucleotide selection and amplification experiment (Sakai *et al*., 2000). The ARR10 DNA binding domain bound to '5-(A/T)GATT-3' in a DNA-binding gel-shift assay. The motif was identified by random oligonucleotide amplification and incubation with ARR10 (Hosoda *et al*., 2002). A truncated ARR11 protein containing the receiver domain and the GARP domain bound preferentially to '5-GGATT-3' in DNA-binding gel shift assays. Mutations decreased DNA binding (Imamura *et al*., 2003). Taniguchi extended the binding motif to 5'-AAGAT(T/C)TTT-3' by bioinformatics comparing gene expression after induced overexpression of ARR1 uninduced plants and to a *arr1 loss-of-function* mutant. ARR1 lacking the receiver domain was shown to bind to the motif by gel mobility shift analysis (Taniguchi *et al*., 2007).

NMR structure resolution revealed a transcription factor-typical helix-turn-helix shape of type-B response regulators (Hosoda *et al*., 2002).

Type-B response regulators localized to the nucleus as expected for transcription factors (Sakai *et al*., 2000; Hwang and Sheen, 2001; Lohrmann *et al*., 2001; Mason *et al*., 2004; Dortay, 2006).

Loss-of-function mutants of single genes caused no or minor phenotypic changes (Sakai *et al*., 2001; Horák *et al*., 2003; Mason *et al*., 2005). For example *arr2* showed a slight insensitivity towards cytokinin and ethylene in hypocotyl elongation (Hass *et al*., 2004). *arr1* had longer roots and *arr21* showed no alterations compared to WT (Sakai *et al*., 2001; Horák *et al*., 2003). The *arr1 arr12* double mutant accumulated less sodium in leaves compared to the WT and the expression of sodium transporter genes was elevated (Mason *et al*., 2010). ARR2 was found to regulate senescence downstream of AHK3. A *loss-of-function* mutant of *ARR2* was less sensitive towards cytokinin in the cytokinin-dependent delay of senescence. The regulation of senescence via ARR2 seems to depend on phosphorylation as overexpression of *ARR2* delayed senescence, but overexpression of a non-phosphorylable version did not (Kim *et al*., 2006). Overexpression of *ARR2* was sufficient to induce cytokinin-dependent SAM proliferation (Hwang and Sheen, 2001). In contrast to the degradation of ARR1, ARR10, ARR12 and ARR18 the proteolysis of ARR2 was cytokinin-regulated dependent on the K90 residue. A K90G *gain-of-function* mutation inhibited the cytokinin-induced degradation. It increased type-A response regulator expression and cytokinin sensitivity (Kim *et al*., 2012b).

Although redundancy in the system was expected to be very high (Mason *et al*., 2005), multiple mutant analysis revealed that ARR1, ARR10 and ARR12 mediate the majority of cytokinin signaling (Yokoyama *et al*., 2007; Argyros *et al*., 2008; Ishida *et al*., 2008b). The *arr10 arr12* double mutant was less sensitive towards cytokinin. The *arr1 arr10 arr12* mutant showed a cytokinin deficiency syndrome as it resembled *wol* and was stunted (Yokoyama *et al*., 2007). The vascular development as well as the light sensitivity, chlorophyll production and cell division in root and shoot were strongly impaired. Plants exhibited exclusively protoxylem instead of metaxylem and had smaller meristems. They flowered later, had a reduced fertility and an increased seed size (Yokoyama *et al*., 2007; Argyros *et al*., 2008; Ishida *et al*., 2008b). The triple mutant was almost completely insensitive towards cytokinin (Mason *et al*., 2005).

Overexpression of type-B response regulators lacking the N-terminal receiver domain led to constitutive active versions causing phenotypic changes. The plant overexpressing constitutively a non-phosphorylable *ARR2* version showed strong phenotypic growth alterations and nearly half of the progeny died (Hass *et al*., 2004). A truncated overexpressed version of *ARR11* lacking the receiver domain caused plants exhibiting atypical growth of cotyledons, carpels and siliques, being hypersensitive towards cytokinin in callus formation and showing a lower apical

dominance (Imamura *et al*., 2003). Overexpression of a truncated version of *ARR20* caused deformed siliques with less seeds. Overexpression of a constitutive active *ARR21* changed the whole morphology. The plant was disordered ending up in callus-like structures as cotyledons or bleached seedlings (Tajima *et al*., 2004). When a truncated version of *ARR14* was overexpressed, the plants showed a reduced apical dominance, leaf-like structures that arose from the cotyledon junction, possibly derived from adventious shoots, and bushy rosette leafs (Tajima *et al*., 2004).

When ARR1 was fused to a dominant SRDX repressor, the plants showed the cytokinin deficiency syndrome. They had smaller leaves, an enhanced root system, reduced cytokinin sensitivity and were resistant towards *PhyB* mediated germination inhibition by far red light (Heyl *et al*., 2008).

Type-B response regulators are involved in auxin signaling. ARR1 activated the expression of *SUPPRESSOR OF HY5/ INDOLE-3-ACETIC ACID PROTEIN 3 (SHY2/IAA3)*, a repressor of auxin response and negative regulator of PIN-FORMED (PIN) distribution. Auxin induced SHY2/IAA3 degradation (Dello Ioio *et al*., 2008).

SHY2/IAA3 can also be transactivated by ARR12. Another point of hormonal crosstalk is the repression of *ARR1* by gibberellic acid via REPRESSOR OF GA1-3 (RGA) (Moubayidin *et al*., 2010).

1.1.5.3.2. The type-A response regulators

Since their discovery several groups identified target genes for the type-B response regulators (reviewed by Schmülling *et al*., 1997; Che *et al*., 2002; Schäfer and Schmülling, 2002; Hoth *et al*., 2003; Rashotte *et al*., 2003; Brenner *et al*., 2005; Kiba *et al*., 2005). One group of cytokinin primary response genes are the type-A response regulators (Brandstatter and Kieber, 1998; Sakakibara *et al*., 1998; Urao *et al*., 1998; Imamura *et al*., 1999). They have been identified in *Arabidopsis* by homology search using bacterial response regulators as template (Imamura *et al*., 1998; Urao *et al*., 1998).

The first type-A response regulator genes to be identified were *ARR3* (Imamura *et al*., 1998), *ARR4/ATRR1/IBC6* (Brandstatter and Kieber, 1998; Imamura *et al*., 1998; Urao *et al*., 1998), *ARR5/ATRR2/IBC7* (Brandstatter and Kieber, 1998; Imamura *et al*., 1998; Urao *et al*., 1998), *ARR6* (Imamura *et al*., 1998), *ARR7* (Imamura *et al*., 1998), *ARR8/ATRR3* and *ARR9/ATRR4* (Urao *et al*., 1998) and the maize response regulator *ZmCip1* (Sakakibara *et al*., 1998). A scheme of type-A response regulators is depicted in Figure 1.7.

Figure 1.7: Model of a type-A response regulator. Type-A response regulators contain receiver domains (RD) with the DDK motif necessary for phosphorylation (Heyl and Schmülling, 2003).

Type-A response regulators are cytokinin primary response genes and their expression is increased upon a cytokinin stimulus. They had different response kinetics (Imamura *et al*., 1999; Kiba *et al*., 1999; D'Agostino *et al*., 2000). The level of *ARR4* and *ARR5* expression increased within minutes (D'Agostino *et al*., 2000), whereas for the maize type-A response regulator *ZmCIP1* the transcript level peaked 30 minutes after treatment (Sakakibara *et al*., 1998). *ARR3-ARR7* were not only induced by cytokinin but also by nitrate resupply (Sakai *et al*., 1998; Taniguchi *et al*., 1998) similar to *ZmCip1* in maize (Sakakibara *et al*., 1998).

Type-A response regulators regulate the cytokinin signaling negatively depending on their ability to be phosphorylated as it was shown by mutation of the phosphor-accepting site (Hwang and Sheen, 2001; To *et al*., 2007; Lee *et al*., 2008). This was observed by overexpression of *ARR7*. Overexpression of *ARR7* caused a repression of nearly all type-A response regulators whereas the overexpression of a non-phosphorylable version caused no alterations (Lee *et al*., 2008). Surprisingly some type-A response regulator acted antagonistically towards each other as To and colleagues described in their publication (Osakabe *et al*., 2002; To *et al*., 2004; To *et al*., 2007). Type-A response regulator genes were not just regulated transcriptionally but also via the stability of the corresponding proteins (To *et al*., 2007; Ren *et al*., 2009).

A subset of type-A response regulators, ARR5, ARR6 and ARR7, was stabilized by cytokinin (To *et al*., 2007) as ARR16 and ARR17 as well as possibly ARR8 and ARR15 (Ren *et al*., 2009).

To elucidate functions of type-A response regulators mutant analysis was performed. Single *knockouts* of type-A response regulators did not show obvious alterations to WT plants but a multiple *knockout* displayed increased cytokinin sensitivity (To *et al*., 2004). Despite high redundancy, some specific functions could be linked to single ARRs by mutant analysis. *ARR4* connected light signaling with cytokinin signaling. It interacted with the active form of PHYTOCHROME B (PHYB) and increased the red light sensitivity by stabilizing the phytochrome (Sweere *et al*., 2001). The corresponding *arr4 loss-of-function* mutant was hypersensitive towards red light treatment (Sweere *et al*., 2001; To *et al*., 2004; Mira-Rodado *et al*., 2007). Type-A response regulators seem to play also a role in the circadian clock (Hanano *et al*., 2006;

Salome *et al*., 2006; Zheng *et al*., 2006). *ARR9* expression was regulated by the circadian clock (Ishida *et al*., 2008a). *ARR4* and *ARR3* l*oss-of-function* mutants displayed a prolonged circadian period without light. In the presence of light, the double mutant looked similar to *phyB* mutants and exhibited a different leading phase (Salome *et al*., 2006). The cytokinin-dependent delay of the circadian phase was also observed by Hanano and colleagues (Hanano *et al*., 2006). The alteration of the circadian clock phase by *ARR4* and *PhyB* also involved the main transcriptional regulators in this process, LATE ELONGATED HYPOCOTYL 7 (LHY7) and CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) (Zheng *et al*., 2006).

Type-A ARRs are involved in the regulation of the meristem maintenance. In the current model *LOG4* generates cytokinin signaling in the epidermis. This is important to regulate *WUS* patterning and maintain the SAM stem cell niche (Chickarmane *et al*., 2012). ARR7 and ARR15 were found to be important for the cytokinin-dependent regulation of *WUSCHEL (WUS)* and *CLAVATA (CLV)* (Buechel *et al*., 2009). *WUS* can be regulated *CLV*-dependent and independent from *CLV* (Gordon *et al*., 2009). WUS itself represses *ARR5*, *ARR6*, *ARR7* and *ARR15* transcription. A constitutive active phosphomimic mutant of *ARR7* exhibited an aberrant SAM. A *loss-of-function* of an ARR homolog caused a larger SAM in maize (Lee *et al*., 2009). *ARR15* expression was selectively impaired in *cre1* mutants. Overexpression caused downregulation of other type-A response regulators (Kiba *et al*., 2002; Kiba *et al*., 2004). *ARR7* and *ARR15* can also be repressed by auxin via AUXIN RESPONSE FACTOR/MONOPTEROS (ARF/MP) (Zhao *et al*., 2010). *ARR7* and *ARR15* were essential to integrate auxin signals into the process of embryo development. Auxin induced *ARR7* and *ARR15* expression. The corresponding proteins repress cytokinin signaling. Cytokinin signaling was important in the hypophysis which gives rise to the root stem cell founder cell. In the precursors of the quiescent centre cytokinin signaling needed to be repressed by *ARR7* and *ARR15* expression to enable proper development (Müller and Sheen, 2008).

In *Arabidopsis*, it was shown that type-A ARRs were connected to other phytohormones as well. *ARR7* and *ARR15* expression was also regulated by ethylene signaling. ETHYLENE INSENSITIVE 3 (EIN3) directly binds to their promoters. The freezing tolerance of *EIN3* overexpressing plants was elevated by additional overexpression of *ARR7* and *ARR15* (Shi *et al*., 2012b).

Type-A response regulators were also found to be involved in pathogen response. They seem to be negative regulators of basal and pathogen-induced salicylic acid-dependent gene expression. Also here type-A response regulators had contrary roles as observed in different multiple *knockout* combinations (Argueso *et al*., 2012).

ARR7 seemed to have a function in temperature stress. The *ARR7* overexpressing plant was more sensitive and the *loss-of-function* mutant *arr7 was* more resistant towards cold temperature (Jeon *et al*., 2010).

Analysis of type-A response regulators have been undertaken in other species than *Arabidopsis* as well. *OsRR1* was shown to play a role in crown root initiation in rice. Auxin induced the *CROWNROOTLESS 5* (*CRL5*) gene and the CRL5-protein itself induced *OsRR1* transcription. OsRR1 acted negatively on cytokinin signaling and enabled crown root initiation (Kitomi *et al*., 2011).

A *loss-of-function* mutation of *ABERRANT PHYLLOTAXY 1* (*ABPHYL1)*, a type-A response regulator, was discovered in maize. The leaves of the mutant were arranged in a decussate pattern instead of an alternate one. *ABPHYL1* expression was induced by cytokinin in the SAM and regulated cytokinin signaling negatively (Giulini *et al*., 2004). Auxin reduced the expression. In the *loss-of-function* mutant reduced auxin and *PIN1* expression levels occurred and leaf initiation was delayed leading to an enlarged SAM and altered leaf pattern (Lee *et al*., 2009).

To identify more functions of type-A response regulators Ren and colleagues performed a transcriptome analysis of *Arabidopsis* plants overexpressing those (Lee *et al*., 2007; Ren *et al*., 2009) and the same was done for *OsRR6* in rice (Hirose *et al*., 2007).

Analyzes of type-A response regulators have been performed in species other than *Arabidopsis*, e.g. in maize (Asakura *et al*., 2003; Muniz *et al*., 2010), *Pinus* (Cortizo *et al*., 2010), poplar (Ramirez-Carvajal *et al*., 2008) and rice (Jain *et al*., 2006b).

1.1.5.3.3. The type-C response regulators

The type-C response regulator family consists of two members in *Arabidopsis,* ARR22 and ARR24, and has also been analyzed. *ARR22* expression was restricted to reproductive organs. Transcript was observed in the junction between funiculus and chalazal tissue (Kiba *et al*., 2004; Gattolin *et al*., 2006). ARR22 localized to the nucleus and was phosphorylated by AHP5. Overexpression caused a dwarfed plant with an underdeveloped root system (Kiba *et al*., 2004; Gattolin *et al*., 2006). A *loss-of-function* mutant exhibited no phenotype but expression of an additional gene copy caused a phenotype similar to plants with a cytokinin deficiency syndrome. This phenotype was not observed when the phospho-accepting aspartate was mutated (Horák *et al*., 2008). *ARR24* was expressed in floral tissue and siliques. Neither single *loss-of-function*

mutants nor the double *knockout* mutant of *ARR22* and *ARR24* showed any phenotype (Gattolin *et al*., 2006).

1.1.6. Cytokinin and transcription beyond the two-component system

Since 2001, several microarray experiments have been conducted to identify cytokinin-regulated genes additional to the type-A response regulators. The analysis was performed using wild type *Arabidopsis* plants in response to different types of cytokinin (Rashotte *et al*., 2003) or separated roots and shoots to detect differences in regulation (Brenner and Schmülling, 2012). Furthermore various mutants of the cytokinin two-component system were examined. This included *loss-of-function* mutants of the receptor *AHK4* (Rashotte *et al*., 2003) and the type-B response regulators *ARR10* and *ARR12* or *ARR1*, *ARR10* and *ARR12* (Yokoyama *et al*., 2007; Argyros *et al*., 2008) as well as *gain-of-function* mutants of *ARR1* (Sakai *et al*., 2001), *ARR7* (Lee *et al*., 2007), *ARR21* and *ARR22* (Kiba *et al*., 2004) and *CKX1* (Brenner *et al*., 2005). Additionally plants expressing a dominant negative version of *ARR1* were examined (Heyl *et al*., 2008). In 2012, Brenner and colleagues compiled all available microarray data to identify genes stably elevated in their expression upon cytokinin treatment (Brenner *et al*., 2012). Type-A response regulators as well as *CKX4*, *CKX5*, *UGT76C2*, *CYP735A2*, *AHK4*, *AHK1*, *CYTOKININ RESPONSE FACTOR 2* (*CRF2*), *CRF5*, *ASSYMETRIC LEAVES 2-LIKE 9* (*ASL9*) (Naito *et al*., 2007b) and *FABULOUS FOUR 3* (*FAF3*) were found to be repeatedly upregulated in their transcription by cytokinin (Brenner *et al*., 2012). Also auxin-related genes exhibited cytokinin-dependent increased transcription as some *SMALL AUXIN-UPREGULATED* (*SAUR*) genes and the AUX/IAA genes *SHY2/IAA3* and *AUXIN-RESISTANT 3/IAA17* (*AXR3/IAA17*) (Brenner *et al*., 2012).

At least two microarray experiments were followed by further analyses of genes identified in this approaches. Rashotte and colleagues found a portion of *ETHYLENE RESPONSIVE ELEMENT-BINDING FACTOR*s (*ERF*s) to show transcriptional upregulation by cytokinin (Rashotte *et al*., 2003), named them *CYTOKININ RESPONSE FACTORS* (*CRFs*) accordingly and subjected them to further analysis (Rashotte *et al*., 2006; Rashotte and Goertzen, 2010; Cutcliffe *et al*., 2011). In 2011 Köllmer and colleagues published the examination of transcription factors showing upregulation of their transcripts by cytokinin in the work of Brenner and colleagues from 2005 (Brenner *et al*., 2005; Köllmer *et al*., 2011).

CRFs represent a monophyletic clade among the ERF transcription factors, possessing an AP2 domain for DNA-binding. The cytokinin-induction of the *CRF* transcription was dependent on the

presence of type-B response regulators (Rashotte *et al*., 2006). The ability to be induced by cytokinin correlated with the presence of an N-terminal CRF domain (Rashotte and Goertzen, 2010). CRFs were shown to be nuclear localized after cytokinin addition (Rashotte *et al*., 2006; Shi *et al*., 2012a). They formed homo- and heterodimers (Cutcliffe *et al*., 2011; Shi *et al*., 2012a) and were shown to interact with the HPTs of the cytokinin signaling system (Cutcliffe *et al*., 2011). Analysis of *loss-of-function* mutants revealed a function for CRFs in the development of cotyledons, the leaves and the embryo (Rashotte *et al*., 2006). As the type-A response regulator induction by cytokinin was strongly decreased in a multiple *CRF loss-of-function* mutants, CRFs are thought to act in concert with the type-B response regulators to mediate cytokinin response, possibly by competing for the phosphate transfer from the AHPs (Rashotte *et al*., 2006).

Köllmer and colleagues focused on a set of cytokinin-regulated transcription factors identified before (Brenner *et al*., 2005; Köllmer *et al*., 2011). *GATA-BINDING FACTOR 22* (*GATA22*), B*ASIC HELIX-LOOP-HELIX TRANSCRIPTION FACTOR 64* (*bHLH64*) and the homeodomain leucine-zipper protein *HAT22* were upregulated in their transcription two-fold by cytokinin whereas *HAT4* transcription was upregulated 13-fold after two hours of cytokinin treatment (Brenner *et al*., 2005; Köllmer *et al*., 2011). Also in other analyses *GATA22* transcription was found to be upregulated by cytokinin and therefore it was named *CYTOKININ RESPONSIVE GATAFACTOR 1* (*CGA1*) (Kiba *et al*., 2005; Naito *et al*., 2007a). Analyses of *loss-of-function* and *gain-of-function* mutants revealed putative functions for those transcription factors downstream of cytokinin signaling. GATA22 and bHLH64 possibly connect cytokinin response with light signaling and gibberellin action concerning hypocotyl elongation and chloroplast development. HAT4 seems to have a function in root architecture whereas HAT22 seems to be involved in regulation of senescence and chlorophyll content (Köllmer *et al*., 2011).

Other factors playing a role in cytokinin action are members of the GLABRA 1 ENHANCER BINDING PROTEIN (GeBP) family (Chevalier *et al*., 2008), leucine-zipper transcription factors. GeBPs form dimers via the leucine-zipper motiv. A triple *loss-of-function* mutant exhibited a lower sensitivity towards cytokinin in senescence and growth but not in cytokinin-mediated root growth inhibition. The transcriptional response of type-A response regulators was elevated in the mutant. It was proposed, that type-A response regulators are repressed by an unknown modulator and GeBPs interfere with cytokinin response by activation of this repressor (Chevalier *et al*., 2008). GeBP proteins were found to be epistatic to CONSTITUTIVE EXPRESSOR OF PATHOGENESIS-RELATED GENES 5 (CPR5) and regulated cell expansion CPR5-dependent (Perazza *et al*., 2011). They were regulated by the KNOTTED1-LIKE HOMEOBOX (KNOX)-

transcription factor KNOTTED-LIKE FROM *ARABIDOPSIS THALIANA* 1 (KNAT1) (Curaba *et al*., 2003)

A role in cytokinin response was also reported for the C_2H_2 transcription factors GLABROUS INFLORESCENCE STEMS 2 (GIS2) and ZINC FINGER PROTEIN 8 (ZFP8) (Gan *et al*., 2007). Both were required for cytokinin-dependent regulation of trichome initiation but just *GIS2* transcription was induced by cytokinin. Cytokinin promoted trichome initiation whereas application of gibberellic acid or mutation in SPINDLY (SPY) inhibited this process. *GLABROUS 1* (*GL1*) transcription was also induced by cytokinin but not required for cytokinin action on trichome development (Gan *et al*., 2007). The authors propose a model, in which gibberellic acid and cytokinin in concert positively regulates the transcription of *ZFP8* and *GIS2* and induces trichome development. SPY functioned upstream of this process, inhibited gibberellin-dependent transcriptional activation and promoted cytokinin-dependent transcriptional activation of those genes. *SPY* itself was inhibited by gibberellic acid application. Trichome initiation via gibberellin-dependent transcriptional activation of *GLABROUS INFLORESCENCE STEMS* (*GIS*) and GL1 was independent from cytokinin (Gan *et al*., 2007).

1.2. Transcriptional regulation in general

Development and most of the reactions to changes in the environment, as phenotypic or metabolic alterations, require specific regulation of gene expression (reviewed in Riechmann, 2002; Mitsuda and Ohme-Takagi, 2009). This is realized e.g. by transcription factors (Riechmann, 2002). A transcription factors is defined as a protein containing a DNA-binding domain (Mitsuda and Ohme-Takagi, 2009). Mostly, transcription factors are modular and contain also a regulator domain (Collado-Vides *et al*., 1991). Transcription factors can be activators or repressors or both (Maniatis *et al*., 1987). Activators often have a domain rich in the acidic amino acids glutamine and proline or contain an AHA motif comprised of acidic amino acids interrupted by aromatic and hydrophobic amino acids e.g. heat shock factors (Mitsuda and Ohme-Takagi, 2009). Repressors can be passive, e.g. function by competition for binding partners or DNA binding sites or active, containing an ERF-associated amphiphilic repression (EAR) motif (Mitsuda and Ohme-Takagi, 2009). Addition of an EAR domain, rich in acidic amino acids and leucine, can turn activators into strong repressors (Hiratsu et al., 2003) and was used to create a dominant negative form of ARR1 (Heyl *et al*., 2008). *Arabidopsis thaliana* possesses about 1500-2000 transcription factors, about 6% of its genome (reviewed in Riechmann, 2002; Mitsuda and

Ohme-Takagi, 2009). I will describe the most common transcription factor families, regulating differential responses. In this work I will exclude those functioning in general transcription as the usual polymerase-associated factors. I will also not include other ways of transcriptional regulation as chromatin remodeling or regulatory RNAs. In most cases, reviews will be cited.

1.2.1. AP2/ERF transcription factors

The first APETALA 2/ETHYLENE RESPONSIVE ELEMENT-BINDING FACTOR (AP2/ERF) discovered was the homeotic APETALA 2 (AP2). Consequently the domain unique to AP2/ERFs was named AP2 domain (Jofuku *et al*., 1994). The AP2/ERF transcription factor family contains e.g. the CRFs that are involved in cytokinin signaling (Rashotte *et al*., 2003; Rashotte *et al*., 2006) and the DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEINs (DREBs) binding to the dehydration-responsive element/C-repeat (DRE/CRT) (Sakuma *et al*., 2002). AP2/ERFs were shown to be involved in the response to abiotic and biotic stress, hormones, sugar, and regulation of flower and seed development and to play a role in cell identity and proliferation (Riechmann, 2002; Mizoi *et al*., 2012).

1.2.2. bHLH transcription factors

BASIC HELIX-LOOP-HELIX (bHLH) transcription factors were shown to bind to the E-box DNA motif and often form homo- and heterodimers (Feller *et al*., 2011). They can be activators or repressors. For instance SPATULA (SPT) was found to be a positive regulator of carpel and fruit development and a negative regulator of seed germination (Feller *et al*., 2011). Some bHLH transcription factors interacted with MYB transcription factors e.g. GLABROUS 3 (GL3) with GL1 to determine the trichome cell fate (Payne *et al*., 2000). Interaction with MYB transcription factors could block protein-protein interaction sites of the MYB-factors or stabilize the protein and prevent ubiquitination (Feller *et al*., 2011). The bHLH transcription factors were shown to be involved e.g. in phytochrome-mediated responses, abiotic stress response (Riechmann, 2002).

1.2.3. MYB transcription factors

MYB transcription factors were named after the *v-MYB* domain of the avian myeloblastosis virus and have their cellular counterpart in *c-MYB* (Peters *et al*., 1987; Mucenski *et al*., 1991). The

DNA-binding domain was shown to mostly consist of 1-3 imperfect MYB repeats (Rosinski and Atchley, 1998). MYB transcription factors represent a large family in *Arabidopsis* making up about 10 % of all transcription factors (Riechmann *et al*., 2000; Dubos *et al*., 2010).

1.2.4. MADS box transcription factors

MINICHROMOSOME MAINTENANCE 1 AGAMOUS DEFICIENS *serum response factor* (MADS) box transcription factors have been shown to function in the control of flowering time or meristem identity e.g. FLOWERING LOCUS C (Yanofsky *et al*., 1990; Ng and Yanofsky, 2001) or APETALA 3 (AP3) (Jack *et al*., 1992). The ABC model explaining floral organ identity was mainly based on MADS box proteins like AP3 (Kater *et al*., 2006).

1.2.5. HD-Zip transcription factors

HOMEODOMAIN-LEUCINE ZIPPER (HD-Zip) transcription factors were found to possess an N-terminal homeodomain for DNA-binding and a C-terminal leucine-zipper domain enabling protein-protein interactions. They were shown to be involved e.g. in responses to abiotic stress, response to abscisic acid, embryogenesis, auxin signaling and lateral organ formation. Examples for HD-Zip transcription factors are PHAVOLUTA (PHV), PHABULOSA (PHB) and REVOLUTA (REV), which were involved in lateral organ formation and meristem maintenance (Elhiti and Stasolla, 2009).

1.2.6. Zinc finger containing transcription factors

Zinc finger containing transcription factors are various in *Arabidopsis*. The largest family among those is the C_2H_2 group, making up 0.7 % of all *Arabidopsis* genes (Englbrecht *et al*., 2004). The DNA-binding zinc finger domains were found to consist of two cysteine and histidine residues bound to a central zinc ion (Pabo *et al*., 2001). If being repressors, they often contained an additional ERF domain (Ciftci-Yilmaz and Mittler, 2008) but they can also function as a repressor or an activator on different promoters lacking this domain. SALT TOLERANCE ZINF FINGER (STZ) for example is a repressor for the EP2-type promoter, a part of the *5-ENOLPYRUVYLSHIKIMATE-3-PHOSPHATE SYNTHASE* (*EPSPS*) promoter (Takatsuji *et al*., 1992). The same protein positively regulated the expression of *ASCORBATE PEROXIDASE 1* and *2* (*APX1* and *2*) (Mittler *et al*., 2006).

Zinc finger transcription factors were shown to play a role in abiotic and biotic stress response and metabolic pathways (Ciftci-Yilmaz and Mittler, 2008).

1.2.7. NAC transcription factors

NAM, ATAF 1,2, CUC 2 (NAC) transcription factors are one of the biggest groups in *Arabidopsis* and were shown to play a role in abiotic and biotic stress response and development. They were found to possess an N-terminal DNA-binding NAC domain and often a C-terminal activation domain (Olsen *et al*., 2005). One example is CUP-SHAPED COTYLEDON 2 (CUC2). A loss of this gene and its homologue CUC1 leaded to fused cotyledons and the absence of a shoot apical meristem, corroborating their role in development (Aida *et al*., 1997).

1.2.8. WRKY transcription factors

The last group of transcription factors I want to mention is the WRKY family, named after the amino acid sequence of the conserved domain (Rushton *et al*., 1996; Eulgem *et al*., 2000; Rushton *et al*., 2010). The WRKY motif was shown to be located N-terminal in the protein whereas the C-terminus contained a zinc finger sequence (Eulgem *et al*., 2000). DNA-binding was found to be realized via the WRKYGQK, which is forming a β-strand entering the DNA major groove and recognizing the W-box DNA motif (Yamasaki *et al*., 2005). WRKY transcription factors are regulators of plant innate immunity and play roles in abiotic stress response (Rushton *et al*., 2010). They were shown to be positive or negative regulators depending on the sequence surrounding the W-box motif (Miao *et al*., 2004).

1.3. Genetic screens using *gain-of-function* approaches

Genetic screens can be used to identify proteins functioning in a selected context. For this purpose, the phenotype of mutated plants can be analyzed under standard or stress conditions or based on molecular differences and compared to wild type plants. In this work I will focus on genetic screens using *gain-of-function* approaches. They are a helpful tool as *loss-of-function* mutants often do not show an informative phenotype (Bouché and Bouchez, 2001). Another advantage is that *gain-of-function* mutations result in dominant phenotypes (Bouché and Bouchez, 2001; Nakazawa *et al*., 2003). So there is no need for homozygous plants for analysis,

which reduces time consumption (Bolle *et al*., 2011). The first *gain-of-function* screens were performed using activation tagging as proposed in 1994 by Walden and colleagues (Walden *et al*., 1994). In this procedure, T-DNAs containing a 35S enhancer or promoter at the right border were transformed into plants and integrated randomly (Walden *et al*., 1994; Weigel *et al*., 2000). Various proteins have been identified and assigned to a functional context this way (Borevitz *et al*., 2000; Ito and Meyerowitz, 2000; van der Graaff *et al*., 2000; Huang *et al*., 2001). Instead of a T-DNA, a transposable element system was also used (Marsch-Martinez *et al*., 2002; Nishal *et al*., 2005). Another possibility to examine unknown gene function is the analysis of enhancer trap lines. A promoterless reporter e.g. *UidA*, *GREEN FLOURESCENT PROTEIN* (*GFP*) or *LUCIFERASE* (*LUC*) was, together with an enhancer sequence, cloned into a T-DNA or a transposable element. After transformation and/or random integration of the construct, plant lines could be analyzed for expression patterns of the reporter or reactions in induction experiments (He *et al*., 2001; Alvarado *et al*., 2004). Enhancers were found to activate gene expression in both directions (Weigel *et al*., 2000; Ichikawa *et al*., 2003).

In 2008, Papdi and colleagues presented a T-DNA-based system using a controlled overexpression effector and a LUC reporter construct (Papdi *et al*., 2008). The effector was GATEWAY™ -compatible and allowed estradiol-inducible ectopic expression of cDNA libraries (Zuo *et al*., 2000). Based on the recombination sites of the GATEWAY™ system, cDNA genes were easy to amplify and identify (Papdi *et al*., 2008). LUC as a reporter enabled non-destructive screening and because of its short half-life of 3 hours, dynamics in expression of the reporter can be analyzed (Koncz *et al*., 1987; Thompson *et al*., 1991; Millar *et al*., 1992). In 2011 Wehner and colleagues performed a high-throughput genetic screen using a transcription factor open reading frame (ORF) library in a GATEWAY™ -compatible overexpression construct and a LUC-reporter. They did not screen whole plants but protoplast in a microtiter-based assay (Wehner *et al*., 2011). As redundancy in transcription factor families is relatively high and single *loss-of-function* mutants often show no phenotype, an *gain-of-function* screen is a suitable tool to analyze their function (Qu and Zhu, 2006; Mitsuda and Ohme-Takagi, 2009). GATEWAY™ -compatible transcription factor ORF libraries are the REGIA collection comprising of 1200 transcription factors (Paz-Ares, 2002; Castrillo *et al*., 2011), the PKU-Yale collection of 1600 transcription factors (Gong *et al*., 2004; Ou *et al*., 2011) and the TF only collection comprising of 1500 transcription factors (Mitsuda *et al*., 2010). As future perspectives for genetic screens Bolle and colleagues suggested media-based screens, e.g. performed in multiwall plates to avoid space consumption in the greenhouse. They proposed future screens to be automated, innovative and non-invasive (Bolle *et al*., 2011).

1.4. Aim of this study

The aim of the presented study was to elucidate functions of cytokinin signaling components that alter the level of cytokinin-induced gene expression. In the first project I wanted to identify and verify non-redundant roles of the type-B response regulator gene *ARR14*. I analyzed expression pattern of the *ARR14* gene and the subcellular localization of the ARR14 protein. To identify specific roles for ARR14, l*oss-of-function* lines were analyzed for their performance in abiotic and biotic stress conditions and *gain-of-function* lines were generated and examined. In the second project I planned to find new modulators of the cytokinin response. For this I developed a new fast and easy-to-use mutant screening method requiring only a plate reader as technical device, being available in the most laboratories. Performing a *gain-of-function* screen on cytokinin response I had expected cytokinin signaling components to be among the candidates, e.g. especially type-B response regulators as they are regulating the cytokinin response under normal conditions. To analyze cytokinin response, I utilized a well characterized cytokinin output reporter in this screen. The usage of an inducible system for ectopic expression of cDNA libraries allowed us, to identify new modulator candidate genes whose constitutive overexpression would have been potentially lethal. This gave us the opportunity to identify unknown modulators being not already assigned to a function in cytokinin response. The screen was developed, conducted and candidate genes tested in a transient protoplast transactivation assay.

2. Material and methods

2.1. Chemicals

Chemicals were obtained from the companies Rapidozym (Berlin, GER), Fluka (Buchs, CH), Peqlab (Erlangen, GER), Roth (Karlsruhe, GER), Merck (Darmstadt, GER), Sigma (Deisenhofen, GER) and PJK (Kleinblittersdorf, GER) unless stated otherwise.

2.2. Organisms

In this study, different organisms were used. *E. coli* was utilized for cloning approaches, *A. tumefaciens* for plant transformation and *A. thaliana* for *in vivo* analysis of the examined genes. *V. longisporum* served as a pathogene for biotic stress experiments. *N. benthamiana* was used for transient expression for subcellular localization studies.

2.2.1. Microorganisms

The microorganism strains used in this study are listed in table 2.1.

Table 2.1: Microorganisms used in this study.

Organism	Strain	Genotype	Reference
E. coli:	DH10B	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G	Calvin and Hanawalt, 1988; Raleigh *et al*., 1988
E. coli:	DB3.1	F^- *gyr*A462 *end*A1 Δ(*sr*1-*rec*A) *mcr*B *mrr* *hsd*S20($r_B^-m_B^-$) *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Sm^r) *xyl*5 Δ*leu mtl*1	Hanahan, 1983; Bernard and Couturier, 1992
A. tumefaciens	GV3101::pM90	*rpoH+ hrcA+*	Schell, 1978
V. longisporum	Isolate 43	wild type	from Karen Zeise, Rostock

2.2.2. Plant material

In plant experiments *A. thaliana* (ecotype Col-0) and *N. benthamiana* were used. The mutant *Arabidopsis* plant lines used in this study are listed in table 2.2.

All mutant plant lines were obtained from The *Arabidopsis* Information Resource (TAIR), http://www.arabidopsis.org/abrc/index.jsp, on www.arabidopsis.org, Jan 18, 2013.

Table 2.2: Mutant plant lines used in this study.

Line	Description	Referece
arr14-1	*Arabidopsis thaliana*, T-DNA insertion mutant, insertion in *ARR14*, insertion in second exon/receiver domain	GABI Kat 147B02 (Ishida *et al*., 2008b)
arr14-2	*Arabidopsis thaliana*, T-DNA insertion mutant, insertion in *ARR14*, insertion in third exon/DNA-binding output domain	SAIL_278_B11
arr14-3	*Arabidopsis thaliana*, T-DNA insertion mutant, insertion in *ARR14*, insertion in 5' UTR	SAIL_630_D09
arr2-4	*Arabidopsis thaliana*, T-DNA insertion mutant, insertion in *ARR2*	SALK_016143 * (Mason *et al*., 2005)
ahk-5	*Arabidopsis thaliana*, T-DNA insertion mutant, insertion in *AHK2*	SALK_037536 * (Riefler *et al*., 2006)
ahk-7	*Arabidopsis thaliana*, T-DNA insertion mutant, insertion in *AHK3*	SALK_069269 * (Riefler *et al*., 2006)
arr2-4	*Arabidopsis thaliana*, T-DNA insertion mutant, insertion in *ARR2*	SALK_016143 (Mason *et al*., 2005)

2.2.3. Transgenic plants generated during this study

The transgenic plants generated during this study are listed in table 2.3.

