Functional characterization of B-type response regulators of *Arabidopsis thaliana*

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1.0 Introduction

1.1 The phytohormone cytokinin and its history

Growth and development of a plant are the result of a defined process wherein cell regeneration and differentiation determine its shape and functionality. These defined processes are coordinated by phytohormones, which are small chemical substances, that already at low concentration, influence plant growth and differentiation. At least six classes of phytohormones play a key role in regulating and modifying plant growth and development. One such class of phytohormones is cytokinin, which is involved in many developmental processes and plays a critical role in numerous physiological responses to changes in the environment (Mok and Mok, 2001). The history of cytokinin dates back to 1913, when Gottlieb Haberlandt discovered that a compound found in phloem had the ability to stimulate cell division in potato parenchyma (Haberlandt, 1913). Later in 1941, the ability of milky endosperm from coconut to stimulate cell division was shown in the growth of plant embryos and tissue cultures (van Overbeek *et al.*, 1941). However, the first cytokinin was isolated from herring sperm in 1955 by Miller and his associates and the compound was named kinetin because of its ability to promote cytokinesis (Miller *et al.*, 1955). Eight years later, the first naturally occurring cytokinin was isolated from immature kernel of *Zea mays* in 1963 by Miller and Witham (Miller and Witham, 1963). In the same year Letham reported zeatin as a factor inducing cell division and later identified it as 6-(4-hydroxy-3-methylbut-trans-2-enylamino)purine (Letham, 1963). Skoog and his associates named kinetin and zeatin-like compounds cytokinins (Skoog *et al.*, 1967). Since then, many more naturally occurring cytokinins have been isolated and their role has been implicated in several aspects of plant growth and development. Some of the well known physiological effects of cytokinins are regulation of shoot and root growth, seed germination, vasculature development, leaf senescence, chloroplast development, transduction of nutrition signals, stress response, pathogen resistance and increased crop productivity (Figure 1; reviewed in Mok and Mok, 2001; Heyl and Schmülling, 2003; Sakakibara, 2006; Argueso *et al.*, 2009).

1.2 Cytokinin biosynthesis and catabolism

Cytokinins have been found in all higher plants as well as mosses, fungi, bacteria, and also in many prokaryotes. In plants, cytokinins are generally found in higher concentrations in meristematic regions and growing tissues. Previous assumptions that the root tip is the major site of cytokinin synthesis and translocated via the xylem to shoots is being supplemented by recent studies showing cytokinin production in a wide range of organs and cell types such as cambium, shoot apex, pollen tubes, lateral root primordia and immature seeds (Miyawaki *et al.*, 2004).

The natural cytokinins are adenine derivatives and are classified by the configuration of their N^6-side chain as isoprenoid or aromatic cytokinins (Mok and Mok, 2001). In plants, the cytokinin levels are regulated by a combination of synthesis, conjugation and catabolism. Cytokinin biosynthesis involves biochemical modification of adenine and the rate-limiting step in this process is the transfer of the isopentenyl group from dimethylallyl diphosphate (DMAPP) to the N^6 position of the adenosine monophosphate (AMP) (reviewed in Heyl *et al.*, 2006; Sakakibara, 2006). This enzymatic process is mediated by an enzyme known as IPT (isopentenyl diphosphate transferase) which is the first cytokinin biosynthetic enzyme identified in *Dictyostelium discoideum*, subsequently from the gall-forming bacterium *Agrobacterium tumefaciens* (Taya *et al.*, 1978; Akiyoshi *et al.*, 1984; Barry *et al.*, 1984). In *Arabidopsis*, seven genes (*AtIPT1*, *AtIPT3*-*AtIPT8*) coding for IPT enzymes were identified and their ability to produce *iso*pentenyladenine (iP) and *trans*-zeatin (tZ) was shown (Kakimoto, 2001; Takei *et al.*, 2001a). Several studies were done in order to elucidate the *in planta* roles of the individual *IPT* genes. For example, *AtIPT3* is a key player in biosynthesis of cytokinins in response to rapid changes in availability of nitrate in the root (Sakakibara, 2003; Miyawaki *et al.*, 2004). Similarly, it was also shown that *AtIPT7* mediated cytokinin biosynthesis is important for maintenance of the shoot apical meristem in *Arabidopsis* and activation of this gene is regulated by the KNOX (Knotted1-like homeobox) proteins (Jasinski *et al.*, 2005; Yanai *et al.*, 2005).

In higher plants, the tZ-type of cytokinins are produced either by hydroxylation of an iP nucleotide or by using an unknown hydroxylated side-chain precursor (reviewed in Heyl *et al.*, 2006; Sakakibara, 2006). The tZ-synthesis via hydroxylation of an iP nucleotide is catalyzed by a cytochrome P450 monooxygenase and in *Arabidopsis* two such enzymes, CYP735A1 and CYP735A2, were identified for this process (Takei *et al.*, 2001b). The active

free base forms of cytokinins are derived from the cytokinin nucleotides either by dephosphorylation or deribosylation. Recently, an enzyme named LOG (LONELY GUY) catalyzing this step was identified in rice (Kurakawa *et al.*, 2007).

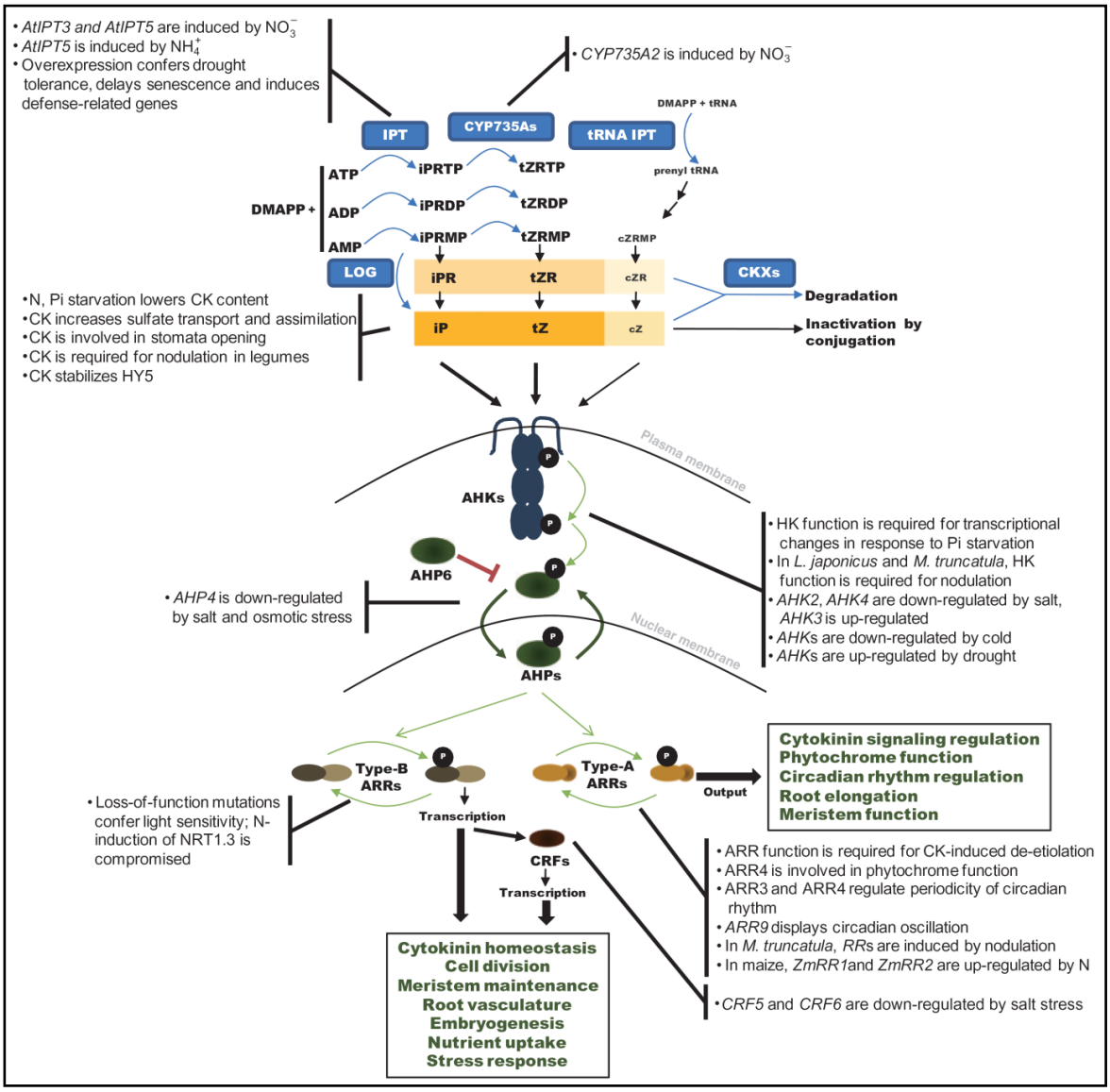

Figure 1: Cytokinin synthesis, signalling and cross talk with other pathways. For the biosynthetic pathway, reactions in which the genes encoding the enzymes have been identified are depicted in blue and the biologically active cytokinins in yellow. Cytokinin signalling is a multi-step phosphorelay, and phosphoryl transfer is indicated by light green arrows. See text for additional details regarding the role of the various steps shown. DMAPP, dimethylallyl diphosphate; tZ, *trans*-zeatin; cZ, *cis*-zeatin; iP, N^6-(Δ^2-isopentenyl) adenine; iPRTP, iP riboside 5'-triphosphate; iPRDP, iP riboside 5'-diphosphate; iPRMP, iP riboside 5'-monophosphate; iPR, iP riboside; tZRTP, tZ riboside 5'-triphosphate; tZRDP, tZ riboside 5'-diphosphate; tZRMP, tZ riboside 5'-monophosphate; tZR, tZ riboside; cZRMP, cZ riboside 5'-monophosphate; cZR, cZ riboside; IPT, isopentenyltransferase; CK, cytokinin; AHKs, *Arabidopsis* histidine kinase receptors; AHPS, *Arabidopsis* histidine phosphotransfer proteins; ARRs, *Arabidopsis* response regulators; CRFs, cytokinin response factors; HK, hybrid sensor kinase receptor (the figure is modified after Argueso *et al.*, 2009).

