Characterization of mediators of the cytokinin transcriptional response

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

Dissertation
Zur Erlangung des akademischen Grades des Doktors in den Naturwissenschaften (Dr. rer. nat.)

eingereicht im Fachbereich für Biologie, Chemie und Pharmazie der Freien Universität Berlin

vorgelegt von

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Erschienen in Berlin, 2013
Disputation Berlin, 03.05.2013

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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 axillary 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 N6-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 monoxygenases 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 Arabidopsis 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. 5-125 ng/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 tZ-adenosinephosphates by CYP735A. LOG leads to the formation of free iP and tZ. 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 cZRMP. This can be converted to cZ and cZ. CYP735A, P450 monoxygenase; IPT, isopentenytransferase; LOG, LONELY GUY; UGT, glycosyltransferase; CKX, cytokinindehydrogenase/-oxidase; ATP/ADP/AMP, adenosine-tri/di/mono-phosphate; Ade, adenosine; iPRT/iPRD/iPRMP, isopentenyladenosine-tri/di/mono-phosphate; tZRTP/tZRDP/tZRMP, trans-zeatinadenosine-tri/di/mono-phosphate; DMAPP, dimethylallyldiphosphate; cZRMP, cis-zeatinadenosinemonophosphate; iPR, isopentenyribose; iP, isopentenyl; tZR, trans-zeatinriboside; tZ, trans-zeatin; cZR, cis-zeatinriboside; cZ, 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 SENECEENCE-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 SENECEENCE 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 irreversibly 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.,
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 CKX 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), Arabidopsis 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 N6-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 iP (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-independent 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 *Arabidopsis*, 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).](image)

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 colleagues 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 leaves (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 RESPONSE REGULATOR-LIKE PROTEIN 5 (ARR2/ARP5) (Sakai et al., 1998; Buchholz et al., 1998) and ARR11/ARP3 (Buchholz et al., 1998).
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-phosphorylatable 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-phosphorylatable 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 adventitious shoots, and bushy rosette leaves (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.
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-phosphorylatable 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 loss-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 PHYLOTAXY 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).

Analyze 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 FACTORS* (ERFs) 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), BASIC 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 motif. 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 HOMEBOX (KNOX)-
transcription factor KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 1 (KNAT1) (Curaba et al., 2003)