Table 2.3: Transgenic plants generated during this study.

Name	Vector	Reference for vector
pARR14 850 bp::GUS	*pCB308*	Xiang *et al*., 1999
35S::ARR14	*pB2GW7*	Karimi *et al*., 2002
pBT10-GUS	*pBT10-GUS*	Sprenger-Haussels and Weisshaar, 2000
pROK219_NAN	*pROK219_NAN*	Kirby and Kavanagh, 2002
35S::GFP-ARR14	*pB7WGF2*	Karimi *et al*., 2002
pARR6 350 kb::LUC+	*pBinLUC+*	Papdi *et al*., 2008
pARR6 1000 kb::LUC+	*pBinLUC+*	Papdi *et al*., 2008
pARR6 2146 kb::LUC+	*pBinLUC+*	Papdi *et al*., 2008
pARR6 2146 kb::LUC+ pER8GW_GONG	*pBinLUC+* *pER8GW*	Papdi *et al*., 2008
pARR6 2146 kb::LUC+ pER8GW_seed/hormone	*pBinLUC+* *pER8GW*	Papdi *et al*., 2008
pARR6 2146 kb::LUC+ pER8GW_ARR2	*pBinLUC+* *pER8GW*	Papdi *et al*., 2008
pARR6 2146 kb::LUC+ pER8GW_CKX1	pBinLUC+ pER8GW	Papdi *et al*., 2008

2.3. cDNA libraries

The cDNA libraries used in this study are listed in table 2.4.

Table 2.4: cDNA libraries used in this study.

Library	Description	Reference
PUK-Yale library	full length ORF transcription factor library from *Arabidopsis thaliana*	Gong *et al*., 2004
seed library	cDNA library from seeds and primary leaves from *Arabidopsis thaliana*	Bürkle *et al*., 2005
hormone library	cDNA library from hormone-treated *Arabidopsis thaliana* seedlings	Bürkle *et al*., 2005

2.4. Culture

2.4.1. Bacteria growth medium

Bacteria were grown in luria broth (LB) media (Bertani, 1951), which was adjusted to pH 7,2-7,5. Solid LB contained 1,2% (petri dishes 9 cm) – 1,6% (bigger plates) agar. For selection, antibiotics were added in the appropriate concentrations after autoclaving (for concentration see table 2.5).

2.4.2. Plant growth

2.4.2.1. Plant growth on media

Plants were grown on murashige and skoog (MS) medium (4,2 g MS-salts + 0,5 g MES + 0,1 g myo-inositol/l + 0,1-1 % sucrose) (Murashige and Skoog, 1962) or ½ MS medium or on *Arabidopsis thaliana* salts (Ats) medium (5 ml KNO_3 [1M], 2,5 ml KPO_4 [1 M], 2 ml $MgSO_4$ [1 M], 2 ml $Ca(NO_3)_2$ [1 M], 2,5 ml Fe-EDTA [20 mM] and 1 ml micronutrients (70 mM H_3BO_3, 14 mM $MnCl_2$, 0,5 mM $CuSO_4$, 1 mM $ZnSO_4$, 100 x Na_2MoO_4, 10 mM NaCl, 1000 x $CoCl_2$)) (Lincoln et al., 1990). Plant media were adjusted to pH 5,7 if not mentioned otherwise. For media for selection on herbicides 0,7% agar or phytagel were added. For root growth experiments on vertical plates 1,2% agar or phytagel were added. For selection, the medium was supplemented with the appropriate antibiotics (for concentrations see table 5) after autoclaving. For osmotic stress experiments, the supplements were autoclaved separately and added after autoclaving except for NaCl. Plants were grown in 16/8 h day/night cycle at 21/18°C in climate chambers.

2.4.2.2. Plant culture on soil

To grow plants on soil, pique earth was used consisting of P- and T-earth (Einheitserde Werkverband e.V. ,Sinntal-Jossa, GER) and Perligran G (Knauf Perlite GmbH, Dortmund, GER) in the ratio 2:2:1. *A. thaliana* was grown in 16/8 h day/night cycle at 21/18°C if not mentioned different. *N. benthamiana* was grown at 14/10 h day/night cycle at 24°C.

2.4.3. Verticillium growth medium

Verticillium longisporum was cultured in czapek-dox medium (30 g sucrose, 3 g Na_2NO_3, 0,5 g $MgSO_4$ x $7H_2O$, 0,5 g KCl und 0,01 g FSO_4 in 1 l bidestilled water) (Warcup, 1950).

2.4.4. Antibiotics and herbicides

The antibiotics and herbicides used in this study are listed in table 2.5.

Table 2.5: Antibiotics and herbicides used in this study.

	Stocks	Final concentration for bacteria	Final concentration for plants
Kanamycin	100 mg/ml	100 µg/ml	
Spectinomycin	50 mg/ml	50 µg/ml	
Carbenecillin	100 mg/ml	100 µg/ml	
Rifampicin	50 mg/ml	50 µg/ml	
Gentamycin	25 mg/ml	25 µg/ml	
Phosphinothricin	25 mg/ml		50µg/ml
Sulfadiazine	7,5mg/ml		5,25mg/l
Hygromycine	50 mg/ml		50µg/ml
Cefotaxine	250mg/ml		125mg/l

2.5. Microbiological methods

2.5.1. Plasmid isolation from bacteria

For small scale plasmid isolation from bacterial cells the Invisorb® Spin Plasmid Mini Two Kit by Invitek (Berlin, GER) or a standard by a protocol involving alcaline lysis modified from Birnboim and Doly (1979) (Birnboim and Doly, 1979) were used. In short 4 ml of an overnight bacteria culture were pelleted by centrifugation and the pellet was resuspended in 150 µl of solution P1 (50 mM Tris-HCl pH 8,0, 10 mM Na_2EDTA, 100 µg/ml Rnase A). 500 µl solution P2 (200 mM NaOH, 1% SDS w/v) were added and the tube inverted several times. By adding 350 µl of solution P3 (3 M K-acetate, pH 5,5 with glacidic acid) and inverting the tube, a white precipitate containing proteins, genomic DNA and cell debris, appeared and was pelleted by centrifugation for 5 minutes. Subsequently 750 µl of the supernatant were transferred into a fresh reaction tube and the plasmid DNA was precipitated by addition of the equal amount of isopropanol and centrifuged for 30 min at 16.000 rpm and 4°C. The pellet was washed with 70% ethanol, dried and dissolved in bidestilled water.

For large scale plasmid isolation the NucleoBond® Xtra Maxi Kit by Macherey&Nagel (Düren, GER) was used. The DNA pellet was dissolved in 100 µl bidestilled water.

2.5.2. Cloning

2.5.2.1. Restriction of plasmids

Restriction digestions were performed for cloning or examination of bacterial plasmids for their identity. Endonucleases were obtained from Fermentas (St. Leon-Rot, GER) and New England Biolabs (Frankfurt, GER). Typically 1-3 µg of plasmid DNA were digested for at least two hours with appropriate enzymes, each 1 unit in their respective buffers. When necessary for downstream applications, enzymes were deactivated according to the manufacturer's instructions. If the digested DNA was used as a vector backbone for cloning alkaline phosphatase was added to the digestion reaction following the manufacturer's instructions to prevent self-religation.

2.5.2.2. Electrophoretic separation of DNA fragments on agarose gels

To analyse the size of DNA-fragments, electrophoretic separation on agarose gels was performed following Sambrook et al. (2001). Depending on the expected size of the DNA fragments the gels had an agarose content of 0,8-2,5 % (w/v) in TAE-buffer (1 l 50 x TAE: 242 g tris base, 57,1 ml acidic acid, 100 ml EDTA [0,5 M], pH 8,0) and contained 0,004% ethidium

bromide. The loading buffer (50 ml) was composed of 40 ml TE-buffer, 33,5 g sucrose and 200 mg OrangeG. As a marker Hyperladder I™ (Bioline USA Inc., Randolph, USA) was used. Small agarose gels (50 ml) were run at 85 V, large gels (200 ml) were run at 120 V. Gels were analyzed for UV fluorescence caused by the ethidium bromide incorporated in the DNA fragments.

2.5.2.3. DNA extraction from gels

Gel extraction was performed with the Zymoclean™ Gel DNA Recovery Kit by Zymo Research (Freiburg, GER) after excision of the desired gel fragments on a UV table following the manufacturer's instructions.

2.5.2.4. Ligation

To ligate DNA fragments 50 ng vector, 3-5x the amount of insert, T4 ligase buffer and 1 u T4 ligase (Fermentas, St. Leon-Rot, GER) were pipetted into one eppendorf tube and incubated at 16°C over night.

2.5.2.5. GATEWAY™ Cloning

The GATEWAY™ cloning is based on site specific recombination of the phage lambda. For GATEWAY™ cloning we followed a procedure modified from the manual from Invitrogen (Version E, september 2003). As donor vector pDONR222 (Invitrogen™, Karlsruhe, GER) was used. A GATEWAY™-compatible *ARR14* ORF was purchased in pENTR™/SD/D-TOPO from The *Arabidopsis* Information Resource (TAIR), http://www.arabidopsis.org/abrc/index.jsp, on www.arabidopsis.org, Jan 18, 2013.

For a modified BP reaction procedure 2 µl (150 ng/µl) of the donor vector were incubated with 2 µl (40-100 fmol) of PCR product, 4 µl BP-buffer and 0,5 µl BP-clonase over night at 25°C. The next day, 1 µl proteinase K was added for proteolysis and the sample first kept at 37°C and then at 68°C each for 10 min to inactivate the proteinase K.

For a modified LR reaction procedure 2 µl (150 ng/µl) of the expression vector were incubated with 2 µl (40-100 fmol) donor vector containing the insert, 4 µl LR-buffer and 0,5 µl LR-clonase over night at 25°C. The next day, 1 µl proteinase K was added for proteolysis and the sample first kept at 37°C and then at 68°C each for 10 min to inactivate the proteinase K.

Buffers, clonases and proteinase K originated from the GATEWAY™ Technology Kit (Invitrogen, USA).

2.5.2.6. Preparation of electrocompetent bacterial cells

For the preparation of electrocompetent *E. coli* or *A. tumefaciens* cells the protocol from Ausubel and colleagues was modified (Ausubel *et al*., 1994). In short, 1 ml of an overnight culture was transferred into 100 ml of LB in an erlenmayer flask and shaken at 150 rpm on 37°C (*E. coli*) or 28°C (overnight, *A. tumefaciens*) until the OD_{600} reached 0,5-0,8. Cells were cooled down on ice, centrifuged at 4.000 rpm for 10 minutes at 4°C and the pellet was resuspended in 30 ml ice cold water. This was repeated under the same conditions and the pellet was dissolved in 20 ml ice cold water and in the next washing step in 20 ml ice cold glycerin [10%]. After an additional centrifugation step, the pellet was dissolved in 1 ml of glycerin [10%] and aliquoted in portions of 50 µl into eppendorf tubes precooled on ice. Competent cells were shockfrozen in liquid nitrogen and subsequently stored in -80°C. Transformation competence was tested by transformation of 20 pg of PUC19-plasmid (Yanisch-Perron et al., 1985).

2.5.2.7. Electro-transformation of bacteria

For electroporation it was necessary to remove salts from the DNA. For dialysis the product of *in vitro* recombination was pipetted onto a Millipore membrane (Millipore Corporation, Bedford, UK) floating on bidestilled water. After 20 minutes, the sample was ready for transformation into the target organism.

The electroporation procedure of Ausubel and colleagues was modified (Ausubel *et al*., 1994). The DNA was added to electrocompetent *E. coli* or *A. tumefaciens* cells which were thawn on ice before and incubated for 30 minutes. The cell-DNA mixture was pipetted into a pre-cooled electroporation cuvette and an electric pulse of 1,7 V (*E. coli*) or 3,6 V (*A. tumefaciens*) was applied. Subsequently 1 ml of LB was added to the transformation and the mixture was shaken in an eppendorf tube for one hour (*E. coli*) or three hours (*A. tumefaciens*) at 37°C (*E. coli*) or 28°C (*A. tumefaciens*). Cells were plated on selection media accordingly.

2.5.2.8. Sequencing

Purified DNA fragments or plasmids were sent for sequencing to GATC Biotech (http://www.gatc-biotech.com/de/index.html, Konstanz, GER) following their instructions.

2.5.2.9. Freezing stocks

For long-term storage, freezing stocks of the generated bacterial cultures were made. 800 µl of a liquid overnight culture were transferred into a cryo tube prefilled with 800 µl sterile glycerol. After mixing the tube was shockfrozen in liquid nitrogene and stored at -80°C.

2.5.3. Polymerase Chain Reaction (PCR)

2.5.3.1. Standard PCR

The following protocol was used for amplification of DNA. It was modified from Saiki *et al*. (1992). A list of oligonucleotides used for PCR can be found in the appendix.

5 µl primer 1 [5 µM]

5 µl primer 2 [5 µM]

2,5 µl dNTPs [5 mM each]

5 µl buffer [10x]

1 µl polymerase

x µl 40-100 ng template

Bidestilled water was added to the final volume of 50 µl

For cloning, adapter primers were used that contained the respective restriction sites or GATEWAY™ extensions. For each PCR reaction the amounts of primers and template were optimized.

2.5.3.2. PCR cycler standard settings

The annealing temperature was adjusted to the respective primer pair, the extension time to the expected product length. Unless mentioned otherwise, the PCR ran for 30 cycles.

Lid 99°C

Initial denaturation	95°C	2 minutes	
Denaturation	94°C	45 seconds	↑ 30x
Annealing	52°C	standard 45 seconds	
Extension	72°C (Taq polymerase) or 68°C (Pfu-polymerase)	standard 2 minutes	
Final elongation	same temperature as extension	10 minutes	

For GATEWAY™ cloning two steps of PCR were performed. The first step consisted of 10-15 cycles of a routine PCR cycler program with gene-specific primers containing GATEWAY™ *att* site extensions. The second step consisted of 20 cycles, GATEWAY™-specific primers were used and the following program was selected for the PCR cycler. The annealing temperature of the first cycle round and the extension times of both were adjusted to the primer pair used and the expected fragment length.

Lid 99°C

Initial denaturation	95°C	2 minutes	
Denaturation	94°C	45 seconds	↑ 5x
Annealing	52°C	standard 45 seconds	
Extension	68°C (Pfu-polymerase)	standard 2 minutes	
Denaturation	94°C	45 seconds	↑ 10-15x
Annealing	54°C	standard 45 seconds	
Extension	68°C (Pfu-polymerase)	standard 2 minutes	
Final elongation	same temperature as extension	10 minutes	

2.5.3.3. PCR genotyping of bacteria

To genotype bacteria 5 µl water was pipetted into PCR tubes and bacteria were transferred into the tube by a toothpick dipped into a bacterial colony. Then the rest of the reaction mixture was added.

1 µl primer 1 [10 µM]

1 µl primer 2 [10 µM]

2,5 µl dNTPs [5 mM each]

2 µl buffer [10x]

1 µl polymerase

7,5 µl bidestilled water

For such PCRs the initial denaturation step was prolonged to 5 (*E. coli*) or 10 (*A. tumefaciens*) minutes.

2.5.3.4. PCR genotyping of plants

This PCR was used to identify homozygocity of in T-DNA mutant alleles in plants.

5 µl DNA extract (see chapter 2.8.4) were pipetted into a PCR tube and

0,3 µl primer 1 [10 µM]

0,3 µl primer 2 [10 µM]

0,4 µl dNTPs [5mM each]

2 µl buffer [10x]

0,75 µl polymerase

11,25 µl bidestilled water

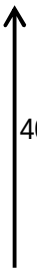
were added.

For genotyping of T-DNA insertion mutant plants, two PCR reactions were set up. The first contained a left gene-specific primer and a right gene-specific primer and an amplificate reflects the WT situation. The second reaction contained a left border T-DNA-specific primer and the right gene-specific primer. An amplificate in this reaction reflects the presence of a T-DNA insertion in the respective position.

2.5.3.5. Reverse Transcription PCR (RT PCR)

To check RNA samples for the presence of transcripts, the QIAGEN® One Step RT-PCR Kit (Qiagen, Hilden, D) was used. The reaction mixture contained primer 1 [6 µM], primer 2 [6 µM], 0,5 µg RNA, 5 µl 5x buffer, 1 µl dNTPs [5 mM], 1µl enzyme mix and was filled up with water to 25 µl volume.

Lid 99°C

First strand synthesis	50°C	30 minutes	
Deactivation of the reverse transcriptase and activation of the DNA polymerase	95°C	15 minutes	
Denaturation	94°C	1 minute	
Annealing	52°C	standard 1 minute	40x
Extension	72°C (Taq polymerase) or 68°C (Pfu-polymerase)	standard 1 minute	
Final elongation	same temperature as extension	10 minutes	

Unless not mentioned otherwise, the PCR ran for 40 cycles.

Annealing temperature was adjusted to the used primer pair and extension time to the expected fragment length.

2.5.3.6. cDNA synthesis

The cDNA synthesis was performed with the help of the SuperScript™ III Reverse Transcriptase Kit by Invitrogen (Karlsruhe, GER).

First a mixture of

1 µl oligo dT N20 primer [50 µl]

1,8 µl random hexamer primer [50 µl]

0,5 µg RNA

2 µl dNTPs [5 mM]

was prepared and filled up to 13 µl with bidestilled water. The sample was incubated at 65°C for 5 min for denaturation and subsequently kept on ice for 1 min. 4 µl first strand buffer, 1 µl DTT [0,1 M] and 1 µl SuperScript™ III reverse transcriptase were added and the sample incubated at 25°C for 10 minutes, followed by a cDNA synthesis step at 50°C for 50 min. The reaction was terminated by heating up the mixture to 85°C for 5 min.

2.5.3.7. Quantitative Real-time PCR (qRT-PCR)

To analyse the abundance of transcript very exact, quantitative Real-time PCR experiments were performed. For this purpose, cDNA was made first and then used in the PCR experiment.

The reaction mix contained

2µl 10X Immolase buffer

0,8 µl MgCl2 [50 mM]

0,4 µl dNTPs [5 mM]

0,2 µl 10x SYBR Green I

0,04 µl ROX [25 µM]

14,28 µl bidest water

0,04 µl Immolase [5 U/µl]

0,12 µl of each Primer [50 µM]

2 µl template cDNA [250 ng/µl – 2 µl of a 1:10 dilution of the cDNA synthesis]

Every sample was assayed in technical triplicates on one PCR plate.

Initial Denaturation	95°C	15 seconds	
Denaturation	95°C	10 seconds	↑ 40x
Annealing	55°C	15 seconds	
Extension	72°C	10 seconds	
Final elongation	Same temperature as extension	1 minute	
Melt curve (continuously) 95°C 1 minute, 70°C 1 minute, 95°C 30 seconds, 35°C 1 second.			

The PCR ran for 40 cycles.

2.5.4. DNA isolation from plants

The DNA isolation is based on the protocol developed by Pruitt and Meyerowitz (1986). About 0,5 cm² of leaf material were harvested in 1,5ml eppendorf tubes filled with 400 µl DNA-extraction buffer (200 mM tris-HCl, pH 7,5, 250 mM NaCl, 25 mM EDTA, 0,5% SDS) and grinded with the help of two steel beads in the Mixamill™ (Retsch, Haan, GER). After grinding, samples were transferred into a centrifuge. After 3 minutes of centrifugation at 16.000 rpm the supernatant was transferred into another eppendorf tube. The DNA was precipitated by mixing the sample with an equal amount of isopropanol and a subsequent centrifugation step of 30 minutes at 16.000 rpm and 4°C. The pellet was washed with 350 µl ethanol [70%], dried and dissolved in 50 µl bidestilled water.

2.5.5. RNA isolation from plants

The RNA isolation method was adapted from Chomczynski and Sacchi (1987). In most of the cases seedlings from liquid culture were used. In this case the material was harvested with the help of a water vacuum pump to remove as much of the liquid as possible to enable efficient grinding of the tissue. After vacuum treatment, the seedlings were shockfrozen in liquid nitrogen in 2ml-eppendorf tubes containing two RNase-free steel beads. If fresh leaves were not directly used for RNA isolation, samples were directly frozen in liquid nitrogen in the eppendorf tubes.

Samples were grinded in precooled attachments in a Mixamill™ (Retsch, Haan, GER) twice for 2 minutes at 30 Hz and subsequently stored in liquid nitrogen again.

1 ml trizol solution (380 µl phenol, 200 µl guanidium-thiocyanat [4 M], 100 µl ammonium-thiocyanat [4 M], 33,4 µl Na-acetat [3 M], 50 µl glycerate, 236,6 µl bidestilled water) was added and the sample vortexed until it appeared homogenous. After a 10 minute incubation at RT the sample was centrifuged for 5 min at 4°C and 16.000 rpm. The supernatant was transferred into a new 2 ml eppendorf tube and inverted with 400 µl chloroform-isoamylalkohol (24:1). After a centrifugation for 5 min at 4°C and 16.000 rpm 700 µl of the upper phase were transferred into a new 1,5 ml eppendorf tube and the RNA was precipitated by addition of each 350 µl of isopropanol and high salt solution [1,2 M NaCl, 800 mM Na-citrate], subsequent inversion of the tube, 10 min incubation at RT and a centrifugation for 10 min at 4°C and 16.000 rpm. The pellet was washed twice with 75% ethanol, let dry and dissolved in 30 µl RNase-free water.

For the application in quantitative Real-time PCR analysis the RNA was purified prior to cDNA synthesis using the Rneasy Kit by Qiagen (Hilden, GER).

2.6. Plant methods

2.6.1. Seed sterilization

The protocol for seed sterilization was modified from Salinas and Sánchez-Serrano (2006). Typically 50 mg seeds were incubated with 70% ethanol+0,01% triton X-100 on a thermomixer at 21°C for 9 min and subsequently washed with 70% ethanol in sterile conditions or seeds were incubated on the thermomixer with 1,2% NaClO+0,01% triton X-100 for 7 minutes and washed with sterile water for 6 times. Afterwards the seeds were dried on a filter paper to put them on medium by toothpicks or were affiliated in 0,1% sterile agarose and dropped on media by pipette.

2.6.2. Crossing of *A. thaliana*

Crossings were performed following the protocol from Salinas and Sánchez-Serrano (2006). For crossings, two parental plants were grown until they reached the stage of flowering. Then in the receptive plant, a flower bud was opened and emasculated. Accordingly anthers of an already opened flower of the pollinating plants were used to fertilize the receptive gynoeceum. Crossings were performed on at least 3 buds per plant and in both directions.

2.6.3. Phenotyping on soil

Plants were grown on soil next to wild type plants for comparison. During all the life cycle photos were made focusing on the rosette size and appearance, the stem height, numbers of flowers or adventious shoots.

2.6.4. Root assays

Root assays were performed similar to Doerner *et al*. (1996) and Riefler *et al*. (2006). MS medium containing 1,2% phytagel or 1,2% agar and different amounts of cytokinin was poured into square petri dishes. For testing the cytokinin sensitivity in root elongation and lateral root development, concentrations of 0 µM, 0,01 µM, 0,05 µM, 0,1 µM and 1 µm benzyladenine were used.

For sensitivity tests upon cold treatment, medium did not contain additional supplements.

The petri dishes were sealed at the sides with scotch tape, the bottom side was enclosed with parafilm and, after stratification at 4°C for two days, placed upright in a climate chamber. At different timepoints, the root length was marked and at the second timepoint, the elongation and the number of lateral roots were counted. For the cold stress experiment, the plants grew in a climate chamber at 11°C (Zhang et al., 2008) during this period.

2.6.5. Scoring of senescence phenotype in short day conditions

Plants were sown out in trays and stratificated in 4°C for two days. After letting them grow for 10 days, they were transferred into single pots and grown until ripening. The 6^{th} and the 10^{th} leaf were marked with small twines and observed for signs of senescence similar to Boyes *et al*. (2001). Experiments were performed in a chamber with an 8h/16h light cycle.

2.6.6. Pathogen response test with *V. longisporum*

V. longisporum inoculation was conducted as described in Häffner *et al*. (2010). *arr14* and wild type plants were grown on soil for 2-3 weeks. Then the soil was removed, the root was cut to a length of 1,5 cm and the plants were inoculated in *V. longisporum* spore solution (10^5-10^6 *Verticillium*-spores/ml) or in czapek-dox media (mock) for 50 min. Subsequently the plants were replanted again and after ripening, the plants were scored for fresh weight, height and adventious shoot formation.

2.6.7. Transformation of *A. thaliana*

Plants were transformed by the floral dip method adapted from Clough and Bent (1998). *A. tumefacies* harboring the chosen plasmid were incubated for 48h in 300 ml of selection media at 28°C 150 rpm. They were pelleted by centrifugation at 4.500 rpm for 15 min and resuspended in 300 ml transformation media (5% sucrose w/v, 0,42% MS salt mixture w/v, 0,02% silvet v/v, pH 5,7). A pot of plants in the developmental stage of flower buds starting to open was submerged with all flower buds for 1-3 minutes and left lying on the side over night. Plants were placed upright again and grown until ripening.

2.6.8. Transient expression in *N. benthamiana*

The protocol for transient expression of proteins in *N. benthamiana* was modified from Witte and colleagues (Witte *et al*., 2004). *A. tumefaciens* harboring the respective plasmid were grown in liquid culture with the respective antibiotics for 2 days at 28°C and 150 rpm. The bacteria were pelleted and washed in infiltration buffer (10 mM MES-NaOH, pH 5,7; 10 mM $MgCl_2$; 150 µM acetosyringone). The pellet was dissolved in infiltration buffer and diluted to an OD_{600} of 0,05 and mixed with bacteria harboring an expression plasmid for p19 (Voinnet et al., 2003) that were treated in the same way. With the help of a syringe the bacterial solution was infiltrated into the lower surface of tobacco leafs. After 5 days the signal was scored.

2.6.9. Confocal microscopy analysis of plants

Leaf discs of *N. tabacum* or *A. thaliana* were placed upside down on a moisturized glass slide and covered with a cover slip. GFP was excited at a wavelength of 488 nm by an argon laser and the signal examined at 509 nm emission in a confocal microscope (Leica TCS SP5, Leica, Solms, GER).

2.6.10. GUS staining and analysis

Samples of *Arabidopsis thaliana* carrying a *pARR14^{850bp}::UidA* construct were fixed in acetone [90%] for 1h and treated with GUS staining buffer (0,1 M NaPO4 pH 7,0, 10 mM EDTA, 0,1% Triton X-100, 1 mM K3Fe(CN)6, 2 mM X-Gluc) overnight as described by Jefferson and colleagues (Jefferson *et al*., 1987). Accordingly the samples were destained twice each overnight with ethanol [70%] and examined by binocular.

2.6.11. Protoplast transactivation assay (PTA) GUS

The protoplast transient assays were modified from Hwang and Sheen (2001). Centrifugation steps were performed in a Haereus Multifuge 3SR+ (Thermo Scientific, Langenselbold, GER). Plants were grown in a climate chamber with low light conditions of 75-100 µEinstein. Shortly before flowering, all intact leafs were cut every 0,5mm with a razor blade at the lower surface of the leaf. The middle vein was cut longitudinal too and the leaf was put into a small petri dish containing enzyme solution (1,25% cellulase R-10, 0,3% macerozyme R-10, 0,4 M mannitol, 20 mM KCL, 20 mM MES, 10 mM CaCl2 pH 5,7, 680 mosm, sterile filtrated and stored at 4°C for not more than one month) with the cut surface facing the bottom of the petri dish. Experiments were performed in triplicate. The petri dishes containing the leaves were incubated overnight in a climate chamber. The next morning protoplasts were harvested by softly shaking the petri dish and pouring the solution through a mesh with an exclusion size of 60 µm. The flow-through was centrifuged for 2 minutes at ascending speed 3, descending speed 1 at 780rpm and room temperature. The pellet was washed carefully with 9 ml of solution W5 (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 2 mM MES pH 5,7, 680 mosm, autoclaved) and after another centrifugation step at the conditions, again dissolved in 9 ml of solution W5. The protoplasts were kept on ice for 5 hours. In the meantime the DNA mixture for the transformation was prepared.

For each transformation a round-shaped 2 ml eppendorf containing the respective DNA mixture was prepared. The mix contained 9 µg of the reporter plasmid *pBT10-GUS* (Sprenger-Haussels and Weisshaar, 2000) with the selected promoter inserted, 3 µg of the transformation control plasmid *pROK219_NAN* (Kirby and Kavanagh, 2002) and 14 µg of the effector plasmid *pB2GW7* (Karimi *et al*., 2002) with the selected effectors inserted. When using two effectors in one transformation mixture, the tubes containing only one effector were filled up with empty effector plamid for comparison.

The protolast solution was centrifuged under the same conditions and the pellet was dissolved in the amount of Mmg solution (0,4 M mannitol, 15 mM MgCl2, 4 mM MES, pH 5,7, 680 mosm, autoclaved) matching the 200µl/transformation mixture. Into each eppendorf tube 200 µl of protolasts were added with a cut pipette tip carefully. The protoplasts were mixed with the DNA by 10x inversion and 220 µl PEG solution (4 g PEG, 3 ml bidestilled water, 2,5 ml mannitol [0,8 M], 1 ml CaCl2 [1 M], sterile filtrated) were added and mixed with the sample by inversion ten times. The mixture was incubated at room temperature for 25 min. Then 800 µl solution W5 were added and again mixed with the sample by inversion. After centrifugation for 2 min at ascending speed 5 and descending speed 5 and 780 rpm at room temperature, the supernatant was carefully removed with a 1ml tip. After another centrifugation at the same conditions the

rest of the supernatant was removed with a 20µl tip and subsequently the pellet was dissolved in 500 µl WI solution (0,5 M mannitol, 4 mM MES, 20 mM KCl, pH 5,7, 680 mosm, autoclaved). Cytokinin was added to a final concentration of 500nM and the samples were incubated in a climate chamber over night.

The next morning, protoplasts were centrifuged and the supernatant was removed like the evening before. The samples were immediately shockfrozen in liquid nitrogen.

150 µl GUS extraction buffer (50 mM phosphate buffer, 10 mM EDTA, 0,1% triton X-100, 0,1/ Na-laurosyl sarcosine, 2 µl/ml mercaptoethanol) pH 7,2 were added and 105 µl of those were mixed with 105µl MUG buffer (GUS extraction buffer pH 7,5, 81,3 mg MUG/50ml buffer, stored at 4°C in the dark for not more than one month). 10 µl out of the remaining 150 µl were mixed with 10 µl MUN buffer (GUS extraction buffer pH 7,0, 1 mM MUN). After 10 min of incubation at 37°C 100 µl of the MUG solution containing samples were transferred into 100 µl 200 mM NaCO3 and 3,3 µl of the MUN solution containing samples were pipetted into 200 µl 330 mM NaCO3. One hour later, this was repeated and all sample-NaCO3 mixtures were read out in the plate reader at 340/360 nm. The relative GUS units were calculated as in Ehlert *et al*. (2006).

2.7. Methods used in the screen for modulators of cytokinin response

2.7.1. Luciferase seedling screening assay

T1 seeds of parental *pARR6^{2kb}::LUC* lines transformed with *pER8GW_PUK-Yale* cDNA library or *pER8GW_seed/hormone* cDNA library constructs (see chapter 2.4) were surface sterilized and plated out on petri dishes containing ½ MS, 1% sucrose, 0,7% agar, 125 mg/l cefotaxin and 50 mg/l hygromycin. Seeds of parental lines were also surface sterilized but put out on non-selective media two days later. All petri dishes were incubated at 4°C for 2 days for stratification. After five to seven days, surviving plants were transferred in black flat bottom 96-well plates containing 100 µl ½ MS 1% sucrose 5 µM beta-estradiol at pH 7,8 in each well. Plates were incubated in the climate chamber overnight. The next day, D-luciferin dissolved in 0,1 N KOH was equilibrated to room temperature and pipetted into the wells to a final concentration of 0,2 mM. After 15 min, the plates were read by the plate reader using luminescence measurement.

Immediately *trans*-zeatin was added to a final concentration of 4,5 µM for induction. After 2 h plates were read again and the readouts were compared to those of the parental lines.

2.7.2. Selection and growth of primary positive plants

Plants with exceptional high luminescence values after induction with estradiol or after *trans*-zeatin treatment were selected for further analyses. Additionally plants showing a high induction of luminescence by cytokinin compared to the parental lines or a decrease in luminescence after cytokinin application were chosen. The selected plants were washed in bidestilled water and transferred to a petri dish containing ½ MS, 1% sucrose, 0,7% agar and 125 mg/l cefotaxin and let grow until they exhibited enough roots to be transplanted onto soil. In the case of contamination the plates, plants were transferred onto new media. To identify the genes being overexpressed in those plants, DNA was extracted, the cDNA region was amplified and the product sent for sequencing.

2.7.3. Protoplast transactivation assay (PTA) LUC

The protoplast isolation and transformation followed the same protocol as for the GUS-PTA (see chapter 2.9.11) but after shock frosting, the samples were treated different.

The frozen protoplast material was dissolved in 150 µl cell culture lysis 1X reagent following manufacturer's instructions of the Luciferase Assay System from Promega (Madison, USA). 10 µl of the samples were treated and assayed as mentioned above for MUN. 100 µl were examined for luciferase activity. For luciferase protoplast transient assays, the *pARR62,4 bp::LUC* construct by Hwang and Sheen was used as reporter (Hwang and Sheen, 2001).

2.7.4. Luciferase leaf disc assay

There were two ways I used leaf disc assays in this study. To test constructs for stable transformation in *Arabidopsis*, they were used for transient expression in tobacco. *Agrobacterial* solutions transformed with the respective vectors were infiltrated in tobacco as described in chapter 2.9.8. Leaf discs were trepanned 3-5 days later and incubated in 50mM MES or MS media (see chapter 2.7.2.1) containing the respective supplements, e.g. cytokinin or DMSO. Leaf discs were assayed for luminescence like seedlings in chapter 2.10.1. The other possibility to use the leaf disc assay was to identify suitable *pER8GW_ARR2* or *pER8GW_CKX1* expressig *Arabidopsis* control plants for the screen. The assay was performed as in tobacco. For the luminescence measurement four leaf discs per plant were placed in media and supplemented with *trans*-zeatin, estradiol or ethanol as estradiol-solvent control. The luciferin addition and readout was the same as for the tobacco leaf discs.

3. Results

3.1 The characterization of the response regulator ARR14

ARR14 is a type-B response regulator thought to be involved in mediation of the cytokinin-induced transcriptional response. It seemed to be an interesting candidate for further analysis because of its previously described interaction with ARR2 and, surprisingly, AHK2 in the yeast two-hybrid system (Dortay *et al*., 2006). Those interactions were also verified by co-affinity purifications (Dortay *et al*., 2006). Additionally earlier analyses indicated a role for ARR14 in development, senescence and pathogen response (Hellmann, 2007). To characterize *ARR14* further, I examined the expression pattern, the subcellular localization and plants overexpressing *ARR14*. To test participation of ARR14 in cytokinin signaling I examined an *Arabidopsis thaliana* T-DNA insertion line of *ARR14* for its cytokinin response and tested the transactivation capacity of ARR14 on the *ARR6* promoter. Additionally I analyzed the insertion line for the performance in abiotic and biotic stress conditions and the senescence progression.

3.1.1 The analysis of *ARR14* expression revealed a specific pattern with a differential temporal change in leaf development

To analyze the expression patterns of genes glucuronidase can be used as a marker. It is coded by the *uidA* gene and acts on 5-bromo-4-chloro-3-indolyl glucuronide resulting in a blue staining (Jefferson *et al*., 1987). For the analysis of expression patterns, the promoter of the examined gene or a promoter-gene fusion is cloned behind a *uidA* gene and stably transformed plants are analyzed for GUS staining (chapter 2.6.10). To create such a construct, one has to decide for a promoter fragment to use in this analysis. This is dependent on the chromosomal context. The *ARR14* gene is located on chromosome 2 in close proximity to two other genes (Figure 3.1). At2g01755 codes for a protein of unknown function. At2g01750 codes for a microtubule-associated protein not further characterized. Mason and Tajima and colleagues used a 1,5-2 kb promoter fragment, respectively, to identify *ARR14* expression patterns. This promoter construct included large portions of the At2g01750 gene. They detected *ARR14* expression mainly in the vasculature of young leaves, in the SAM and at the tip of the gynoceum (Mason *et al*., 2004; Tajima *et al*., 2004). The promoter fragment used in their experiments contained the full gene At2g01755 and parts of the gene At2g01750 including intronic regions. It was known that introns can act as enhancers depending on their sequence and position relative to the transcriptional start site (Mascarenhas *et al*., 1990; Rose and Beliakoff, 2000; Rose, 2002, 2004; Rose *et al*., 2008). As it is also known that the majority of *cis*-regulatory elements is located in the first 300-

400 bp downstream of the transcriptional start site (Geisler *et al*., 2006), I decided to reanalyze *ARR14* expression using a promoter fragment not containing parts of the gene At2g01750.