This *LOG* gene encodes a phosphoribohydrolase that directly and specifically converts cytokinin-5`-monophosphates to the free base form as illustrated in Figure 1. The expression of *LOG* is localized to the shoot apical meristem and it was concluded from the *log* mutant phenotype that it plays a pivotal role in regulating meristem activity (Kurakawa *et al.*, 2007).

Apart from the iP and tZ-type cytokinins, plants also contain substantial amounts of *cis*-zeatin (cZ)-type cytokinins. The degradation of tRNA is considered as the source for the production of cZ-type cytokinins. The first step in this process as illustrated in Figure 1 is catalyzed by the tRNA-isopentenyltransferase (tRNA-IPT) enzyme (Figure 1; Sakakibara, 2006). The role of cZ-type cytokinins in plants has not been fully characterized. However, cytokinin receptors from maize were able to respond to cZ, suggesting a relevant role of cZ at least in some plant species (Yonekura-Sakakibara *et al.*, 2004).

The levels of active cytokinin in plants are also regulated by conjugation to sugars and degradation by CKXs (cytokinin oxidases/dehydrogenases). The active cytokinin can be inactivated both irreversibly and reversibly. The irreversible inactivation of cytokinin is achieved by glucosylation of cytokinin at N^3-, N^7- and N^9-position of the adenine ring, whereas the reversible inactivation is carried out by conjugation at the hydroxyl group of the side chains of tZ, DZ (dihydrozeatin) and cZ as *O*-glucosides or *O*-xylosides (Sakakibara, 2006). Two proteins (UGT76C1 and UGT76C2) capable of *N*-glucosylation of different cytokinins at the N^7- and N^9-position were identified in *Arabidopsis* (Hou *et al.*, 2004) but their physiological relevance in plants still remains unclear. However, the major pathway to reduce the pool of active cytokinins is mediated by CKXs via selective degradation of cytokinins with unsaturated isoprenoid side chains (i.e. Z- and iP-type cytokinins) (Mok and Mok, 2001). CKX proteins are encoded by a small multigene family, which was identified in monocots, dicots and in lower plants. *CKX*-like genes were also detected in the genome of prokaryotes like *Rhodococcus fascians* (Schmülling *et al.*, 2003). While the genome of *Arabidopsis* codes for seven *CKX* genes, an analysis of the rice genome revealed 11 *CKX* homologues (Schmülling *et al.*, 2003). Cytokinin bases and nucleosides were identified as preferred substrates for CKX proteins and experimental evidence from *Arabidopsis* revealed that these enzymes can be targeted to the vacuole or the endoplasmic reticulum/extracellular space (Bilyeu *et al.*, 2001; Werner *et al.*, 2003; Werner *et al.*, 2006). In *Arabidopsis*, *CKX* genes are upregulated by cytokinins and their expression was localized to the shoot meristem (*AtCKX1* and *AtCKX2*), auxiliary buds, the procambial region of the root meristem

(*AtCKX5*), stomata cells (*AtCKX4*, *AtCKX6*), trichomes, the root cap (*AtCKX4*) and vascular tissue (*AtCKX6*) (Brenner *et al.*, 2005; Kiba *et al.*, 2005; Werner *et al.*, 2006). Studies on the loss-of-function of individual *AtCKX* gene family members indicate a high degree of functional redundancy (Dr. Batrina, personal communication). Recently, a major quantitative trait locus (QTL) for grain production in rice was identified as a loss-of-function mutation in *OsCKX2* (Ashikari *et al.*, 2005). Reduced expression of *OsCKX2* cause an increase in cytokinin content in the inflorescence meristem, which led to an increase in number of reproductive organs and thereby increased grain yield. In contrast, ectopic expression of *CKX* genes in transgenic plants leads to a strong reduction of endogenous cytokinin levels and causes aberrant phenotypic traits which are collectively termed "cytokinin deficiency syndrome". The main features of this syndrome are the formation of stunted shoots with small leaves, an enhanced root system and bigger seed size (Werner *et al.*, 2001; Werner *et al.*, 2003; Galuszka *et al.*, 2004). To sum up, the steady state levels of active cytokinins are regulated by a combination of biosynthesis, conjugation and degradation. And cytokinin acts as positive and negative regulator for shoot and root growth, respectively (Werner *et al.*, 2001; 2003; 2006).

1.3 Cytokinin signal transduction

In recent years, significant progress has been made towards in understanding how the cytokinin signal is perceived and transduced (Heyl and Schmülling, 2003; Kakimoto, 2003; Mizuno, 2004; Heyl *et al.*, 2006; Hwang and Sakakibara, 2006; Müller and Sheen, 2007; Argueso *et al.*, 2009). In the current model, which has been developed mainly in *Arabidopsis thaliana*, the hormone is perceived by membrane-bound hybrid histidine kinase receptors (AHKs), which auto-phosphorylate upon binding of the hormone ligand. After trans-phosphorylation within the receptor, the phosphate group is transferred to histidine phospho-transfer proteins (AHPs), which subsequently translocate to the nucleus, where they activate B-type response regulators (ARRs) via phosphorylation. These transcription factors then regulate the transcription of their target genes, one group of which codes for A-type response regulators. A negative feedback on the cytokinin signaling pathway was shown to be mediated by members of this protein class (Figure 1; Hwang and Sheen, 2001; To *et al.*, 2004).

1.3.1 The *Arabidopsis* histidine kinase receptors (AHKs)

In *Arabidopsis,* eight two-component input regulator genes have been identified. Out of these, three (*AHK2, AHK3* and *CRE1/AHK4*) are involved in cytokinin perception, two (*ETR1* and *ERS1*) encode ethylene receptors, one (*AHK1*) acts as osmosensor, one (*CKI1*) in female gametophyte development and one (*AHK5/CKI2*) plays a role in stomatal signalling (Grefen and Harter, 2004; Tran *et al.*, 2007; Desikan *et al.*, 2008). Different cytokinins are perceived by the cytokinin receptors AHK2, AHK3 and CRE1/AHK4 at the plasma membrane and this activates a multi-step phosphorelay as indicated in Figure 1. The first cytokinin receptor (*AHK4*/*CRE1*/*WOL*) was identified as a mutant in a screen, where it failed to respond to exogenous cytokinin in a callus greening assay (Inoue *et al.*, 2001). Subsequently, two close homologs, *AHK2* and *AHK3,* were also identified (Suzuki *et al.*, 2001; Yamada *et al.*, 2001; Higuchi *et al.*, 2004). All three cytokinin receptors have an extracellular cytokinin-binding CHASE (cyclase/His kinase-associated sensing extracellular) domain. These three AHKs have been shown to act as functionally overlapping positive regulators of cytokinin signalling (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.*, 2006). Knockout studies of single receptor genes did not show any obvious plant phenotype, whereas in the triple receptor knockout mutant plant growth is severely impaired and exhibited complete insensitivity to cytokinins in different biological assays (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.*, 2006).

1.3.2 The *Arabidopsis* histidine phospho-transfer (AHP) proteins

The AHP proteins are encoded by a small multi-gene family with six members in *Arabidopsis*. Out of these six, five AHPs (AHP1-AHP5) contain the conserved amino acids required to function as a histidine phospho-transfer protein (HPt) and the remaining one (AHP6) is considered to be a pseudo-AHP, as it lacks the conserved His residue that is required for phosphorylation (Suzuki *et al.*, 2000; Mähönen *et al.*, 2006). Because of their ability to interact with receptor histidine kinases and response regulators (RRs) they act as mediators of cytokinin signalling (Heyl and Schmülling, 2003; Heyl *et al.*, 2006). Recent genetic studies revealed that the *AHP*s act as positive regulators of cytokinin signaling and that they are redundant in their function (Hutchison *et al.*, 2006). Knockouts of single *ahp* mutants did not display any altered cytokinin sensitivity or any obvious effect on growth or development. However, the higherorder *ahp* mutants showed increased resistance to

cytokinin in various assays for cytokinin sensitivity of the shoot and the root (Hutchison *et al.*, 2006). The quintuple *ahp1 ahp2 ahp3 ahp4 ahp5* mutant showed a reduced cytokinin-mediated induction of A-type *ARR* transcripts and resemble the triple receptor mutant in its primary root phenotype, indicating that AHPs are required for cytokinin primary signal transduction (Hutchison *et al.*, 2006). Interestingly, AHP6, the pseudo AHP of the AHP family has been identified as a negative regulator of cytokinin signaling (Figure 1; Mähönen *et al.*, 2006). It was shown that the *ahp6* loss-of-function mutations can partially suppress the *wol* mutation of CRE1/AHK4 and confer hypersensitivity to cytokinin in root vascular differentiation. It was also discovered that the negative regulation of cytokinin signalling by AHP6 is done by interfering with the phosphorelay between components of this signalling system (Mähönen *et al.*, 2006). Together, the results of these studies indicate that the AHPs (AHP1-AHP5) are redundant positive elements of cytokinin signalling, whereas AHP6 negatively regulates cytokinin signalling.