A role in cytokinin response was also reported for the C2H2 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 C2H2 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, loss-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.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli:</td>
<td>DH10B</td>
<td>F mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 endA1 aroD139 Δ(ara, leu)7697 galU galK λ rpsL nupG</td>
<td>Calvin and Hanawalt, 1988; Raleigh et al., 1988</td>
</tr>
<tr>
<td>E. coli:</td>
<td>DB3.1</td>
<td>F gyrA462 endA1 Δ(sr1-recA) mcrB mrr hsdS20(r6m159) supE44 ara14 galK2 lacY1 proA2 rpsL20(Sm) xyl5 Δleu ml1</td>
<td>Hanahan, 1983; Bernard and Couturier, 1992</td>
</tr>
<tr>
<td>A. tumefaciens</td>
<td>GV3101::pM90</td>
<td>rpoH+ hrcA+</td>
<td>Schell, 1978</td>
</tr>
<tr>
<td>V. longisporum</td>
<td>Isolate 43</td>
<td>wild type</td>
<td>from Karen Zeise, Rostock</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Line</th>
<th>Description</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>arr14-1</td>
<td><em>Arabidopsis thaliana</em>, T-DNA insertion mutant, insertion in ARR14, insertion in second exon/receiver domain</td>
<td>GABI Kat 147802 (Ishida et al., 2008b)</td>
</tr>
<tr>
<td>arr14-2</td>
<td><em>Arabidopsis thaliana</em>, T-DNA insertion mutant, insertion in ARR14, insertion in third exon/DNA-binding output domain</td>
<td>SAIL_278_B11</td>
</tr>
<tr>
<td>arr14-3</td>
<td><em>Arabidopsis thaliana</em>, T-DNA insertion mutant, insertion in ARR14, insertion in 5' UTR</td>
<td>SAIL_630_D09</td>
</tr>
<tr>
<td>arr2-4</td>
<td><em>Arabidopsis thaliana</em>, T-DNA insertion mutant, insertion in ARR2</td>
<td>SALK_016143 *             (Mason et al., 2005)</td>
</tr>
<tr>
<td>ahk-5</td>
<td><em>Arabidopsis thaliana</em>, T-DNA insertion mutant, insertion in AHK2</td>
<td>SALK_037536 *             (Riefler et al., 2006)</td>
</tr>
<tr>
<td>ahk-7</td>
<td><em>Arabidopsis thaliana</em>, T-DNA insertion mutant, insertion in AHK3</td>
<td>SALK_069269 *             (Riefler et al., 2006)</td>
</tr>
<tr>
<td>arr2-4</td>
<td><em>Arabidopsis thaliana</em>, T-DNA insertion mutant, insertion in ARR2</td>
<td>SALK_016143 *             (Mason et al., 2005)</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Name</th>
<th>Vector</th>
<th>Reference for vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>pARR14 850 bp::GUS</td>
<td>pCB308</td>
<td>Xiang et al., 1999</td>
</tr>
<tr>
<td>35S::ARR14</td>
<td>pB2GW7</td>
<td>Karimi et al., 2002</td>
</tr>
<tr>
<td>pBT10-GUS</td>
<td>pBT10-GUS</td>
<td>Sprenger-Haussels and Weisshaar, 2000</td>
</tr>
<tr>
<td>pROK219_NAN</td>
<td>pROK219_NAN</td>
<td>Kirby and Kavanagh, 2002</td>
</tr>
<tr>
<td>35S::GFP-ARR14</td>
<td>pB7WGF2</td>
<td>Karimi et al., 2002</td>
</tr>
<tr>
<td>pARR6 350 kb::LUC+</td>
<td>pBinLUC+</td>
<td>Papdi et al., 2008</td>
</tr>
<tr>
<td>pARR6 1000 kb::LUC+</td>
<td>pBinLUC+</td>
<td>Papdi et al., 2008</td>
</tr>
<tr>
<td>pARR6 2146 kb::LUC+</td>
<td>pBinLUC+</td>
<td>Papdi et al., 2008</td>
</tr>
<tr>
<td>pARR6 2146 kb::LUC+ pER8GW_GONG</td>
<td>pBinLUC+</td>
<td>Papdi et al., 2008</td>
</tr>
<tr>
<td>pARR6 2146 kb::LUC+ pER8GW_seed/hormone</td>
<td>pBinLUC+</td>
<td>Papdi et al., 2008</td>
</tr>
<tr>
<td>pARR6 2146 kb::LUC+ pER8GW.ARR2</td>
<td>pBinLUC+</td>
<td>Papdi et al., 2008</td>
</tr>
<tr>
<td>pARR6 2146 kb::LUC+ pER8GW.CXX1</td>
<td>pBinLUC+</td>
<td>Papdi et al., 2008</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Library</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUK-Yale library</td>
<td>full length ORF transcription factor library from <em>Arabidopsis thaliana</em></td>
<td>Gong et al., 2004</td>
</tr>
<tr>
<td>seed library</td>
<td>cDNA library from seeds and primary leaves from <em>Arabidopsis thaliana</em></td>
<td>Bürkle et al., 2005</td>
</tr>
<tr>
<td>hormone library</td>
<td>cDNA library from hormone-treated <em>Arabidopsis thaliana</em> seedlings</td>
<td>Bürkle et al., 2005</td>
</tr>
</tbody>
</table>