To analyze the expression pattern of *ARR14*, 850 bp of its promoter were cloned in front of a *uidA* gene and the construct was integrated stably into the *Arabidopsis thaliana* genome. The promoter was amplified with the primer pair P3 and P4 (Apendix) and subsequently cloned into PCB308 (Xiang *et al*., 1999) by GATEWAY™ cloning (chapter 2.5.2.5). Plants were transformed by floral dip (chapter 2.6.7) and the progeny assayed for GUS signals (chapter 2.6.10). I analyzed six lines for expression patterns of ARR14.

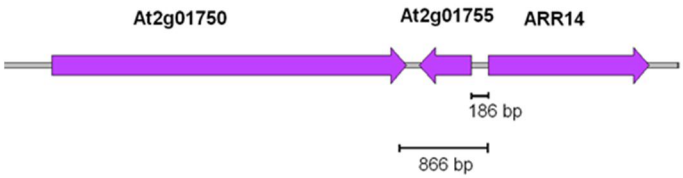


Figure 3.1: Genomic context of the *ARR14* gene. In close proximity, an intronless unknown protein coding gene (At2g01755) and gene coding for a microtubule associated protein (At2g01750) are located. The arrows show the transcriptional direction. Scheme was displayed using VectorNTI (www.invitrogen.com).

Seedlings, leaves in in all leaf stages, siliques and inflorescences were analyzed for GUS signals. *pARR14::uidA* expression was detected in the whole leaf area of newly developed leaves (Figure 3.2). In older leaves, the signal was restricted to the leaf margins (Figure 3.2a-c, e-g). In senescent leaves, the expression pattern of p*ARR14::uidA* was patchy (Figure 3.2d). The veinature was accentuated but a staining solely in the veinature as reported by Tajima and colleagues (Tajima *et al*., 2004) was not observed (Figure 3.2c). The shoot meristem showed a strong signal in agreement with the publication by Mason and colleagues (Mason *et al*., 2004) (Figure 3.2a). *pARR14::uidA* expression was also detected in the upper part of the root (Figure 3.2a). Siliques showed expression of p*ARR14::uidA* at the tip and at the abscission zone (Figure 3.2h).

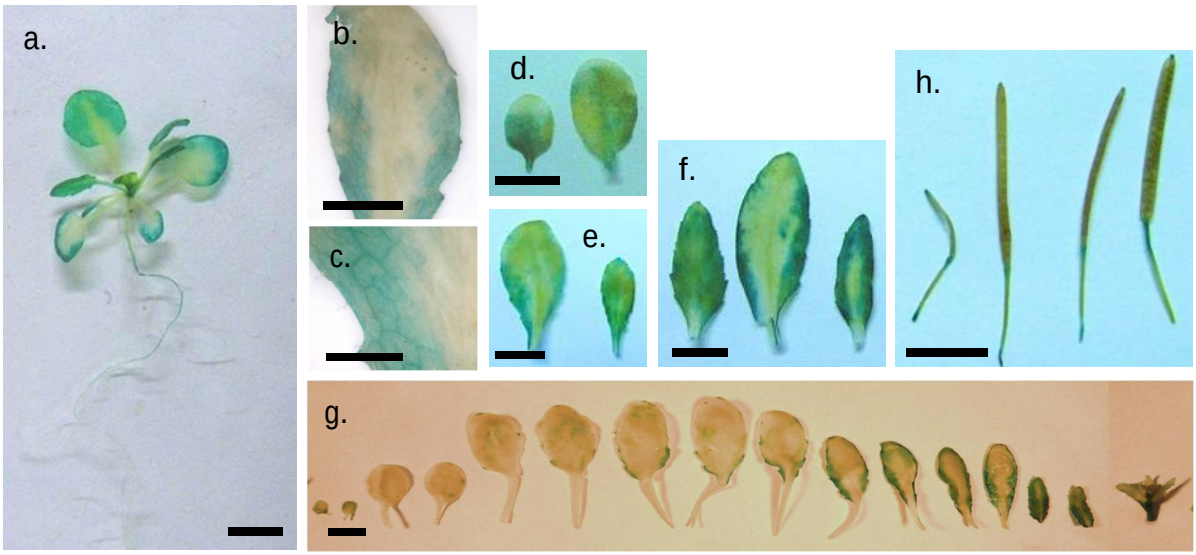


Figure 3.2: Analysis of *ARR14* expression using p*ARR14*^{850bp}*::uidA Arabidopsis* lines. (a.) A whole seedling, 15 DAG, (b.), (f.) a cauline leaf, (c.) a close-up of the leaf margin, (d.) primary leaves and (e.) rosette leaves of a 4 week old plant are shown. (g.) Developmental row of leaves of one rosette and shoot apexis of a 3 week old plant. (h.) Mature siliques were analyzed for *pARR14*^{850bp}*::uidA* expression (chapter 2.6.10). Staining was observed in young leaves, in older leaves at the leaf margin, in a patchy pattern in senescent leaves and at the abscission zone and tip of siliques. Scale bars, 0,5 cm; in (c.) scale bar, 0,2 cm

In the inflorescence *pARR14::uidA* expression was detected in the sepals and at the tip of the gynoecium. During developing into a young silique, the signal in the sepals decreased whereas a stronger signal appeard at the abscission zone of the silique. The anthers showed expression of *pARR14::uidA* at the tip (Figure 3.3 a-c).

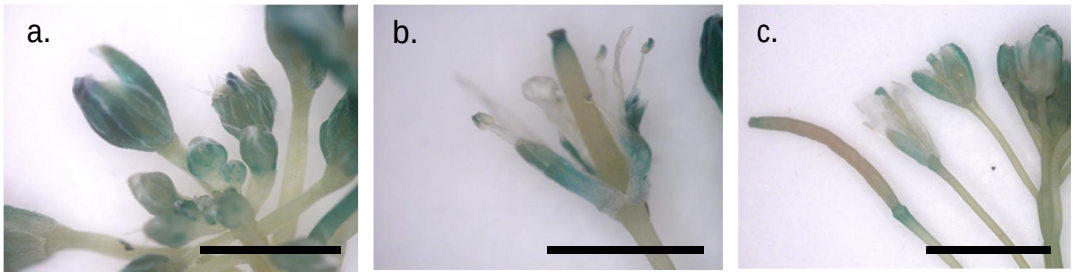


Figure 3.3: Analysis of *pARR14*^{850bp}*::uidA* expression in inflorescences. (a.) Flower buds, (b.) a close-up of a flower with organs unfolded and (c.) a developmental row from flower to young silique were analyzed for GUS signals (chapter 2.6.10). Staining was observed in sepals, the tip of the gynoecium and later at the tip of the silique and the abscission zone. Scale bars, 0,5 cm

3.1.2 ARR14 localized to the nucleus

In 2006, ARR14 was proven to localize to the nucleus by transient expression studies in bombarded onion cells (Dortay *et al*., 2006). To examine the subcellular localization of ARR14 in *Arabidopsis thaliana*, I constructed a binary *35S::GFP-ARR14* fusion as well as *35S::ARR14-GFP.* For this purpose, I amplified the ARR14 cDNA with primer pair P1 and P2 (Appendix) without a stop codon for preparation of the c-terminal GFP fusion protein (chapter 2.8.3.1 and 2.8.3.2). ARR14 and $ARR14_{without\ stop}$ were subsequently cloned into *pB7WGF2* and *pB7GWF2* via GATEWAY™ cloning (chapter 2.5.2.5). Those constructs were first tested in transient expression analysis in *Nicotiana benthamiana* (chapter 2.6.8) and stably introduced into *Arabidopsis thaliana* (chapter 2.6.7). The tobacco and *Arabidopsis* plants were analyzed by confocal microscopy (chapter 2.6.9) and a GFP-signal was detected in the nucleus (Figure 3.4). A *35S::ARR14-GFP* construct was not able to produce any GFP-signal neither in tobacco nor in *Arabidopsis*.

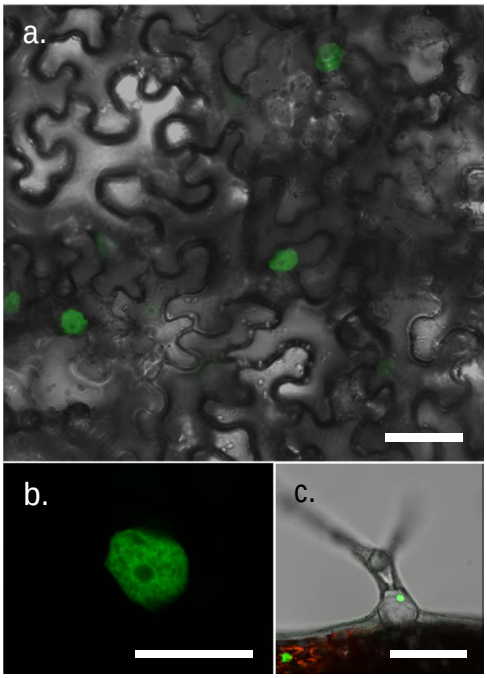


Figure 3.4: Subcellular localization of a *35S::GFP-ARR14* encoded fusion protein by confocal microscopy (chapter 2.6.9). (a.) Transient localization in *N. benthamiana* cells (chapter 2.6.8) resulted in a nuclear localization of the of the ARR14 fusion protein. Scale bar, 40 µm. (b.) Close-up on the nucleus in transient localization of the ARR14 fusion protein in *N. benthamiana.* Scale bar, 20 µm. (c.) In stably transformed *A. thaliana* lines (chapter 2.6.7) the ARR14 fusion protein also localized in the nucleus e.g. of trichomes. Scale bar, 200 µm.

3.1.3 Overexpression of N-terminal GFP-tagged *ARR14* lead to various phenotypes

A widely used approach to get insights into possible functions of a gene is the examination of plant lines overexpressing the respective gene. To establish transgenic lines that overexpress *ARR14*, *Arabidopsis* plants were transformed with the previously tested *35S::GFP-ARR14* construct (chapter 3.1.2, chapter 2.6.7). Additional *Arabidopsis* plants were transformed with *35S::ARR14* to overexpress an untagged protein for comparision.

In several transformation procedures, I established only six lines of *35S::GFP-ARR14* plants showing a GFP-signal. T1 *35S::GFP-ARR14* plants exhibited a stunted growth and round-shaped leaves (Figure 3.5 a,b). All other WT-like transformants did not show a GFP-signal.

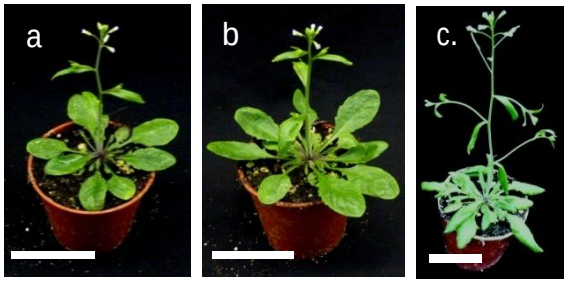


Figure 3.5: Typical general phenotype of *35S::GFP-ARR14* lines in the T1 generation. Depicted are lines (a.) *35S::GFP-ARR14*-4 and (b.) *35S::GFP-ARR14*-5 and (c.). a wild type plant The rosettes were smaller and the leaves had a round shape and longer petioles. Please note that there was no selection pressure on the wild type plant whereas the T1 plants were sprayed with ppt. Plants were four weeks old. Scale bars, 5cm.

I generated two stable homozygous lines exhibiting a growth phenotype and a GFP-signal (Figure 3.6b,d) and one line as an example for having lost the signal in the next generation and showing no phenotype compared to WT (Figure 3.6 a,c).

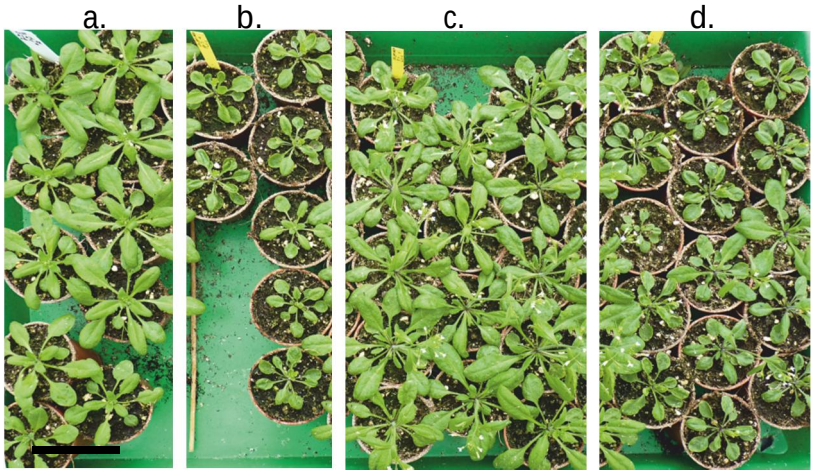


Figure 3.6: Growth of homozygous lines stably transformed with *35S::GFP-ARR14* compared to WT. (a.) WT. (b.) *35S::GFP-ARR14*-1. (c.) *35S::GFP-ARR14*-3. (d.) *35S::GFP-ARR14*-2. *35S::GFP-ARR14*-1 and -2 exhibited a growth phenotype compared to WT. They had smaller rosettes and round-shaped leaves with longer petioles. *35S::GFP-ARR14*-3 looked similar to WT. In line *35S::GFP-ARR14-2* variation was high but more plants set seeds. Plants were three weeks old. Scale bar, 10 cm

Homozygous plants overexpressing *35S::GFP-ARR14* exhibited a round leaf shape, longer petioles, were stunted and sometimes more bushy later in development (Figure 3.6, 3.7). Rosettes were smaller. The variance in rosette size was lower in *35S::GFP-ARR14*-1 and higher in *35S::GFP-ARR14*-2 (Figure 3.6b,d). Because plants of line 2 set more seeds than line 1 they seemed to be suited better for further analysis. *35S::GFP-ARR14*-3 did not show a difference in phenotype compared to WT (Figure 3.6c).

Plants expressing *35S:: ARR14* exhibited no phenotype compared to WT (Figure 3.7d,e.).

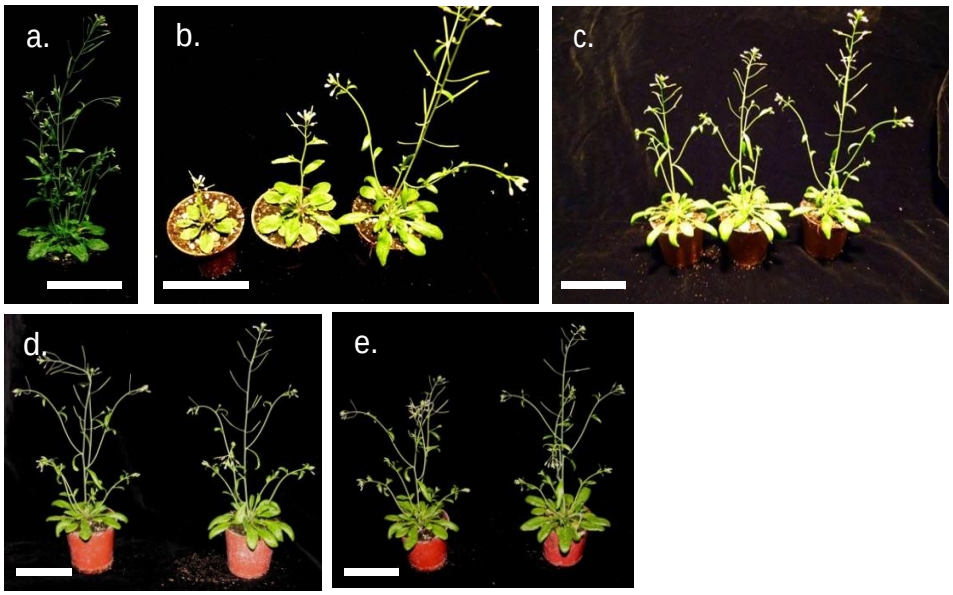


Figure 3.7: Phenotypes of *ARR14*-overexpressing *A. thaliana* lines. (a.) *35S::GFP-ARR14-1*, (b.) *35S::GFP-ARR14-2*, (c.) *35S::GFP-ARR14-3* and (d.) *35S::ARR14-1* in the age of 5 weeks. (e.) *35S::ARR14*-2. In (b.) - (e.) the mutant is depicted on the left side whereas the wild type is shown on the right side. *35S::GFP-ARR14* plants exhibited smaller rosettes, a stunted growth and sometimes a bushy appearance (a.). Overexpression of *35S::ARR14* caused no phenotype Plants were four to five weeks old. (d.-e.). Scale bars, 10 cm.

To examine a potential correlation between phenotype and *ARR14* expression level, quantitative realtime PCR analysis was performed with plants of different appearance (Figure 3.8). Leaf samples of plants were taken and RNA was extracted (chapter 2.5.5) After cDNA synthesis (chapter 2.5.3.6) samples were analyzed for the level of *ARR14* transcript in a Real-time PCR analysis (chapter 2.5.3.7). For amplification of *ARR14* transcript primer pair P59 and P60 were used (Appendix). The Houskeeping gene was amplified with primer pair P61 and P62 (Appendix). The WT transcript level was set to one.

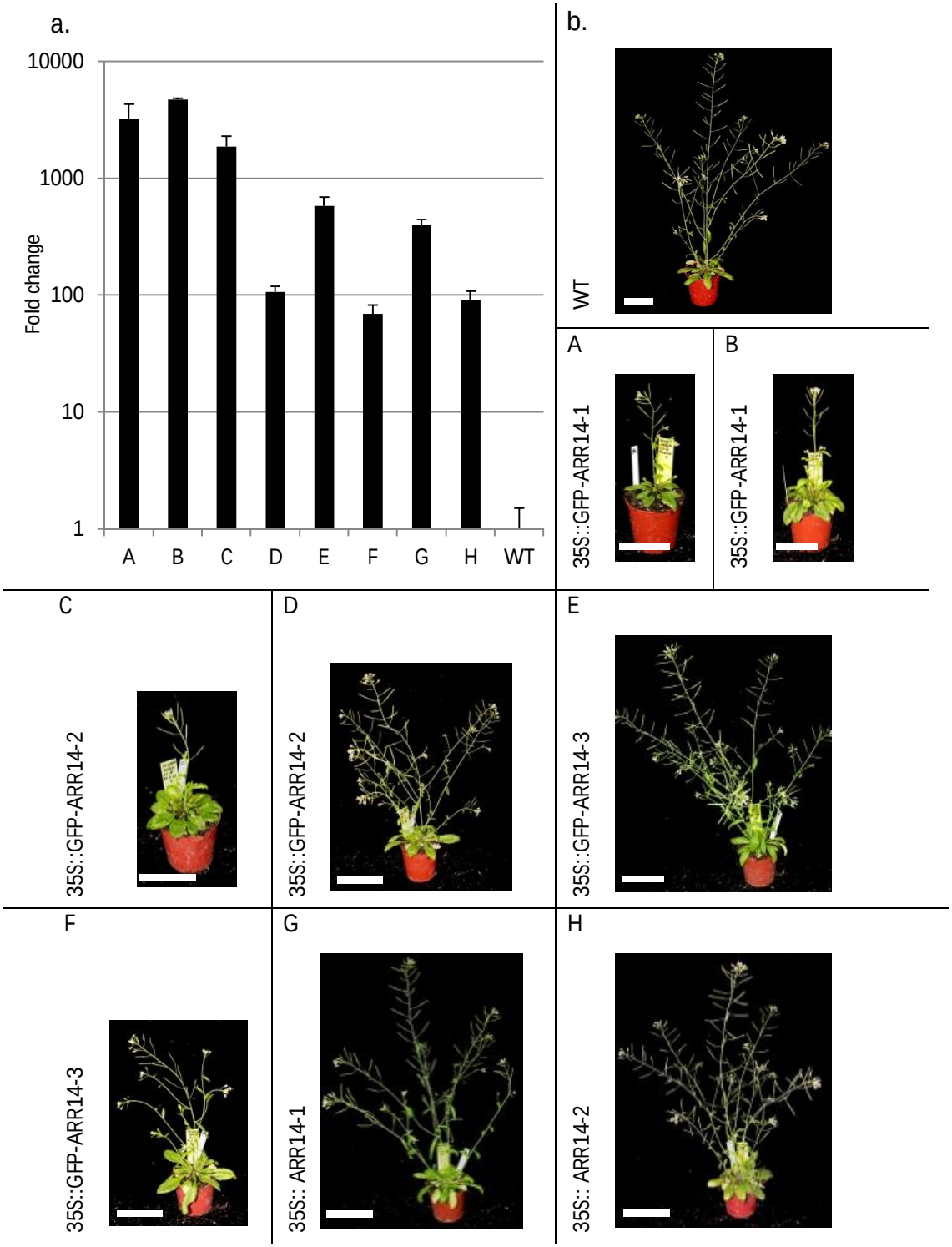


Figure 3.8: Level of overexpression of *ARR14* and phenotype of individual plants. (a.) ARR14 transcript levels in leaves of individual plants were examined by Real-time PCR analysis (chapter 2.5.3.7). The plants represented by the characters A-H are depicted in the table (b.). Small plants expressing *35S::GFP-ARR14-1* (A+B) exhibited a more than 1000-fold increase in the *ARR14* transcript level as well as a small plant of line-2 (C). A bigger plant expressing *35S::GFP-ARR14-2* (D) had a 100-fold higher *ARR14* transcript level than WT. *35S::GFP-ARR14-3* expressing plants (E+F) showed a 100-700-fold higher *ARR14* transcript level compared to WT. *35S::ARR14*-1 and -2 expressing plants (G+H) also showed an increase of *ARR14* transcript of 700- and 100-fold. Plants were seven weeks old. Scale bars. 10 cm

Stunted plants with a strong phenotype from 35S::GFP-ARR14-1 showed the highest transcript level, a more that 1000-fold overexpression of *ARR14* (Figure 3.8). This was also true for a smaller plant with a strong phenotype of 35S::GFP-ARR14-2. The bigger plant of that line exhibited lower levels of *ARR14* transcript but still 100 times higher than the WT. Since the variation was high in one population and also in the next one, probably the expressivity of *GFP-ARR14* is different. Classifying those plants into phenotypical groups would have been very artificial because changes were gradual. Therefore no quantification was done. The 35S::GFP-ARR14-3, which was not showing any GFP-signal, exhibited *ARR14* transcript levels of 100-700-fold of the WT-level. A smaller plant (Figure 3.8 plant F) did not show a higher *ARR14* expression level than a WT-like plant. This argues against a correlation between phenotype and ARR14 expression level. Two lines of the *35S::ARR14* expressing plants were tested. However, although having an *ARR14* expression level of 100-400-fold of the WT level no phenotype was visible.

3.1.4 The *arr14 loss-of-function* line did not show an obvious phenotype

The *ARR14* overexpressing lines exhibited an up to more than 1000-fold increased *ARR14* level in their leaves compared to WT. The level of ARR14 overexpression might correlate with the intensity of the phenotype (Figure 3.8). Another way to examine the function of a protein is to knock out the respective gene. To learn more about the function of ARR14 I also did *loss-of-function* analyses. Three different *Arabidopsis* lines with T-DNA insertions in *ARR14* were selected and analyzed for *ARR14* transcript. *arr14-1* was shown to have an insertion in the second exon within the receiver domain and is identical with the line published by Ishida and colleagues (Ishida *et al*., 2008b).

arr14-2 has an insertion in the third exon within the DNA-binding domain and in *arr14-3* the insertion is located in the *5'UTR* region of *ARR14* (Figure 3.9).

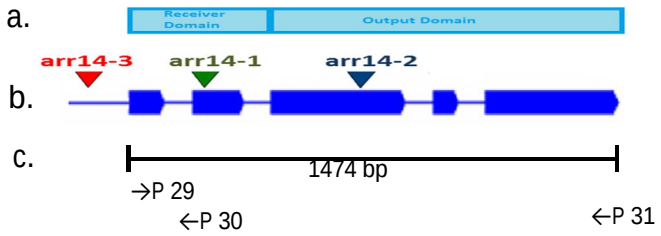


Figure 3.9: Model of the *ARR14* gene. (a.) Protein domain structure of ARR14. (b.) Gene structure of *ARR14* with positions of T-DNA insertions modified from "The Arabidopsis Information Resource" (TAIR, www.arabidopsis.org). Boxes represent exons, lines introns. The T-DNA positions in the different *Arabidopsis* lines are marked with arrows. Line *arr14-3* carries an insertion in the 5'UTR, line *arr14-1* has an insertion in the receiver domain (Ishida *et al*., 2008b) and line *arr14-2* carries an insertion in the DNA-binding domain; (c.) Vorresponding primer pairs for the reverse transcription-PCR (chapter 2.5.3.5). Primer pair P 29 and P 30 amplified the region between the start of *ARR14* and the T-DNA insertion in *arr14-1*, primer pair P 29 and P 31 amplified the full length *ARR14* transcript.

The three lines were examined for *ARR14* transcript by reverse transcription (RT)-PCR (chapter 2.5.3.5) (Figure 3.10) and the product was analyzed on an agarose gel (chapter 2.5.2.2). In lane 1 and 4 primers P 29 and P 31 were used (primers in Appendix). In lane 2 and 5 primers P 29 and P 30 were used (Figure 3.10). In lane 3 and 6 primers P 27 and P 28 were used to amplify the *ACTIN2* gene product for verification of the RNA quality. I observed PCR products in WT for full length *ARR14* (fl, primer P 29 and P 31), as well as for the short transcript (s, primer P 30 and P 29) and for ACTIN2. In the *arr14-1* line I observed PCR products for the short *ARR14* transcript (s, primer P30 and P29) in front of the T-DNA insertion and for *ACTIN2* but not for the full length *ARR14* fragmen (fl). Therefore I assume the line *arr14-1* to be a *loss-of-function* line. This has been verified by the publication of Ishida and colleagues in 2008 (Ishida et al., 2008b).
The other two lines, *arr14-2* and *arr14-3* showed transcripts with the *ARR14* full length primer pair and were excluded from analysis (data not shown).

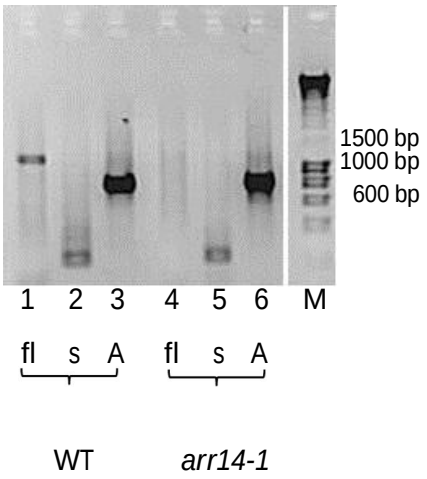


Figure 3.10: Agarose gel of the transcript analysis for *ARR14.* Lane 1-3: WT; lane 4-6: *arr14-1*. In lane 1 and 4 *ARR14* full length primers were used to amplify an about 1100 bp fragment (fl). In lane 2 and 4 primers that span the region in front of the T-DNA insertion were used to amplify a fragment of about 200 bp (s) and in lane 3 and 6 *ACTIN2* primers were used to amplify a fragment of about 800 bp (A) in the RT-PCR (chapter 2.5.3.5). M represents the marker HyperLadder™ 1kb from Bioline (Appendix).

By observing growth and development of *arr14-1* plants I could not detect any obvious phenotype (Figure 3.11). A delay in shoot development of two to seven days was already known from Hellmann (2007). In these experiments the rosette growth of *arr14-1* was slightly delayed as well as the inflorescence growth (Hellmann, 2007).

Figure 3.11: Habitus of *arr14-1*. WT (right) and *arr14-1* (left) plants are shown. No obvious phenotype was observed during the whole development. Plants were seven weeks old. Scale bar, 10 cm

3.1.5 Double mutants of *arr14* with *arr2*, *ahk2* and *ahk3* did not reveal any obvious phenotype

In a yeast two-hybrid analysis Dortay and colleagues discovered an interaction of ARR14 with ARR2 and with AHK2. This was also proven by a co-affinity purification experiments (Dortay, 2006). An interaction with AHK2 could represent a shortcut in cytokinin signaling by bypassing the AHPs. To verify the interactions, split-YFP experiments in tobacco were performed but no interaction could be detected (data not shown). To study the possible genetic interaction *in vivo* and *in planta*, the *arr14-1 loss-of-function* line was crossed with *loss-of-function* mutants of ARR2 (*arr2-4*, Mason *et al*., 2005) and AHK2 (*ahk2-5*, Riefler *et al*., 2006). To test if signaling via a possible AHK2-ARR14 interaction might be important, *arr14-1* was also crossed with *ahk3-7* (Riefler *et al*., 2006). If the interaction was relevant *arr14 ahk3* should have looked similar to the semi-dwarfed *ahk2 ahk3* mutant (Nishimura *et al*., 2004; Riefler *et al*., 2006). *arr14-1* was not crossed with *ahk4* because ARR14 and AHK4 are closely genetically linked. After crossing (chapter 2.6.2) the T2 generation was analyzed for the presence of T-DNA insertions. For this, PCRs (chapter 2.5.3.4) with the respective primer pairs (Appendix) were performed and analyzed on agarose gels (chapter 2.5.2.2).

None of the resulting isolated double mutants exhibited any obvious aberration in growth or development compared to WT (Figure 3.12). For a more detailed analysis *arr14 ahk2* was included in experiments for cytokinin sensitivity and stress resistance.

Figure 3.12: General phenotype of homozygous double mutants with *arr14-1. arr14-1* was crossed with *loss-of-functio*n lines of its potential interactors and homozygous progeny was compared to WT (right). (a.) *arr2 arr14*. (b.) *ahk2 arr14*. (c.) *ahk3 arr14.* Plants were about four weeks old. Homozygous mutants were identified by PCR (chapter 2.5.3.1., 2.5.3.2 and 2.5.3.4) on DNA extractions (chapter 2.5.4) with primer pairs binding in the T-DNA and surrounding the T-DNA (Appendix). There were no abnormalities in segregation. Scale bars, 10 cm

3.1.6 Cytokinin-related functional characterization of ARR14

ARR14 is a type-B response regulator and might therefore be involved in cytokinin signaling as transcription factor. The transactivation capacity of ARR14 on the *ARR6* promoter was tested. Additionally assays for determination of the cytokinin-sensitivity were performed with the *arr14-1* line.

3.1.6.1 ARR14 transactivated the *ARR6* promoter to a lower extent than *ARR12*

The transactivation capacity of ARR14 on the *ARR6* promoter was tested in protoplast transient assays and compared to the promoter without effector (empty effector vector) and with ARR12 as effector (chapter 2.6.11). In the experiments a 350 bp (Figure 3.13a) or 1000 bp (Figure 3.13b) fragment of the *ARR6* promoter were coupled to a *uidA* gene and used as reporter. All experiments were performed with 500 nm *trans*-zeatin (induced) and without *trans*-zeatin (uninduced). ARR14 exhibited no transactivation activity on a *uidA* gene coupled to the multiple cloning site of the vector (data not shown). The transactivation capacity of ARR14 without cytokinin on the 350 bp fragment of the *ARR6* promoter was about 50 % lower than for ARR12 and slightly above vector control. ARR14 showed no transactivation capacity on the 1000 bp fragment of the *ARR6* promoter with cytokinin. In the In contrast to ARR12, ARR14 increased the *ARR6* promoter output independent of cytokinin supply. The increase was between 40 % on the 350 bp fragment of the *ARR6* promoter and 30 % on the 1000 bp fragment of the *ARR6* promoter compared to the control (Figure 3.13). p*ARR6*^{350bp} was found to be sufficient for the full cytokinin response in protoplasts (Ramireddy, 2009).

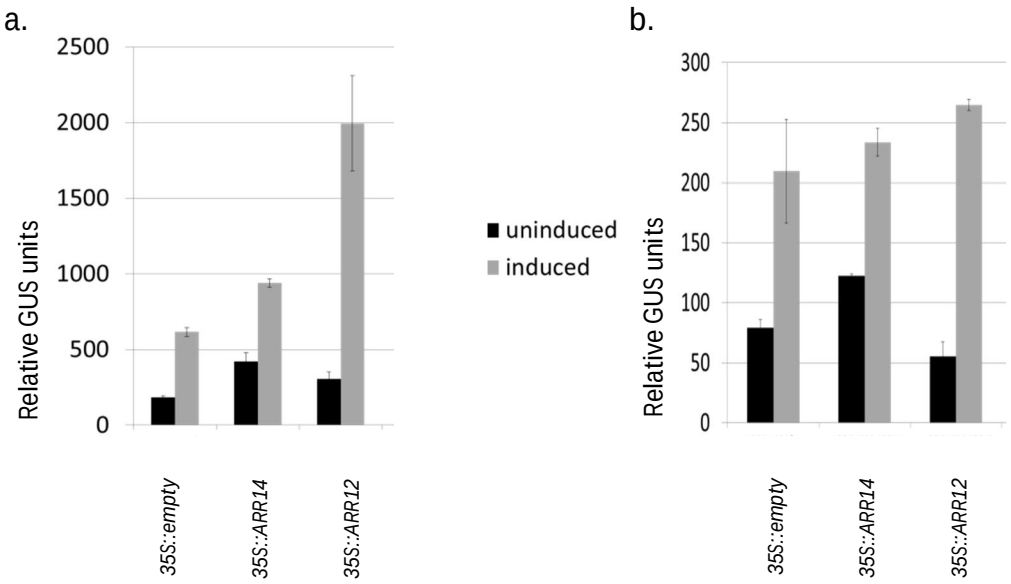


Figure 3.13: Tran**sactivation capacity of ARR14** on the *ARR6*-promoter i**n protoplast transient assays. (**a.) Transactivation capacity on *pARR6*^{350bp}. **(b.)** Transactivation capacity on *pARR6*^{1000bp}. Transactivation capacity of *ARR14* was compared to overexpression of the empty effector vector and *ARR12*. Overexpression of the empty vector with the *pARR6* promoter represents the endogenous reaction without effector. All protoplast transient assays were performed without (uninduced) and with (induced) 500 nm *trans*-zeatin and in triplicate (chapter 2.6.11). The calculation of relative GUS values was performed as by Ehlert (Ehlert *et al.*, 2006).

To test the involvement of ARR14 in cytokinin signaling, the root length and lateral root induction on media containing different cytokinin concentrations were assayed in the *arr14-1* mutant line. Cytokinin inhibits root elongation and lateral root development with increasing concentrations. A difference in this inhibition compared to WT indicates altered cytokinin sensitivity and a function of the examined protein in cytokinin signaling or metabolism.

I assayed root elongation and lateral root number of plants grown for ten days on media containing 0 µM, 0,01 µM, 0,05 µM, 0,1 µM and 1 µm benzyladenine. On none of the cytokinin concentrations *arr14-1* showed differences to the WT. The only significant change was the increase in lateral root number of *arr14* and *arr14 ahk2* without cytokinin (Figure 3.14 and 3.15).

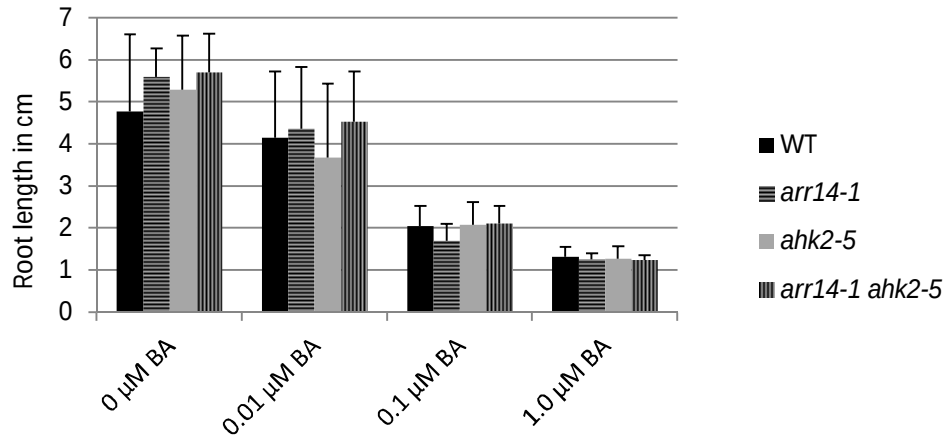


Figure 3.14: Root length on different cytokinin concentrations. Root growth on media containing 0 µM, 0,01 µM, 0,05 µM, 0,1 µM and 1 µm benzyladenine was assayed after ten days of growth on vertical MS plates. *arr14-1* and *ahk2-5*, as well as the double mutant were compared to the wild type. In none of the tested conditions *arr14-1*, *ahk2* or a*rr14 ahk2* exhibited differences to WT in root length. n_{WT} = 63, n_{arr14} = 21, n_{ahk2} = 21, $n_{arr14\ ahk2}$ = 21 for each condition. The experiment was repeated with a similar result.