1.3.3 The response regulators (RRs)

The response regulator proteins contain a receiver domain, in which a phospho-accepting, invariant Aspertate (Asp) residue is located (Figure 2A). On the basis of their structural designs the RR protein family can be further classified into two major subgroups, the A-type and B-type RRs. In *Arabidopsis*, there are 10 members of A-type RRs and each contains the phospho-accepting receiver domain with a short carboxy-terminal extension. A-type RRs are considered as primary response genes to cytokinin and have been shown to be involved in a negative feedback mechanism of the cytokinin signalling (Figure 1; Hwang and Sheen, 2001; To *et al.*, 2004). The B-type RRs are 11 in number, and in addition to a receiver domain they also contain a DNA-binding domain (Myb-class DNA-binding domain), which is highly conserved (Figure 2A; Riechmann *et al.*, 2000). The 11 B-type *RR* genes are not induced by cytokinin and are considered as transcription factors of cytokinin signalling as they can activate the transcription of cytokinin primary response genes (Figure 1; Hwang and Sheen, 2001). In *Arabidopsis*, another set of RRs, termed pseudo-RRs was also identified. There are seven pseudo-RRs, which contain a receiver domain, but they do not have the essential phospho-accepting Asp site as it is substituted by a glutamate (Figure 2A; reviewed in Mizuno *et al.*, 2004). Five of these *Arabidopsis* pseudo-RRs (i.e. APRR1, APRR3, APRR5, APRR7 and APRR9) can be grouped into a small family on the basis of a common

signature motif (CCT motif) in their carboxy-terminal ends (Figure 2A; Mizuno *et al.*, 2004). The remaining two (APRR2 and APRR4) contain a Myb-DNA-binding domain in their carboxy-terminal ends, similar to the B-type RRs. It is assumed that the pseudo-RRs might not necessarily be involved in the canonical His-Asp phosphorelay pathway, because they cannot undergo phosphorylation *in vitro* (Matsushika *et al.*, 2001). Recent studies indicate that the *Arabidopsis* pseudo-RRs are involved in circadian clock-associated functions (Mizuno and Nakamichi, 2005; McClung, 2006).

1.3.3.1 The B-type response regulators

B-type RRs were also identified in poplar (11), rice (7) and maize (3) and they were shown to be implicated in cytokinin signalling (Figure 2B; Asakura *et al.*, 2003; Du *et al.*, 2007; Ramírez-Carvajal *et al.*, 2008). The phylogenetic analysis based on full-length protein sequence similarities of B-type RRs of *Arabidopsis* revealed that the B-type ARRs can further be classified into three subclasses. The biggest subclass consists of seven members (ARR1, ARR2, ARR10, ARR12, ARR11, ARR14 and ARR18) and two other subclasses consist each of two members (ARR13/ARR21 and ARR19/ARR20) (Figure 2B). Most of the B-type RRs of poplar, rice, and maize are also placed in the biggest subclass of ARRs (Figure 2B). The expression pattern of B-type *ARR*s similarly reflects their subdivision revealed by phylogenetic analysis (Figure 3). While the members of the largest subgroup are expressed in almost all tissues, the other two subgroup members are expressed specifically in reproductive organs (Figure 3A; reviewed in Heyl *et al.*, 2006). The tissue-specific expression of *ARR19* and *ARR21* was recently reported to be in the chalazal region of the endosperm (Tiwari *et al.*, 2006; Day *et al.*, 2008). The expression of B-type ARRs during different developmental growth stages is also similar to their tissue-specific expression (Figure 3B). While the members of the largest subgroup are expressed with different strengths in every developmental stage (genevestigator database), members of smaller subgroups (*ARR19* and *ARR21*) are expressed specifically during endosperm development (Figure 3B; Day *et al.*, 2007). However, within the members of the large subgroup, highly localized expression can also be detected. For example, the pair *ARR10*/*ARR12* is closely related and both are expressed together during primary root development. Interestingly, *ARR12* was specifically expressed during lateral root development, whereas the expression of *ARR10* was not detected at this stage (Yokoyama *et al.*, 2007).

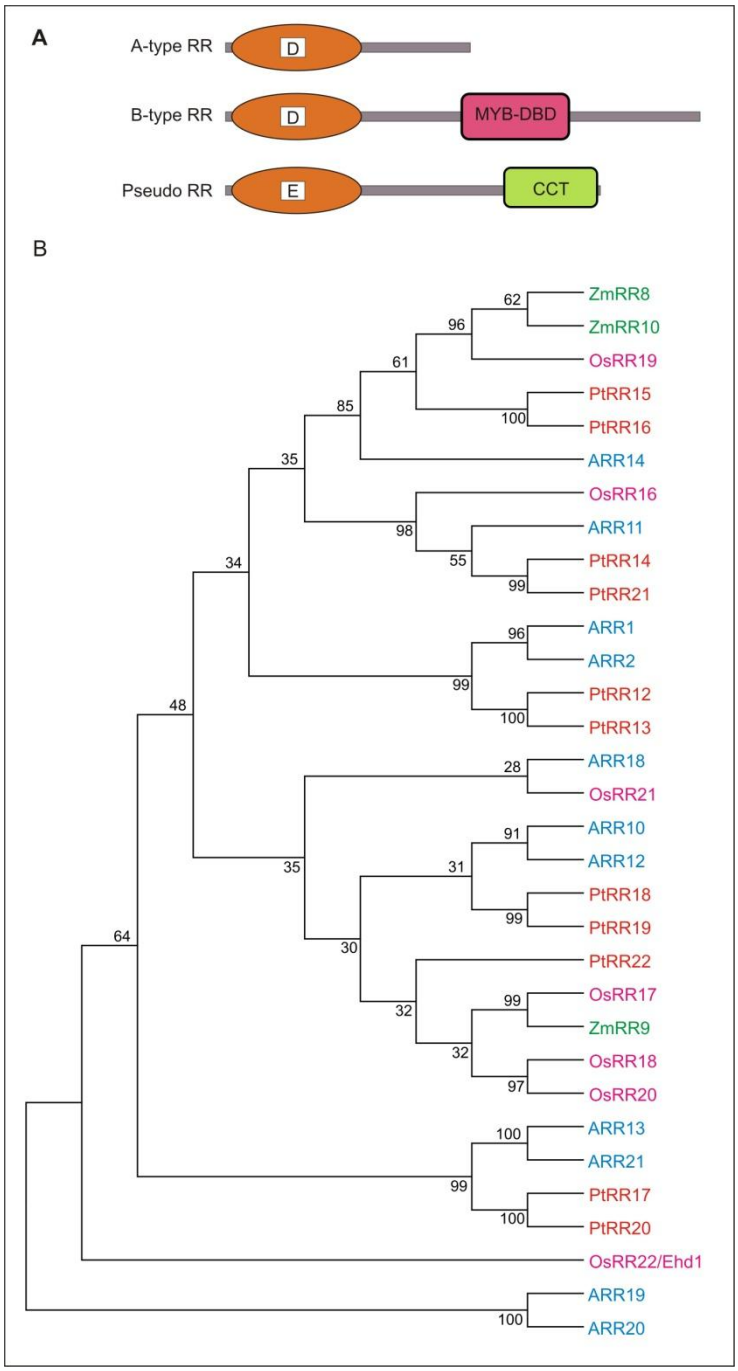

Figure 2: Schematic representation of common domain structures of RRs and phylogenetic analysis of B-type RRs. A. Common characteristic features of RR proteins. The receiver domain and Myb-DNA-binding domains are indicated by oval and rectangular shaped structures, respectively. The phospho-accepting aspartate (D) in receiver domains of RRs is conserved, whereas in pseudo-RRs this residue has been replaced by a glutamate (E). Five of the pseudo-RRs contain CCT motifs at their N-terminal end and the remaining two contains Myb-DNA-binding domain. B. Phylogenetic analysis of known B-type RRs of *Arabidopsis*, poplar, rice and maize. Full-length protein sequences of the RRs were obtained from respective genome databases of *Arabidopsis*, poplar and rice and aligned using the ClustalW program (Thompson *et al.*, 1994; Finn *et al.*, 2006). A phylogenetic tree was constructed using the MEGA 4.0 program (Tamura *et al.*, 2007). Different B-type RRs are indicated by different colours. The bootstrap values (n = 1000 iterations) are indicated at the internodes of the tree. RRs: Response regulators; MYB-DBD: MYB-DNA-binding domain.

For *ARR13* and *ARR20*, no tissue- and developmental-specific expressions were available based on array experiments in the genevestigator database, however RT-PCR experiments showed their expression in young leaves and in the floral organs, respectively (Figure 3A; Mason *et al.*, 2004).