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 KNO₃ [1M], 2,5 ml KPO₄ [1 M], 2 ml MgSO₄ [1 M], 2 ml Ca(NO₃)₂ [1 M], 2,5 ml Fe-EDTA [20 mM] and 1 ml micronutrients (70 mM H₃BO₃, 14 mM MnCl₂, 0,5 mM CuSO₄, 1 mM ZnSO₄, 100 x Na₂MoO₄, 10 mM NaCl, 1000 x CoCl₂)) (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 Na₂NO₃, 0.5 g MgSO₄·7H₂O, 0.5 g KCl und 0,01 g FSO₄ 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>
<thead>
<tr>
<th>Antibiotics</th>
<th>Stocks (mg/ml)</th>
<th>Final concentration (µg/ml) for bacteria</th>
<th>Final concentration (µg/ml) for plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Carbenecillin</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Gentamycin</td>
<td>25</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Phosphinothricin</td>
<td>25</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>7.5</td>
<td>5.25</td>
<td></td>
</tr>
<tr>
<td>Hygromycine</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Cefotaxine</td>
<td>250</td>
<td>125</td>
<td></td>
</tr>
</tbody>
</table>
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 Na₂EDTA, 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 glacialic 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 OD₆₀₀ 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 thawed 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 nitrogen 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

<table>
<thead>
<tr>
<th>Initial denaturation</th>
<th>95°C</th>
<th>2 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>45 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>52°C</td>
<td>standard 45 seconds</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C (Taq polymerase) or 68°C (Pfu-polymerase)</td>
<td>standard 2 minutes</td>
</tr>
<tr>
<td>Final elongation</td>
<td>same temperature as extension</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>

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.
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

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>First strand synthesis</td>
<td>50°C</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Deactivation of the reverse transcriptase and activation of the DNA polymerase</td>
<td>95°C</td>
<td>15 minutes</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Annealing</td>
<td>52°C</td>
<td>standard 1 minute</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C (Taq polymerase) or 68°C (Pfu-polymerase)</td>
<td>standard 1 minute</td>
</tr>
<tr>
<td>Final elongation</td>
<td>same temperature as extension</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>15 seconds</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>10 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>15 seconds</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>10 seconds</td>
</tr>
<tr>
<td>Final elongation</td>
<td>Same as extension</td>
<td>1 minute</td>
</tr>
</tbody>
</table>

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 1.5 ml 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-thiocyanate [4 M], 100 µl ammonium-thiocyanate [4 M], 33.4 µl Na-acetate [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 adventitious 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 6th and the 10th 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 (10⁵-10⁶ 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 adventitious shoot formation.
2.6.7. Transformation of A. thaliana

Plants were transformed by the floral dip method adapted from Clough and Bent (1998). A. tumefaciens 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 MgCl2; 150 µM acetosyringone). The pellet was dissolved in infiltration buffer and diluted to an OD600 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 pARR14850bp::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 leaves 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 plasmid 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 protoplasts 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-lauroyl 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\textsuperscript{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 gynoecium (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 (Appendix) 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.](Image)

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 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 pARR14::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 pARR14::uidA at the tip and at the abscission zone (Figure 3.2h).
Figure 3.2: Analysis of ARR14 expression using pARR14\(^{850bp}\)::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 apexes 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 appeared 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\textsubscript{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](image_url)

**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 comparison.

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.](image)

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 than 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 fragment (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).

![Agarose gel of the transcript analysis for ARR14](image)

**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).
3.1.5 Double mutants of \textit{arr14} with \textit{arr2}, \textit{ahk2} and \textit{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 \textit{in vivo} and \textit{in planta}, the \textit{arr14-1 loss-of-function} line was crossed with \textit{loss-of-function} mutants of ARR2 (\textit{arr2-4}, Mason \textit{et al}., 2005) and AHK2 (\textit{ahk2-5}, Riefler \textit{et al}., 2006). To test if signaling via a possible AHK2-ARR14 interaction might be important, \textit{arr14-1} was also crossed with \textit{ahk3-7} (Riefler \textit{et al}., 2006). If the interaction was relevant \textit{arr14 ahk3} should have looked similar to the semi-dwarfed \textit{ahk2 ahk3} mutant (Nishimura \textit{et al}., 2004; Riefler \textit{et al}., 2006). \textit{arr14-1} was not crossed with \textit{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 \textit{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-function 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 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). pARR6\textsuperscript{350bp} was found to be sufficient for the full cytokinin response in protoplasts (Ramireddy, 2009).
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 arr14 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.](image)