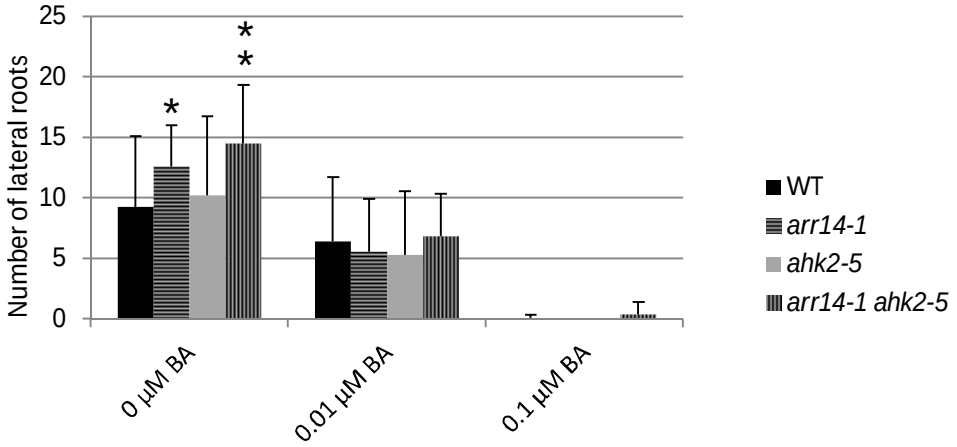


Figure 3.15: Lateral root number on different cytokinin concentrations. Lateral root numbers on media containing 0 µM, 0,01 µM, 0,05 µM, 0,1 µM and 1 µm benzyladenine were assayed after ten days of growth on vertical MS plates. *arr14-1* and *ahk2-5*, as well as the double mutant were compared to the wild type. *arr14-1* and *arr14 ahk2* plants had significantly more lateral roots than WT and *ahk2* on media without cytokinin. Asterisks represent statistically relevant differences to WT. * $p < 0,05$. ** $p < 0,005$. n_{WT} = 63, n_{arr14} = 21, n_{ahk2} = 21, $n_{arr14\ ahk2}$ = 21 for each condition . The experiment was repeated with a similar result.

3.1.6.2 The expression of the type-A response regulator genes *ARR15* and *ARR17* was strongly reduced in the *arr14 loss-of-function* line

The putative transcription factor ARR14 showed a minor transactivation capacity on a 350 bp fragment of the *ARR6* promoter. To test if ARR14 might be involved in type-A response regulator gene regulation other than *ARR6,* seedlings of the *loss-of-function* line *arr14-1* were analyzed for the transcriptional profile in a Real-time PCR experiment (chapter 2.5.3.7, for primer pairs see Appendix). Type-A response regulators represent common target genes of type-B response regulators (Argyros *et al*., 2008; Heyl *et al*., 2008; Ishida *et al*., 2008b). For ARR14 a function in senescence was indicated by earlier experiments (Hellmann, 2007). Therefore I examined also the expression of the senescence indicator *SAG12.* Additionally, I analyzed the expression of the auxin transport protein *PIN5* to examine a possible crosstalk of cytokinin and auxin signaling (recently reviewed by Hwang *et al*., 2012). The majority of the type-A response regulators tested did not exhibit significant changes in expression in *arr14-1* but the expression of two type-A response regulators was significantly reduced. The transcript level of *ARR15* was strongly decreased to about ten % of the wild type level as well as for *ARR17* which was decreased to about 25 % of the wild type level. A minor decrease in gene expression of about 50 % in *arr14-1* was observed for *ARR5*, *ARR6*, *ARR7* and *ARR16*. *PIN5* expression was about 75 % lower in *arr14-1* compared to WT. *SAG12* expression was slightly reduced in *arr14-1* to about 50 % of the wild type level (Figure 3.16).

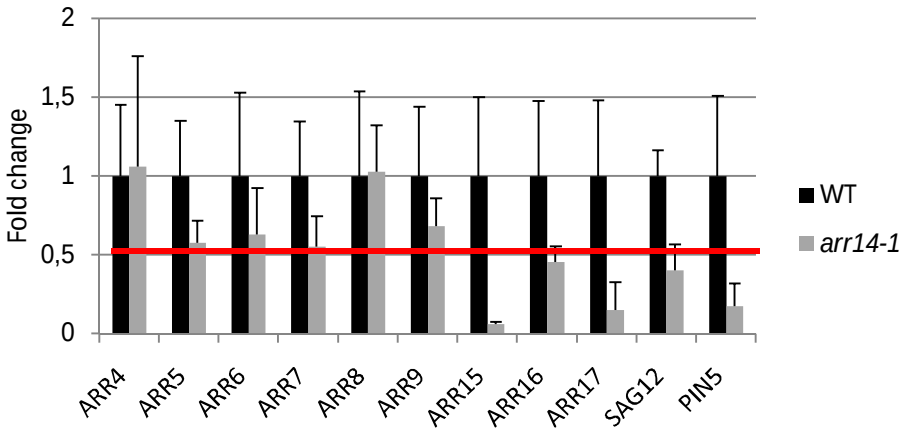
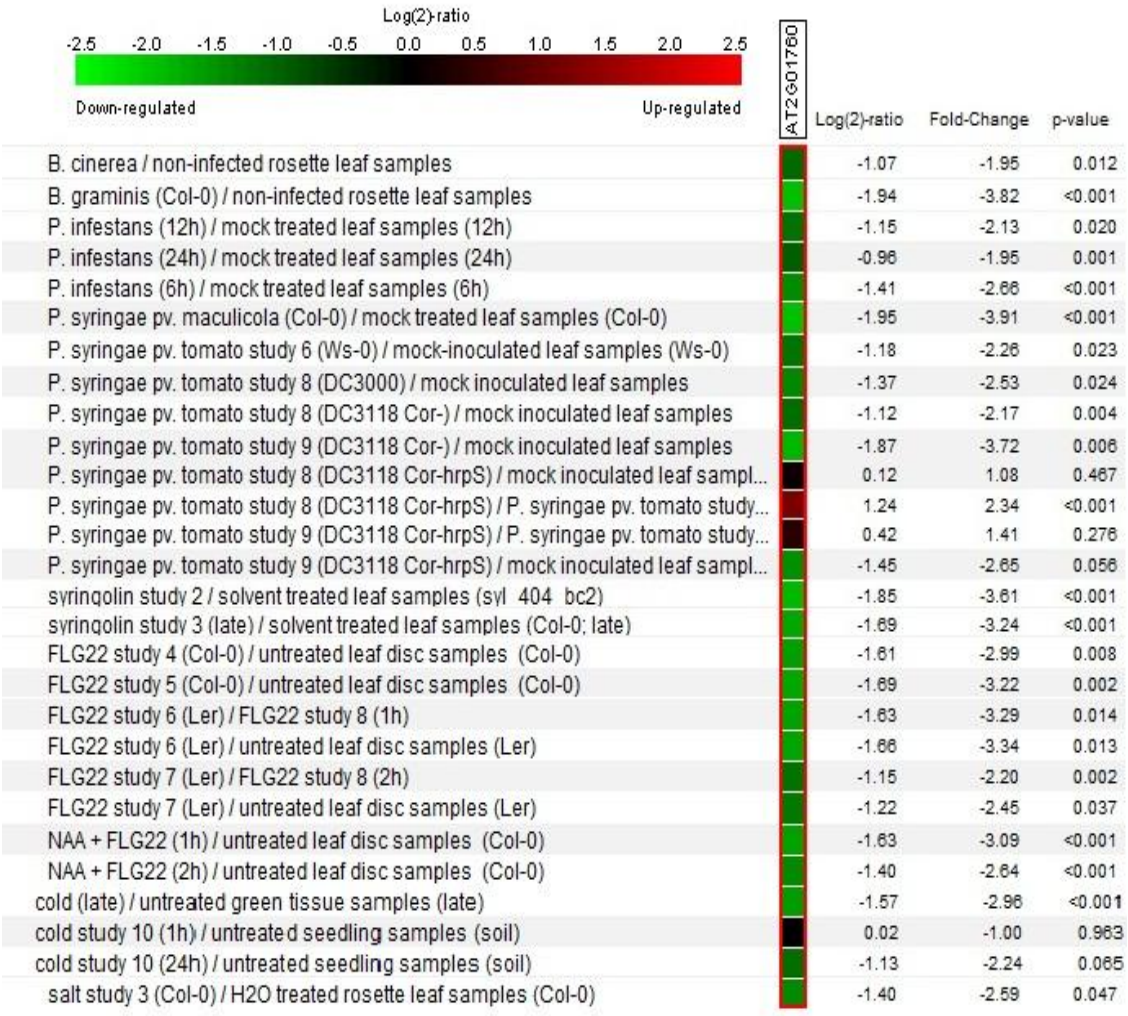


Figure 3.16: Transcript level of selected genes in *arr14-1* seedlings. The expression of several type-A response regulators, *SAG12* and *PIN5* in five day old seedlings was examined by Real-time PCR analysis (chapter 2.5.3.7). The WT expression level for the genes was set on 1 and used as reference for regulation in *arr14-1*. The red line represents the fold change 0,5 that is the threshold for down-regulation. Strong reductions in expression were observed in *arr14-1* for *ARR15*, *ARR17* and *PIN5*. *SAG12* expression seemed to be slightly reduced. Experiments were performed in technical triplicates and biological duplicates.

3.1.7 Functions of ARR14 beyond cytokinin signaling

To obtain clues of possible functions of ARR14 I analyzed expression data available on the current version of Genevestigator Response Viewer *in silico* (Hruz *et al*., 2008). *ARR14* transcription was shown to be regulated by exposure to pathogens like *Botrytis cinerea*, *Blumeria graminis*, *Phytophtora infestans*, *Pseudomonas syringae* and the elicitors syringolin and flagellin 22 (FLG22). Additionally the data indicated a regulation by cold stress and salt stress (Figure 3.17). Earlier studies revealed that ARR14 possibly is also involved in senescence (Hellmann, 2007). Preleminary results had indicated a delay in senescence and a changed resistance to *Verticillium longisporum* in the *arr14-1* mutant (Hellmann, 2007).

Based on those data the *arr14-1* line was tested for the performance in osmotic stress, cold stress, senescence progression and resistance towards *V. longisporum*.



	Log(2)-ratio	Fold-Change	p-value
B. cinerea / non-infected rosette leaf samples	-1.07	-1.95	0.012
B. graminis (Col-0) / non-infected rosette leaf samples	-1.94	-3.82	<0.001
P. infestans (12h) / mock treated leaf samples (12h)	-1.15	-2.13	0.020
P. infestans (24h) / mock treated leaf samples (24h)	-0.96	-1.95	0.001
P. infestans (6h) / mock treated leaf samples (6h)	-1.41	-2.66	<0.001
P. syringae pv. maculicola (Col-0) / mock treated leaf samples (Col-0)	-1.95	-3.91	<0.001
P. syringae pv. tomato study 6 (Ws-0) / mock-inoculated leaf samples (Ws-0)	-1.18	-2.26	0.023
P. syringae pv. tomato study 8 (DC3000) / mock inoculated leaf samples	-1.37	-2.53	0.024
P. syringae pv. tomato study 8 (DC3118 Cor-) / mock inoculated leaf samples	-1.12	-2.17	0.004
P. syringae pv. tomato study 9 (DC3118 Cor-) / mock inoculated leaf samples	-1.87	-3.72	0.006
P. syringae pv. tomato study 8 (DC3118 Cor-hrpS) / mock inoculated leaf sampl...	0.12	1.08	0.467
P. syringae pv. tomato study 8 (DC3118 Cor-hrpS) / P. syringae pv. tomato study...	1.24	2.34	<0.001
P. syringae pv. tomato study 9 (DC3118 Cor-hrpS) / P. syringae pv. tomato study...	0.42	1.41	0.276
P. syringae pv. tomato study 9 (DC3118 Cor-hrpS) / mock inoculated leaf sampl...	-1.45	-2.65	0.056
syringolin study 2 / solvent treated leaf samples (syl 404 bc2)	-1.85	-3.61	<0.001
syringolin study 3 (late) / solvent treated leaf samples (Col-0; late)	-1.69	-3.24	<0.001
FLG22 study 4 (Col-0) / untreated leaf disc samples (Col-0)	-1.61	-2.99	0.008
FLG22 study 5 (Col-0) / untreated leaf disc samples (Col-0)	-1.69	-3.22	0.002
FLG22 study 6 (Ler) / FLG22 study 8 (1h)	-1.63	-3.29	0.014
FLG22 study 6 (Ler) / untreated leaf disc samples (Ler)	-1.66	-3.34	0.013
FLG22 study 7 (Ler) / FLG22 study 8 (2h)	-1.15	-2.20	0.002
FLG22 study 7 (Ler) / untreated leaf disc samples (Ler)	-1.22	-2.45	0.037
NAA + FLG22 (1h) / untreated leaf disc samples (Col-0)	-1.63	-3.09	<0.001
NAA + FLG22 (2h) / untreated leaf disc samples (Col-0)	-1.40	-2.64	<0.001
cold (late) / untreated green tissue samples (late)	-1.57	-2.96	<0.001
cold study 10 (1h) / untreated seedling samples (soil)	0.02	-1.00	0.963
cold study 10 (24h) / untreated seedling samples (soil)	-1.13	-2.24	0.065
salt study 3 (Col-0) / H2O treated rosette leaf samples (Col-0)	-1.40	-2.59	0.047

Figure 3.17: Expression data of *ARR14* in stress conditions. *In silico* expression analysis revealed, that *ARR14* transcript seems to be mostly regulated via a reduction in transcript level. Regulation occurred by pathogens, elicitors, cold stress and salt stress. Modified from Genevestigator Response Viewer (Hruz *et al*., 2008).

3.1.7.1 The *arr14 loss-of-function* line was more resistant towards cold stress

To examine if ARR14 is involved in abiotic stress response, I analyzed root elongation and lateral root number in cold stress conditions and germination and growth in osmotic stress conditions.

For cold stress conditions plants were grown on vertical agar plates and after five days transferred to eleven °C (similar to Zhang *et al*., 2008). Three days later root elongation and the number of lateral roots were assayed. The experiment was conducted twice giving similar results. *arr14-1* plants performed significantly better than the WT. They exhibited an increased root elongation compared to WT. In contrast to *arr14, ahk2 arr14* roots were significantly shorter (Figure 3.18a). However, cold stress had no impact on lateral root number in this assay (Figure 3.18b).

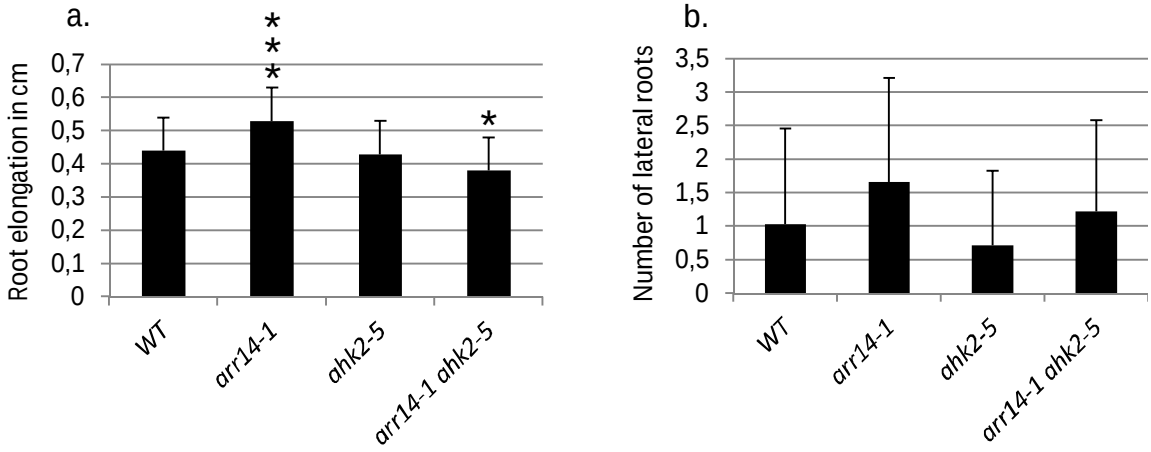


Figure 3.18: Performance of mutants plants in cold stress conditions. *arr14*, *ahk2* and the *arr14 ahk2* double mutant were analyzed for root elongation (a.) and number of lateral roots (b.) in cold stress conditions. Plants were grown on vertical plates and transferred to 11 °C after five days. Three days later, root elongation and lateral root number were measured. *arr14-1* roots were significant longer than WT roots after cold treatment. Roots of *arr14 ahk2* were significantly shorter. There were no significant differences in lateral root number for all phenotypes. Asterisks represent statistically relevant differences to WT. * $p < 0,05$, *** $p < 0,0005$. $n_{WT} = 121$, $n_{arr14} = 100$, $n_{ahk2} = 110$, $n_{arr14\ ahk2} = 90$. The experiment was repeated with a similar result.

The germination rate can be inhibited by osmotics (Lee and Zhu, 2010). To analyze a possible function of ARR14 in osmotic stress response, the germination rate of *arr14-1* on different glucose concentrations was examined (Figure 3.19) after three days. None of the glucose concentrations caused a different germination rate of *arr14-1* plants compared to WT. At 7,5% glucose, the germination rate was about 80% for both genotypes. At 10 % glucose the germination of both, *arr14-1* and WT, was strongly impaired and decreased to five to ten % compared to control conditions.

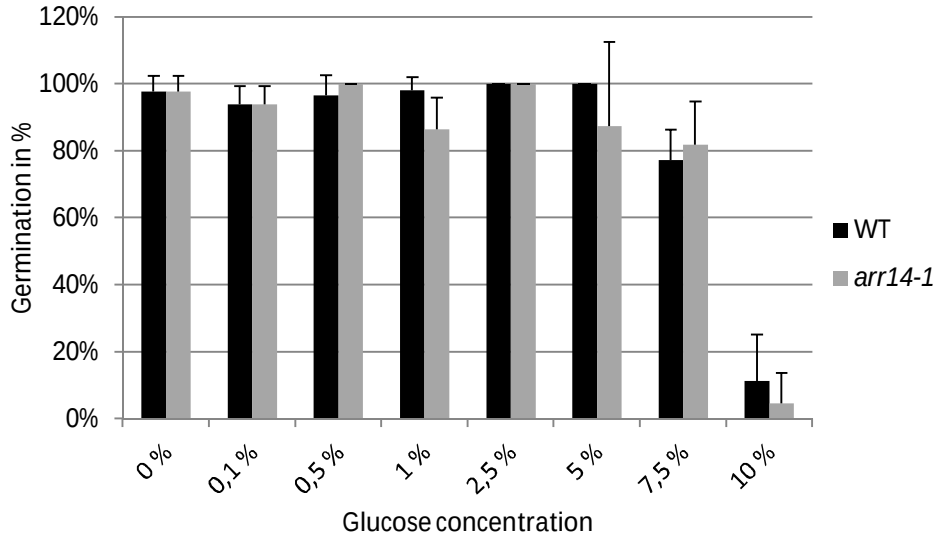


Figure 3.19: Germination rate of *arr14-1* and WT on different glucose concentrations. Seeds were plated on media containing 0 – 10 % glucose. The germination rate after three days on glucose concentrations lower than 7,5% glucose was close to 100% for WT and *arr14-1*. At 7,5% glucose, the germination rate dropped to about 80% and at 10% glucose to about 10%. There was no significant difference between *arr14-1* and WT. $n_{WT} = 44$, $n_{arr14} = 44$ for each condition.

I also analyzed root growth in osmotic stress conditions. Here fore I supplied 2 %, 4 % and 6 % glucose, mannitol or polyethyleneglucol (PEG) to the growth media of *arr14-1* and WT to induce osmotic stress. Root length and lateral root number were determined after ten days of growth. None of the stress treatments had a significant effect on lateral root number. The results for the root length were not repeatedly significant (data not shown).

Glucose can induce osmotic stress in plants but it can also act as a signal to influence senescence (Wingler *et al*., 2006) and growth (Zheng, 2009) by modulation of the C-N-ratio. To test if ARR14 is involved in these processes, the dry weight of *arr1-14* and WT grown on different glucose concentrations for 2 weeks was measured (Figure 3.21).
There was no significant difference observed in dry weight of *arr14-1* compared to WT at any glucose concentration.

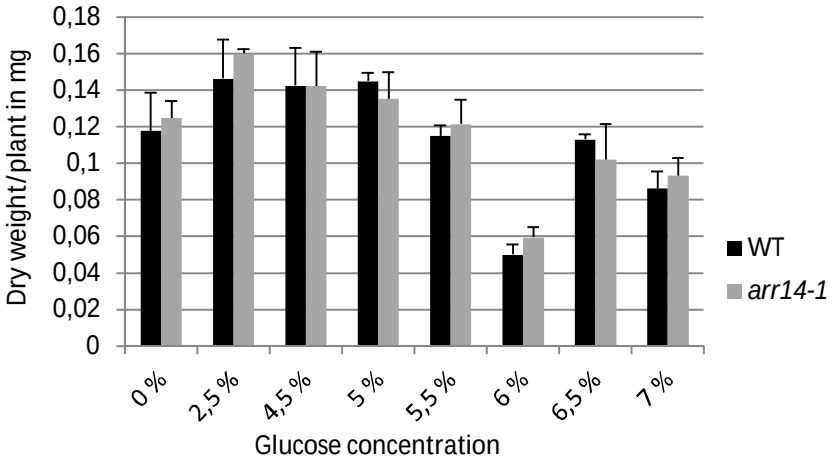


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Figure 3.21: Dry weight of *arr14* and WT on different glucose concentrations. Plants were grown for two weeks on media containing 0 % - 7 % glucose and the dry weight was measured. There was no significant difference in dry weight between *arr14-1* and WT. $n_{WT} = 4$, $n_{arr14} = 4$ for each condition.

3.1.7.2 The *arr14 loss-of-function* line exhibited a delay in senescence in short day conditions

Preliminary results indicated a function of ARR14 in senescence onset and progression. The senescence of *arr14-1* plants was delayed for up to seven days in short day conditions (Hellmann, 2007). To examine this further, the progression of senescence of the 6th and the 10th leaf were analyzed in three independent experiments in short day conditions (chapter 2.6.5). The portions of leaves in the observed senescence stages: margin, 1/4 , 1/3, 1/2, 2/3, 3/4 and 1 (whole leaf area) were determined (Figure 3.22).

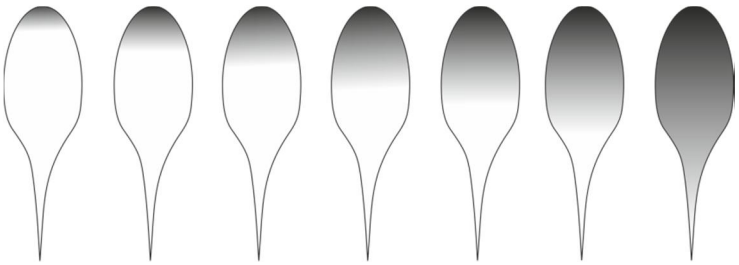


Figure 3.22: Definition of senescence stages. From left to right the stages were named margin, 1/4 , 1/3, 1/2, 2/3, 3/4 and 1 (whole leaf area). Grey represents the yellowing of the leaf.

The senescence progression of the 6th leaves is depicted as an example in figure 3.23a (WT) and 3.23b (*arr14*). It was not possible to combine all three experiments performed in one graph representing the different leaf stages. The time until the last 10th leaves were yellow varied within the experiments, possibly due to seasonal effects.

arr14-1 showed a delay in senescence in all experiments. This can be seen e. g. from day 44 after germination (44 DAG) until day 54 and on day 68 after germination in leaf six. Until day 54 DAG the portion of completely green leaves was about 80 % in *arr14-1* whereas in the WT it was about 70 %. On day 68 DAG e.g. 70 % of *arr14* leaf six were completely senescent whereas in WT this is the case for 80 % of leaf 6. The senescence onset of *arr14-1* leaves was delayed about 4 days as well as the time point of 95 % yellowing was delayed about two days in the experiment depicted.

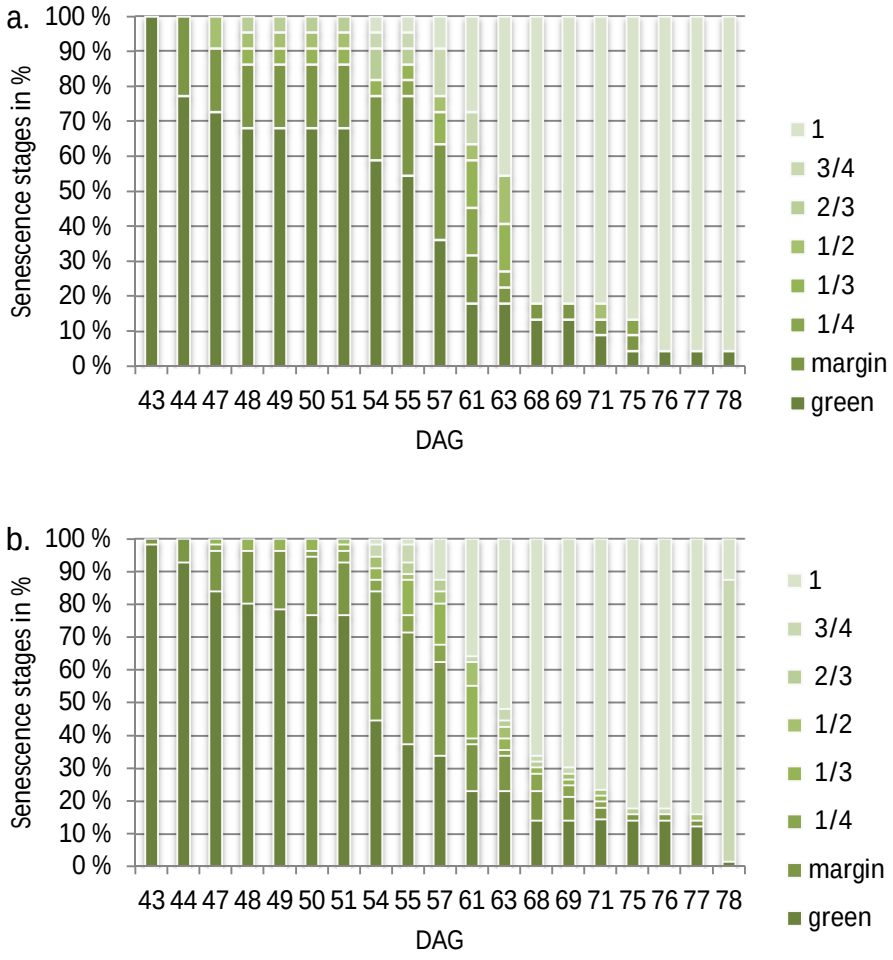


Figure 3.23: Senescence stages of WT and *arr14-1* leaf six in short day conditions. Depicted is one example experiment under short day conditions. Leaf senescence stages (Figure 3.22) were analyzed from day 43 after germination to day 78 after germination (chapter 2.6.5). Senescence stages of (a.) WT and (b.) *arr14-1. arr14-1* leaves stayed completely green longer and the portion of completely yellowed leaves was lower compared to the WT. Until 54 days after germination (DAG) the portion of green leaves was about 80% for *arr14-1* and 70% for the WT. From 54 DAG to 63 DAG the portion of green leaves was lower in *arr14-1* and the portion of yellow leaves higher compared to WT. From then on to 78 DAG the portion of yellow leaves was slightly lower in *arr14-1* compared to WT. $n_{WT} = 22$, $n_{arr14} = 56$

For a more detailed analysis of senescence progression, the portion of senescent leaves was plotted over the days after germination. To compare the three independent experiments, trend lines were plotted (Figures 3.24 and 3.25). The polynomic functions of those trend lines were used to extrapolate a mean trend line for three experiments and indirectly compare them that way (Figures 3.26 and 3.27).

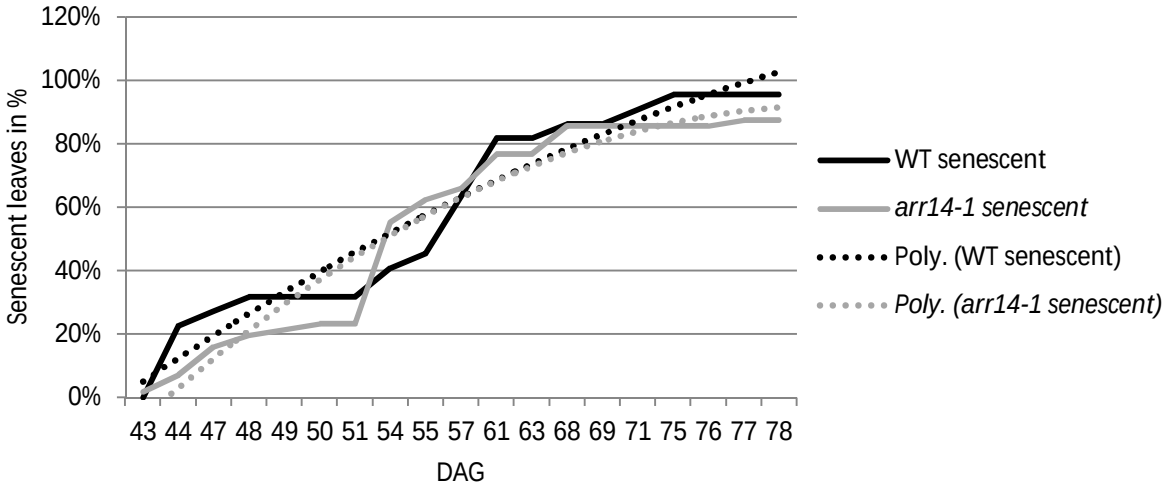


Figure 3.24: Percentage of leaves six of WT and *arr14-1* showing senescence in short day conditions. Depicted is one exemplary experiment. Polynomic trend lines are represented by dotted lines and were named Poly.(WT senescent) and Poly.(*arr14-1 senescent*). The polynomic function of the trend line for WT is $y=-0{,}0011x^2+0{,}0773x-0{,}0254$ and the polynomic function for *arr14* is $y=-0{,}0026x^2+0{,}105x-0{,}1708$. Between 44 DAG and 54 DAG and between 71 DAG and 78 DAG the portion of senescent leaves was lower in *arr14-1* than in WT. This was also reflected by the trend lines. $n_{WT} = 22$, $n_{arr14} = 56$

For leaf six there seemed to be differences in the portion of senescent leaves between *arr14-1* and WT. Until 54 DAG *arr14-1* senescence was delayed. From then onwards the portion of senescent leaves was higher or similar to WT until 71 DAG. Then *arr14-1* had a lower percentage of senescing leaves. Thus senescence in *arr14-1* was delayed again. This means as well the onset of senescence as also the culmination in complete yellowing was delayed.

The trend lines of *arr14* and WT were different (Figure 3.24). Until day 54 and again from day 63 onwards the trend line for WT was above the trend line of *arr14*. In the beginning the ascent for the *arr14* trend line was higher, from day 63 onwards; the ascent of the WT trend line was higher. For the interjacent time, the lines were congruent.

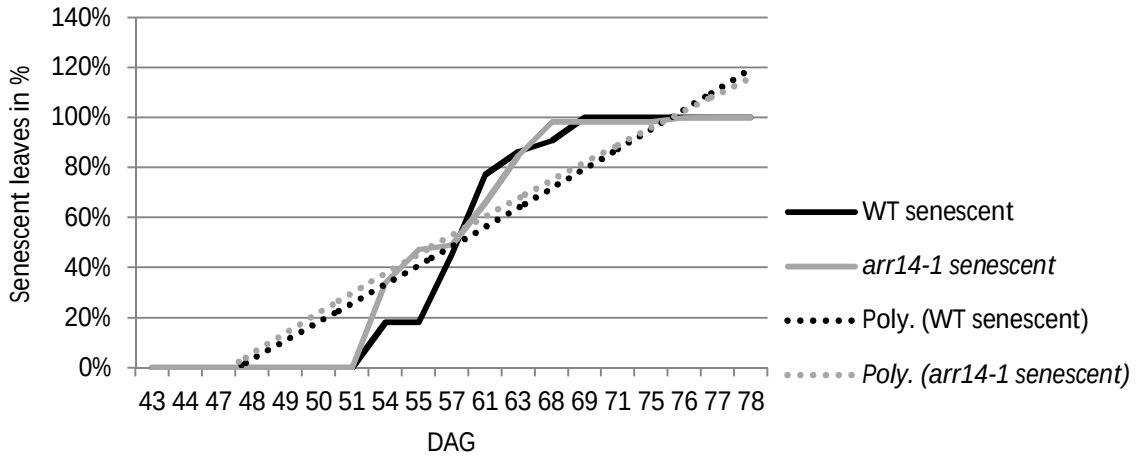


Figure 3.25: Percentage of leaves ten of WT and *arr14-1* showing senescence in short day conditions. Depicted is one exemplary experiment. Polynomic trend lines are represented by dotted lines and were named Poly.(WT senescent) and Poly.(*arr14-1 senescent*). The polynomic function of the trend line for WT is $y=0{,}0003x^2+0{,}0716x-0{,}2559$ and the polynomic function for *arr14* is $-0{,}0006x^2+0{,}0867x-0{,}28$. On 54 DAG - 57 DAG the percentage of senescent leaves in *arr14-1* was higher than for WT. From then onwards, there was no difference between *arr14-1* and WT observed. This was also reflected by the trend lines that appear nearly identical. $n_{WT} = 22$, $n_{arr14} = 56$

The senescence progression for leaf ten appeared different compared to the progression for leaf six. The portion of senescent leaves was higher in *arr14-1* than in WT from 54 DAG to 57 DAG. Later, there was no difference observed between the percentage of senescent leaves in *arr14-1* and WT (Figure 3.25).

To combine the results of all three experiments, I calculated an extrapolated trend line by computing the mean and the standard deviation from the three trend lines of the three independent experiments using their polynomic functions. The numbers on the X-axis represent time but not DAG. The number one in the y-axis represents 100% yellowing. The combination of trend lines for leaf six resulted in similar mean trend lines as in one experiment depicted in Figure 3.24. The trend line of leaf 6 from *arr14-1* was lower than in WT in the first third of the function. The trend lines for *arr14-1* and WT leaf ten were nearly identical (Figure 3.26, 3.27).

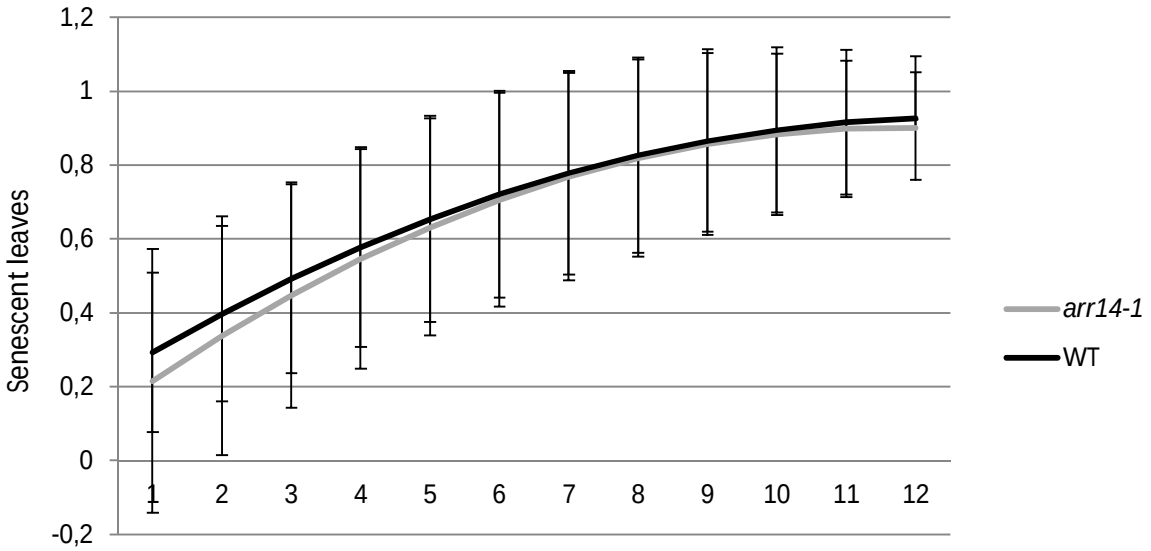


Figure 3.26: Extrapolated trend lines of the senescence progression of leaf six in short day conditions. The trend lines for three independent experiments for the senescence progression of leaf six in *arr14-1* and WT were combined. The numbers on the X-axis are the values used for calculation of the extrapolated trend line from the polynomic functions of the single trend lines. The number one in the y-axis represents 100% of yellowing. From datapoint 1-6 the portion of senescent leaves of *arr14-1* was lower than in WT. $n_{WT} = 68$, $n_{arr14} = 131$

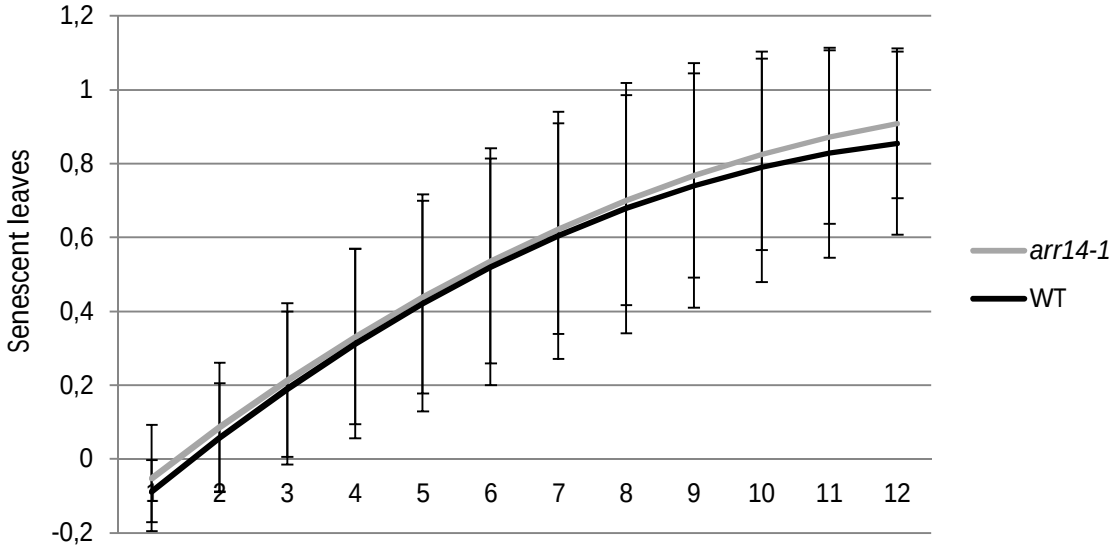


Figure 3.27: Extrapolated trend lines of the senescence progression of leaf ten in short day conditions. The trend lines for three independent experiments for the senescence progression of leaf ten in *arr14-1* and WT were combined. The numbers on the X-axis are the values used for calculation of the extrapolated trend line from the polynomic functions of the single trend lines. The number one in the y-axis represents 100% of yellowing. The combined trend lines of *arr14-1* and WT are nearly identical. $n_{WT} = 68$, $n_{arr14} = 131$

From 44 DAG to at least 56 DAG the portions of senescencent leaves six in *arr14-1* and WT were significantly different from each other in a Chi²-Test (when numbers of senescent/green plants ≥5) or a Fisher's exact test with a hypergeometric distribution analysis (Table 3.1). For leaf ten this was not the case to that extent

Table3.1: p-values of the statistical analysis of senescence in short day conditions for leaf six. Depicted are the experiments, the days after germination (DAG) and the corresponding p-values. Significance values derive from Fisher's Exact Tests. Asterisks mark Chi^2-tests.