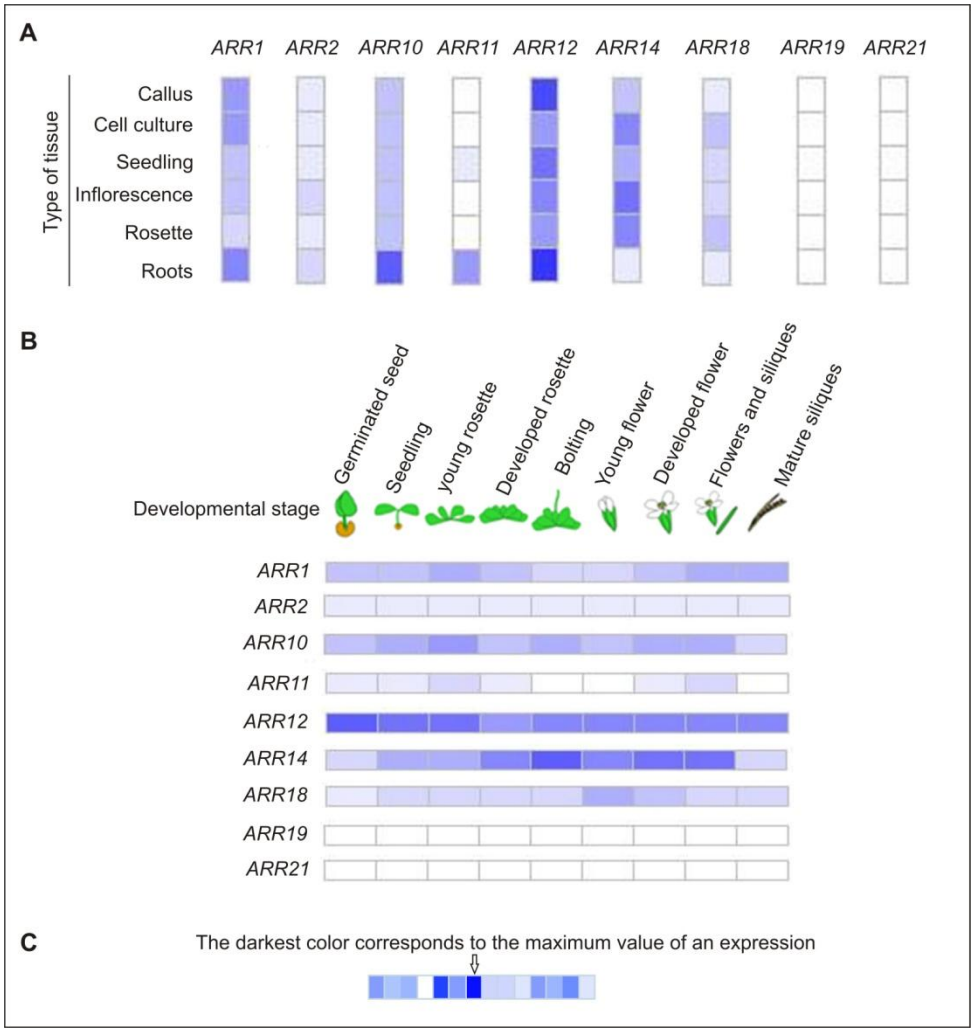

Figure 3: Tissue- and developmental stage-specific expression of the *Arabidopsis* B-type *ARR* genes. The data for the tissue-specific (A) and developmental stage-specific (B) expression of B-type *ARR*s were obtained from Genevestigator database (https://www.genevestigator.com/gv/index.jsp; Hruz *et al.*, 2008). C. Color coding used in the A and B. The expression of each gene is shown as heat map in a blue-white color coding. The darkest blue color represents the maximum level of expression for a given gene across all measurements available in the database for this gene. The B-type *ARR*s *ARR13* and *ARR20* were not included in the figure as there was no data available for these genes.

The sub-cellular localization of all tested B-type ARRs was in the nucleus, which is consistent with their role as transcription factors (Hwang and Sheen, 2001; Lohrmann *et al.*, 2001; Mason *et al.*, 2004; Dortay *et al.*, 2008). Analysis of protein interactions within the cytokinin signalling pathway revealed that all the B-type ARR proteins tested were

interacting with AHPs. Also some new interacting partners belonging to different functional categories were identified (Dortay *et al.*, 2006; Dortay *et al.*, 2008). Several studies reported loss-of-function mutants of the B-type *ARR* (*ARR1*, *ARR2*, *ARR10*, and *ARR11*) genes, and their role in cytokinin signalling has been recognized (Table 1 and references therein).

Table 1. Key features of B-type ARRs of the cytokinin signalling pathway.

Gene	AGI code	Features
ARR1	AT3G16857	Transcription factor activity, activates *ARR6* (Sakai *et al.*, 2000; Sakai *et al.*, 2001; Hwang and Sheen, 2001); plays a role in root meristem size determination (Dello Ioio *et al.*, 2007); binds to optimal DNA sequence 5'-GAT(T/C)-3' (Sakai *et al.*, 2000; Taniguchi *et al.*, 2007).
ARR2	AT4G16110	Transcription factor activity, activates *ARR6* (Sakai *et al.*, 2000; Hwang and Sheen, 2001); acts as a signalling component functioning downstream of ETR1 in the ethylene signal transduction (Hass *et al.*, 2004); participates in AHK3-mediated control of leaf longevity in *Arabidopsis* (Kim *et al.*, 2006); binds to optimal DNA sequence 5'-GAT(T/C)-3' (Sakai *et al.*, 2000).
ARR10	AT4G31920	Characterization of three-dimensional structure of DNA binding domain by NMR spectroscopy; binds to optimal DNA sequence 5'-AGATT-3' (Hosoda *et al.*, 2002); implicated in cytokinin-mediated regulation of protoxylem differentiation in roots of *Arabidopsis* (Yokoyama *et al.*, 2007).
ARR11	AT1G67710	Overexpressing the C-terminal DNA-binding domain caused unusual growth of cotyledons and reduced apical dominance; binds to optimal DNA sequence 5'-GGATT-3' (Imamura *et al.*, 2003).
ARR12	AT2G25180	Implicated in cytokinin-mediated regulation of protoxylem differentiation in roots of *Arabidopsis* (Yokoyama *et al.*, 2007).
ARR13	AT2G27070	No functional studies available.
ARR14	AT2G01760	Overexpressing the C-terminal DNA-binding domain of ARR14 in *Arabidopsis* causes anomaly at early vegetative developmental stage (Tajima *et al.*, 2004).
ARR18	AT5G58080	The *arr18* knockout plants did not display any physiological alteration of their morphological phenotype (Mason *et al.*, 2005).
ARR19	AT1G49190	No functional studies available.

ARR20	AT3G62670	Overexpressing the C-terminal DNA-binding domain of ARR20 in *Arabidopsis* causes anomaly at late reproductive stage (Tajima *et al.*, 2004).
ARR21	AT5G07210	The *ARR21* insertion mutant (*arr21-1*) did not cause any alterations in viability and fertility, flowering time, sensitivity to ethylene, cytokinin or red light (Horák *et al.*, 2003); overexpressing the C-terminal DNA-binding domain of ARR21 causes extremely anomalous development in *Arabidopsis* (Tajima *et al.*, 2004).

Knockout mutants of *arr1* and *arr21* did not display any major alteration in their phenotypes, supporting the notion that B-type ARRs are functionally redundant (Sakai *et al.*, 2001; Horák *et al.*, 2003). However, the *arr2* knockout mutant showed a slight insensitivity towards cytokinin and ethylene in a hypocotyl elongation assay. So, a role for ARR2 in the ethylene signalling pathway was suggested in addition to cytokinin signalling (Hass *et al.*, 2004). Similar to the knockout studies, overexpression of ARR1 and ARR2 did not cause dramatic phenotypic changes (Sakai *et al.*, 2001; Hass *et al.*, 2004). However, overexpression of the C-terminal DNA-binding domains of the proteins ARR11, ARR14, ARR20 and ARR21 caused different degrees of anomal plant phenotypes during the vegetative stage (ARR11/ARR14) or the reproductive developmental stage (ARR20/ARR21) (Table 1 and references there in). B-type ARRs regulate the transcription of their target genes in response to cytokinin treatment (Sakai *et al.*, 2000; Hwang and Sheen, 2001; Lohrmann *et al.*, 2001; Imamura *et al.*, 2003). Several B-type ARRs have been shown to bind to the same or very similar sequence motifs. In case of ARR1, it has been shown that the DNA-binding domain of ARR1 binds *in vitro* to a specific DNA sequence containing the 5'-GAT(T/C)-3' motif (Sakai *et al.*, 2000). For ARR10 and ARR11 the optimal *in vitro* binding was observed to a DNA sequence containing the 5'-AGATT-3' motif and 5'-GGATT-3' motif, respectively (Hosada *et al.*, 2002; Imamura *et al.*, 2003). This is consistent with this a similar sequence, 5'-GATCTT-3', has been found frequently in the upstream regions of cytokinin response genes (Rashotte *et al.*, 2003). However, studies regarding the *in planta* function of the binding motifs identified for some of the B-type ARRs are still lacking.

Very few functional studies on B-type RRs of rice and poplar have been reported compared to *Arabidopsis*. One of the rice B-type RRs, *Early heading date1* (*Ehd1*/*OsRR22*), was analyzed and shown to participate in the regulation of photoperiodic flowering (Doi *et al.*, 2004). Similarly, one of the B-type RRs of poplar, *PtRR13*, was also studied in greater

detail and shown that it acts as a negative regulator of adventitious root development (Ramírez-Carvajal *et al.*, 2009).

1.3.3.2 The A-type response regulators

The expression of the A-type *RR*s in *Arabidopsis* is induced rapidly and specifically in response to exogenous cytokinin and phylogenetic analysis based on their receiver domains revealed that they fall into five very similar pairs (D'Agostino *et al.*, 2000; To *et al.*, 2004). The gene expression pattern differs among the A-type *ARR*s. The transcript accumulation in response to cytokinin was as early as 15 min after induction in case of *ARR5, ARR6, ARR7,* and *ARR15,* but it was delayed more than 30 min in case of *ARR4, ARR8,* and *ARR9* (D'Agostino *et al.,* 2000; Rashotte *et al.,* 2003; Brenner *et al.*, 2005). Genetic analysis of single, double and higher order mutants of A-type *ARR*s indicated that these are partially redundant negative regulators of cytokinin signalling (To *et al.,* 2004). The redundancy among A-type *ARR*s observed in cytokinin responses is consistent with their generally overlapping patterns of expression under cytokinin induced conditions (To *et al.,* 2004). However, tissue-specific roles for A-type *ARRs* was revealed in recent experiments and some of these tissue-specific roles involve antagonistic interactions between different A-type *ARR*s (reviewed in To and Kieber, 2008). For example, *ARR3* and *ARR4* play cytokinin-independent roles in the control of circadian period. The *arr3 arr4* double mutants exhibited an altered circadian rhythm and this phenotype seemed to be suppressed by the *arr8 arr9* double mutants, suggesting antagonistic roles for these A-type *ARR*s (Salomé *et al.*, 2006). Similarly, recent experiments on the AHK1/ATHK1-mediated osmotic response in *Arabidopsis* also suggested anatgonistic role for *ARR3, ARR4, ARR8,* and *ARR9* in osmotic and water stress regulation (Wohlbach *et al.*, 2008). The results from this study also indicate that the *arr8 arr9* mutation can suppress the *arr3 arr4* double mutant phenotype during osmotic stress, thereby implicating the antagonistic roles of these A-type *ARR*s (Wohlbach *et al.*, 2008).

The subcellular localization of most of A-type ARRs was found to be in the nucleus (Hwang and Sheen, 2001; Dortay *et al.,* 2008). All tested A-type ARRs were shown to interact with all AHPs in *Arabidopsis* except for AHP4 (Dortay *et al.,* 2008). In addition to the AHPs, A-type ARRs are known to interact with various *Arabidopsis* proteins and thus mediate the cross-talk between cytokinin signalling and other cellular signalling pathways.