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 \textit{in silico} (Hruz et al., 2008). ARR14 transcription was shown to be regulated by exposure to pathogens like \textit{Botrytis cinerea}, \textit{Blumeria graminis}, \textit{Phytophthora infestans}, \textit{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). Preliminary results had indicated a delay in senescence and a changed resistance to \textit{Verticillium longisporum} in the \textit{arr14-1} mutant (Hellmann, 2007).

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

![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).](image-url)
3.1.7.1 The \textit{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. \textit{arr14-1} plants performed significantly better than the WT. They exhibited an increased root elongation compared to WT. In contrast to \textit{arr14}, \textit{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.** \textit{arr14}, \textit{ahk2} and the \textit{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. \textit{arr14-1} roots were significant longer than WT roots after cold treatment. Roots of \textit{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\text{WT} = 121, n\text{arr14} = 100, n\text{ahk2} = 110, n\text{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 \textit{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 \textit{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, \textit{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 polyethylene-gluconol (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.
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 senescence 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

Table 3.1: 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 Chi²-tests.

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<th>Experiment 3</th>
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*Chi²-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 adventitious 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, adventitious shoot number and percent of colonization.

![Figure 3.28](image)

**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 adventitious 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 symptoms. 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*<sub>WT mock</sub> = 50, *n*<sub>arr14 mock</sub> = 56, *n*<sub>WT inoc.</sub> = 64, *n*<sub>arr14 inoc.</sub> = 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\text{WT mock} = 50, n\text{arr14 mock} = 56, n\text{WT inoc.} = 64, n\text{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 greenhouse. 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 consists 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 promoter was chosen 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 pARR62146bp::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\(2146\)::uidA A. thaliana plants. pARR6::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 HindIII 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.

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<tr>
<td>4</td>
<td>T-box promoter motif</td>
<td>Light-regulation</td>
<td>Chan et al., 2001</td>
</tr>
<tr>
<td>5</td>
<td>MYB4 binding site</td>
<td>UV-protection</td>
<td>Chen and Provart, 2002</td>
</tr>
<tr>
<td>6</td>
<td>Box II promoter motif</td>
<td>Light-regulation</td>
<td>Le Gourrierec et al., 1999</td>
</tr>
</tbody>
</table>
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\textsuperscript{350bp}, pARR6\textsuperscript{1000bp} and pARR6\textsuperscript{2kb} 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 laboratory. None of the pARR6\textsuperscript{350bp} reporter plants showed a cytokinin induction. Just one pARR6\textsuperscript{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\textsuperscript{350bp} and pARR6\textsuperscript{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 luminescence 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, \textit{pARR6}^{350\text{bp}}::LUC and \textit{pARR6}^{1000\text{bp}}::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 \textit{pARR6}^{2\text{kb}}::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 \textit{ARR1} overexpression (Figure 3.37). \textit{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 \textit{ARR1} and cytokinin. Reporter plant B showed a strong mainly \textit{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 \textit{ARR1}. R2 I selected to also find cytokinin-independent activators of the \textit{ARR6} promoter in the screen.
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).
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 (tZ), 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\textsubscript{untreated} = 4, n\textsubscript{tZ} = 28, n\textsubscript{EtOH} = 8, n\textsubscript{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\textsubscript{untreated} = 4, n\textsubscript{tZ} = 28, n\textsubscript{EtOH} = 8, n\textsubscript{Estr} = 8.