Experiment 1		Experiment 2		Experiment 3	
DAG	p-value	DAG	p-value	DAG	p-value
44	0,00333317	43	not significant	41	1,0548E-11 *
46	3,439E-07 *	44	0,00283689	42	1,9548E-08 *
48	1,3655E-06 *	47	4,9944E-09 *	44	7,2402E-08 *
50	8,5849E-06 *	48	4,79E-09 *	47	4,8311E-08 *
52	2,7842E-06	49	7,0339E-09 *	49	1,3496E-08 *
55	7,856E-06	50	9,5399E-09 *	51	1,1244E-09
57	8,1172E-08	51	9,5399E-09 *	54	2,6237E-09
59	1,4391E-07	54	4,785E-09 *	56	3,9635E-09
62		55	2,7669E-09 *	58	
64		57	1,667E-08 *	61	
66		61	1,2526E-05	63	
69		63	1,2526E-05	68	
		68	8,2237E-06	70	
		69	8,2237E-06	72	
		71	6,8058E-07	75	
		75	7,0381E-08	77	
		76	7,0381E-08	79	
		77	9,9388E-08	82	
		78	9,9388E-08	84	

*Chi^2-test

3.1.7.3 The *arr14 loss-of-function* line was more resistant towards *V. longisporum*

As described in chapter 3.1.7 *ARR14* expression was regulated by pathogen attack. Preliminary results indicated that *ARR14* might play a role in *V. longisporum* infection. The previous test was performed in the greenhouse and *arr14-1* seemed to be less resistant towards *V. longisporum* compared to WT (Hellmann, 2007).

Susceptibility towards *V. longisporum* was assayed in two ways. Firstly the symptoms of the infection were scored. *V. longisporum* caused stunting, earlier senescence and more adventious shoots (Figure 3.28). The biomass can be influenced, so fresh weight was measured. Secondly the colonization of stems was analyzed. Stem segments were cut after the infection experiment and put on malt agar plates until *V. longisporum* grew out of the stem segments (Häffner *et al*., 2010). For the analysis of *arr14-1* susceptibility towards *V. longisporum* plant I scored height, fresh weight, adventious shoot number and percent of colonization.

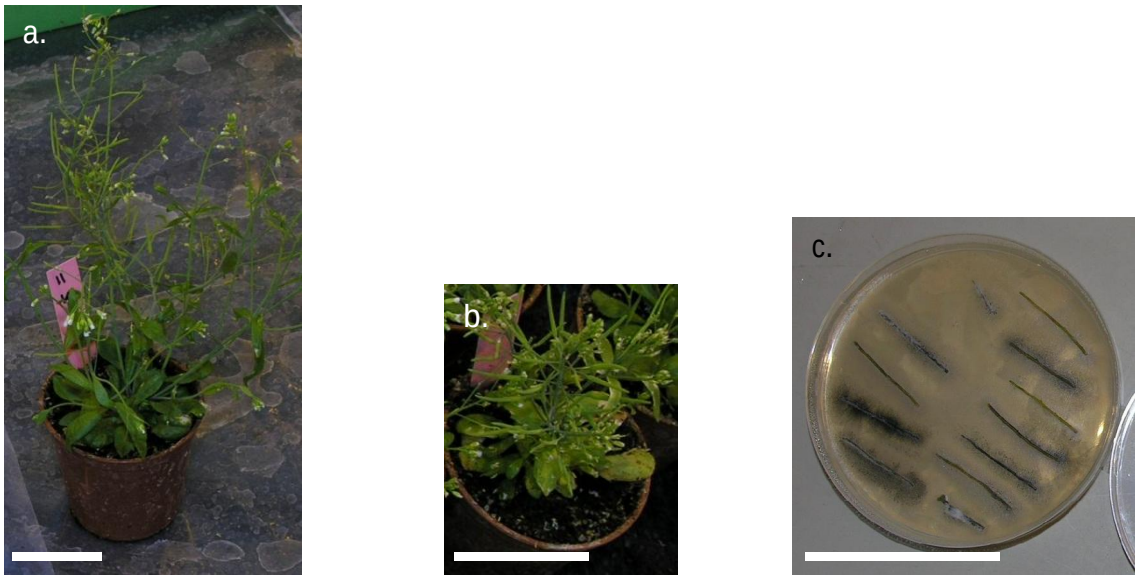


Figure 3.28: Observation of *V. longisporum* infection symptoms in *A. thaliana.* Plants were grown in climate chambers and photographed two weeks after incubation. After harvesting hypocotyl fragments were plated on malt agar and assayed for *Verticillium* growth after two weeks. (a.) *arr14* solvent control treated. (b.) *arr14* treated with *V. longisporum* spore suspension. Plants were stunted and often exhibited smaller rosettes, a lower fresh weight and more adventious shoots. (c.) Stem segments on malt agar plates and growth of *V. longisporum* indicating colonization.

I performed three independent experiments on the susceptibility of *arr14-1* and WT towards *V. longisporum*. One experiment was conducted in the greenhouse with less stable conditions. To reduce variability in growth of the plants the other two trials were performed climate chambers with autoregulating temperature and humidity.

Plants were grown on soil and after two weeks the root was cut. Subsequently the plants were inoculated for one hour with a spore solution of *V. longisporum* or a solvent control (mock)

(chapter 2.6.6). After ripening of the first siliques, the plants were scored for symptomes. Criteria were plant height, fresh weight, adventious shoot number and percentage of colonization. The only criteria showing a consistent difference in all experiments was the height of the main stem. *arr14-1* plants were significantly higher than the WT after treatment (Figure 3.29).

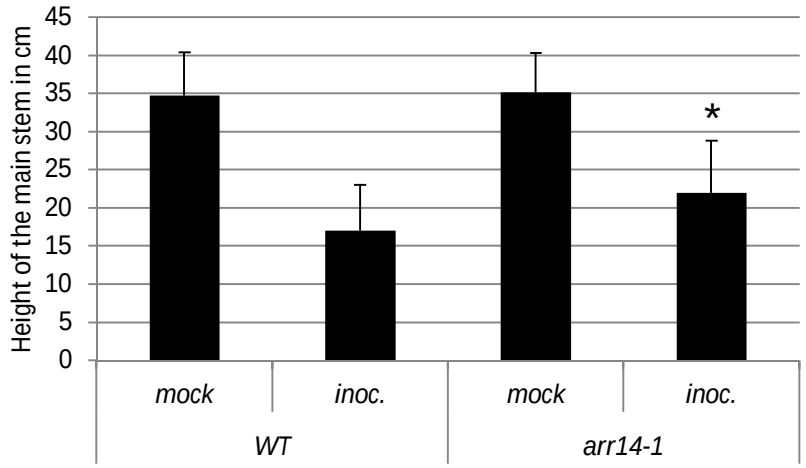


Figure 3.29: Height of the main stem of WT and *arr14-1* in the *Verticillium* experiment. Results of the experiments in the climate chamber were combined. Two to three week old plants were mock-treated (mock) or inoculated with *V. longisporum* spore suspension (inoc.) (chapter 2.6.6). The height of the main stem was measured after ripening of the first siliques in the sample set. Main stems of *arr14-1* were significantly higher after inoculation than those of WT. Mock-treated *arr14-1* and WT plants did not differ in height. Asterisks represent statistically relevant differences to WT. The experiment was repeated and showed similar results *; $p<0{,}00005$. $n_{WT\ mock} = 50$, $n_{arr14\ mock} = 56$, $n_{WT\ inoc.} = 64$, $n_{arr14\ inoc.} = 82$

A difference in the height of the main stem between *arr14-1* and WT might be caused by a difference in symptom development or colonization of the stem. To test this, the colonization of stem segments was examined. WT and *arr14-1* showed similar colonization levels. Colonization was about 60 %-97 % of all stem segments in *arr14*-1 and in WT in different experiments. WT colonization was set to 100%. The colonization of *arr14-1* plants was 104 % of WT colonization (Figure 3.30). This means that the increased resistance of *arr14-1* is due to a change in symptom development and not due to a decreased colonization.

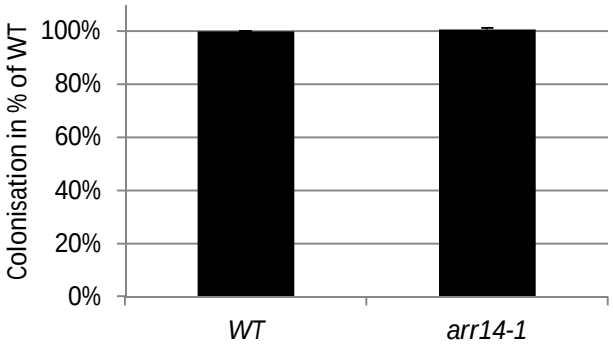


Figure 3.30: Colonization of stem segments of WT and *arr14-1* by *V. longisporum*. The colonization was examined after ripening of the first siliques in the sample set. Stem segments were cut and plated on malt agar. After two weeks the colonization was scored. The percentage of stem segments colonized by *V. longisporum* in *arr14-1* and WT was similar. Colonization in different experiments was between 60 % of all stems and 97 % of all stems. WT colonization was set to 100%. Colonization of *arr14-1 stem* segments is shown in percentage of WT colonization. $n_{WT\ mock} = 50$, $n_{arr14\ mock} = 56$, $n_{WT\ inoc.} = 64$, $n_{arr14\ inoc.} = 82$

3.2 A screen for modulators of the cytokinin response

To identify new modulators of the transcriptional cytokinin response, I developed and performed a genetic screen. The screen was planned to be medium to high throughput, fast and easy to use. As it should be widely applicable, I designed the screen to be moderately priced and not based on expensive laboratory equipment except for a microplate reader. As a reporter gene *luciferase (LUC)* was chosen because it allows fast and easy quantification of promoter activity in plants in a non-destructive manner. To accelerate the speed of the screen, I planned to use an overexpression system instead of e.g. EMS (ethyl metanesulfonate) mutagenized plants. In an overexpression approach, mutations are dominant and plants can be assayed for reporter activity already in the first transformed generation. To overcome space limitations and accelerate the speed, the screen was planned to be performed on small seedlings. If a T1 generation in a young stage is tested, all plants not showing a response in the screen can be sorted out quickly and just the selected candidates consume space in the green house. The overexpression of cDNAs enables the detection of redundant modulators for which the corresponding *loss-of-function* mutants do not show any effects e.g. lots of transcription factors (Wehner *et al*., 2011). In an overexpression approach, one can decide for a cDNA library suited for the research goal. The cDNAs in candidate plants will be easy to identify because the borders of the vector are known and along with this also primer binding sides. For my screen I used vectors that are GATEWAY™ compatible, enabling easy shuttling of the cDNA into other vectors. Usage of cDNA overexpression in an *in vivo* system and has the disadvantage, that

overexpression of some modulators might cause lethality. Therefore an inducible overexpression system was selected for the screen.

A vector combination convenient for the planned screen was the two vectors published by Papdi and colleagues (Papdi *et al*., 2008). It consistes of a luciferase reporter fused to a selected promoter and a vector enabling an estradiol-inducible overexpression of cDNA libraries. The effector was GATEWAY™ technology compatible and allowed to transfer GATEWAY™ compatible cDNA libraries efficiently into the vector (Figure 3.31).

The efficient transfer of GATEWAY™ compatible libraries was shown before (Bürkle *et al*., 2005).

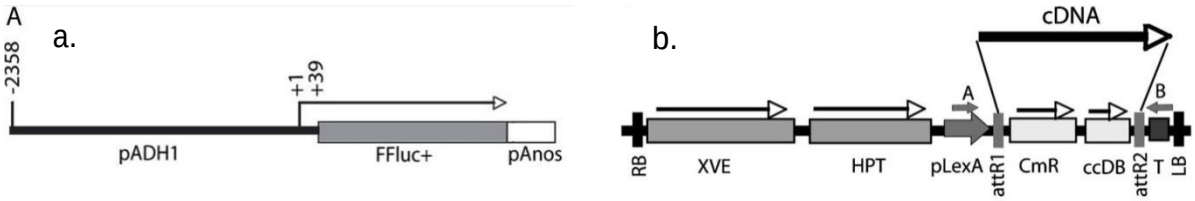


Figure 3.31: The two vectors utilized in the screen. (a.) The reporter vector carrying the selected promoter (in this case *pADH1*) driving a luciferase reporter gene (*FFluc+*). (b.) The effector vector enabling the estradiol-induced overexpression of a selected cDNA-library. *XVE* provides the estradiol-binding transcription factor which is binding to the *LexA*-promoter to activate cDNA transcription. *attR1 and attR2*-sites mark GATEWAY™ technology attachment sites for site specific recombination. *ccDB* is a bacterial suicide gene. RB and LB stand for right and left border for *Agrobacterium*-facilitated T-DNA integration into plants. *HPT* stands for hygromycin resistance. pAnos and T are terminators (Papdi *et al*., 2008).

3.2.1 The *ARR6* promotor was choosen as reporter of the cytokinin response

The next step was the selection of a suitable promoter enabling quantification of the cytokinin response. Type-A response regulator transcription is known to respond to cytokinin treatment. One of the best studied type-A response regulator genes is *ARR6*. To and colleagues showed the cytokinin induction in *pARR6*^{2146bp}*::uidA Arabidopsis* plants (Figure 3.32) (To *et al*., 2004). Seedlings in an age of five to seven days were planned to be examined in the screen. To check if *ARR6* is expressed in that stage I used the eFP browser for an *in silico* analysis. Combined microarray data in the eFP browser showed, that *ARR6* was ubiquitously expressed and was also expressed in young seedlings (Figure 3.32) (Winter *et al*., 2007).

Analysis by Ramireddy had revealed that the *ARR6* promoter is a suitable tool to analyze cytokinin response. A 350 bp promoter fragment was sufficient for nearly the full cytokinin induction in protoplast transient assays (Ramireddy, 2009).

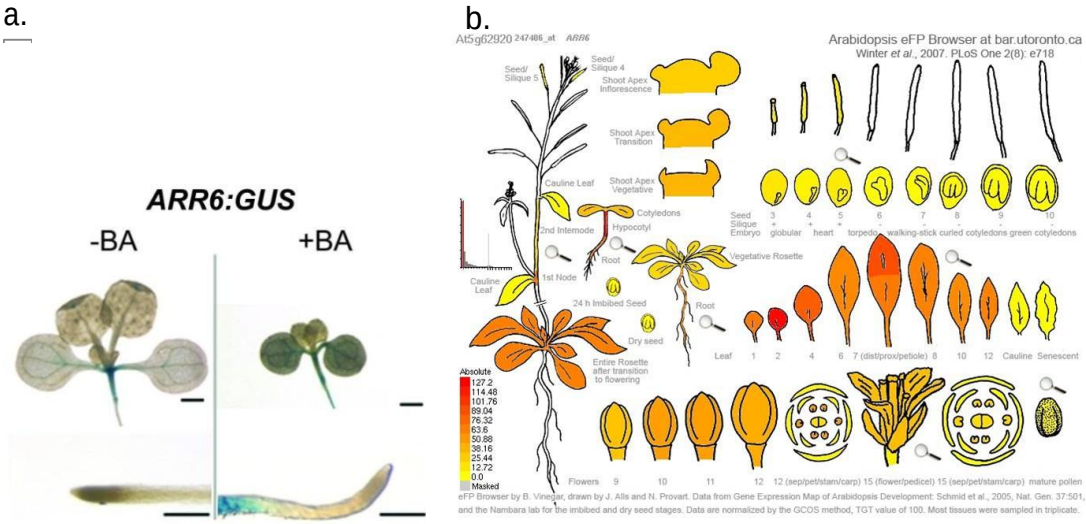


Figure 3.32: The *ARR6* promoter as a reporter for cytokinin response. (a.) Cytokinin induction of *ARR6* using *pARR6*^{2146bp}*::uidA A. thaliana* plants. p*ARR6::uidA* expression increased in the whole leaf area after cytokinin treatment (To et al., 2004). (b.) eFP browser compilation of available microarray results for *ARR6*. *ARR6* is expressed relatively ubiquitously including young seedlings (Winter *et al*., 2007).

According to Ramireddy (2009) the promoter fragments of 350 bp and 1000 bp upstream of the transcriptional start site of *ARR6* are sufficient to enable nearly full induction by cytokinin (Ramireddy, 2009). Therefore those fragments were chosen to drive the reporter gene in the screen. The idea was to keep the promoter as short as possible. Often in the first 300-400 bp of a promoter, the main *cis*-regulatory sequences of the corresponding gene are located. Additional ones, fine-tuning the expression or allowing reaction to more diverse stimuli, are located more distant to the transcriptional start site in the most cases (Geisler *et al*., 2006).

Type-A response regulator genes are also regulated by other factors than cytokinin, e.g. nitrogen and environmental stimuli (Taniguchi *et al*., 1998). By choosing a short promoter fragment I hoped to exclude possible modulators that regulate *ARR6* in reaction to environmental changes, e.g. connected to the screening procedure such as pH changes. In order to restrict the outcome of the screen to cytokinin-associated modulators I selected the shortest promoter fragment of *ARR6* known to show the complete cytokinin response. I also cloned the 1000 bp fragment of the *ARR6* promoter and the 2146 bp fragment as a backup (To *et al*., 2004) into the reporter vector using the *Hind*III endonuclease and ligation with the T4 ligase (chapters 2.5.2.1 and 2.5.2.4). In the promoter fragments of 1000 bp and 2146 bp parts of another gene, At5g62930, are included (Figure 3.34).

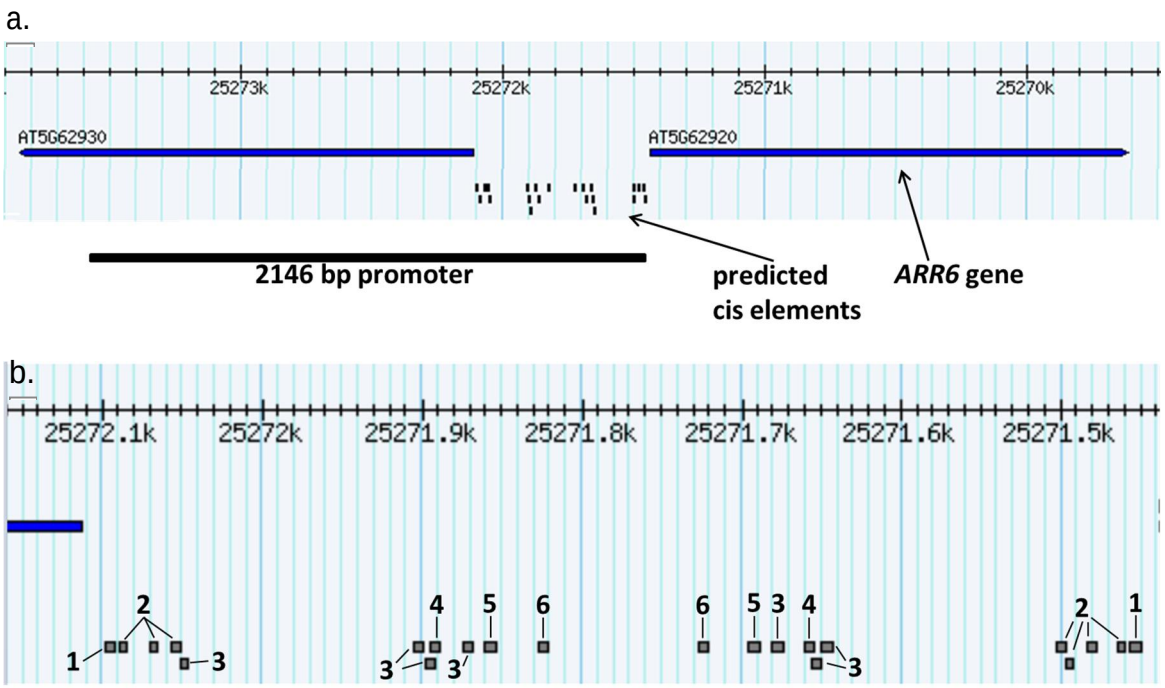


Figure 3.34: Genomic context of the *ARR6* promoter. 2146bp is the promoter length proven to be cytokinin-inducible by To and colleagues (To *et al*., 2004). (a.) In close proximity, there is another gene located, At5g623930, that shares the promoter with *ARR6*. (b.) Putative *cis*-elements of the ARR6 promoter. The description of those *cis*-elements can be found in table 3.2. Pictures are taken from http://arabidopsis.med.ohio-state.edu/AtcisDB/

In the ARR6 promoter, there are located several putative binding sites for transcription factors. In the Atcis database, binding sites for LFY, MYB4 and WRKYs are listed (Figure 3.34). Additionally other binding sites mediating light regulation (SORLIP, T-box, box II) were depicted (http://arabidopsis.med.ohio-state.edu/AtcisDB/) (Table 3.2).

Table 3.2: Putative *cis*-elements in the *ARR6* promoter. In the first column, numbers corresponding to those in figure 3.34 are listed. In column two the *cis*-regulatory element is named and its function is noted in column three. In the last column references concerning the *cis*-element are listed.

Number	*cis*-regulatory elements	Functional context	Reference
1	LFY consensus binding site	Regulation of AP3 homeotic gene	Lamb *et al*., 2002
2	SORLIP2	Light-regulation	Hudson and Quail, 2003
3	W-box promoter motif	WRKY binding site	Yu *et al*., 2001
4	T-box promoter motif	Light-regulation	Chan *et al*., 2001
5	MYB4 binding site	UV-protection	Chen and Provart, 2002
6	Box II promoter motif	Light-regulation	Le Gourrierec *et al*., 1999

Having cloned the 350 bp and the 1000 bp promoter fragments into the reporter vector I wanted to test the cytokinin induction in protoplasts (chapter 2.7.3). This was not successful, possibly because of the size of the luciferase reporter constructs, which were about 14 kbp long. Another option to test the constructs was to transiently express them in tobacco and score luciferase activity in a leaf disc assay. The luminescence observed was not much above background levels for both promoter lengths used (data not shown). Therefore I decided to add the *ARR6* promoter of 2146 kb to my experiments, which was already described to be induced after cytokinin application (To *et al*., 2007). This will be referred to as the 2 kb *ARR6* promoter in this study. After one hour cytokinin induction, tobacco leaf discs infiltrated with the 1000 bp promoter-construct as well as the 2 kb-construct exhibited elevated luminescence. For the 1000 bp promoter, the fold induction was about 1,5, for the 2 kb promoter it was about 2 (Figure 3.35). Unfortunately DMSO had the same effect on the induction of the promoter in leaf discs (Figure 3.35). Therefore it was decided to use *trans*-zeatin hydrochloride in the assay as this can be dissolved in water. *Trans*-zeatin hydrochloride was able to induce a luminescence response in seedlings (data not shown). I tested also the stability of the luciferin in the system. For this I measured the luminescence of the samples three hours after cytokinin application. Then I added luciferin and measured samples again (Figure 3.35). There was no difference in luminescence before and after addition of new luciferin (Figure 3.35). Therefore I assumed that the level of luciferin was saturating.

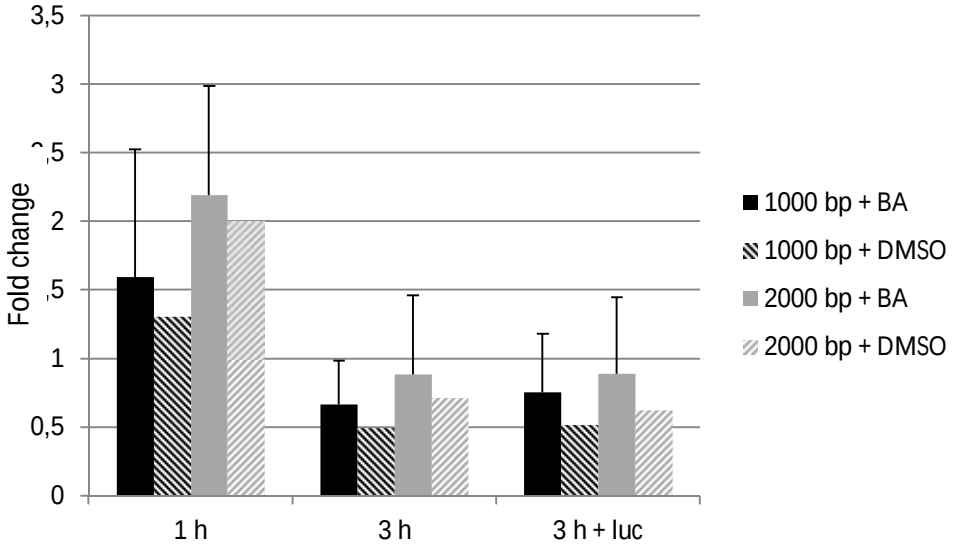


Figure 3.35: Luminescence of tobacco leaf discs. Tobacco was infiltrated with *Agrobacteria* bearing a 1000 bp or 2000 bp *ARR6* promoter-luciferase fusion construct. Leaf discs were incubated with 5 µM benzyladenine (BA) for one and three hours. Luminescence after three hours induction was measured before and after supply of additional luciferin. n = 8 for each condition.

The *pARR6*^{350bp}, *pARR6*^{1000bp} and *pARR6*^{2kbp} vectors were transformed into *Agrobacterium* (chapter 2.5.2.7). Subsequently *Arabidopsis thaliana* plants were transformed by floral dip (chapter 2.6.7). Among transformed *Arabidopsis* plants, the number of primary positive plants was low for the shorter promoter fragments but the transformants surviving the selection procedure were tested for cytokinin induction of the reporter gene. There were 15 plants with the 350 bp promoter, 50 with the 1000 bp promoter and 120 with the 2 kb promoter of *ARR6* fused to the luciferase reporter tested in seedling assays (chapter 2.7.1) or leaf disc assays (chapter 2.7.4). The induction was done with *trans*-zeatin dissolved in DMSO because the same was done for Real-time PCR analyses in the lab before. Possibly seedlings might not be as sensitive to DMSO as leaf discs are. Additionally there was no *trans*-zeatin dihydrochlorid available at that timepoint in the labroratory. None of the *pARR6*^{350bp} reporter plants showed a cytokinin induction. Just one pARR6^{1000bp} reporter plant exhibited a luminescence signal above background. To examine if the cytokinin induction did work in that assay, leaves of two plants, *pARR6*^{350bp} and *pARR6*^{1000bp}, were subjected to Real-time PCR experiments. In those the transcriptional responses of the *ARR6* and the *luciferase (LUC)* to cytokinin were tested and compared. For the experiment leaves were incubated for one hour with 5 µM *trans*-zeatin or DMSO as a control, harvested and the RNA was extracted (chapter 2.5.5). cDNA was prepared and assayed in a Real-time PCR analyses for *ARR6* and *luciferase* transcripts (chapter 2.5.3.6 and 2.5.3.7). Both plants showed an induction of the endogenous *ARR6* transcription of about seven-fold but the *LUC*-transcript was not increased after cytokinin treatment (Figure 3.36). The plant transformed with the 1000 bp ARR6 promoter showed an increased *LUC*-transcript before and after induction. This might be due to a general activation of *LUC* transcription by the place of insertion. The higher *LUC* transcription correlated with a luminescene above background in the assay of this plant before (data not shown). As the luciferase expression by the plants tested did not reflect the endogenous reaction of *ARR6* upon cytokinin treatment, I decided to use the 2 kb promoter fragment of *ARR6* to drive the luciferase reporter in my screen. As plants transformed with the 2 kb promoter fragment of *ARR6* fused to the *LUC* gene exhibited cytokinin-inducible luminescence, I did not test them in a Real-time PCR analysis.

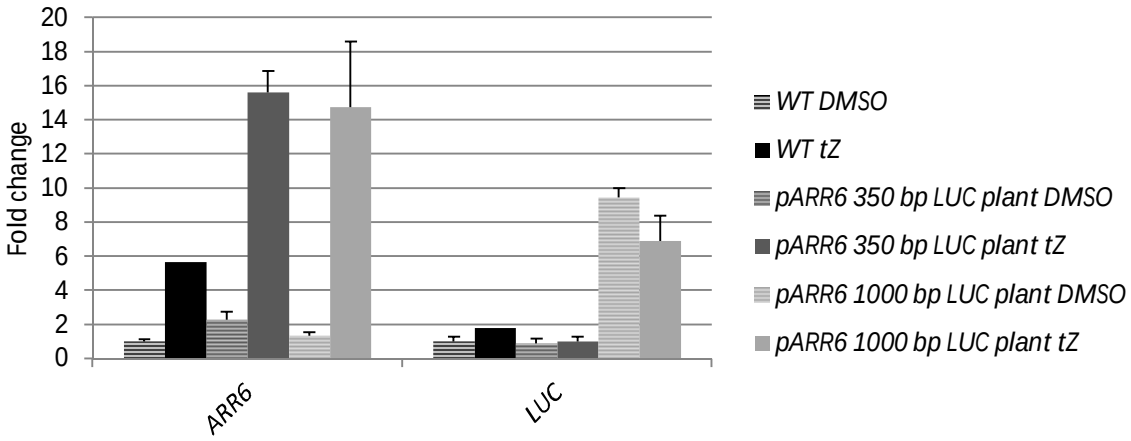


Figure 3.36: Expression level of *ARR6* and *LUC* in WT, *pARR6*^{350bp}*::LUC* and *pARR6*^{1000bp}*::LUC*. Leaf samples were treated with DMSO control solution or 5 µM *trans*-zeatin, RNA was extracted (chapter 2.5.5) and cDNA synthesized (chapter 2.5.3.6). Real-time PCR analysis (chapter 2.5.3.7) revealed that the endogenous *ARR6* transcript level of WT and mutant lines increased after cytokinin treatment compared to DMSO-treated WT. The luciferase transcript level was not influenced by cytokinin application. This experiment was performed in technical triplicates.

Among the pARR6^{2kb}::LUC plants tested for cytokinin induction, several potential reporter lines were identified with the help of a leaf disc assay (chapter 2.7.5). Reporter plant B luminescence values for instance increased 140-fold by cytokinin application. The best four candidates were tested in protoplast transactivation assays (see chapter 2.7.3) for cytokinin induction and reaction to *ARR1* overexpression (Figure 3.37). ARR1 served as a test model for the expected candidates that I wished to find in the screen. The protoplast transactivation assays could be performed just once as I did not want to remove all leaves of the potential reporter plants. So results should be seen as a hint and not a proof. Reporter plant A exhibited a medium response to cytokinin but was induced about three-fold by addition of ARR1 and cytokinin. Reporter plant B showed a strong mainly ARR1-dependent induction of luminescence of about five-fold (Figure 3.37a). For reporter plant C the situation was similar but the fold change was about 23 – 33. For reporter plant D no induction in the protoplast transactivation assay at all was observed (Figure 3.37b). I selected reporter plant A and reporter plant C to use them in the screen. Those will be referred to as R1 (reporter plant A) and R2 (reporter plant C). R1 seemed to be suited to find strong activators in the screen such as ARR1. R2 I selected to also find cytokinin-independent activators of the *ARR6* promoter in the screen.

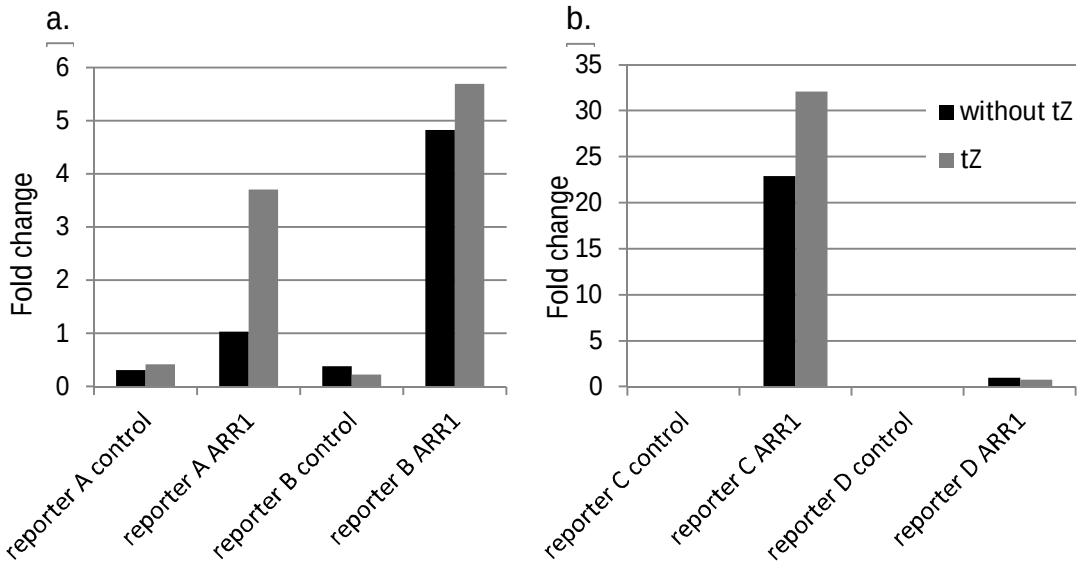


Figure 3.37: Protoplast transactivation assays with four potential reporter plants. Protoplasts of mature reporter plants A and B (a.) or C and D (b.) were used for transformation with ARR1. Half of the protoplasts were treated with *trans*-zeatin overnight. On the next day they were assayed for luciferase activity using the Promega Luciferase Assay System as described in chapter 2.7.3.

After having identified the reporter plants for my screen for modulators of the cytokinin response, I tested other important matters of the assay. First I examined the kinetics of the luminescence graph in a leaf disc assay (chapter 2.7.4) after addition of luciferin purchased from SIGMA and from PJK. Luminescence reached a maximum level at 45 minutes (Figure 3.38). Addition of each of the luciferins resulted in a similar graph. The luciferin from PJK reached higher absolute luminescence values. As this one was also less expensive, it was the one selected for the assay. Based on the outcome of this test, luminescence should have been measured after 30-45 min. This is not practicable because luciferin has to be equilibrated already to room temperature for one hour, so the time-consumption would be high. Additionally this graph shows that luminescence decreased after one hour (Figure 3.38). This can be due to two possible assumptions. The first assumption would be that the luciferase or the luciferin degraded. The second possibility might be that the leaf discs are dying and therefore luciferase activity decreased. As in bigger tobacco leaf discs I observed a stable luminescence after 3 hours that was above background, I think that the leaf discs were starting to die. They were exposed to a harsh pH of 7,8, so this would not be surprising. Luciferin was dissolved in 0,1 N KOH. The change of the pH by luciferin addition was measured. The final pH was about 8, which is perfect for the luciferase (data not shown).