One such interaction was shown in the case of ARR4, which was found to interact with phyB and to play a positive role in red-light signalling (Sweere *et al.,* 2001). Consistent with this, several experiments have also indicated an involvement of the cytokinin signalling components in red light signalling (To *et al.,* 2004; Riefler *et al.,* 2006).

1.3.3.3 New players in cytokinin signalling

Recent studies indentified some new components of the cytokinin signalling system. One set of the new components are the cytokinin response factors (CRFs), which have been implicated in the response to cytokinin. Similar in function as the B-type ARRs, the CRFs act as transcription factors and mediate the transcriptional response to cytokinin (Rashotte *et al.*, 2006). The *Arabidopsis* genome encodes six CRFs and they are belonging to a subfamily of the AP2 (APETALA2)-like superfamily of transcription factors. Out of these six, three *CRF*s (*CRF2, CRF5* and *CRF6*) are induced in response to cytokinin in a B-type ARR-dependent manner. Loss-of-function studies of a subset of *CRF* genes indicated that a significant portion of the early transcriptional response to cytokinin is altered (Rashotte *et al.,* 2006). Recently, GeBP-like (Glabrous1 enhancer-binding protein) proteins have also been implicated in cytokinin responsiveness and it was reported that these GeBP-like proteins are likely to act as antagonist of the negative feedback of the A-type *ARR*s in cytokinin signalling (Chevalier *et al.*, 2008).

1.4 Transcriptional regulation by repressors

The regulation of gene expression is controlled by transcriptional activators and repressors in an opposite manner. Transcriptional repressors and co-repressors have been identified as important regulators in switching off genes during developmental processes of eukaryotes. Repressor proteins such as the lac and tryptophan repressor were first identified in bacterial systems, and their role in *lacZ* gene regulation in *E.coli* was well studied (Monad and Jacob, 1961). Unlike in the bacterial system, where transcriptional regulation is mostly mediated by repressors, both activators and repressors play significant roles in the regulation of gene expression in eukaryotes. The function of repressors in gene regulation is especially well documented in mammals, *Drosophila* and yeast (Hanna-Rose and Hansen, 1996; Thiel *et al.*, 2004). However, the mode of their action in impairing the transcriptional activation is different among the eukaryotic organisms and often is achieved by chromatin modification

such as histone deacetylation or by interacting with the activators of gene expression (Thiel *et al.*, 2004). Until recently, very few transcriptional repressors have been reported in plants (Liu *et al.*, 1998; Raventós *et al.*, 1998; Fujimoto *et al.*, 2000; Ohta *et al.*, 2001). However, many members of transcription factor families that act as transcriptional repressors in plants were identified in recent studies. The repressor activity of these transcription factors is mediated by the EAR (ERF-associated amphiphilic repression) domain (Ohta *et al.*, 2001; Hiratsu *et al.*, 2002; Tiwari *et al.*, 2004; He *et al.*, 2005a; Kam *et al.*, 2008).

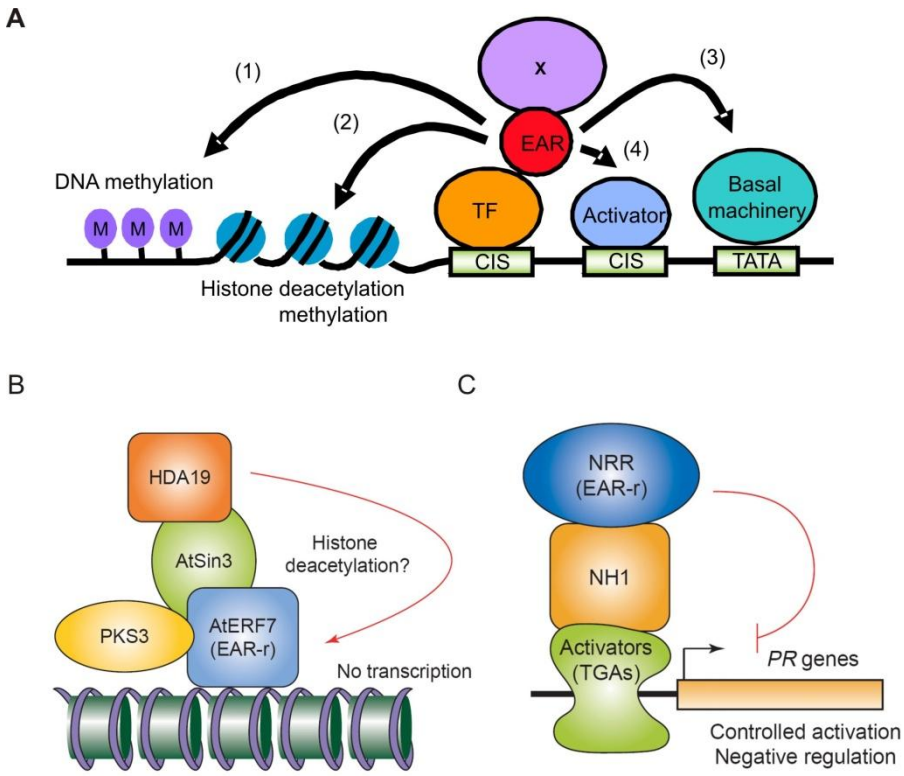

Figure 4: A possible mechanism of EAR-repressor mediated transcriptional regulation. A. Schematic representation of possible modes of action for EAR mediated repression. The EAR domain might regulate the transcription by chromatin remodeling. It can be achieved by recruiting either methyl transferase (1) or histone deactylase (2). A protein factor that interacts with EAR interferes with an assembly of the basal machinery (3) or with a specific activator (4) of transcription. B. *AtERF7* probably suppresses gene expression through chromatin modification mediated by histone deacetylation (see text for details). C. NRR mediated negative regulation of *PR* gene expression in rice is achieved by interfering with transcriptional activators (see text for details). Figure 4A is courtesy from Dr. Masaru Ohme-Takagi, and Figure 4B, 4C are modified after Kazan, 2006.

In *Arabidopsis*, the EAR motif has been found in various proteins, which include AUX/IAA (Auxin/Indole-3-acetic acid) proteins, BZR1 (Brassinazole-resistant 1), class II ERF (Ethylene response factors) and Q-type C_2H_2 zinc finger subfamily proteins (reviewed in Kazan, 2006). In a recent study, a novel group of transcriptional repressor domain was

identified and 29 *Arabidopsis* transcription factors, which include members of the RAV, ARF, Hsf and MYB families, contain the novel repression domain (Ikeda and Ohme-Takagi, 2009). Active transcriptional repressors identified in plants are involved in many aspects of plant growth and development, including phytohormone signalling, organ formation and regulating many biotic and abiotic stress responses (Kazan, 2006). Apart from the repressor proteins, recent studies identified a large number of Groucho/Tup1 family co-repressors in *Arabidopsis* which revealed their functionality in regulating key developmental processes in plants, such as floral organ identity specification, embryo apical-basal fate determination and stem cell maintenance at the shoot apex (reviewed in Liu and Karmarkar, 2008). However, one should bear in mind that the studies pertaining to the mechanism of transcriptional repressors in plants are still in a preliminary stage. Based on some recent publications, a possible mode of action by EAR repressor mediated transcription repression is illustrated in Figure 4A. Histone deacetylation is considered as one of the active mechanisms used by transcriptional repressors in mammals, *Drosophila* and yeast (Thiel *et al.*, 2004). Two recent findings in *Arabidopsis* suggested that in plants, the EAR domain containing transcriptional repressors also use histone deacetylation as a mechanism in gene regulation (Song *et al.*, 2005; Long *et al.*, 2006; Szemenyei *et al.*, 2008). For example, in *Arabidopsis*, *ERF7* an EAR-domain containing transcriptional repressor is involved in the regulation of ABA and drought stress response (Song *et al.*, 2005). The active repression by ERF7 might be achieved by recruiting the AtSIN3 (*Arabidopsis* homologue of *S*WI-independent3) and HDA19 (Histone deacetylase 19), a co-repressor and a histone deacetylase, respectively, to the transcription unit (Figure 4B; Song *et al.*, 2005). At the same time, it was also suggested that the phosphorylation of ERF7 by Protein Kinase 3 (PKS3) can be a potential way to relieve the effect of suppression (Song *et al.*, 2005; Kazan, 2006). Similarly, the EAR domain containing AUX-IAA protein IAA12/BDL, represses the transcription of ARF5 (Auxin response factor 5) by recruiting the Groucho/Tup1 family co-repressor called TOPLESS (TPL). Later, it was shown that mutations in a histone acetyltransferase suppress the *tpl* phenotype, suggesting that the possible mechanism of EAR- and TPL-mediated repression by chromatin remodeling (Long *et al.*, 2006; Szemenyei *et al.*, 2008). Another possible mechanism for EAR domain mediated repression comes from studies on regulation of pathogen-responsive *PR* gene expression in rice and *Arabidopsis* (Chern *et al.*, 2005). The NRR (Negative Regulator of Resistance), which contains an EAR domain, negatively

regulates the expression of *PR* genes in rice. The negative regulation is achieved by interacting with NH1 (NPR1 Homolog 1), which in turn interacts with the activators (i.e. TGA-type transcription factors) of *PR* gene expression in rice (Chern *et al.,* 2005; Kazan, 2006).