Finally homozygous reporter lines transformed with the pARR6\textsuperscript{2kb}: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 luminescence to untransformed reporter plants by cytokinin induction, 3) high luminescence 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 \textit{pARR6}^{2kb::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 tZ, 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 outlier 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 outliers and the CKX1-plant outliers 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 outliers 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).

<table>
<thead>
<tr>
<th>Transformation of</th>
<th>Number of T1 screened</th>
<th>Number of primary positives selected</th>
<th>% of primary positives in the screened T1</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1/R2 with cDNA library</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>R1 seed/hormone</td>
<td>2944</td>
<td>532</td>
<td>18.1</td>
</tr>
<tr>
<td>R2 seed/hormone</td>
<td>3544</td>
<td>129</td>
<td>3.6</td>
</tr>
<tr>
<td>R1 PKU-Yale</td>
<td>2768</td>
<td>227</td>
<td>8.2</td>
</tr>
<tr>
<td>R2 PKU-Yale</td>
<td>2264</td>
<td>43</td>
<td>1.9</td>
</tr>
<tr>
<td>Total</td>
<td>11502</td>
<td>931</td>
<td>8.1</td>
</tr>
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</table>

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.

<table>
<thead>
<tr>
<th>Gene number</th>
<th>Gene name/description</th>
<th>LUC-units before cytokinin</th>
<th>LUC-units after cytokinin</th>
<th>At number</th>
<th>Name/description</th>
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<td></td>
<td></td>
<td>- tZ</td>
<td>+ tZ</td>
<td></td>
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</tr>
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<td>4439</td>
<td>3551</td>
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<td>Winged helix-turn-helix transcription repressor</td>
<td>5312</td>
<td>7037</td>
<td>At1g77570</td>
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<td>Gene 3</td>
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<td>15351</td>
<td>3099</td>
<td>At3g51910</td>
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<td>2740</td>
<td>933</td>
<td>At3g54990</td>
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<td>Gene 5</td>
<td>LEAFY COTYLEDON 1-LIKE (L1L or LEC1-like)</td>
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<td>5391</td>
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<td>Gene 6</td>
<td>BEL1-LIKE HOMEODOMAIN 5 (BLH5)</td>
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<td>546</td>
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<td>Gene 7*</td>
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<td>1868</td>
<td>1887</td>
<td>At1g27730</td>
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<tr>
<td>Gene 8</td>
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<td>2813</td>
<td>4236</td>
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<tr>
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<tr>
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<td>2512</td>
<td>2127</td>
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<tr>
<td>Gene 11</td>
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<td>2040</td>
<td>3601</td>
<td>At5g67450</td>
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<tr>
<td>Gene 12*</td>
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<td>11030</td>
<td>9860</td>
<td>At5g59613</td>
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<tr>
<td>Gene 13*</td>
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<td>97</td>
<td>577</td>
<td>At1g49120</td>
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</tbody>
</table>

* 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 pARR\(_{6}^{2,4kb}::\text{LUC}\) (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 in 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).

![Transactivation capacity of 13 candidate genes on the ARR6 promoter.](image)

**Figure 3.44 Transactivation capacity of 13 candidate genes on the ARR6 promoter.** The cDNAs were isolated out of the primary positive *Arabidopsis* 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 pARR\(_{6}^{2,4kb}::\text{LUC}\) (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 transactivation 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 occurred regularly 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 outliers 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 (-\(tZ\)/+\(tZ\)) 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 (-\(tZ\)/+\(tZ\)). 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