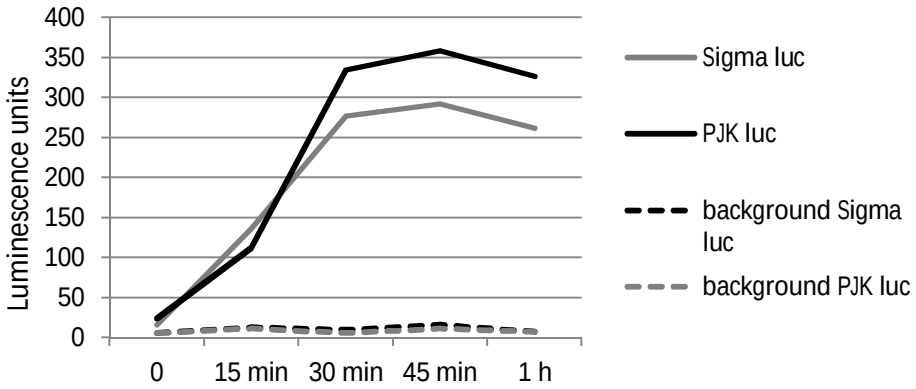


Figure 3.38: Luminescence deviation of a potential reporter plant in a leaf disc assay. Leaf discs of one plant transformed with the 2 kbp *ARR6* promoter luciferase reporter fusion were assayed for luminescence (chapter 2.7.4) after addition of different luciferins from Sigma (Sigma luc) and PJK (PJK luc).

I planned to screen seedlings using multiwell plates and assay luminescence in the plate reader. For this I tested if black microtiter plates can be reused. After removal of the samples, two washing steps with 10 % ethanol to remove estradiol and cleaning in the dish washer, plates exhibited no luminescence signals anymore and could be reused (data not shown).

The next experiment for the development of the screen concerned the estradiol application. Estradiol was used to activate the expression of the cDNA libraries in the *pER8GW* effector vector. It was dissolved in ethanol as all other possible solvents appeared even more harmful to plants. Estradiol (Estr) and ethanol (EtOH) addition were tested additional to *trans*-zeatin (*t*Z), also in a leaf disc assay (chapter 2.7.4). *Arabidopsis* leaf discs were incubated with the respective supplement and assayed after 2,5 hours of incubation. This time period was selected to find a compromise between death of leaf discs (starts according to Figure 3.38 after about 45 minutes) and realistic conditions (induction was thought to be applied overnight). After 2,5 hours *trans*-zeatin-treated leaf discs exhibited a 1,5-2 fold induction of luminescence compared to untreated samples. Ethanol led to luminescence induction similar to that. Estradiol application seemed to reflect situation of the untreated sample (Figure 3.39).

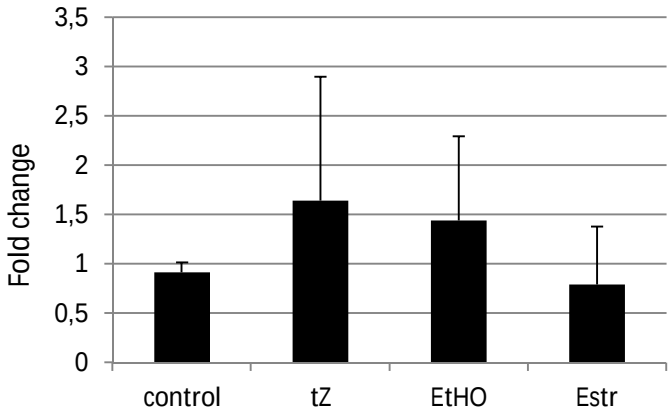
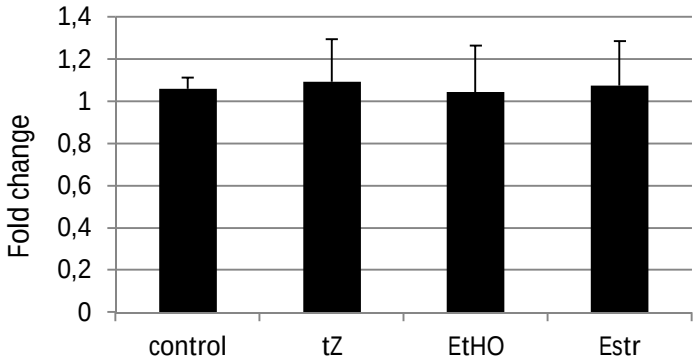


Figure 3.39: Fold change of *Arabidopsis* leaf discs luminescence levels. Leaf discs of a plant line transformed with 2 kb of the *ARR6* promoter fused to *LUC* were supplemented with 4,5 µM *trans*-zeatin (tZ), ethanol (EtOH) or 5 µM estradiol (Estr) and assayed subsequently (chapter 2.7.4). Luminescence levels were compared to untreated samples. Ethanol seemed to have a similar effect as *trans*-zeatin. This seemed to be suppressed by Estradiol. $n_{untreated} = 4$, $n_{tZ} = 28$, $n_{EtOH} = 8$, $n_{Estr.} = 8$.

To examine if the luciferin concentration was still saturating also in *Arabidopsis* after 2,5 hours, I added luciferin and assayed the leaf discs again. The fold change comparing the luminescence of the samples before and after luciferin supply was about one in all cases (Figure 3.40). This means the luciferin level was still saturated.



3.40: Fold change of luminescence of *Arabidopsis* leaf discs after luciferin resupply. Leaf discs (chapter 2.7.4) of a plant line transformed with 2kb of the *ARR6* promoter fused to *LUC* were incubated for 2,5 hours with 4,5 µM *trans*-zeatin (tZ), ethanol (EtOH) or 5 µM estradiol (Estr). Luminescence was measured. After luciferin resupply luminescence was measured again. $n_{untreated} = 4$, $n_{tZ} = 28$, $n_{EtOH} = 8$, $n_{Estr.} = 8$.

Finally homozygous reporter lines transformed with the *pARR6^{2kbp}::LUC* constructs were tested for their ability to be induced by cytokinin in a seedling assay (chapter 2.7.1) and for the variation within one population. The two reporter lines R1 and R2 showed unequal cytokinin induction (Figure 3.41). R1 exhibited higher luminescence values and a stronger inducibility by cytokinin. The fold induction in the individual seedlings after cytokinin application reached from

zero to 24. This reporter plant was suited to find e.g. repressors of the cytokinin response. R2 showed lower absolute values and the fold induction by cytokinin application was much lower. The fold induction reached from zero to four. In this line the variation within the response was much lower. This reporter line was selected to find modulators that induce a high level of reporter activity with or without cytokinin. Plants showing no induction occurred regularly during the screen. This could be due to the harsh pH conditions in the media or a wounding by transfer into the microtiter plates with a forceps. I would assume that the findings from the seedling assay are more reliable than those from the leaf disc assay. The seedling assay reflects the situation in the screen and seedlings might be more resistant towards a pH of 7,8-8 than leaf discs.

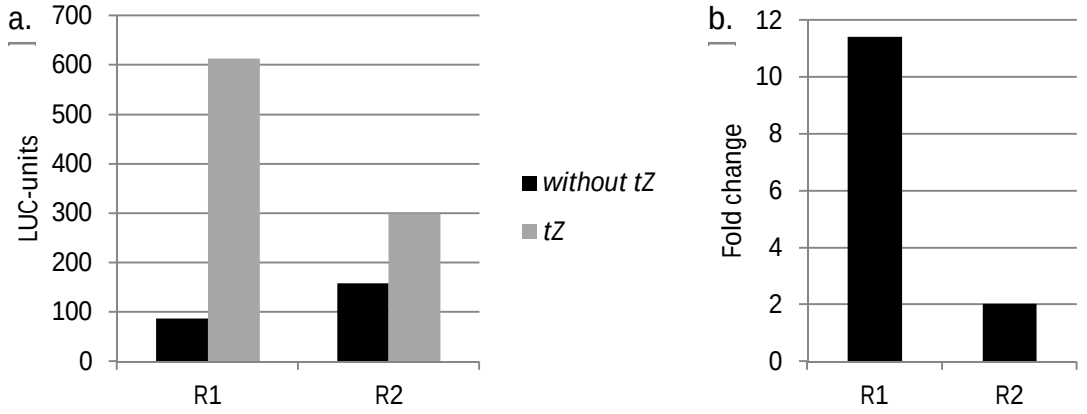


Figure 3.41: Induction of the two selected reporter lines by cytokinin. Homozygous reporter plants were examined in a seedling assay (chapter 2.7.1) (a.) Absolute luminescence values and (b.) fold change of LUC-units by induction with 4,5 µM *trans*-zeatin are depicted. Line R1 exhibited much higher values and also a stronger induction by cytokinin but the variation in the population was high (data not shown). The fold chance by cytokinin reached from zero to 24. R2 showed lower absolute values and a lower induction by cytokinin but the variation among the population was lower. The variation reached from zero to four. n = 48. Repetition of the experiment resulted in similar trends.

3.2.2 Three libraries were selected and cloned into the effector plasmid

The cDNA-libraries used in this setup were derived either from hormone-treated seedlings library , a seeds and primary leaves library, each with about 10^6 primary clones (Bürkle *et al*., 2005) or from a collection of 1282 full-length transcription factors of *A. thaliana* cloned into the pENTR TOPO vector system (Gong *et al*., 2004).

3.2.3 The setup of the screen allowed a high throughput search for genes that modify the cytokinin response

The following screen procedure was developed (Figure 3.42).

Reporter plants R1 and R1 were transformed by *Agrobacterium*-mediated floral dip (chapter 2.6.7). The progeny was plated on selection media and grown for five days. Surviving plants were placed in black 96 well microtiter plates containing estradiol dissolved in ½ MS. The microtiter plates containing the plants were incubated in a growth chamber overnight in a 16 h / 8 h light cycle to enable recovering from the transfer and cDNA expression induced by estradiol. Luciferin was equilibrated, added to the wells and luminescence of the samples subsequently was measured in the plate reader. Afterwards cytokinin was added and the plate incubated for two hours. A longer incubation time was not practicable as it would have been time consuming. With a two hour cytokinin induction period, it was possible to screen twelve plates per day. A second measurement of luminescence was then performed to quantify reporter gene activity after cytokinin induction. Plants that showed a high steady state level of luminescence, a high fold change by cytokinin induction compared to the R1 and R2 or a repression instead of an induction were selected. They were grown further on MS agar plates. Since the luciferase seedling assay was not performed sterile and I worked with plants of a T1 generation, often colonized by bacteria, 125 µg/l cefotaxim were added to the media to avoid contamination. When the plants had enough roots they were planted onto soil. After the plants had enough leaves, one was harvested, the DNA extracted and a PCR with specific primers for the sequence surrounding the cDNA was performed (chapter 2.5.4, 2.5.3.1 and 2.5.3.2, primer in Appendix). PCR products were checked on an agarose gel for the existence of single bands, purified and sent for sequencing (chapters 2.5.2.2, 2.5.2.3 and 2.5.2.8). Identified genes were sorted, evaluated for their potential to be followed after and 14 candidates were selected. The criteria for this selection were 1) deviation in luminescence to reporter plants (R1 and R2) without cytokinin induction, 2) deviation in fold change in luminenscence to untransformed reporter plants by cytokinin induction, 3) high luminencence level compared to untransformed reporter plants after cytokinin induction, 4) full-length clone, 5) absence of frameshifts, point mutations, deletions or insertions in the sequence. I selected candidates for further analyses mostly from the PUK-Yale cDNA library. Their advantage was to be full length, in the correct frame and in the GATEWAY™ system. The pENTR clones were available in the laboratory and GATEWAY™ cloning was used to shuttle the respective genes into overexpression vectors for protoplast transactivation assays. In the protoplast transactivation assays I tested if the candidate genes transactivated the *pARR6*^{2,4kb}*::LUC* construct (Hwang and Sheen, 2001).

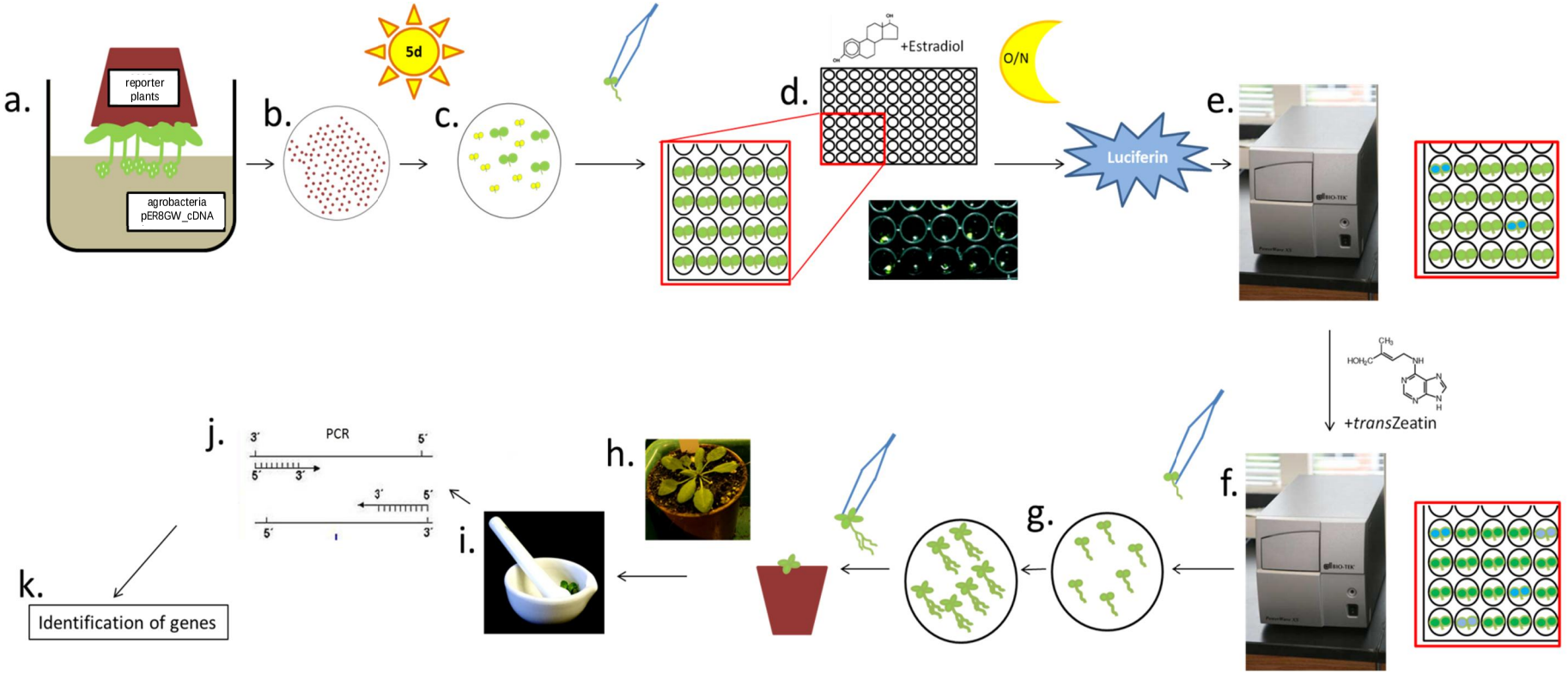


Figure 3.42 Screening procedure for modulators of the cytokinin response. (a.) Transformation of the effector into transgenic plants harboring the reporter construct. (b.) Plating and (c.) selection of T1 seeds. (d.) Transfer of seedlings in 96-well plates and incubation with estradiol. (e.) First luciferase measurement and incubation with *trans*-zeatin, (f.) second luciferase measurement and (g.) transfer of seedlings on petri dishes for recovery. (h.) Transfer and growth of seedlings on soil. (i.) DNA-extraction, (j.) PCR on DNA and (k.) identification of candidate genes.

3.2.4 *ARR2* and *CKX1* were utilized as controls for the screen

To assess potential candidate genes, the range of the signal to be expected should be tested. As suitable candidates to increase or to decrease activity of the *ARR6* promoter I selected *ARR2* and *CKX1*. *ARR2* overexpression is known to increase the *ARR6* signal independent from cytokinin in protoplast transactivation assays (Heyl *et al*., 2008). CKX1 decreases cytokinin output due to its function as cytokinin dehydrogenase/oxidase (Werner *et al*., 2003). *ARR2* and *CKX1* were cloned into *pER8GW* by GATEWAY™ cloning (chapter 2.5.2.5). I generated plants overexpressing estradiol inducible *ARR2* or *CKX1* and tested also the T1 in the seedling assay corresponding to the situation in the screening procedure (Figure 3.43) (chapter 2.7.1).

R1 exhibited much higher absolute values than R2. Absolute luminescence values were about 500 units whereas luminescence values in R2 were in the range of 200 units. Both reporter plants showed a low cytokinin induction of the reporter gene. The transformed plants were not directly comparable to the reporter lines because they underwent hygromycin selection. Hygromycin treatment leads to plants with smaller cotyledons (Duan *et al*., 2011) and impaires root growth (Duan and Ding, 2007), so the plants are smaller and show a lower luminescence signal. Plants overexpressing *ARR2* showed higher LUC-values than plants with *CKX1* being overexpressed. The median was about four times higher in R1 ARR2 plants compared to R1 CKX1 plants with and without cytokinin induction (Figure 3.43a). In R2 ARR2 the median was about three times higher than in R2 CKX1. R2 ARR2 plants exhibited a three times induction by 4,5 µM *t*Z, similar to R1 ARR2 and R1 CKX1. The fold induction of R2 CKX1 was about two times. The variation in plants transformed with XVE::ARR2 was high and single outliner values in the box plots went up to four to seven times of the median values (Figure 3.43). This can be due to transformation effects. T-DNA insertions integrate relatively unspecific (Krysan *et al*., 2002). Expression levels therefore are dependent on the chromosomal context. This leads to T1 generations being very variable in their level of expression of the transgene. Additionally sometimes plants are transformed with a T-DNA construct and show very little or no expression as well as plants wounded by transfer into the microtiter plates. One has therefore to keep in mind that possibly some genes might have to be expressed more often in one screen until a true signal occurs. As *ARR2*-transformed plants showed much higher outliners and the *CKX1*-plant outliners are in the range of the wild type situation in the reporter plants, I would estimate the probability of false positive candidate plants lower than for false negative signals. Outliners of *ARR2* transformation would have matched the selection criteria for the screen and would have been selected for further analysis. Outliners of *CKX1* transformation could have been overseen

as a low signal is in most of the cases not due to a repression but to a plant having decreased luminescence levels because it is dying.

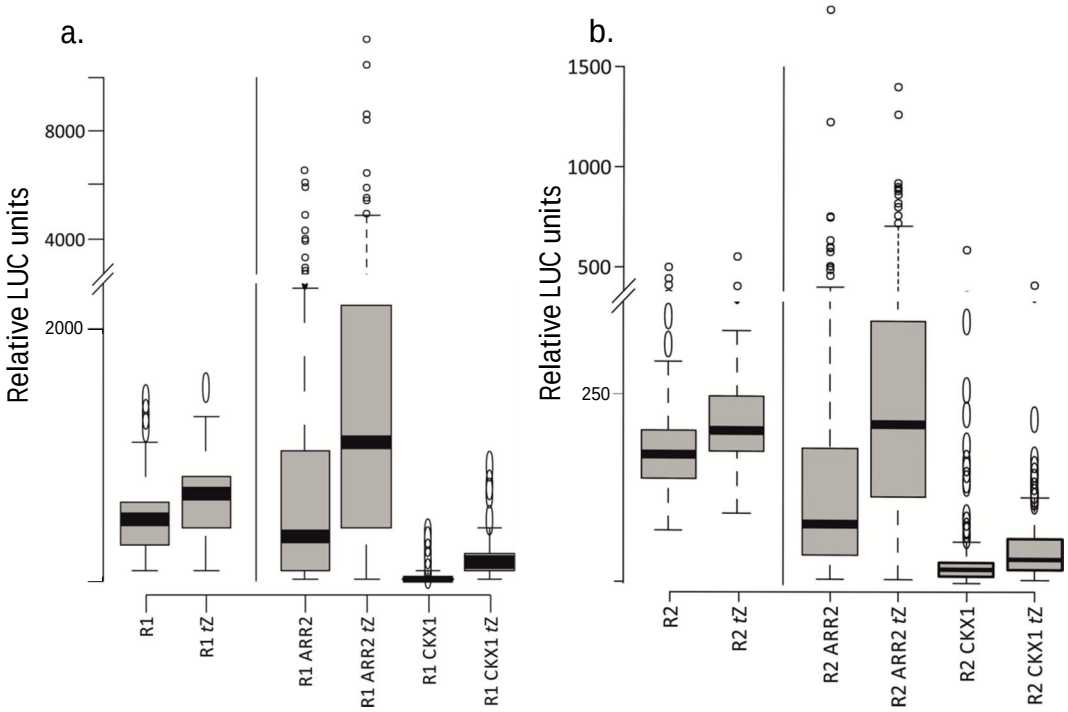


Figure 3.43: Box-plots of seedling assays of the reporter plants and of the control plants. Control plants were transformed with *XVE::ARR2* or *XVE::CKX1* and assayed with and without 4,5 µM *trans*-zeatin induction in the T1 generation (chapter 2.7.1). R1 (a.) and R2 (b.) were grown on non-selective media, the other plants were grown on hygromycin-containing media to select for positives. Please note that the absence of selection pressure led to an unequal size and development. The box includes 50 % of data points, the thicker line is the median. The whiskers include 95% of all data points, all outliners are marked with small circles. In both reporter backgrounds, the fold change by cytokinin application was about four-times in *ARR2* transformed plants and R2 CKX1 plants. *CKX1* overexpression decreased the absolute luminescence values to about ¼ of the *ARR2* transformed plants as well as the fold change induction in R2 CKX1. Lower part of the plot was magnified.

3.2.5 Out of the primary positive plants 14 genes were identified and used for transactivation assays

Altogether 11502 plants were screened and 931 primary positive plants were selected (Table 3.3). Among those the most plants were selected because of high luminescence levels after estradiol-induction or after cytokinin-treatment. Also plants showing an increased induction of the reporter gene were selected as well as plants exhibiting a repression of luminescence by cytokinin application. From those 56 plants underwent DNA extraction (chapter 2.5.4). 45 sequences could be identified (chapter 2.5.2.8). 14 genes being full length and exhibiting no frameshift were selected to be tested in protoplast transient assays.

Table 3.3: Statistics of the screen. The number of plants screened and assigned as primary positive from the screen (Figure 3.42) is listed as well as the origin of the candidate cDNA. R1 and R2 are the selected reporter plants. Seed/hormone and PKU-Yale are the cDNA libraries used in the screen (chapter 2.3).

Transformation of R1/R2 with cDNA library	Number of T1 screened	Number of primary positives selected	% of primary positives in in the screened T1
R1 seed/hormone	2944	532	18,1
R2 seed/hormone	3544	129	3,6
R1 PKU-Yale	2768	227	8,2
R2 PKU-Yale	2264	43	1,9
Total	11502	931	8,1

The seedling screen for modulators of the cytokinin response led to 931 primary positive plants. Plants that showed a high steady state level of luminescence, a high fold change by cytokinin induction compared to the reporter plants or a repression instead of an induction were selected. Although most of the selected primary positive plants derived from R1 plants transformend with the seed/hormone cDNA library, I proceeded with R1 plants and R2 plants transformed with the PKU-Yale cDNA library, to simplify the cloning procedure. Those were GATEWAY™ compatible and full length clones in the right reading frame. 13 of the candidates were cloned from their *pENTR* clones into the overexpression vector *pB2GW7* by GATEWAY™ cloning (chapter 2.5.2.5). Gene 13 was first cloned into *pDONR222* by BP reaction and then cloned into *pB2GW7* as it was not a clone from the PKU-Yale cDNA library but from the seed/hormone cDNA library.

In table 3.4 the selected primary positive genes are listed. The luminescence values and the AGI codes as well as a short description or the name are shown. Gene 12 was found twice in the screen, gene 13 four times.

Table 3.4: Candidate genes expressed in the selected primary positive plants identified by the screen. The gene name, the LUC-values in the screen before and after cytokinin application, the At number and the description are listed. All genes, except for gene 13, derived from the PUK-Yale cDNA library. If not mention otherwise, the genetic background was reporter plant R1.

Gene number	LUC-units		At number	Name/description
	- tZ	+ tZ		
Gene 1	4439	3551	At4g01060	*CAPRICE-LIKE MYB 3 (CPL3)*
Gene 2	5312	7037	At1g77570	Winged helix-turn-helix transcription repressor
Gene 3	15351	3099	At3g51910	*HEAT SHOCK TRANSCRIPTION FACTOR A7A (HSFA7A)*
Gene 4	2740	933	At3g54990	*SCHLAFMUTZE (SMZ)*
Gene 5	6673	5391	At5g47670	*LEAFY COTYLEDON 1-LIKE (L1L* or *LEC1-like)*
Gene 6	17	546	At2g27220	*BEL1-LIKE HOMEODOMAIN 5 (BLH5)*
*Gene 7**	1868	1887	At1g27730	*SALT TOLERANCE ZINC FINGER (STZ* or *ZAT10)*
Gene 8	2813	4236	At2g28200	C_2H_2-type zinc finger protein
Gene 9	204	2636	At2g17560	*HIGH MOBILITY GROUP B4 (HMGB4)*
Gene 10	2512	2127	At4g21050	*DNA-BINDING WITH ONE FINGER 4.4 (DOF4.4)*
Gene 11	1018	453	At5g60570	F-Box/Kelch repeat protein
Gene 12	2600	3601	At5g67450	*ARABIDOPSIS ZINC-FINGER PROTEIN 1 (AZF1)*
*Gene 13**	11030	9860	At5g59613	Unknown protein in mitochondrial ATP synthase complex
*Gene 14**	97	577	At1g49120	*CYTOKININ RESPONSE FACTOR 9 (CRF9)*

*in background of reporter plant R2

3.2.6 Six genes were identified to modulate the cytokinin response in protoplast transactivation assays

Out of the 14 genes, 13 were cloned into the *pB2GW7* overexpression vector by GATEWAY™ cloning (chapter 2.5.2.5) and analyzed for their transactivation capacity on *pARR6*^{2,4kb}*::LUC* (Hwang and Sheen, 2001) in protoplast transactivation assays (chapter 2.7.3). I was not successful in cloning *CRF9* into *pB2GW7*. The reporter vector used in the protoplast transactivation assays was easier to transform into protoplasts than the reporter construct used in the seedlings. An additional advantage is a different backbone of both vectors. This might reduce the probability to pull candidates out of the screen only acting on the transgene. Such transgenes might not activate the reporter gene is this combination anymore and might be excluded from further experiments. For six genes, an effect on cytokinin response in the protoplast transactivation assay was observed (Figure 3.44).

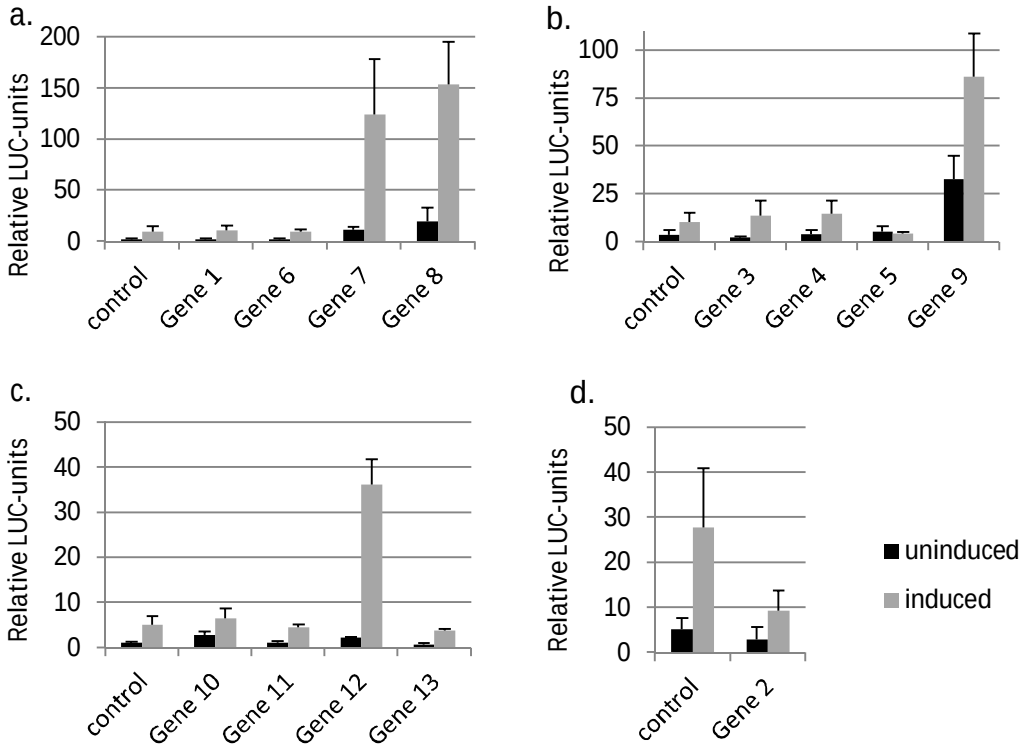


Figure 3.44 Transcactivation capacity of 13 candiate genes on the *ARR6* promoter. The cDNAs were isolated out of the primary positive *Arabidopisis* plants identified in the screen (chapter 2.5.4, 2.5.3.1, 2.5.3.2, 2.5.3.8) and subsequently shuttled into *pB2GW7* by GATEWAY™ cloning (chapter 2.5.2.5) for constitutive overexpression. Those constructs have been utilized in protoplast transactivation assays (chapter 2.7.3) to examine the transactivation capacity of the cDNAs on the *ARR6* promoter. The reporter construct was *pARR6*^{2,4kb}*::LUC* (Hwang and Sheen, 2001). Transactivation capacity of the candidate genes was analyzed with and without cytokinin application. (a.) Gene 1,6,7 and 8 (b.) Gene 3, 4, 5, 9. (c.) Gene 10, 11, 12, 13 (d.) Gene 2. Genes 2, 8, 9 and 12 exhibited transactivation capacity, genes 2 and 5 reduced the endogenous transativation of the promoter after cytokinin treatment. Experiments were performed in triplicate.

Gene 2 and gene 5 mediated a repression of the cytokinin response. Induction of the reporter gene was half of the control levels. The repression concerned just the cytokinin induction of the promoter (Figure 3.44d, b). Genes 7 and 8 mediated a higher reporter activity with cytokinin of about 12 times of the control level (Figure 3.44a). In both cases, but more pronounced for gene 8, also the level of reporter activity without cytokinin was higher than in the control. Gene 9 mediated an increase of reporter activity with cytokinin of about seven-fold (Figure 3.44b). Without cytokinin the increase was nearly ten-fold compared to the control. Gene 12 caused a moderate increase with cytokinin of about five-fold of the control and no difference without cytokinin (Figure 3.44c). The genes modifying cytokinin response in the protoplast transient assay were counted as secondary positive candidates.

Gene 1, *gene 3*, *gene 6*, *gene 10* and *gene 11* were excluded from further analysis. The T2 progeny plants of the remaining secondary positive genes were tested in the seedling assay (chapter 2.7.1) (Figure 3.45). Plants transformed with *XVE::gene 5* and gene *XVE::gene 8* did not give rise to progeny. The progeny of plants transformed with *XVE::gene 2* and *XVE::gene 9* showed very low steady state levels of luminescence in the seedling assay, also the induction by cytokinin was nearly absent. Higher luminescence levels were observed for plants transformed with *XVE::gene 4*, *XVE::gene 7*, *XVE::gene 12* and *XVE::gene 14*. The cytokinin-induced induction of plants transformed with *XVE::gene 7* and *XVE::gene 14* was about three-fold whereas the induction in plants transformed with *XVE::gene 4* and *XVE::gene 12* was nearly absent. The reporter plants cannot be directly compared to the *XVE::cDNA*-transformed plants because they lacked the selection process on hygromycin and were less stressed therefore (Figure 3.45a, b) as mentioned before. The induction by cytokinin was absent in reporter plants of line R2. Possibly those plants were grown on contaminated plates and were infected by bacteria or fungus before the assay. This might have influenced the ability to be induced by cytokinin. It was not possible to test if the plants were contaminated before because the screening procedure is non-sterile from the addition of the luciferin onwards and contamination occured regularely after this timepoint. *Gene 14* was not tested in the protoplast transactivation assay because I was unable to shuttle it into the expression vector *pB2GW7*. Anyhow, it was tested in the seedling assay because CRFs are already known to be connected to cytokinin (Rashotte *et al*., 2003; Rashotte *et al*., 2006; Rashotte and Goertzen, 2010; Cutcliffe *et al*., 2011; Shi *et al*., 2012a).

Summed up, the progeny of plants transformed with *XVE::gene 2*, *9* and *13* seems to repress cytokinin response. For the other genes, an induction by cytokinin was clearly visible.

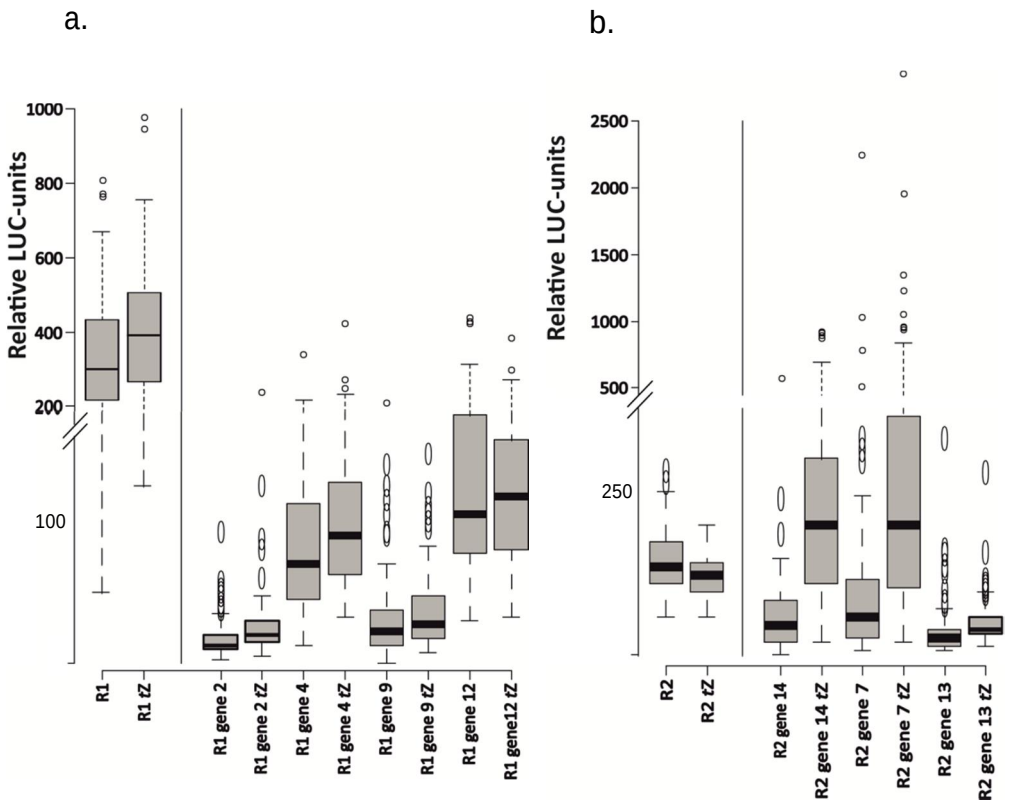


Figure 3.45: Box-plots of seedling assays of the progeny of candidate plants. The plants assayed with a seedling assay (chapter 2.7.1) were the progeny of plants inducible overexpressing *genes 2*, *4*, *7, 9*, *12*, *13* or *14.* There was no progeny of plants transformed with *XVE::gene 5* and *XVE::gene 8* plants existing. *Genes 2*,*4*,*9*, and *12* were inducible overexpressed in the background of reporter plant line R1 (a.) and *genes 7*, *13* and *14* in the background of reporter plant line R2 (b.). R1 and R2 were grown on non-selective media, the other plants were grown on hygromycin-containing media. Please note a difference in growth and development due to that influencing the values. The box includes 50 of data points, the thicker line is the median. The whiskers include 95% of all data points, all outliners are marked with small circles. Lower part of the plot was magnified. $n_{reporter\ plants} = 48$, $n_{mutants} = 96$

For a better comparison of the effects caused by overexpression of the candidate genes, the data of the different assays were compiled in table 3.5. Absolute luminescence values varied from one experiment to the other and cannot really be compared to each other. Absolute values have to be compared to values of the reporter lines of the same experiment. Therefore I assigned trends of the luminescence levels to the genes.