1.4.1 The Chimeric REpressor Silencing Technology (CRES-T)

Even though the regulatory mechanisms of EAR domain-containing repressors are not yet well understood, the EAR-motif has already been used as a tool in functional genomics. By using the EAR repressor domain, a scientific group led by Dr. Masaru Ohme-Takagi developed a technology called chimeric repressor silencing technology (Hiratsu *et al.*, 2003). This technology has been helpful in studying the consequences of silencing of the target genes of single transcription factors and has also been used to overcome the experimental limitations caused by functional redundancy of transcription factor families. It was known from the animal field, that the targeted repression of genes of interest can be achieved by using chimeric repressors, in which a DNA-binding domain or a transcription factor is fused to a repression domain (John *et al.,* 1995; Beerli *et al.,* 1998; Beerli *et al.*, 2000; de Haan *et al.,* 2000). For example, repressor domains from the Krüppel-associated box (KRAB) of the human estrogen receptor, Engrailed (En) of *Drosophila* or the mSIN3 interaction domain (Sid) of humans have been used effectively and shown that such chimeric repressors act dominantly in mammalian cells (John *et al.,* 1995; Beerli *et al.,* 1998, 2000; de Haan *et al.*, 2000). Taking cues from these studies a similar targeted repression system was developed for plants using the EAR repressors. When the EAR motif was fused to activators of transcription, the resultant chimeric transcription factors acted as strong repressors and the repressive activity was dominant over both intra- and inter-molecular activating activities in transient expression assays in *Arabidopsis* (Hiratsu *et al.,* 2002; Ohta *et al.,* 2001). Later, it was also shown in stably transformed *Arabidopsis* plants, that the fusion of the EAR motif to transcriptional activators converts them into dominant repressors, even in the presence of the original activator domain. Interestingly, these dominant repressors may repress not only the transcription of their own target genes, but also the expression of target genes of other members of their respective gene family and thus overcome functional redundancy (Hiratsu *et al.*, 2003). For example, CUC1 and its homolog CUC2 are functionally redundant transcriptional activators and regulate shoot apical meristem formation and organ separation

during embryogenesis in *Arabidopsis* (Aida *et al.*, 1997; Takada *et al.*, 2001). Single knockout mutant of either *cuc1* or *cuc2* did not display any alteration in their cotyledon development, whereas loss-of-function *cuc1 cuc2* double mutant plants resulted in the formation of cup-shaped cotyledons (Aida *et al.*, 1997; Takada *et al.*, 2001). When the SRDX repressor domain (a modified version of the EAR motif), which comprises 12 amino acids (LDLDLELRLGFA), was fused to the C-terminal end of CUC1, the resultant CUC1-SRDX was able to reproduce the cup-shaped cotyledon phenotype in transgenic plants (Hiratsu *et al.*, 2003). Similarly, *AtMYB23* is a positive regulator of trichome development in *Arabidopsis* and has been shown to act partially redundant with *GL1* (GLABRA1) (Kirik *et al.*, 2001). Transgenic plants with antisense constructs directed against the *AtMYB23* gene have no visible abnormal features. But transgenic plants that expressed the chimeric protein consisting of AtMYB23 and SRDX (AtMYB23-SRDX) showed disruption in trichome development (Hiratsu *et al.*, 2003). The strong dominant-negative phenotypes that were observed in *35S:CUC1-SRDX* and *35S:AtMYB23-SRDX* transgenic plants are the result of the suppression of target genes of functionally redundant transcription factors (Hiratsu *et al.*, 2003). Thus, CRES-T is a simple method for generating loss-of-function phenotypes in functionally redundant gene families and provides an alternative to the construction of multiple mutant combinations.

1.5 Aim of the present study

The aim of this thesis was to contribute to an improved understanding of the cytokinin signalling system by characterizing the functions of transcription factors of cytokinin signalling i.e. the B-type ARRs. The analysis of B-type *ARR* mutants has revealed their involvement in cytokinin signalling, but also a high degree of functional redundancy (see 1.3.3.1). In order to explore the functions of B-type response regulators and overcome their functional redundancy, the chimeric repressor silencing technology (CRES-T) was used. Current knowledge indicates that cytokinin has a prominent role in different developmental and physiological processes (Figure 1). Furthermore, there are a number of experimental results that indicate links between cytokinin and other signalling systems. Thus it was the objective of this study to contribute to an understanding of the interaction network, which was done by finding target genes of B-type ARRs and thereby revealing the specific

roles of these response regulators in cytokinin signalling. More specifically, the aim of this study was achieved through the following objectives:

(i) *Characterization of transgenic* 35S:ARR1-SRDX *plants*

A complete characterization of numerous morphological and physiological features of *35S:ARR1-SRDX* transgenic plants compared to wild-type plants. This should yield information about the roles played by B-type response regulators in various physiological and developmental processes of plants.

(ii) *Characterization of other* ARR1 *mutant plants*

In addition to the *35S:ARR1-SRDX* plants, other mutant variants of the *ARR1* gene such as an *ARR1* loss-of-function mutant (*arr1*), and a line overexpressing the *ARR1* gene constitutively (*35S:ARR1*) were characterized. Simultaneously, in order to get more insight into the regulation of ARR1 at the protein level, selected interactions of the ARR1 protein were studied. These experiments should provide more information about the biological roles of ARR1.

(iii) *Expression profile analysis by microarray experiments and identification of* cis*-acting elements*

The expression profiles of the different mutant lines of *ARR1* have been compared. More specifically, the immediate-early transcriptional cytokinin response of *35S:ARR1-SRDX* plants, *arr1* mutants and *35S:ARR1* overexpressers were compared. After identifying potential target genes by microarray analysis, the promoter of one target gene was analysed to identify *cis*-acting elements by reporter gene assays (GUS fusions) and deletion analysis.

By working on the above listed objectives, the thesis was aimed to contribute to an improved understanding of the B-type ARRs in cytokinin signalling and thereby in plant development.

2.0 Materials and Methods

2.1 Materials

2.1.1 Experimental organisms

2.1.1.1 Plants

(i) *Arabidopsis thaliana*:

Arabidopsis thaliana is a small annual weed which belongs to the Brassicaceae family. The Columbia-0 (Col-0) ecotype of the *Arabidopsis thaliana* was used as wild-type in all experiments. The seeds were obtained from NASC (Nottingham Arabidopsis Stock Centre) (NASC; http://arabidopsis.info/; (Scholl *et al.*, 2000).

(ii) Tomato (*Solanum lycopersicum*):

Solanum lycopersicum, formerly known as *Lycopersicon esculentum*, is an annual vegetable crop belonging to the Solanaceae family. The *Solanum lycopersicum* cv Micro-Tom was used as wild-type in this study. Micro-Tom seeds were provided by Lázaro Peres (University of Sao Paulo, Brazil).

(iii) Tobacco (*Nicotiana benthamiana*):

Nicotiana benthamiana is an annual herbaceous plant belonging to the Solanaceae family. The seeds were obtained from Icon Genetics™ GmbH (Halle/Saale, Germany).

2.1.1.2 Bacteria

(i) *Escherichia coli*:

The different *E.coli* strains used in this study are listed in Table 2, along with their genotype and respective references.

Table 2. *E.coli* strains.

Strain	Reference	Genotype
DB3.1	(Hanahan, 1983; Bernard and Couturier, 1992)	F^- *gyr*A462 *end*A1 Δ(*sr*1-*rec*A) *mcr*B *mrr* *hsd*S20($r_B^-m_B^-$) *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Sm^r) *xyl*5 Δ*leu* *mtl*1
DH10B	(Calvin and Hanawalt, 1988; Raleigh *et al.*, 1988)	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G
Xl1Blue	Stratagene™	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F` *pro*AB $lacI^qZ$ΔM15 Tn10 (Tet^r)]

(ii) *Agrobacterium tumefaciens*:

The *Agrobacterium* strain GV3101 was used in this study (Koncz and Schell, 1986). The pMP90 plasmid of this strain contains the *vir* region as well as the genes for gentamycin and rifampicin resistance markers. The strain was used for the stable transformation of *Arabidopsis thaliana*, *Solanum lycopersicum* and transient transformation of *Nicotiana benthamiana* leaves.

2.1.1.3 Yeast

The strain L40ccU3 of *Saccharomyces cerevisiae* was used in the yeast two-hybrid experiments. The genotype of the strain is *MATa* his3Δ200 *trp1-901 leu2-3,112 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)$_8$-lacZ ADE2::(lexAop)$_8$-URA3 GAL4 gal80 can1 cyh2* (Goehler *et al.*, 2004).

2.1.2 Culture media

2.1.2.1 Plant culture media

The plant growth media was prepared as described (Murashige and Skoog, 1962).

i) *Arabidopsis:*

Soil composition	65% compost soil (Einheiterdewerk™, Untersen, Germany) 25% Sand 10% Granulate (Knaufperlite™ GmbH, Dortmund, Germany)
MS Medium	4.33 g/L MS-vitamin mixture (Duchefa, Haarlem, Netherlands) 0.5 g/L MES 1% (w/v) Sucrose 0.1 g/L myo-Inositol 0.8-1.0% Agar pH adjusted to 5.7 with 1 N KOH

ii) Tomato:

MS Medium	4.33 g/L MS salt-vitamin mixture 0.5 g/L MES 3% (w/v) Sucrose 0.1 g/L myo-Inositol 112 mg/L B5-Gamborg vitamin mix 0.8% Agar pH adjusted to 5.7 with 1 N KOH

2.1.2.2 Bacteria culture media

The different kinds of bacterial and yeast media were prepared as described (Bertani, 1951; Sambrook *et al.*, 1989; Rose *et al.*, 1990).

LB Medium:
5 g/L Bacto Yeast extract
10 g/L Bacto Tryptone
5 g/L NaCl
1-1.5% Agar (for solid media)

YEP Medium**:**
10 g/L Peptone
10 g/L Yeast extract
5 g/L NaCl
1.5% Agar (for solid medium)
pH adjusted to 7.0 with 5 N NaOH

SOC Medium:
20 g/L Bacto Tryptone
5 g/L Yeast extract
0.5 g/L NaCl
2.5 mL/L 1 M KCL
pH adjusted to 7.0 with 5 N NaOH
before use add 20 mL/L 1 M Glucose

The LB or YEP selection media were prepared by adding antibiotics in the required concentrations to the autoclaved media, when it was cooled down to a temperature of ~55ºC. The final concentrations of the different antibiotics are listed in Table 3.

Table 3. The antibiotic concentrations and stock solutions.