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>T1 seedling assay</th>
<th>Luminescence level and induction</th>
<th>PTA -(tZ)/+(tZ)</th>
<th>T2 seedling assay -(tZ)/+(tZ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene 1</td>
<td>CAPRICE-LIKE MYB 3 (CPL3)</td>
<td>High</td>
<td>↑</td>
<td>/ →/ →</td>
<td>-</td>
</tr>
<tr>
<td>Gene 2</td>
<td>Winged helix-turn-helix transcription repressor</td>
<td>High</td>
<td>↑</td>
<td>/ →/ ↓</td>
<td>→/ ↓</td>
</tr>
<tr>
<td>Gene 3</td>
<td>HEAT SHOCK TRANSCRIPTION FACTOR A7A (HSFA7A)</td>
<td>High</td>
<td>↓</td>
<td>/ →/ →</td>
<td>-</td>
</tr>
<tr>
<td>Gene 4</td>
<td>SCHLAFMUTZE (SMZ)</td>
<td>High</td>
<td>↓</td>
<td>/ →/ →</td>
<td>↑/↑</td>
</tr>
<tr>
<td>Gene 5</td>
<td>LEAFY COTYLEDON 1-LIKE (L1L or LEC1-like)</td>
<td>High</td>
<td>→</td>
<td>/ →/ ↓</td>
<td>†</td>
</tr>
<tr>
<td>Gene 6</td>
<td>BEL1-LIKE HOMEODOMAIN 5 (BLH5)</td>
<td>↑</td>
<td>→/ →</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Gene 7</td>
<td>SALT TOLERANCE ZINC FINGER (STZ or ZAT10)</td>
<td>High</td>
<td>→</td>
<td>/ ↗/↑</td>
<td>→/↑</td>
</tr>
<tr>
<td>Gene 8</td>
<td>C2H2-type zinc finger protein</td>
<td>High</td>
<td>↑</td>
<td>/↑/↑</td>
<td>†</td>
</tr>
<tr>
<td>Gene 9</td>
<td>HIGH MOBILITY GROUP B4 (HMGB4)</td>
<td>↑</td>
<td>/↑/↑</td>
<td>→/↓</td>
<td></td>
</tr>
<tr>
<td>Gene 10</td>
<td>DNA-BINDING WITH ONE FINGER 4.4 (DOF4.4)</td>
<td>High</td>
<td>→</td>
<td>/ →/ →</td>
<td>-</td>
</tr>
<tr>
<td>Gene 11</td>
<td>F-Box/Kelch repeat protein</td>
<td>↓</td>
<td>→/ →</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Gene 12</td>
<td>ARABIDOPSIS ZINC-FINGER PROTEIN 1 (AZF1)</td>
<td>High</td>
<td>↑</td>
<td>/↑</td>
<td>↑/↑</td>
</tr>
<tr>
<td>Gene 13</td>
<td>Unknown protein in mitochondrial ATP synthase complex</td>
<td>High</td>
<td>→</td>
<td>/ →/ →</td>
<td>→/ →</td>
</tr>
<tr>
<td>Gene 14</td>
<td>CYTOKININ RESPONSE FACTOR 9 (CRF9)</td>
<td>↑</td>
<td>-</td>
<td>→/↑</td>
<td></td>
</tr>
</tbody>
</table>

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 co-affinity 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 \textit{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 \textit{ARR14} in the vasculature is likely, because the other type-B response regulators examined, \textit{ARR1} and \textit{ARR2}, are also expressed in the vasculature and in the shoot apical meristem as it was reported for \textit{ARR14} (Mason et al., 2004; Tajima et al., 2004). Reverse transcription experiments for \textit{ARR14} expression revealed a high amount of transcript in younger leaves, whereas in older leaves the \textit{ARR14} transcript abundance was lower (Mason et al., 2004). This would fit to the results of the \textit{ARR14} expression pattern observed in my experiments. To examine the expression of \textit{ARR14} further, \textit{in situ}-hybridization of leaf tissue with \textit{ARR14} probes would be a suitable tool.

The most interesting result of the expression analysis of \textit{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 \textit{CYCLIN D3,1} and \textit{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 \textit{ARR16} was shown (Pillitteri et al., 2011).

Conversely, for \textit{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 leaves.
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 pARR6 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 \textit{arr14-1} line was more resistant towards \textit{V. longisporum} infection than the wild type (Figure 3.28-3.30). \textit{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 \textit{Verticillium}.