Table 3.5: Summary of the responses in the different assays. Depicted are the gene name and description, the effects of induced overexpression in the T1 plant and reaction of the reporter gene upon cytokinin treatment in the seedling assay (chapter 2.10.1). In column four, the transactivation capacity of the genes in the protoplast transactivation assay (PTA) (chapter 2.10.3) without and with cytokinin (-*t*Z/+*t*Z) is shown. In column five the trend of the luminescence levels and the reaction in the T2 generation of the plants selected in the T1 screen is depicted without and with cytokinin (-*t*Z/+*t*Z). Luminescence was compared to values of the reporter plants. ↑, increase at least two-fold, →, same level as before/reporter line, ↓, decrease, ↗, slight increase (below two-fold), ↘, slight decrease (below two-fold), -, not tested, †, no progeny

Gene name	Description	T1 seedling assay Luminescence level and induction	PTA -*t*Z/+*t*Z	T2 seedling assay -*t*Z/+*t*Z
Gene 1	*CAPRICE-LIKE MYB 3 (CPL3)*	High ↗	→/→	-
Gene 2	Winged helix-turn-helix transcription repressor	High ↗	→/↓	→/↓
Gene 3	*HEAT SHOCK TRANSCRIPTION FACTOR A7A (HSFA7A)*	High ↓	→/→	-
Gene 4	*SCHLAFMUTZE (SMZ)*	High ↓	→/→	↑/↗
Gene 5	*LEAFY COTYLEDON 1-LIKE (L1L* or *LEC1-like)*	High →	→/↓	†
Gene 6	*BEL1-LIKE HOMEODOMAIN 5 (BLH5)*	↑	→/→	-
Gene 7	*SALT TOLERANCE ZINC FINGER (STZ* or *ZAT10)*	High →	↗/↑	→/↑
Gene 8	C_2H_2-type zinc finger protein	High ↗	↑/↑	†
Gene 9	*HIGH MOBILITY GROUP B4 (HMGB4)*	↑	↑/↑	→/↓
Gene 10	*DNA-BINDING WITH ONE FINGER 4.4 (DOF4.4)*	High →	→/→	-
Gene 11	F-Box/Kelch repeat protein	↓	→/→	-
Gene 12	*ARABIDOPSIS ZINC-FINGER PROTEIN 1 (AZF1)*	High ↗	→/↑	↑/↗
Gene 13	Unknown protein in mitochondrial ATP synthase complex	High →	→/→	→/→
Gene 14	*CYTOKININ RESPONSE FACTOR 9 (CRF9)*	↑	-	→/↑

Overexpression of the candidate genes, identified in the primary positive plants in the screen did not always cause the same effects on the reporter gene expression in the different assays. Overexpression of *gene 2* led to an increase in luminescence in the T1 seedling assay whereas in

the protoplast transactivation assay repression of cytokinin induction of the *ARR6* promoter occurred. In the T2 seedling assay also a repression of reporter activity was observed. *Gene 4* overexpression led to plants with a high level of luminescence which decreased after cytokinin application in the T1 seedling assay. In the PTA no effect was observed whereas in the T2 seedling assay reporter activity was high and increased slightly after cytokinin application. *Gene 7* overexpression caused a high reporter activity in all assays. In the T1 seedling assay the luminescence did not increase after cytokinin application whereas in the PTA and in the T2 seedling assay an induction of reporter gene expression was observed. An overexpression of *gene 9* caused an increase in reporter gene activity by cytokinin application in the T1 seedling assay and in the PTA. In the T2 seedling assay a reduction in luminescence occurred after cytokinin-application. The reporter gene regulation by overexpression of *gene 12* was similar in all three assays. In both seedling assays, the luminescence was relatively high already in the beginning. In all three assays the reporter gene activity increased after cytokinin-application. *Gene 13* does not code for the complete mitochondrial ATPase subunit. It produces just a very short peptide. The effect in the T1 seedling assay was enormous. It was identified four times in the screen due to the high reporter gene activity before cytokinin-application. In the PTA no effect was observed. Possibly the action in the T2 seedling assay is a repression of cytokinin response. For *gene 14* no PTAs were done due to problems with cloning. In both seedling assays performed, the reporter gene activity increased strongly after cytokinin-application in plants overexpression *gene 14*. *Gene 5* and *gene 8* could not be analyzed in a T2 seedling assays as T1 plants overexpressing those gene did not produce any progeny. *Genes 1*, *3*, *6*, *10* and *11* were excluded from the T2 seedling assays as I had to decide for a subset of genes to follow further due to time limitations. The overexpression of those genes did not cause any differences in reporter gene activity in the protoplast transactivation assay compared to the reporter plants.

4. Discussion

The cytokinin response is mediated at least in part by transcriptional regulation. This is realized mainly by the type-B response regulators (Argyros *et al*., 2008; Heyl *et al*., 2008; Ishida *et al*., 2008b). To analyze transcriptional cytokinin responses, microarray experiments have been performed. In those, cytokinin-induced seedlings were compared to untreated samples or mutants of cytokinin signaling components were compared to wild-type plants (Sakai *et al*., 2001; Rashotte *et al*., 2003; Kiba *et al*., 2004; Brenner *et al*., 2005; Kiba *et al*., 2005; Lee *et al*., 2007; Yokoyama *et al*., 2007; Argyros *et al*., 2008; Brenner *et al*., 2012; Brenner and Schmülling, 2012). In another microarray approach a dominant-negative type-B response regulator version was expressed in *Arabidopsis thaliana* and differential gene regulation was examined (Heyl *et al*., 2008). To learn more about factors that mediate the cytokinin response on the level of transcription, I analyzed the function of the type-B response regulator ARR14 in *Arabidopsis thaliana*. In a second approach I developed and performed a screening procedure to identify unknown modulators of the cytokinin response. I will first discuss the analysis of ARR14.

4.1. The characterization of the response regulator ARR14

Although it is known that the major cytokinin response is mediated by the type-B response regulators ARR1, ARR10 and ARR12 (Mason *et al*., 2005; Yokoyama *et al*., 2007; Argyros *et al*., 2008; Ishida *et al*., 2008b), ARR14 was selected for analysis for the following reasons: In a yeast two-hybrid approach, a homodimerization of ARR14, a heterodimerization of ARR14 with ARR2 and an unexpected interaction with the cytokinin receptor AHK2 were observed. This was verified also by coaffinity purification (Dortay, 2006). In addition preliminary results indicated a role for ARR14 in development, senescence and pathogen response (Hellmann, 2007). To learn more about the function of ARR14 I performed an expression analysis, subcellular localization and created *gain-of-function* mutants. The *loss-of-function* mutant *arr14-1* was examined for reactions in cold stress, pathogen response and senescence. The function of ARR14 in the cytokinin response was analyzed by performing root assays with *arr14-1* on media containing cytokinin and with quantitative real-time PCR experiments focused on the transcriptional response of type-A regulator genes.

4.1.1. *ARR14* showed a peculiar expression pattern similar to an auxin biosynthesis gene

To examine the *ARR14* expression I utilized 850 bp of the *ARR14* promoter fused to a *uidA* gene and analyzed the pattern after GUS staining of transformed plants. In contrast to others, who employed 1,5-2 kb long promoter fragments (Mason *et al*., 2004; Tajima *et al*., 2004), I used a shorter promoter because of the following reasons: In close proximity to *ARR14* two other genes are located. One gene is located on the same strand, ending about 850 bp in front of the *ARR14* transcriptional start site. The other short and intronless gene is located within the 850 bp upstream of *ARR14* on the opposite strand (Figure 3.1). Geisler and colleagues discovered that most *cis*-regulatory elements are situated within the first 300-400 bp upstream of the transcriptional start site of a gene (Geisler *et al*., 2006). Therefore usage of the 850 bp fragment should include most of the regulatory elements of *ARR14* expression. I detected *ARR14* expression in the shoot apical meristem and at the tip of the gynoecium (Figure 3.3). The same was reported by Mason, Tajima and colleagues (Mason *et al*., 2004; Tajima *et al*., 2004). In contrast to these publications I did not see an elevated expression in the vasculature of young leaves. Using the short promoter, *ARR14* expression was detected in the whole leaf area of young leaves. Later in the leaf development, the signal became restricted to the leaf margins (Figure 3.2).

The discrepancy between my observations and the published results concerning expression in the vasculature of young leaves was probably due to different promoter lengths used in the experiments. It might be that factors promoting the expression in the vasculature bind to the ARR14 promoter in a greater distance than 850 bp. Possibly a regulation of *ARR14* expression by such factors was missing in my experiments. Another possibility is that introns of the gene At2g01750 caused a difference in the observed expression patterns. This is the gene located on the same strand as *ARR14* ending in 850 bp distance to the *ARR14* transcriptional start site (Figure 3.1). Intronic parts of At2g01750 are included in the promoter fragment used by Tajima, Mason and colleagues but not in the *ARR14* promoter fragment I used in my experiments (Tajima *et al*., 2004; Mason *et al*., 2004). As reported by several researchers, introns can function as transcriptional enhancers (Mascarenhas *et al*., 1990; Rose, 2002, 2004; Rose *et al*., 2008). Therefore one cannot exclude that the detected expression of *ARR14* in the vasculature is an artifact caused by neighboring intronic regions out of their context. Another difference to the experiments published is that I used a promoter-gene fusion whereas Tajima and Mason and colleagues examined plants transformed with promoter-gene-*uidA* fusions (Mason *et al*., 2004;

Tajima *et al*., 2004). Keeping this in mind, another possible explanation for the observed differences might be that introns of the *ARR14* gene are essential for expression in the vasculature. Factors assigned to the same function often show a similar expression pattern as it was described for highly redundant MADS box proteins (Gu *et al*., 1998; Liljegren *et al*., 2000). One could argue that an expression of *ARR14* in the vasculature is likely, because the other type-B response regulators examined, *ARR1* and *ARR2,* are also expressed in the vasculature and in the shoot apical meristem as it was reported for *ARR14* (Mason *et al*., 2004; Tajima *et al*., 2004). Reverse transcription experiments for *ARR14* expression revealed a high amount of transcript in younger leaves, whereas in older leaves the *ARR14* transcript abundance was lower (Mason *et al*., 2004). This would fit to the results of the *ARR14* expression pattern observed in my experiments. To examine the expression of *ARR14* further, *in situ*-hybridization of leaf tissue with *ARR14* probes would be a suitable tool.

The most interesting result of the expression analysis of *ARR14* was the specific expression pattern in leaves and the changes in expression during leaf development.

Cytokinin is involved in cell division (Riou-Khamlichi *et al*., 1999). A point of crosstalk between cell cycling and cytokinin are the cyclins. The expression of type-D cyclins as *CYCLIN D3,1* and *CYCLIN D2,1* was shown to be regulated by cytokinin (Soni *et al*., 1995; Riou-Khamlichi *et al*., 1999; Richard *et al*., 2002). Cyclins mediate the cytokinin effects in apical growth and development (Dewitte *et al*., 2007) and their binding to cyclin-dependent kinases is necessary for cell cycle progression (reviewed in Doree and Galas, 1994). In leaves, the expression shows a dot-like pattern which is stronger at the leaf base, marking meristematic zones (Donnelly *et al*., 1999; Andriankaja *et al*., 2012). Meristematic zones in leaf growth occur distributed in the leaf blade, in the vasculature and in meristemoid structures giving rise to the stomata (Donnelly *et al*., 1999; Pillitteri *et al*., 2011).

I would have expected type-B response regulator expression in those meristematic zones, influencing the cell division activity by mediating the cytokinin response. At least for meristemoid cells of the leaves a peculiar cytokinin response in terms of a specific expression of the type-A response regulator *ARR16* was shown (Pillitteri *et al*., 2011).

Conversely, for *ARR14* the expression signal in all leaves examined exhibited the same pattern: First there was expression observed in the whole leaf area of younger stages. Then the signal was restricted more and more to the margins in older leafs.

Similar expression patterns were observed for *INDOLE-3-ACETIC ACID CARBOXYLMETHYLTRANSFERASE 1* (*IAMT1)* and also for *DR5::uidA* reporter plants treated with naphthylphthalamic acid (NPA) (Qin *et al*., 2005; Petricka and Nelson, 2007). IAMT1 is an auxin-methyltransferase converting free indole-3-acetic acid (IAA) to methyl indole-3-acetic acid (MeIAA) (Qin *et al*., 2005). MeIAA was shown to be even more potent than IAA in hypocotyl assays for auxin effects (Qin *et al*., 2005). The expression of the methyltransferase *IAMT1* matches perfectly with the expression of *ARR14* in the flowers as well as in the leaves. There was first expression detected in the whole leaf area. In older leaves the signal was restricted to the leaf margins (Qin *et al*., 2005) (Figure 3.2).

A second expression pattern similar to the *ARR14* expression in leaves was the one observed in NPA-treated *DR5::uidA* seedlings. NPA is an auxin transport inhibitor and *DR5* is used as a synthetic auxin-signaling reporter (Ulmasov *et al*., 1997). The places of *DR5::uidA* expression were interpreted as spots of auxin biosynthesis (Petricka and Nelson, 2007). It could be that the site of auxin biosynthesis is even more restricted and the *DR5* expression pattern in a broader part of the leaf reflects the *IAMT1* expression. *IAMT1* expression would lead to more MeIAA which would activate auxin-signaling (Qin *et al*., 2005). Additionally MeIAA might diffuse through cell membranes easily because of being unpolar and therefore might increase the area of *DR5* expression further (Qin *et al*., 2005).

I performed coexpression analysis with *ARR14* and *IAMT1* using the GeneCAT tool (Mutwil *et al*., 2008). *ARR14* and *IAMT1* were not coexpressed. Subsequently I checked the expression pattern of *IAMT1* in the *eFP* browser (Winter *et al*., 2007) compiling available microarray data. The highest expression was observed in the seeds. In contrast to the published pattern the expression level of IAMT1 in leaves was low (Qin *et al*., 2005). In the publication, a 2,7 kb promoter fragment of *IAMT1* fused to the *uidA* gene was used for the expression analysis (Qin *et al*., 2005). Differences between microarray data and promoter-*uidA* analysis might be due to regulatory introns of the gene or UTR parts missing in the promoter-*uidA* fusion. Possibly the plants analyzed in the microarrays and the GUS assay were grown in different conditions influencing the expression pattern. A point for the *pIAMT1::uidA* expression pattern reflecting the true situation *in planta* is that it fitted to the auxin reporter signal. *In situ* hybridizations might enlighten the actual localization of *IAMT1* expression.

ARR14 seems to be coexpressed with an auxin metabolism gene and an auxin signaling output reporter at least in later stages of leaf development. This is restricted to promoter-*uidA* analyzes and did not occur in microarrays.

4.1.2. ARR14 localized to the nucleus and stably transformed *Arabidopsis gain-of-function* plants of *ARR14* exhibited a growth phenotype

To test the functionality of *35S::GFP-ARR14* and *35S::ARR14-GFP* constructs I prepared for stable overexpression in *Arabidopsis thaliana*, both constructs were transiently expressed in *N. benthamiana*. The leaves were assayed for GFP signals after five days.

In plants expressing an N-terminal GFP fusion, I observed a signal in the nucleus as it was expected for ARR14 as transcription factor. Nuclear localization of ARR14 was already shown in transiently transformed onion cells (Dortay, 2006) (Figure 3.4). I was not able to detect any signal using C-terminal *ARR14-GFP* fusion constructs. The sequence of the construct had been verified by sequencing before. Possibly the ARR14-GFP fusion protein was partially degraded by proteinases or just a truncated version was expressed lacking the *GFP*. It could also be that a correct protein folding was not possible with a C-terminal GFP attached. Also in this case the fusion protein might have been degraded quickly. In eukaryotic cells misfolded proteins are degraded via the 26S proteasome (reviewed in Goldberg, 2003). The presence of ARR14 protein could be tested with protein blots using ARR14-specific antibodies.

Among the stably transformed T1 35S::GFP-ARR14 *Arabidopsis* plants I identified lines showing a GFP-signal but not among the T1 35S::ARR14-GFP plants. It seemed as if the C-terminal ARR14-GFP-fusion protein was not expressed, was degraded or the *GFP* was not translated in tobacco as well as in *Arabidopsis*. Further analysis of the *Arabidopsis* plants expressing the N-terminal *35S::GFP-ARR14* revealed, that most of the plants did not show a GFP signal in the next generation. Those were excluded from the analysis leaving three lines for phenotypical monitoring. In those plants I observed phenotypical aberrations compared to WT and to plants expressing *35S::ARR14*. Plants expressing *35S::GFP-ARR14* were smaller, bushy and had round-shaped leaves (Figure 3.6 and 3.7). The level of overexpression could be correlated by to the strength of the phenotype (Figure 3.8) with the exception of plant F of the line 35S::GFP-ARR14-3. Plant F might be a plant germinated later or being smaller because of natural variation. To be sure about the correlation of transcript level and phenotype of the *35S::GFP-ARR14* expressing plants, more plants should be analyzed.

The increase in *ARR14* transcript was higher in plants expressing *35S::GFP-ARR14* than in plants expressing *35S::ARR14*. *35S::ARR14* expressing mutants displayed a wild type-like phenotype. As ARR14 has an autoinhibitory N-terminus, the fusion with an N-terminal GFP might interfere with ARR14 function. One could hypothesize that this might happen via structural changes by the attached GFP and might create a constitutive active ARR14 version, stabilize the ARR14 protein

or mask protein protein interaction sites. The phenotype observed for *35S::GFP-ARR14* expressing plants resembled plants overexpressing the constitutive active *ARR14* version (Tajima *et al*., 2004) or the stunted cytokinin-deficient plants (Werner *et al*., 2003; Riefler *et al*., 2006; Heyl *et al*., 2008). One could speculate that expression of *35S::GFP-ARR14* might mimic constitutive cytokinin supply and activate transcription of type-A ARRs. Type-A ARRs act as inhibitors on cytokinin signaling and their overexpression might lead to a phenotype similar to that of plants being deficient in cytokinin or cytokinin signaling.

Summarized I could show a nuclear localization for ARR14 and detected a growth phenotype in plants expressing *35S::GFP-ARR14* which was absent in plants expressing *35S::ARR14*.

4.1.3. ARR14 functions as transcription factor but does not regulate the typical cytokinin response marker *ARR6* and *ARR7*

ARR14 is a type-B response regulator and therefore predicted to act as a transcription factor for cytokinin primary response genes. To test the function of ARR14 in cytokinin signaling I examined the transactivation capacity of ARR14 on a potential cytokinin primary response gene. Herefore *ARR14* was overexpressed in *Arabidopsis* protoplasts together with an *ARR6* promoter-*uidA* reporter fusion. ARR14 was able to transactivate the *ARR6* promoter and function as a transcription factor in cytokinin signaling. The transactivation capacity on the 350 bp promoter fragment of *ARR6* was in a low range of about half the level of ARR12 transactivation capacity (Heyl *et al*., 2008) after cytokinin application (Figure 3.13). It was to a very low extent dependent on cytokinin. Without cytokinin application the transactivation of the 350 bp and 1000 bp fragment of the *ARR6* promoter increased by ARR14 addition already to levels that were 40 % respectively 30% higher than for the controls. One could hypothesize that ARR14 is not a main player in type-A response regulator transcriptional activation. Even if the transactivation capacity on p*ARR6* was very low one cannot conclude that ARR14 has no function in cytokinin signaling. ARR12 exhibits a very low transactivation capacity on the *ARR6* promoter as well compared to ARR1 or ARR2 (Ramireddy, 2009) but as multiple *loss-of-function* mutants of *ARR12* with *ARR1* and *ARR10* are strongly impaired in growth and cytokinin response, it seems to play an important role in cytokinin signaling anyway (Mason *et al*., 2005; Yokoyama *et al*., 2007; Argyros *et al*., 2008; Ishida *et al*., 2008b). It might be that transcriptional activation of type-A response regulators is very specific for each type-B response regulator. The *ARR6* promoter might not be the main target of ARR14. The lack of a strong cytokinin-dependent increase of transcriptional activation could be interpreted as a very low dependence of ARR14 from

phosphorylation derived from the cytokinin signaling cascade concerning its function as transcription factor.

To get further insights in the biological role of ARR14 as transcription factor, possible target gene expression was analyzed in Real-time PCR experiments. In the *loss-of-function* line *arr14-1* a strongly reduced expression of *ARR15, ARR17* and *PIN5* (*PIN-FORMED 5*) to ten % respectively 25 % and 25 % of wild type level could be observed (Figure 3.16). *SAG12* (*SENESCENCE-ASSOCIATED GENE 12*) and *ARR16* expression was reduced to about 50 % of wild type level. *SAG12* is a senescence marker gene (Weaver *et al*., 1998) and PIN5 is an atypical ER-localized auxin-transporter, regulating auxin distribution within one cell (Mravec *et al*., 2009). *ARR15* transcription was found to be induced by auxin and repressed by cytokinin in the basal cell in embryo development together with *ARR7* transcription (Müller and Sheen, 2008). Its expression was also shown to be repressed by auxin in the shoot apical meristem (Zhao *et al*., 2010). *ARR15* and *ARR16* appear to be activated by cytokinin via *AHK4* (Kiba *et al*., 2002). *ARR17* expression increases by auxin and decreases by NPA treatment (Winter *et al*., 2007)
Surprisingly ARR14 seems to regulate elements that are connected to auxin and a senescence marker instead of the typical cytokinin response genes *ARR5* or *ARR6*.

Based on these results one could hypothesize that *ARR14* has no main function in the induction of the transcriptional cytokinin response involving *ARR6*. As *ARR6* does not seem to be a target of ARR14 it is not surprising that there was no high transactivation capacity observed in the protoplast transactivation assay on the *ARR6* promoter. ARR14 might mediate the transcriptional regulation of a small very specific branch of type-A response regulators, *ARR15*, *ARR16* and *ARR17*.

4.1.4. In cytokinin signaling, ARR14 seems to be redundant

To learn more about possible functions of ARR14, *loss-of-function* mutants of the gene were examined. I analyzed three independent T-DNA insertion lines. Solely line *arr14-1* (Ishida *et al*., 2008b) did not exhibit any *ARR14* full length transcript and was therefore selected for further analysis. I did not observe any obvious phenotype which was expected for a single *loss-of-function* mutant of the redundant type-B response regulators (Figure 3.11) (Sakai *et al*., 2001; Horák *et al*., 2003; Mason *et al*., 2005). The absence of additional *loss-of-function* mutants of *ARR14* complicates conclusions about ARR14. Results can serve as hints but need to be verified using other *loss-of-function* lines and complemented mutants. As there was no growth

phenotype observed in the *loss-of-function* line a possible complementation experiment could be the instauration of specific type-A response regulator expression level by *ARR14* expression in *arr14-1.*

ARR14 was found to interact with ARR2 and AHK2 in the yeast two-hybrid system and *in vitro* coaffinity purification (Dortay *et al*., 2006). *In silico* analysis using the *eFP* browser compiling available microarray data revealed that *ARR2* is expressed at a very low level ubiquitously and shows a high expression in mature pollen. *AHK2* is also expressed ubiquitously with an increased transcript level in young leaves. *ARR14* expression can be detected in all tissues but is increased in the shoot apical meristem and flower buds (Winter *et al*., 2007). This means that *AHK2* and *ARR2* are not coexpressed with *ARR14* but there is an overlap in expression e.g. in leaves, where an interaction might occur. To examine the possible genetic interaction I crossed *loss-of-function* mutants of *ARR2* and *AHK2* as well as of *AHK3* with *arr14-1*. If there was an essential genetic interaction between ARR14 and AHK2 I would have expected the double mutant *arr14 ahk3* to look similar to the semi-dwarfed *ahk2 ahk3* mutant (Nishimura *et al*., 2004; Riefler *et al*., 2006). None of the double mutants did show any obvious phenotype, probably due to compensation and redundancy in the cytokinin signaling system (Figure 3.12). If there was a true interaction between the proteins, it seems to be not essential or has another functional relevance not revealed here.

To get further insight into the functions of ARR14 I examined *arr14-1* plants for their root growth on different concentrations of cytokinin and compared them to WT, *ahk2-5* and the double mutant *arr14-1 ahk2-5.* There was no significant change in all mutants examined in lateral root number and also in root elongation (Figure 3.14 and 3.15).

ARR14 seems to play no major role in cytokinin signaling in the roots and/or the redundant signaling system seems to compensate for the loss.

4.1.5. In cold stress, senescence and pathogen response, ARR14 functions as a regulator.

Based on *in silico* analysis using the response viewer (Zimmermann *et al*., 2004) and earlier results (Hellmann, 2007), I analyzed the *arr14-1* line for its reaction to cold stress, in senescence and in the pathogen response (Figure 3.17). The data of the response viewer revealed in most of the experiments (Figure 3.17) a regulation of *ARR14* towards a reduction of expression. This

makes the analysis of ARR14 actions using *loss-of-function* lines more difficult. Because of the reduction of *ARR14* expression in the experiments the wild type becomes more similar to *arr14* mutants. Therefore in the experiments one has to expect very subtle differences in *arr14* reactions compared to the wild type.

In cold stress conditions *arr14-1* did not show any significant change in lateral root number. In root elongation, *arr14-1* was significantly more resistant towards cold stress (Figure 3.18). Thus ARR14 seems to play a role in the sensitivity towards cold stress. The double mutant *arr14-1 ahk2-5* was significantly less resistant than the wild type in root elongation in cold stress conditions. Other mutants impaired in cytokinin signaling showed the opposite effect. For *ahk2 ahk3* and *ahk3 ahk4* an enhanced freezing tolerance was reported (Jeon *et al*., 2010). Some type-A response regulators, e.g. *ARR7*, showed a cold induction in their expression (Argueso *et al*., 2009; Jeon *et al*., 2010). Overexpression of *ARR7* led to hypersensitivity towards cold stress, *loss-of-function* mutants were more resistant (Jeon *et al*., 2010). The cold-induction of type-A response regulators is dependent on the AHPs. *Loss-of-function* mutants of the AHPs showed a lower transcriptional induction of type-A response regulators by cold stress. Partially the induction is mediated by ARR1. Addition of cytokinin to an *ARR1 gain-of-function* mutant enhanced its freezing tolerance (Jeon and Kim, 2012). This hints for a function of ARR1 as a positive regulator of cold stress response whereas ARR14 seems to be a negative regulator of cold stress response. Opposite roles for two type-B response regulators are not surprising as cold stress response seems to be controlled in a complex manner. Decreased cytokinin signaling (receptor *loss-of-function*, *ARR7 loss-of-function*) led to enhanced cold resistance whereas increased cytokinin signaling (*ARR7 gain-of-function*, *ARR1 gain-of-function* plus cytokinin) led not automatically to a decreased resistance as one could have expected (Jeon *et al*., 2010; Jeon and Kim, 2012). Interestingly application of cytokinin caused an increased resistance against cold stress (Jeon and Kim, 2012). Application of cytokinin would additionally lead to an increase in type-A response regulator levels, among them *ARR7*, decreasing the cold resistance again.

Since 2007 there were hints from Hellmann that ARR14 might play a role in senescence (Hellmann, 2007). To check, if ARR14 is involved in senescence I examined *arr14-1* for the start and the progression of senescence of the 6th and 10th leaf. *arr14-1* showed a delay in the start of senescence of the 6th leaf (Figure 3.23-3.27). Thus ARR14 seems to be a positive regulator of the onset of senescence. This is also corroborated by its regulatory function in the transcriptional regulation of *SAG12*. ARR14 being a positive regulator of senescence is surprising because

cytokinin is known to delay senescence via ARR2 and AHK3 (Kim *et al*., 2006). One could hypothesize that ARR14 might be a positive regulator of senescence by inhibiting cytokinin signaling by transcriptional activation of *ARR15*. In embryonic patterning *ARR7* and *ARR15* expression restrict cytokinin signaling to the hypophysis to enable proper root and shoot development (Müller and Sheen, 2008).

The cytokinin response of *ARR5* expression during senescence in general seems to progress stably as observed in *ARR5::uidA* reporter plants (Kudryakova *et al*., 2008). The steady state signaling level, displayed by *ARR5::uidA* expression, decreased with the progression of senescence (Kudryakova et al., 2008). The intensity of cytokinin response increased with the age of the leaf. This means, the more senescent a leaf is, the more intense external stimuli are needed to rescue the leaf but the stronger is the reaction (Kudryakova *et al*., 2008). Possibly this is why *arr14-1* plants caught up with the WT during the senescence progression. In wild type, there might be more cytokinin signaling going on to prevent senescence progression, appearing as 'natural progression of senescence'. In *arr14-1* mutants, the signaling cascade was impaired. One might interpret the gaining of senescence progression of *arr14-1* on the wild type as an accelerated progression at this stage.

Cytokinin was connected to pathogen response in several publications (for reviews see Choi *et al*., 2011; Naseem and Dandekar, 2012; Naseem *et al*., 2012; Pieterse *et al*., 2012). Some pathogens intervene in the levels of cytokinin and auxin in their host to increase their proliferation rate (Jameson, 2000; Pertry *et al*., 2010). In the hosts, higher cytokinin levels and signaling mostly correlated with higher resistance. E.g. ARR2 interacts with TGACG SEQUENCE-SPECIFIC BINDING PROTEIN 1 A-RELATED GENE 3 (TGA3) to bind to the *PATHOGENESIS-RELATED 1* (*PR1*) promoter and increases resistance against *Pseudomonas syringae* (Choi *et al*., 2010). Increased cytokinin contents led to increased antimicrobial phytoalexin synthesis and increased resistance against *Pseudomonas syringae* via this pathway (Grosskinsky *et al*., 2011). In this study I analyzed the response of *Arabidopsis thaliana arr14-1* mutants to *Verticillium longisporum* infection. *Verticillium* is a fungal pathogen attacking cruciferous hosts (Karapapa *et al*., 1997). It enters via the root from the soil into the plant and spreads from there systemic in the xylem (Zhou *et al*., 2006). Some genes mediating *Verticillium* resistance have been identified in *Arabidopsis thaliana* e.g. the ethylene receptor *ETR1* (*ETYLENE RESISTANT 1*) (Pantelides *et al*., 2010).

One of the experiments was performed in the greenhouse, two more in growth chambers. In all experiments the *arr14-1* line was more resistant towards *V. longisporum* infection than the wild type (Figure 3.28-3.30). *arr14-1* was about ten % higher than the wild type after inoculation (Figure 3.29). The percentage of colonization by the pathogen was the same but plants were significantly less stunted when scored after yellowing of the first siliques. Therefore ARR14 is likely to be involved in symptom development and not in colonization by *Verticillium*.
ARR14 seems to be also a positive regulator in infection *Verticillium* of host plants contrary to ARR2 in *Pseudomonas* infection.

4.1.6. ARR14 possibly functions in auxin signaling

ARR14 codes for a nuclear localized protein (chapter 4.1.2) showing a similar expression pattern as *IAMT1* and as *DR5::uidA* when auxin transport is inhibited (chapter 4.1.1). It might be that expression of *35S::GFP-ARR14* causes a constitutive active ARR14 version leading to a phenotype with round-shaped leaves and bushy stunted plants (chapter 4.1.2). ARR14 seems to play a minor role in cytokinin signaling in roots or transactivation of *ARR6* but it regulates *ARR15*, *ARR16*, *ARR17, SAG12* and *PIN5* expression (chapter 4.1.3). It might play a role in negative regulation of cold tolerance (chapter 4.1.5). ARR14 seems to be a positive regulator of senescence and a negative regulator of resistance towards *V. longisporum* (chapter 4.1.5).

ARR14 action seems to be contradictory to the actions of other cytokinin signaling factors e.g. ARR2. A very speculative interpretation of the ARR14 data is a possible involvement of ARR14 in auxin-cytokinin-interaction. ARR14 regulates the transcription of some type-A ARRs, possibly starting a negative feedback loop on cytokinin-signaling, among those *ARR15. ARR15* was thought to strongly inhibit cytokinin signaling in concert with ARR7 in the basal cell in embryo development enabling a proper root growth (Müller and Sheen, 2008) but another group questioned this and showed functional redundancy for ARR15, ARR7 and the other type-A ARRs (Zhang *et al*., 2011).
A second pathway could be the regulation of auxin distribution in the cell via regulation of *PIN5* expression. A higher *PIN5* expression could lead to more auxin signaling and a stronger inhibition of cytokinin signaling.
ARR14 and *IAMT1* showed a similar expression pattern. This could point to the possibility of a common regulator or a transcriptional regulation of *IAMT1* by ARR14. One could hypothesize that this might represent a third pathway of regulation of cytokinin signaling via increased auxin signaling not dependent on PINs.

Increased auxin signaling caused by *ARR14* expression might explain the obverse behavior of *ARR14* indicated in the experiments. This concerns typical cytokinin actions as inhibition of senescence as well as the resistance towards pathogens by higher cytokinin levels. I will discuss the functions of ARR14 in a possible auxin context. Please note that the proposed ARR14 functions are deduced by very small differences comparing *arr14-1* and wild type plants and a connection to auxin is highly speculative.

Cytokinin signaling might be blocked by intensified auxin signaling. ARR14 could possibly regulate the auxin distribution via regulation of *PIN5* expression. Hou and colleagues found out, that auxin induced *SAUR* (*SMALL AUXIN-UP*) gene expression, e.g. *SAUR36* which is a positive regulator of senescence (Hou *et al*., 2012). On the other hand the auxin biosynthesis gene *YUC6* (*YUCCA 6*) increased the auxin content and led to a delay in senescence (Kim *et al*., 2011). The action of auxin in senescence is not definite until now so a function of ARR14 in an auxin context concerning senescence remains unclear.

Auxin is also involved in cold stress response. The PIN proteins are directly affected by cold stress and auxin transport is inhibited leading to a loss of auxin gradients in the root (Shibasaki *et al*., 2009). When the auxin maximum is lost by cold stress, root growth is inhibited (Rahman, 2012). One could hypothesize that ARR14, acting on *PIN5* expression, might interfere with root growth in cold stress. In this study the root growth of *arr14-1* plants was increased in cold stress. Those were mutants exhibiting a strongly reduced *PIN5* level. So the increase in *arr14-1* root growth compared to the wildype is contrary to what would be expected for a mutant with decreased PIN accumulation (Rahman, 2012). Possibly PIN5 was not responsible for root growth. It is an atypical PIN protein, located in the endoplasmatic reticulum acting on intracellular auxin compartmentalization (Mravec *et al*., 2009; Dal Bosco *et al*., 2012).

In pathogen infection, the response of *arr14-1* plants would fit to known auxin effects. Lots of pathogens produce not only cytokinin (Pertry *et al*., 2010) but also auxins (Robert-Seilaniantz *et al*., 2007). Exogenous application of auxin increases the susceptibility towards *Pseudomonas syringae* and auxin signaling mutant plants exhibited an increased resistance (Navarro *et al*., 2006; Chen *et al*., 2007; Wang *et al*., 2007). This means auxin positively regulates *Pseudomonas* infection.

ARR14 seems to be a positive regulator of *Verticillium* infection of plants, possibly via regulation of auxin signaling (*PIN5*) (Figure 4.1). It remains unclear if also infections by other pathogens are regulated by ARR14 with a similar result.

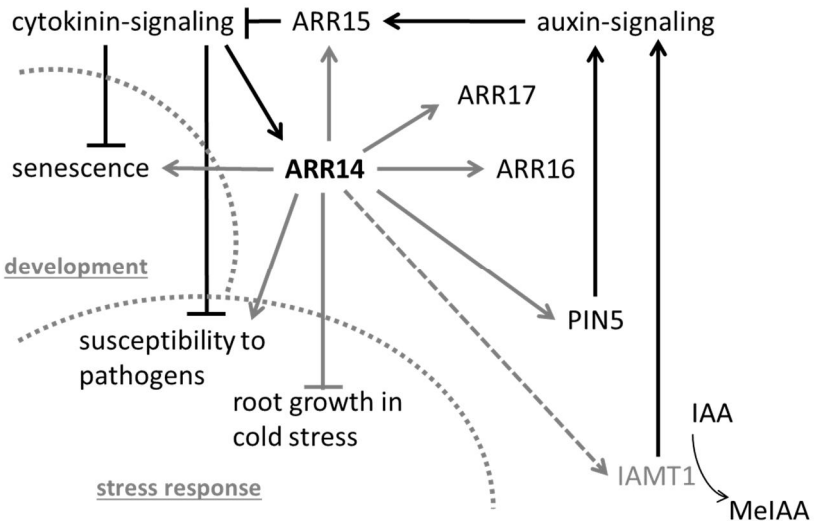


Figure 4.1: Hypothetical model of ARR14 function. Published data are depicted in black arrows, results of my work in grey arrows. The hypothetic connection to IAMT1 is depicted in a dashed line, the topics 'stress response' and 'development' are confined by dotted lines. ARR14 might counteract cytokinin by increasing auxin signaling in different ways. ARR14 induces type-A-response regulators that are known to inhibit cytokinin-signaling (Figure 3.16) (Hwang and Sheen, 2001; To et al., 2007; Lee et al., 2008). ARR14 is also involved in transcriptional regulation of *PIN5* (Figure 3.16). *PIN* expression regulates auxin signaling (reviewed in Krecek *et al*., 2009). As *ARR14* and *IAMT1* show a similar expression pattern in promoter-*uidA* fusion analysis (Figure 3.2 and 3.3), one could speculate that ARR14 might regulate *IAMT1* expression or they might be under the control of a common regulator. *IAMT1* expression would then positively regulate auxin signaling. Cytokinin blocks senescence (e.g. Kim *et al*., 2006) and pathogen susceptibility (e.g. Choi *et al*., 2010; Grosskinsky *et al*., 2011). ARR14 counteracts cytokinin in these processes. It remains unclear whether ARR14 acts directly on those processes or if it counteracts cytokinin by increasing auxin signaling.