Antibiotic	**Stock solution**	**Final concentration**	
		E.coli	***Agrobacterium***
Ampicillin	50 mg/mL	50 µg/mL	50 µg/mL
Carbenicillin	50 mg/mL	50 µg/mL	50 µg/mL
Spectinomycin	30 mg/mL	30 µg/mL	60 µg/mL
Kanamycin	100 mg/mL	100 µg/mL	100 µg/mL
Gentamycin	50 mg/mL	50 µg/mL	50 µg/mL
Rifampicin	50 mg/mL	50 µg/mL	50 µg/mL

2.1.2.3 Yeast culture media

SD Medium:
6.7 g/L Yeast nitrogen base (without amino acids)
12 g/L Agar
pH adjusted to 5.8 with HCl

When the autoclaved media was at 55ºC temperature, 50 mL of 40% glucose was added. The amino acids were dissolved in water to prepare 0.2% stock solutions. The final concentrations in the media are listed in Table 4.

Table 4. The amino acids concentrations in SD medium.

Component	Final concentration in medium
Leucine	100 mg/L
Tryptophan	20 mg/L
Uracil	20 mg/L
Histidine	20 mg/L

YPD Medium:
12 g/L Bacto Yeast extract
20 g/L Bacto Peptone
20 g/L Glucose
20 g/L Agar (for solid media)
pH adjusted to 6.5

2.1.3 Buffers and solutions

10x PCR buffer:
200 mM Tris/HCL of pH 8.8
100 mM KCl 100 mM $(NH4)_2SO_4$
20 mM $MgSO_4$
1 mg/mL Nuclease-free BSA
1% Triton X-100

50x TAE buffer:
2 M Tris Base
1 M Glacial acetic acid
50 mM EDTA, pH adjusted 8.0

10x TBE buffer:
0.89 M Tris base
0.89 M Boric acid
20 mM EDTA, pH adjusted 8.4

10x MOPS buffer:
0.2 M MOPS
10 mM EDTA
5 mM Sodium acetate
pH adjusted to 7.0 with NaOH

TE buffer:
10 mM Tris of pH 8.0
1 mM EDTA of pH 8.0

10x Loading buffer (50 ml):	40 ml TE-Buffer 33.5 g Sucrose 200 mg OrangeG
TRIzol reagent:	38% Phenol 800 mM Guanidinium thiocyanate 400 mM Ammonium thiocyanate 100 mM Sodium acetate 5% Glycerol
10x Second strand buffer:	100 mM Tris-HCl, pH 6.9 450 mM KCl 23 mM MgCl2 0.75 mM β-NAD 50 mM $(NH4)_2SO_4$
Leaf digestion solution:	1.25% Cellulase (Yakult Pharmaceuticals™, Tokyo, Japan) 0.3% Macerozyme (Yakult Pharmaceuticals™, Tokyo, Japan) 0.4 M Mannitol 20 mM KCl 20 mM MES (pH 5.6) 10 mM $CaCl_2$ sterile filtrate using membrane filters (40 µM size)
MMg solution:	0.4 M Mannitol 15 mM $MgCl_2$ 4 mM MES-KOH (pH 5.6) sterile filtrate using membrane filter (40 µM size)
PEG solution (40%):	4 g PEG 4000 3 mL H_2O 2.5 ml of 0.8 Mannitol 1 M $CaCl_2$ sterile filtrate using membrane filter (40 µM size)
W5 solution:	154 mM NaCl 125 mM $CaCl_2$ 5 mM KCl 2 mM MES-KOH (pH 5.6) sterile filtrate using membrane filter (40 µM size)
WI solution:	0.5 M Mannitol 4 mM MES-KOH (pH 5.6) 20 mM KCL sterile filtrate using membrane filter (40 µM size)

GUS extraction buffer (50 ml): 50 mM Phosphate buffer (pH 7.0, 7.2 and 7.5)
10 mM EDTA
0.1% Triton X-100
0.1% Na- Laurosyl sarcosine
25 µL of β-Mercaptoethanol was added before use.

2.1.4 Materials for microarray

Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany)

Qiagen RNase-Free DNase kit (Qiagen, Hilden, Germany)

QiaQuick PCR purification kit (Qiagen, Hilden, Germany)

MEGA script T7 transcription kit (Ambion, Darmstadt, Germany)

Type 7* mirror slides (Amersham, Little Chalfont, UK)

RNase Out (Invitrogen, Karlsruhe, Germany)

E. coli DNA-Ligase (Invitrogen, Karlsruhe, Germany)

E. coli DNA-Polymerase I (Invitrogen, Karlsruhe, Germany)

E. coli RNase H (Invitrogen, Karlsruhe, Germany)

Random-Nonamers N9 (Metabion, Martinsried, Germany)

HT7T-Primer: HPLC-purified, 200 ng/µL concentration

Poly-dT35 (Metabion^{TM}, Martinsried, Germany)

SuperScript II RT RNase H (Invitrogen, Karlsruhe, Germany)

10 mM dNTP-Mix (Amersham Pharmacia Biotech)

-C-dNTP-Mix (Amersham Pharmacia Biotech)

Cy3-dCTP for Microarrays (Amersham, Little Chalfont, UK)

Cy5-dCTP for Microarrays (Amersham, Little Chalfont, UK)

Microarray hybridization buffer (Amersham, Little Chalfont, UK)

1.7 M Trehalose (Sigma, München, Germany)

2.1.5 Commonly used kits

The commonly used kits are listed below in Table 5 along with their manufacturers.

Table 5. Commonly used kits.

Name	Purpose	Manufacturer
One Step RT-PCR Kit	RT-PCR	Qiagen, Hilden, Germany
RNeasy Mini Kit	RNA purification	Qiagen, Hilden, Germany
Quick Change Site Directed Kit	Mutagenesis	Stratagene, Amsterdam, Netherlands
QIAquick Gel Extraction Kit	Cloning	Qiagen, Hilden, Germany
Nucleobond Xtra Maxi	Maxi preparation of plasmid	Macherey-Nagel, Düren, Germany
Invisorb Spin Plasmid Mini Kit	Mini preparation of plasmid	Invitek, Berlin, Germany
QIAquick PCR Purification Kit	Cloning	Qiagen, Hilden, Germany
QIAquick Nucleotide Removal Kit	Cloning	Qiagen, Hilden, Germany

2.1.6 Primers and oligonucleotides

All primers and oligonucleotides were ordered from Invitrogen™ (Karlsruhe, Germany). Lyophilized oligonucleotides were resuspended in sterile ddH_2O to a final concentration of 100 µmol/µL and were stored at -20°C. Please refer to the Appendix for a list of primers (Table 17 in Appendix) used.

2.1.7 Vectors

The following vectors were used in this study. Please see section 5.1 in the Appendix for vector maps.

Table 6. Vectors used in the present study.

Vector	Purpose	Source
pDONR221	Gateway™ Entry-Vector	Invitrogen™
pDONR201	Gateway™ Entry-Vector	Invitrogen™
pDONR207	Gateway™ Entry-Vector	Invitrogen™
pACT2	Prey vector for Yeast two-hybrid system	Clontech™
pBTM116	Bait vector for Yeast two-hybrid system	Clontech™
pB2GW7	Plant expression vector (35S promoter)	(Karimi *et al.*, 2002)
pK2GW7	Plant expression vector (35S promoter)	(Karimi *et al.*, 2002)
pBT10:GUS	Reporter vector for protoplast transactivation assay	(Sprenger-Haussels and Weisshaar, 2000)

2.2 Methods

2.2.1 Growth conditions and culture methods

2.2.1.1 Plants

i) Arabidopsis:

Plants of the Columbia (Col-0) accession of *Arabidopsis thaliana* were grown in the greenhouse on soil (see 2.1.1) at 22°C under long-day conditions (16 h light/8 h dark). For *in- vitro* experiments, seeds were surface sterilized with a saturated calcium hypochlorite solution. 100-150 seeds of *Arabidopsis* were taken into a microcentrifuge tube, 1 ml of calcium hypochlorite solution was added and incubated for 5-7 min on an orbital shaker. After incubation seeds were washed with sterile water 2-3 times by brief centrifugation. Seeds were air dried on sterile Whatman paper and placed on MS media (Murashige and Skoog, 1962) plates. The plates were kept at 4ºC for 3 d in the dark for stratification treatment and then exposed to white light (75 µE). Seedlings were grown at 22ºC in long day conditions on MS medium, unless specified otherwise.

ii) Tomato and tobacco:

The surface sterilization of tomato seeds was done according to Meissner *et al.* (Meissner *et al.*, 1997). The seeds of tomato were put into a sieve and dipped for 1-2 min in 70% ethanol. The same sieve was dipped 2-3 times in sterile water and placed 8-10 min in a beaker containing 10% hypochlorite solution. After that, seeds were washed with sterile water by placing the sieve in the water beaker for 5 min. The seeds were dried by placing them on a sterile Whatman paper. The air dried seeds were placed in a glass beaker containing MS medium (see 2.1.2.1) and kept in the dark at 25ºC for 4 days. The germination was induced by placing the seeds under white light (16 h light/8 h dark) at 25ºC. After 8-10 days the seedlings were transferred to the green house. Tobacco seeds were sown directly on soil without any sterilization steps. Both tomato and tobacco were grown at 25-26ºC under long day (16 h light/8 h dark) conditions in the green house.

2.2.1.2 Microbial culture

The microbial cultures were grown under standard conditions as described (Bertani, 1951; Sambrook *et al.,* 1989; Rose *et al.,* 1990). The *E.coli* strains were grown at 37ºC on a shaker for overnight (O/N), whereas the *Agrobacterium* strain was usually grown at 28ºC on a shaker at 150-180 rpm for 2-3 days. The bacterial cultures were grown in liquid LB or YEP

media (see 2.1.2.2) containing the appropriate antibiotics. The final concentrations of different antibiotics that were used are described in chapter 2.1.2.2. The yeast strain was grown at 30ºC. Liquid cultures were always grown while shaking at the respective temperatures. Selective components were added to the medium according the plasmid requirements.