ARR14 seems to be also a positive regulator in infection \textit{Verticillium} of host plants contrary to ARR2 in \textit{Pseudomonas} infection.

\subsection*{4.1.6. ARR14 possibly functions in auxin signaling}

\textit{ARR14} codes for a nuclear localized protein (chapter 4.1.2) showing a similar expression pattern as \textit{IAMT1} and as \textit{DR5::uidA} when auxin transport is inhibited (chapter 4.1.1). It might be that expression of \textit{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 \textit{ARR6} but it regulates \textit{ARR15}, \textit{ARR16}, \textit{ARR17}, \textit{SAG12} and \textit{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 \textit{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 \textit{ARR15}. \textit{ARR15} was thought to strongly inhibit cytokinin signaling in concert with \textit{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 \textit{ARR15}, \textit{ARR7} and the other type-A ARRs (Zhang \textit{et al}., 2011).

A second pathway could be the regulation of auxin distribution in the cell via regulation of \textit{PIN5} expression. A higher \textit{PIN5} expression could lead to more auxin signaling and a stronger inhibition of cytokinin signaling.

\textit{ARR14} and \textit{IAMT1} showed a similar expression pattern. This could point to the possibility of a common regulator or a transcriptional regulation of \textit{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 wildtype 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 C2H2-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 MITOGEN ACTIVATED PROTEIN KINASE 3 and 6 (MPK3 and MPK6) (Nguyen et al., 2012).

**Gene 8**

At2g28200, a C2H2-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 GOUPT 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 C2H2-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
A 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 pARR6::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.
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 SAUR homologs 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 PTAs 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 deviations 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 yet 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


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


8. Acknowledgements

First of all I want to express my gratitude to Professor Schmülling and Dr. Heyl for giving me the opportunity to work on two interesting projects and for the supervision during all those years. I thank you for the guidance, the discussions and the motivation.

I am also much obliged to Professor Schuster, Professor Werner and Dr. Leuendorf for being part of the graduation committee and for hints and discussions.

I am very grateful also to Dr. Diederichsen and Dr. Häffner for the introduction into the Verticillium experiments and the help with the evaluation.

I want to thank Cordula Braatz and Dave Farnell who helped me with the experiments as well as student workers.

For the introduction into R for drawing box-plots and rescuing our vector database several times I want to thank Dr. Brenner.

Special thanks go to the current and former Lab106 people. You significantly increased fun and were so helpful to me in discussions (so you had a kind of pleiotropic effect).

I am grateful to all members of the Institute for being a community instead of a competition.

Thank you for discussions and for so many answers during all those years.

I thank my friends for their support, for having been patient and I am glad that they still call me when I lock myself in a room for a while to e.g. write a thesis.

Finally I want to thank my family who always supported me and believed in me, often much more than I did. Thank you for being so sure that everything will go its way.

I am grateful to the Elsa-Neumann-Stipendium des Landes Berlin, the Frauenförderung der FU Berlin, the DCPS and the DRS for financial support.
9. Appendix

9.1. Abbreviations

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<th>Abbreviation</th>
<th>Definition</th>
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<td>bp</td>
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<td>fw</td>
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<td>kilobasepairs</td>
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<td>rev</td>
<td>reverse</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
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<td>h</td>
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133
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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


Bertani, G. (1951). Studies on lysogenesis


Hiratsu, K., Matsui, K., Koyama, T., and Ohme-Takagi, M. (2003). Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in Arabidopsis. Plant J 34, 733-739.


Qin, G., Gu, H., Zhao, Y., Ma, Z., Shi, G., Yang, Y., Pichersky, E., Chen, H., Liu, M., Chen, Z., and Qu, L.J. (2005). An indole-3-acetic acid carboxyl methyltransferase regulates Arabidopsis leaf development. The Plant Cell 17, 2693-2704.