To analyze a possible connection between ARR14 and auxin, one should examine auxin signaling output in *arr14-1* and test for a regulation of *IAMT1* in *arr14-1.*

4.2. A screen for modulators of the cytokinin response

The second project was the development of a screening method to identify new modulators of the cytokinin response. The screen was planned to be easy to use, fast, inexpensive and non-destructive. As it was planned to use conditional overexpression of cDNA-libraries and a luciferase reporter, the vector system presented by Papdi and colleagues was utilized (Papdi *et al*., 2008). *ARR6* was selected as a cytokinin reporter. Because 350 bp and 1000 bp fragments of the *ARR6* promoter did not show cytokinin induction *in planta*, the 2 kb promoter was used and

reporter lines could be selected. As cDNA libraries, the PUK-Yale transcription factor library (Gong *et al*., 2004) and a mixture of a seed and primary leaves cDNAs and a hormone treated seedling library (Bürkle *et al*., 2005) were selected and cloned into the respective vector. About 10000 plants were screened.

4.2.1. Nine genes were identified as secondary positive candidates

Out of 14 candidate genes (Table 3.4) identified from the screen nine did show a difference towards the controls in the protoplast transient assay or in the seedling assay of the T2 generation (Figure 3.44 and 3.45). Four genes showed the same pattern of regulation in the T1 assay and in the protoplast transactivation assay, for two genes, there was no progeny to examine in the T2 assay.

Gene 2

AT1g77570 encodes a winged helix-loop-helix-transcriptional repressor. It was identified in the screen because of very high luminescence values. In the protoplast transactivation assay it caused a two-fold repression of the luminescence signal. In the T2 seedling assay plants exhibited a low level of luminescence and were not induced by cytokinin.

Gene 4

At3g54990, *SMZ* (*SCHLAFMÜTZE*), was identified in the screen because of a high start level and a repression of the luciferase signal following cytokinin treatment. In the protoplast transactivation assay there was no difference to the control but in the T2 seedling assay the start luminescence values were again high without cytokinin. Overexpression of this gene led to a low level of induction of the reporter by cytokinin. *SMZ* encodes a repressor of flowering and a target of miR172. It is an AP2-like transcription factor and directly regulates *FT* (*FLOWERING LOCUS T*) dependent on *FLM* (*FLOWERING LOCUS M*) (Mathieu *et al*., 2009).

Gene 5

At5g47670, *LEAFY COTYLEDON 1-LIKE* (*LEC1-like*), was selected in the screen because of high luminescence values that decreased slightly after cytokinin addition. In the protoplast transactivation assay a repression of the luminescence signal was observed after addition of *trans*-zeatin. The plants had no progeny. *LEC1-like* belongs to the *LEC*-type clade of the *HAP3* subunit of the CCAAT-BINDING FACTOR (*CBF, NF-Y*) together with *LEAFY COTYLEDON 1* (*LEC1*)

(Lee *et al*., 2003). Overexpression causes aberrant seedlings as well as *loss-of-function*, which might explain the absence of progeny (Kwong *et al*., 2003).

Gene 7

At1g27730, *SALT TOLERANCE ZINC FINGER/ZINC FINGER OF ARABIDOPSIS THALIANA 10* (*STZ/ZAT10*), was selected in the screen due to the high level of luminescence. In the protoplast transactivation assay the level of reporter activity was already increased without cytokinin but after cytokinin addition the signal was about 15 times higher than in the control. In the T2 seedling assay an about 12-fold induction of luminescence by cytokinin could be observed. STZ is a C_2H_2-type zinc finger protein acting as a nuclear localized transcriptional repressor regulating the transactivation capacity of other transcription factors. Overexpression is lethal in most of the cases (Sakamoto *et al*., 2004; Mittler *et al*., 2006). STZ is activated by MITOGENE ACTIVATED PROTEIN KINASE 3 and 6 (MPK3 and MPK6) (Nguyen *et al*., 2012).

Gene 8

At2g28200, a C_2H_2-type zinc finger protein, was not further characterized until now. The luminescence values in the initial screen were high and even increased after cytokinin addition. It caused an increase of the reporter signal of about five-fold in the protoplast transactivation assay without cytokinin and about 20 times with cytokinin. The plant had no progeny.

Gene 9

At2g17560, *HIGH MOBILITY GOUP B4* (*HMGB4*), was selected because the fold change of luminescence after cytokinin addition was more than ten-fold. In the protoplast transactivation assay the signal was increased about five times, both with and without cytokinin. The gene is highly expressed in flowers and roots and contributes to regulation of transcription and recombination (Kwak *et al*., 2007). In meristematic cells the protein is localized to the nucleus, in cortex cells, it shuttles between nucleus and cytoplasm (Pedersen and Grasser, 2010).

Gene 12

At5g67450, *ARABIDOPSIS ZINC FINGER PROTEIN 1* (*AZF1*), was selected because of high luminescence values in the screen and those even increased after cytokinin induction. *AZF1* was found twice in the screen. In the protoplast transactivation assay the signal increased about five times with cytokinin addition. In the T2 seedling assay the luminescence start values were already high and the induction by cytokinin low. *AZF1* belongs to the same gene family as *STZ.*

(Sakamoto *et al*., 2004). Overexpression is difficult, plants are dwarfed and leaves curly. AZF1 regulates the *SAUR* genes (*SMALL AUXIN UP-REGULATED genes*) and truncated a AFZ1 protein binds to their promoter (Kodaira *et al*., 2011).

Gene 13

The candidate was not a complete gene, but a fragment of *At5g59613*, encoding an unknown protein that is part of the mitochondrial ATP synthase complex. T1 plants in the screen exhibited extremely high luminescence signals. Gene 13 was identified four times in the screen. In the protoplast transactivation assay, there was no difference to WT. In the T2 seedling assay there was no increase in signal at any time point. Gene 13 does not code for the complete protein. It might produce a short peptide. The same clone was also identified in a yeast two-hybrid screen for AHK4 (Dortay *et al*., 2008).

Gene 14

At1g49120, *CYTOKININ RESPONSE FACTOR 9* (*CRF9*), was selected in the T1 seedling because of a high fold change of the reporter by cytokinin application. I was not able to clone *CRF9* into the constitutive overexpression vector, so there are no results for the protoplast transactivation assay. In the T2 seedling assay there was a strong induction of the signal by cytokinin addition. CRF9 has a CRF domain, an AP2/ERF domain and a putative MAPK phosphorylation site (Rashotte and Goertzen, 2010; Shi *et al*., 2012a) and is mainly expressed in the vasculature (Zwack *et al*., 2012). Some CRFs were shown to interact with AHPs (Cutcliffe *et al*., 2011).

4.2.2. The screen revealed new players in the cytokinin response awaiting further analysis

The first fact catching ones eye was the overrepresentation of zinc finger family genes among the candidate genes identified in the screen. Out of nine genes, four genes coded for zinc finger proteins, all of the C_2H_2-type, which represents the largest group among the zinc finger family (Ciftci-Yilmaz and Mittler, 2008). They can at the same time act as an activator or repressor, depending on the target promoter (Ohta *et al*., 2001; Ciftci-Yilmaz and Mittler, 2008). Most of the zinc finger proteins identified in the screen were positive regulators of cytokinin signaling. Zinc finger proteins were found to be involved in response to biotic and abiotic stress and those candidates identified by the screen might represent a link between cytokinin and abiotic/biotic stress response (Ciftci-Yilmaz and Mittler, 2008). Surprisingly no member of the MYB transcription factor family was identified in the screen. MYB transcription factors are the largest

group of transcription factors (Riechmann, 2002). It is surprising that so many different factors seem to influence the cytokinin response. This might be due to the long promoter fragment used and also might be due to indirect actions apart from direct activation of the *ARR6* promoter. The expression of the cDNAs was induced about ten hours before the first measurement. Therefore also factors that e.g. stimulate type-B response regulator akkumulation might have an effect on the *ARR6* promoter activity via type-B response regulator dependent transcriptional activation. Similar, other indirect effects could influence the p*ARR6::LUC* activity as e.g. inhibition by auxin-signaling. This will be discussed in chapter 4.2.3.

Most of the candidate genes are poorly described in the literature until now. Two plants selected did not generate progeny. Those plants, respectively the cDNAs, most probably would not have been found in a constitutive overexpression screen.

The discovery of a candidate gene not representing a protein is interesting. The candidate 'gene 13' was identified four times. Plants always exhibited extremely high luminescence values and died in two of the four cases immediately after the assay. In the protoplast transactivation assay, there was no effect on the *ARR6* promoter observed. Plants of the next generation did not show high luminescence values. Possibly already silencing had appeared as overexpression seemed to be lethal. The absence of large parts of the gene might hint produce a regulatory mRNA or short peptide. If this is the case, it remains confusing, that the same fragment was identified twice in a yeast two-hybrid screen with AHK4 (Dortay *et al*., 2008). In the yeast two-hybrid system, the existence of a protein is prevalent for the screen. It might be that in my experiments an artificial regulatory mRNA was generated by accident and interfered with the transcription of the original gene. Inhibiting an ATPase might generate problems in energy metabolism and lead to the death of the plant as I observed it, but this is highly speculative and should be analyzed further.

In my opinion the most interesting candidates for future research are those causing repeatedly the same change in reporter gene expression and in general those, which are normally lethal when overexpressed and do not have an effect as *loss-of-function* mutants e.g. *STZ* and *AZF1*. They are difficult to find in another screen design and therefore probably mostly uncharacterized concerning cytokinin action. The appearance of *CRF9* is a hint that the screen is able to also identify cytokinin-related elements. However I would have expected more cytokinin-related candidates, as e.g. type-B response regulators, to pop up in the screen.

The induction of the reporter by cytokinin in the inducible ARR2 *gain-of-function* control plants was much lower than expected. In protoplast transactivation assays performed with ARR2, the increase of reporter gene activity was higher than observed in the seedling assay. Phenotypic alterations in constitutive *gain-of-function* plants of type-B response regulators were also minor (Kim *et al*., 2006; Jeon and Kim, 2012). The existence of the regulatory N-terminal receiver domain in type-B response regulators might be the reason for that. Phosphorylation of the type-B response regulators might be a bottleneck for cytokinin response in intact plants. This is corroborated by the obvious phenotypical changes of mutants expressing versions of constitutively active type-B response regulators lacking the N-terminal receiver domain (Tajima *et al*., 2004; Liang *et al*., 2012). Although plants lacking the N-terminal receiver domain of ARR2 would have been a nice control for a strong increase in luminescence values, I decided to use plants expressing the full length ARR2 as controls in the screen. To my opinion, this might reflect the situation in the screen more realistic, at least for screens using full length ORF libraries. Possibly type-B response regulator-expressing plants were overseen in the seedling screen.

4.2.3. AZF1 might connect cytokinin response to auxin regulation

The candidate with the best potential to work on further is in my opinion *AZF1*. In all experiments, the data were consistent and with *STZ* an additional member of the family was found. AZF1 was shown to repress *SAUR* gene expression (Kodaira *et al*., 2011). *SAUR* genes are a multigene family consisting of more than 70 members in *Arabidopsis*. They were shown to be induced by auxin fast similar to *Aux/IAAs* and seem to be regulated by a short lived repressor (Jain *et al*., 2006a). *SAUR39* e.g. regulates auxin synthesis and transport negatively in rice (Kant *et al*., 2009; Kant and Rothstein, 2009). One could hypothesize about a possible regulation of cytokinin signaling via *SAUR* gene mediated changes in auxin-signaling.

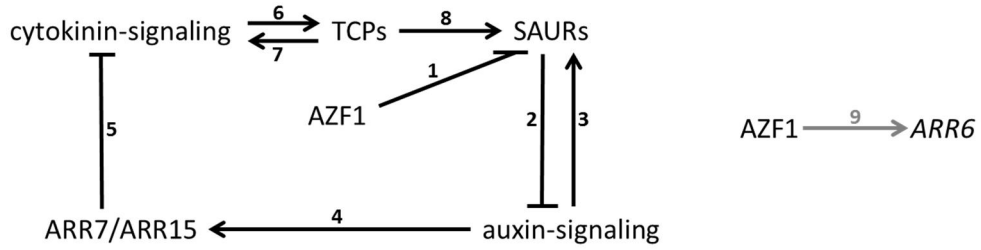


Figure 4.2: Regulations of cytokinin- and auxin-signaling and *AFZ1*. Published data are depicted in black arrows, results from my work in grey arrows. (1) AZF1 represses the expression of some *SAUR* gene family members (Kodaira *et al*., 2011). (2) SAUR39 was shown to negatively regulate auxin-signaling (Kant *et al*., 2009; Kant and Rothstein, 2009) and (3) *SAUR* gene expression is induced by auxin (Jain *et al*., 2006a). (4) Auxin induces *ARR7* and *ARR15* and (5) ARR7 and ARR15 repress, among other type-A response regulators, cytokinin signaling (Müller and Sheen, 2008). (6) It was discussed that cytokinin regulates the expression of *TCP14* and *TCP15* (Steiner *et al*., 2012). (7) TCP14 and TCP15 promote cytokinin signaling via interaction with SPINDLY (Steiner *et al*., 2012). (8) TCP3 regulates a *SAUR39* homolog in Arabidopsis (Koyama *et al*., 2010) and TCP4 regulates the expression of *SAUR-AC1* and the SAUR gene *At5g18060* (Sarvepalli and Nath, 2011). *SAUR39* expression was increased by cytokinin application (Kant and Rothstein, 2009). This might be due to a regulation via TCPs. (9) In my experiments AZF1 was found to increase the cytokinin response which is contradictory to what was expected.

Cytokinin application positively regulates the expression of *SAUR39*. Overexpression of *SAUR39* leads to a decrease in auxin transport and auxin synthesis (Kant and Rothstein, 2009). SAUR39 regulates *PIN6* and some *Aux/IAA* genes (Kant *et al*., 2009). TEOSINTE BRANCHED 1, CYCLOIDEA AND PCF TRANSCRIPTION FACTORs (TCPs) act upstream of *SAUR* genes and *Aux/IAA*. E.g. TCP3 was shown to directly bind to the promoters of *SAUR39 h*omologs and of *IAA/SHY2* and regulate their expression (Koyama *et al*., 2010) and TCP4 was shown to regulate *SAUR-AC1* expression (Sarvepalli and Nath, 2011) (Figure 4.2).

One could hypothesize the following model (Figure 4.2): *AZF1* overexpression might decrease SAUR gene expression. A decreased *SAUR* expression could lead to a lower level of SAUR proteins interfering in auxin signaling. Auxin signaling would increase. This could possibly activate the transcription of *ARR7* and *ARR15*. ARR7 and ARR15 might inhibit cytokinin signaling as an indirect consequence of *AZF1* overexpression. If this might be a pathway in which AZF1 influences cytokinin signaling, the question remains: Why cytokinin signaling was elevated in *AZF1 gain-of-function* plants? The regulatory influence of AZF1 on cytokinin signaling might by much more complex and further research should be done to unravel this pathway.

4.2.4. Further research possibilities for the screen

The screen resulted in the identification of 14 genes possibly having a function in modulation of the cytokinin response. Additionally there were a number of cDNA identified, more candidate DNA extractions await identification of the respective cDNA and there are more T1 seeds to screen available.

The screen cannot be completely evaluated because not all data needed for this are available until now. All progeny plants need to be tested on their endogenous *ARR6* levels. Also the ability to be cytokinin-induced needs to be checked to be sure that effectors are not solely influencing the transgene. Subsequently *loss-of-function* plants should be analyzed for cytokinin responses and, if possible, constitutive *gain-of-function* plants. For some plants, both kinds of mutants are available in the laboratory.
Based on this, one could estimate the rate of true positive genes identified in the screen.

In the current situation, I can just evaluate the procedure of the screen itself.
I will first name some drawbacks that should be kept in mind using the screen. For some candidate genes there were clear differences in the results of the single assays. This could be due to the identification of false positives or might be connected with the assays used. In the T1 seedling assay, the luminescence signal might be not true because of sole activation of the transgene. To address this possible pitfall, the genes were tested in protoplast transactivation assays utilizing a different reporter vector with a different vector backbone from the one used in the reporter plants. Additionally transformation with T-DNAs can cause chromosomal rearrangements near the insertion site or also hit a gene, leading to a *loss-of-function* mutant (Gheysen *et al*., 1990; De Neve *et al*., 1997; Nacry *et al*., 1998). The majority of *loss-of-function* mutants will have no phenotype in the T1 generation examined in the screen. Mostly they are recessive. In the T2 generation, homozygous insertion mutants might cause phenotypes and differences in the luminescence levels if the insertion locus has an effect on the *ARR6* promoter. Another possibility for different results in the seedling assays and the PTA might be the existence of an additional transgene in the T1 plants influencing reporter gene activity. Plants were examined for a second fragment after amplification of the cDNA, but it might be that the chromosomal position of another insertion was more difficult to access by polymerase and primers and so just one of the insertions produced a fragment which was sent for sequencing. If the transgenes are located in close proximity they might also cause no changed segregation in a germination analysis. Differences in the results of the seedling assays and the PTAs might be also

due to the requirement of other factors not present in protoplasts. One possibility is the requirement of proteins being expressed not in the leaves in general or the developmental leaf stage of leaves used for the PTA. Another possibility are factors moving within the plant as peptides, regulatory RNAs or whole pathways being required for the action of the candidate gene on the reporter gene. A difference in the two seedling assays might be due to mechanic stress. In the T1 seedling assay just one plant is analyzed and stress by transfer might influence the result. In the T2 seedling assay, 96 plants are examined and the mechanical damage of one plant would not influence the result calculated from all of the plants very much. Also the pH of 7,8 in the media can stress the plants, but seems to be the best condition for the luciferase to work. Aberrations in luminescence levels in seedling assays using the T2 generation compared to those of the T1 generation might be due to compensation or silencing effects in the T2 generation (Mlotshwa *et al*., 2010). Candidates for this are genes that cause lethality when being overexpressed.

In my opinion the screen is a well suited method to access genes involved in cytokinin signal that have not been assigned to a function in cytokinin response until now. As the results show, it was possible, to also find potential modulators of the cytokinin response that do not show a phenotype in *loss-of-function* mutants and are lethal in *gain-of-function* mutants. The screen is non-destructive and easy to do. The only technical requirement to assay the luminescence values is a plate reader, which is available in most of the institutes by now in contrast to an expensive CCD camera. The plate reader also has the advantage that a readout already provides a quantification. Other non-destructive screens based on GFP for example often are time-consuming qualitative screens or require quantification by estimation. This massively increases impreciseness. Another possibility is to use a PAM fluorimeter. Depending on the promoter used, fluorescence or luminescence levels are often too low for PAM fluorimetry.

The screen I developed does not require much space as plants are grown on plates and candidates are selected very early. It is fast, because plants are screened in the T1 generation five days after sowing. Except for the sowing on plate and the transfer of the seedlings into the microtiter plates, it is possible to screen fully automated if the machines are available. This would also overcome the limitation of about twelve plates per day and one could screen complex libraries in a short time. So the screen fits all the requirements Bolle and colleagues articulated in their publication about the future of genetic screens (Bolle *et al*., 2011). It is non-invasive, innovative and can be automated.

The screen could be used for various applications, e.g. for a test with other promoters or a test for reaction on chemicals.

5. Summary

Cytokinins are essential phytohormones involved in numerous processes during the plant life cycle. In concert with other factors, they mediate growth and development, as well as responses to the environment. Cytokinins are perceived by transmembrane receptors. Those, upon ligand binding, activate an intracellular signaling cascade, which culminates in activation of transcription factors. Type-B response regulators, transcriptional activators, are the most prominent proteins that mediate the primary response to the cytokinin stimulus. Recently additional protein families, for example the CYTOKININ RESPONSE FACTORs (CRFs), have also been identified as transcription factors in the cytokinin signaling pathway.

Among the type-B response regulators, the mostly uncharacterized ARR14 has some unique features. It was found to interact with one of the receptors and with another type-B response regulator. Additionally there were hints that ARR14 might be involved in the progression of senescence and pathogen response. ARR14 was therefore an interesting candidate for further studies and was selected as my first project in this work.

Reactions of plants to their environment are mostly regulated by diverse effectors, allowing a fine-tuning of responses. Unknown factors, or factors known from a different functional context, might influence a pathway that they are not jet connected to. To identify unknown modulators of the cytokinin response, I developed and conducted a screening procedure as my second project.

For *ARR14* I could show a specific expression pattern in leaves similar to the area of auxin biosynthesis. ARR14 was localized to the nucleus and seems to be not involved in the regulation of the typical cytokinin primary response gene *ARABIDOPSIS RESPONSE REGULATOR 6 (ARR6)* but in the transcriptional activation of *ARR15, ARR16* and *ARR17*. Additionally, it appears to regulate *SENSESCENCE-ASSOCIATED GENE 12 (SAG12)* and *PIN-FORMED 5 (PIN5)* on the transcriptional level. By *loss-of-function* analysis I identified possible functions of ARR14. Contradictory to typical cytokinin actions, ARR14 seems to be a negative regulator of root growth in cold stress conditions, a positive regulator of senescence onset and positive regulator in pathogen susceptibility towards *Verticillium*.

The screen for modulators of the cytokinin response was based on a vector system composed of an inducible overexpression effector vector and a *luciferase (LUC)* reporter

vector. Utilization of an overexpression system allowed a fast screening of plants in the first transformed generation. An inducible system has the advantage to avoid lethality often caused by constitutive overexpression. To use a LUC reporter construct enabled non-destructive screening of plants. For assembly of a cytokinin response reporter, the promoter of *ARR6* was cloned in front of the *LUC* gene. A transcription factor full length ORF cDNA library and a cDNA library of primary leaves, seeds and hormone-treated seedlings were shuttled into the effector vector. Reporter plants were established and transformed with the effector vector containing the cDNA libraries. The resulting T1 progeny was assayed in a newly designed luciferase seedling assay in microtiter plates with a microplate reader. Luminescence was measured before and after cytokinin treatment and candidates exhibiting deviances to the reactions of untransformed reporter plants were selected for analysis and grown further. DNA was extracted and the respective candidate genes were identified. Accordingly, those genes were reanalyzed for their regulative function on the *ARR6* promoter by protoplast transactivation assays and a seedling assay of the respective plant progeny. Several candidate genes not jet connected to cytokinin were identified and verified. Additionally, the screen resulted in the identification of a *CRF*. CRFs were already shown to regulate cytokinin response.

This study was performed to learn more about the transcriptional regulation and modulation of the cytokinin response. It shed a light on new functions of the type-B response regulator and transcription factor ARR14 and identified novel factors modulating the cytokinin response.

6. Zusammenfassung

Cytokinine sind essentielle Phytohormone, die an verschiedensten Prozessen während des Lebenszyklus von Pflanzen beteiligt sind. Gemeinsam mit anderen Faktoren vermitteln sie sowohl Wachstum und Entwicklung als auch Reaktionen der Pflanze auf ihre Umwelt. Cytokinine werden von Transmembranrezeptoren wahrgenommen. Bindet das Cytokinin an den Rezeptor, erfolgt eine intrazelluläre Signalkaskade, die in der Aktivierung von Transkriptionsfaktoren kulminiert. Typ-B *Response* Regulatoren sind transkriptionelle Aktivatoren und stellen die bekannteste Gruppe, der Cytokinin-aktivierten Transkriptionsfaktoren dar. Auch die CYTOKININ RESPONSE FACTORen (CRFs) vermitteln einen Teil der Cytokininantwort.

Unter den Typ-B *Response* Regulatoren wurde bisher nur für ARR14 eine Interaktion mit einem der Rezeptoren und einem weiteren Typ-B Response Regulator nachgewiesen. Dies und Hinweise auf eine Funktion des bisher weitestgehend uncharakterisierten ARR14 in der Seneszenz und der Pathogenanwort machten ARR14 zu einem interessanten Kandidaten für weitere Studien. Das erste Projekt meiner Arbeit war deshalb die Charakterisierung von ARR14.

Die Reaktionen von Pflanzen auf ihre Umwelt sind in den meisten Fällen durch diverse Effektoren reguliert, die eine Feineinstellung der zellulären Antwort ermöglichen. Unbekannte Faktoren, sowie Faktoren, die bisher in einem anderen funktionellen Kontext gesehen wurden, könnten Signalwege beeinflussen, in die sie bisher nicht eingeordnet wurden. Um unbekannte Modulatoren der Cytokininantwort zu finden, habe ich ein einem zweiten Projekt eine *Screening*prozedur entwickelt und durchgeführt.

ARR14 zeigte in meinen Untersuchungen ein spezifisches Expressionsmuster, das dem Muster der Auxinbiosynthese ähnelt. Das Protein ist kernlokalisert und scheint nicht das typische Cytokininantwortgen *ARABIDOPSIS RESPONSE REGULATOR 6 (ARR6)* zu regulieren. Jedoch beeinflusst es die Transkription von *ARR15, ARR16* und *ARR17*. Zudem scheint ARR14 an der transkriptionellen Regulation von *SENSESCENCE-ASSOCIATED GENE 12 (SAG12)* und *PIN-FORMED 5 (PIN5)* beteiligt zu sein. In *loss-of-function* Experimenten konnte ich ARR14 mögliche Funktionen zuordnen. Es scheint ein negativer Regulator von Wurzelwachstum unter Kältestress zu sein, ein positiver Regulator des Seneszenzbeginns und ein positiver Regulator der Empfindlichkeit gegenüber *Verticillium*.

Für den *Screen* nach Modulatoren der Cytokininantwort wurde ein Vektorsystem aus einem in Effektorvektor zur induzierbaren Überexpression und einem *Luziferase (LUC)*-Reportervektor verwendet. Die Verwendung eines Systems zur Überexprimierung erlaubte eine schnelle *Screening*prozedur, da bereits die Transformanden der ersten Generation untersucht werden konnten. Die induzierbare Überexpression ermöglicht es, Gene zu untersuchen, die bei konstitutiver Überexpression lethal sind. Mit einem *LUC*-basierten Reportervektor können Pflanzen nicht-destruktiv untersucht werden. Um einen Reportervektor herzustellen, der auf Cytokininapplikation reagiert, wurde der Promotor von *ARR6* vor das *LUC*-Gen kloniert. In den Effektorvektor wurden cDNA-Bibliotheken kloniert. Das waren zum einen eine Transkriptionsfaktor-cDNA-Bibliothek aus Klonen in voller Länge und zum anderen eine cDNA-Bibliothek aus hormonbehandelten Keimlingen, Primärblättern und Samen. Nachdem Reporterpflanzen etabliert waren, wurden diese mit dem cDNA-Effektorvektor transformiert und die Nachkommen in einem neu entwickelten Keimlingsassay untersucht. Hierfür wurde die Luciferaseaktivität der Keimline in Mikrotiterplatten in einem *Microplate Reader* vor und nach Cytokininzugabe gemessen. Pflanzen mit erhöhten Lumineszenzwerten oder Abweichungen in der Reaktion auf Cytokinin verglichen mit untransformierten Reporterpflanzen wurden herangezogen und die DNA isoliert. Die daraufhin identifizierten Kandidatengene wurden in Protoplasten-Transaktivierungsversuchen erneut auf ihren Einfluss auf den *ARR6* Promoter untersucht. Zudem wurde auch die Nachkommenschaft der Ursprungspflanze im Keimlingsassay untersucht. Es konnten mit Hilfe des *Screens* einige Gene identifiziert werden, die die Cytokininantwort beeinflussen zu scheinen. Neben unbekannten Genen wurde auch ein *CRF* identifiziert. Für CRFs ist eine Beteiligung an der Cytokininantwort bekannt.

Diese Arbeit beschäftigte sich mit der transkriptionellen Regulation und Modulation der Cytokininantwort. Es wurden neue Erkenntnisse gewonnen über den Typ-B *Response* Regulator und Transkriptionsfaktor ARR14 und es wurden neue Faktoren identifiziert, die an der Regulation der Cytokininantwort beteiligt zu sein scheinen.

7. Publications

Hellmann, E., Gruhn, N., and Heyl, A. (2010). The more, the merrier: Cytokinin signaling beyond *Arabidopsis*. Plant Signaling and Behaviour 5, 1365 - 1371.

Cutcliffe, J.W., Hellmann, E., Heyl, A., and Rashotte, A.M. (2011). CRFs form protein-protein interactions with each other and with members of the cytokinin signalling pathway in *Arabidopsis* via the CRF domain. Journal of Experimental Botany.

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9. Appendix

9.1. Abbreviations

bp	basepairs
fw	Forward
kb	kilobasepairs
p	promoter
Pfu	DNA-Polymerase aus *Pyrococcus furiosus*
qRT	Quantitative Real-time
rev	reverse
rpm	rounds per minute
h	hours
WT	wild type
LUC	Luciferase

9.2. Oligonucleotide list

#	Lab identifier	Name	Sequence	Application
1	338	*ARR14* fw	AAAAAGCAGGCTTGATGCCGATCAAC GATCAGTTTCC	cloning
2	466	*ARR14* without stop rev	CAAGAAAGCTGGGTCTCTTTGTCTTGS SGATC	cloning
3	463	*ARR14* promoter 850 bp fw	TACAAAAAAGCAGGCTTCTCTCCTTGA AGGCTTGAAAA	cloning
4	465	*ARR14* promoter rev	TACAAGAAAGCTGGGTCTAAGCTTTGG TGTGAGTATTGA	cloning
5	4	GW attB1	GGGGACAAGTTTGTACAAAAAAGCAG GCT	cloning
6	5	GW attB2	GGGGACCACTTTGTACAAGAAAGCTG GGT	cloning
7		Screen fw	GCTTGGGCTGCAGGTCGAGGCTAA	cloning
8		Screen rev	CTGGTGTGTGGGCAATGAAACTGATG	cloning

			C	
9		Screen seq	ATAAGGAAGTTCATTTCATTTGGAGAG GAC	sequencing
10	563	*ARR2* T-DNA P1	TTGCTGATGTTCTTGTTGTGC	analysis T-DNA mutants
11	564	*ARR2* T-DNA P2	TCTGTTGAATTGCATCAGCAG	analysis T-DNA mutants
12	460	*AHK2* T-DNA P1	AGTGTACCCGGGGATTATATGTGCAAC AAGTAACGC	analysis T-DNA mutants
13	461	*AHK2* T-DNA P2	GTACCATAGCGGCCGCAATATCCTTCT CCTTGGTATTTTAG	analysis T-DNA mutants
14	844	*AHK3* T-DNA P1	GGTCGGCTAAAGCTTTGCTA	analysis T-DNA mutants
15	805	*AHK3* T-DNA P2	CGCAAGCTATGGAGAAGAGG	analysis T-DNA mutants
16	489	*ARR14* – 1 T-DNA P1	TATCTCCAACATCGCCATTTC	analysis T-DNA mutants
17	490	*ARR14* – 1 T-DNA P2	CAACAGTCTCGAGGCTACCAC	analysis T-DNA mutants
18	475	*ARR14* – 2 T-DNA P1	CTCAGAAGTGCGTTTGAAACC	analysis T-DNA mutants
19	476	*ARR14* – 2 T-DNA P2	GTGGTAGCCTCGAGACTGTTG	analysis T-DNA mutants
20	477	*ARR14* – 3 T-DNA P1	CACAAGCTCCATGGTTGATTC	analysis T-DNA mutants
21	478	*ARR14* – 3 T-DNA P2	TATCTCCAACATCGCCATTTC	analysis T-DNA mutants
22	491	T-DNA GABI-KAT	ATATTGACCATCATACTCATTGC	analysis T-DNA mutants
23	479	T-DNA SALK	ATTTTGCCGATTTCGGAAC	analysis T-DNA mutants
24	480	T-DNA SAIL	GCCTTTTCAGAAATGGATAAATAGCCT TGCTTCC	analysis T-DNA mutants
25	714	*ARR2* probe fw	GTTTCAAGCTCCTTGAACACG	RT-PCR
26	713	*ARR2* probe rev	GCTGCTCTGAACATTCTGTCC	RT-PCR
27	131	*Actin* probe fw	TACAACGAGCTTCGTGTTGC	RT-PCR
28	132	*Actin* probe rev	GATTGATCCTCCGATCCAGA	RT-PCR

29	406	*ARR14* probe fw	ATGCCGATCAACGATCAGTTTC	RT-PCR
30	562	*ARR14* probe rev short	AGCACCAAATCAAAACTGTC	RT-PCR
31	616	*ARR14* probe rev	CTATCTTTGTCTTGAAGATCT	RT-PCR
32	850	*LUC* probe fw	AATCTCACGCAGGCAGTTCT	qRT-PCR
33	849	*LUC* probe rev	CCAGGGATTTCAGTCGATGT	qRT-PCR
34	RT 7	*ARR4* probe fw	CCGTTGACTATCTCGCCT	qRT-PCR
35	RT 8	*ARR4* probe rev	CGACGTCAACACGTCATC	qRT-PCR
36	RT 15	*ARR5* probe fw	CTACTCGCAGCTAAAACGC	qRT-PCR
37	RT 16	*ARR5* probe rev	GCCGAAAGAATCAGGACA	qRT-PCR
38	RT 27	*ARR6* probe fw	GAGCTCTCCGATGCAAAT	qRT-PCR
39	RT 28	*ARR6* probe rev	GAAAAAGGCCATAGGGGT	qRT-PCR
40	RT 13	*ARR7* probe fw	CTTGGAACCAATCTGCTCTC	qRT-PCR
44	RT 14	*ARR7* probe rev	ATCATCGACGGCAAGAAC	qRT-PCR
45	RT 273	*ARR8* probe fw	CAACCCGAGAAGCCACTA	qRT-PCR
46	RT 274	*ARR8* probe rev	ACGATGTTGCTGCGGTAT	qRT-PCR
47	RT 275	*ARR9* probe fw	GATAGAGCACGTCCTAGATTCG	qRT-PCR
48	RT 276	*ARR9* probe rev	CTGCATTCCCTACTGAAACC	qRT-PCR
49	RT 277	*ARR15* probe fw	GAGAGGTGGTGAAGCTGAA	qRT-PCR
50	RT 278	*ARR15* probe rev	GATGGAGTGTCGTCATCAAG	qRT-PCR
51	RT 303	*ARR16* probe fw	TCAGGAGGTTCTTGTTCGTCTT	qRT-PCR
52	RT 304	*ARR16* probe rev	AACCCAAATACTCCAATGC	qRT-PCR
53	RT 305	*ARR17* probe fw	TTCTTGCAAAGTGACAACTGC	qRT-PCR
54	RT 306	*ARR17* probe rev	TCAATGAATCAGTCTGTTGTGG	qRT-PCR
55	RT 293	*SAG12* probe fw	TCTGGTGTGTTCACTGGAGAGT	qRT-PCR
56	RT	*SAG12* probe rev	ATCCGTTAGTAGATTCGCCGTA	qRT-PCR

	294			
57	RT 283	*PIN5* probe fw	CTAAGCACAGCGTAAGTCCA	qRT-PCR
58	RT 284	*PIN5* probe rev	GAATAAACTCCAGAGCTGCG	qRT-PCR
59	1064	*ARR14* probe fw	TCCTGGAAACTCGAAGAAGTCACG	qRT-PCR
60	1063	*ARR14* probe rev	AGAATCCGCTTTGGTACAGCTTTG	qRT-PCR
61	RT 3	Housekeeping At3g25800 fw	CCATTAGATCTTGTCTCTCTGCT	qRT-PCR
62	RT 4	Housekeeping At3g25800 rev	GACAAAACCCGTACCGAG	qRT-PCR
63	770	*ARR6* prom 1000 bp hindIII fw	CAGAACTATCTCCGGCCTCA	qRT-PCR
64	768	*ARR6* prom 350 bp hindIII fw	CAAGTTATTGAATATCGGTTTGG	qRT-PCR
65	863	*ARR6* prom 2000 bp hindIII fw	GCATTTCTTCGGGAGAGAGCCAAG	qRT-PCR
66	864	*ARR6* prom 2000 bp hindIII	TCGTAAGCTTGGATTTGGAAGAAAAA GGAA	qRT-PCR

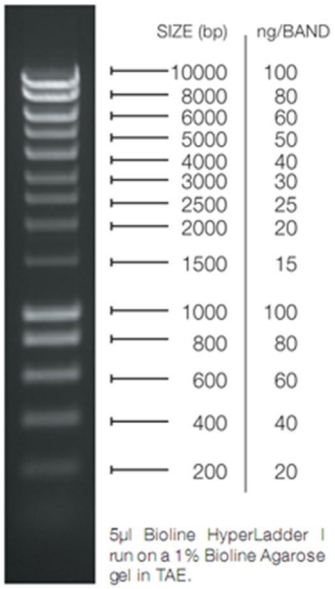
T-DNA-insertions in plants were identified as follows:

Mastermix 1(identifies WT-allel): forward primer + reverse primer

Mastermix 2 (identifies T-DNA insertion): T-DNA primer + reverse primer

Primers were selected using http://signal.salk.edu/tdnaprimers.2.html

9.3. Hyperladder I



HyperLadder™ 1kb, (Bioline USA Inc., Randolph, USA)

Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation in allen Teilen selbständig verfasst und keine anderen als die von mir angegebenen Quellen verwendet habe.

Berlin, den

_____

Eva Hellmann

10.Literature

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