2.2.2 Microbial transformation methods

2.2.2.1 Preparation of E.coli *electrocompetent cells*

A single colony of DH10B strain of *E. coli* was taken and inoculated into 3 mL of LB medium for O/N culture. On the next day 100 mL of LB medium (pre-heated to 37ºC) was inoculated with 1 mL of the O/N culture. The culture was incubated at 37ºC on a shaker until the culture OD_{600} reached 0.35-0.55. After that, the culture flask was chilled on ice for 30 min. The culture was dispensed into two 50 mL pre-chilled falcon tubes and centrifuged for 15 min, 4,000 rpm, at 4ºC. The pellet was washed and dissolved twice in 20 mL of ice cold water. The tubes were incubated 15 min on ice and subsequently centrifuged for 15 min, 4,000 rpm, at 4ºC. The cell pellet was dissolved in 20 ml of 10% glycerol. This step was repeated and the pellet was dissolved in 1-2 mL of 10% glycerol. From the cell suspension, 50 µL aliquots were prepared in pre-chilled microcentrifuge tubes. The tubes were shock frozen in liquid N_2 and stored at -80ºC.

2.2.2.2 Preparation of A. tumefaciens *electrocompetent cells*

The *Agrobacterium* strain GV3101 was used for preparing electrocompetent cells. A single colony of this strain was inoculated into 5 mL of pre-culture and grown at 28ºC for 2 days. The following day, the pre-culture was inoculated into 300 ml of LB media with appropriate antibiotics and grown in the same condition for another 24 hours until the OD_{600} reached 0.5-0.8. From this step onwards the same procedure was followed as described (see 2.2.2.1) for the preparation of *E.coli* competent cells.

2.2.2.3 Transformation of DNA into bacterial host cells

For plasmid transformation 1-2 µL (approximately 20-100 ng) of DNA was used. Shortly before transformation the competent cells were thawed on ice for 5 min followed by the addition and incubation of DNA for 20 min. Later plasmid DNAs were transformed into

host cells (either *E. coli* or *A. tumefaciens*) by electroporation with GenePulserII (Bio-Rad, München, Germany) with the following settings: 200 Ω, 1.8 kV, 2.5 to 5 mS. The cells were collected in 1 mL SOC medium (see 2.1.2.2) and incubated on a shaker at 37ºC for 1 h (*E. coli*) or 28ºC for 2 h (*A. tumefaciens*). The appropriate amount of the bacterial culture was then plated on solid LB agar plates containing selective marker, incubated at 37ºC over night in case of *E. coli* and at 28ºC for 2 days for *A. tumefaciens*.

2.2.2.4 Heat shock transformation

The microcentrifuge tubes containing 100 µL of chemical competent cells (Stratagene™, Amsterdam, Netherlands) were thawed on ice for 5 min before transformation. 10-50 ng of DNA was added to the competent cells and incubated on ice for 30 min. The transformation mixture was heat-pulsed at 42ºC for 45 seconds and placed on ice for 2-3 min. 900 µL of pre-heated SOC medium (see 2.1.2.2) was added and incubated at 37ºC for 1 h on a shaker at 200-225 rpm. The appropriate volume of the transformation mixture was plated on the LB selection medium and incubated O/N at 37ºC.

2.2.2.5 Transformation of yeast

Yeast transformation was performed after Elble (Elble, 1992) with minor modifications. The yeast strain was grown in YPD medium at 30ºC for 2-3 days as starting culture. 1-2 mL of the starting culture was transferred into fresh pre-heated (at 30ºC) 30 mL of YPD media and grown at 30ºC until the OD_{600} reached 0.6-0.8. The yeast cells were centrifuged at 2,000 rpm for 5 min and the pellet was resuspended in 10 ml of mix 1, containing 0.1 M LiAc, 1 M Sorbitol, 5 mM Tris-HCl (pH 7.5) and 0.5 mM EDTA. The suspension mixture was washed twice with mix 1 by centrifugation and finally suspended in 1-3 ml of mix 1. The transformation mixture was prepared in a fresh 1.5 mL tube by adding 1 µg of plasmid DNA, 50 µg of herrings sperm DNA, 230 µL of mix 2 (0.1 M LiAc, 40% PEG 3350, 10 mM Tris-HCl pH 7.5 and 1 mM EDTA) in a 50 µL of mix1 suspension. The DNA and yeast cell suspension was incubated for 30 min at 30ºC. After incubation, 30 µL of DMSO was added and heat shock transformation was performed at 42ºC for 20 min. The tubes were centrifuged for 3 min, 4,000 rpm, at room temperature (RT) and the cell pellet was dissolved in 200 µL of sterile water. The transformed cell suspension was plated on SD media and incubated at 30ºC for 3-5 days.

2.2.2.6 Preparation of glycerol stocks

After overnight growth in LB medium supplemented with antibiotics, 800 µL of cell suspension was mixed with 800 µL of sterile glycerol and directly frozen in liquid nitrogen. The glycerol stocks were labeled properly and stored at -80ºC.

2.2.3 Plant transformation methods

2.2.3.1 Stable transformation of Arabidopsis thaliana

Transformation of Col-0 plants was carried out by the floral dip method according to the protocol by Clough and Bent (Clough and Bent, 1998). A single colony of *Agrobacterium* bearing the desired binary vector was inoculated into 5 mL of LB medium containing selective antibiotics and grown at 28ºC as starting culture. After 20-24 h, the starting culture was transferred into 300 mL YEP (see 2.1.2.2) selection medium and grown for 24-40 h at 28ºC. Bacteria were centrifuged at 6,000 ×g for 15 min at 4ºC and resuspended in an infiltration medium (50 g/L sucrose, 2.19 g/L MS salts, and 300 µl/L Silwet 77) so that the OD_{600} of the solution should be 0.8-1.0. *Arabidopsis* flowering stalks were dipped in infiltration medium for 15-30 seconds and left in humid, low light conditions for 24 h. The transformants were then transferred to the greenhouse and grown directly on soil. When the plants were 2-3 week old glufosinate ammonium (BASTA) a commercial herbicide (Bayer Crop Science, Monheim, Germany), was sprayed. Seedlings were sprayed with a final concentration of 0.1% BASTA. The BASTA spray was carried out two times in a 3-day interval. Resistant seedlings were transferred to individual soil pots and grown under standard conditions as described in 2.2.1.1.

2.2.3.2 Stable transformation of tomato

The stable transformation of tomato cultivar Micro-Tom was done according to Sun *et al.,* (Sun *et al.,* 2006). Seeds were surface sterilized and germinated on MS medium (see 2.2.1.2). After sowing, seeds were kept in the dark for 4 days and then transferred to light (16 h of photoperiod) for another 4 days at 25ºC. Cotyledons of 8-9-day-old seedlings were excised. Every cotyledon was cut into two pieces and the basal and proximal ends were removed to facilitate infiltration of the bacterial suspension into the cotyledons. The cotyledon pieces were placed on Petri dishes containing MS medium with hormones (2 mg/L zeatin; 0.1 mg/L IAA), in a manner that the abaxial surfaces of the cotyledons were in

contact with the medium. *Agrobacterium* cells containing the chosen construct were grown in LB medium with antibiotics at 28ºC O/N. The following day *Agrobacterium* cells were centrifuged at 4,000 rpm for 15 min, RT and the pellet was resuspended in liquid MS medium until OD_{600} reached 0.2. To this suspension 100 µM acetosyringone was added. The *Agrobacterium* suspension mixture was added to the explants and incubated for 10 min. After incubation, the suspension culture was removed from the explants by a sterile filter paper. The explants were cultivated on the same plates for 48 h at 25ºC in the dark. After 48 h of co-cultivation the explants were transferred to MS medium with hormones and the selective marker kanamycin. To suppress growth of *Agrobacterium*, augmentin (375 mg/L) was added to the medium. After two weeks the explants were again sub-cultured to fresh MS selection media with hormones. After 2-3 weeks of culture, the transformed elongated shoots were transferred to medium containing only IAA to induce roots. The fully regenerated plant grown seedling was then transferred to the greenhouse.

2.2.3.3 Transient transformation of tobacco leaves

The transient transformation of tobacco was done according to Walter *et al.*, (Walter *et al.*, 2004). The *A. tumefaciens* cells transformed with a binary vector containing fluorescent protein fusion constructs were grown in 50 mL YEP medium (see 2.1.2.2) supplemented with appropriate antibiotics at 28ºC with 180 rpm shaking for 24 hours. The following morning, cells were collected by centrifugation at 4,000 ×g for 10 min and resuspended in 10 mM MES-KOH (pH 5.6) containing 10 mM $MgCl_2$ and 150 µM of acetosyringone. 4-5 weeks old *N. benthamiana* plants were used for infiltration. Co-infiltration of *Agrobacterium* strains containing fluorescent protein fusion constructs and the p19 silencing plasmid was carried out at an OD_{600} of 0.7:0.7:1.0 respectively. The suspension was infiltrated into the abaxial side of leaves with a needleless 10 mL syringe until the tissue was filled with liquid. The plants were kept in greenhouse for 4-5 days for the accumulation of fluorescent protein.

2.2.3.4 **Arabidopsis** *mesophyll protoplast transformation*

Protoplast isolation and transformation was performed according to the method described by Hwang and Sheen (Hwang and Sheen, 2001). The plants were grown 4-5 weeks in Percival plant chambers (CLF Plant Climatics, Emersacker, Germany) under long day

conditions (16 h light/8 h dark), low light intensities (50-60 µE) and ~70% RH, at 23ºC. Leaves from the same growth stage from different plants were taken for protoplast isolation. The lower side of the leaves were cut and placed in the leaf digestion solution (see 2.1.3) in a way that the lower side was in contact with the solution. The digestion of the leaves was done O/N at 23ºC. The following day protoplasts were released from the leaves by gentle shaking of the Petri dish containing the digestion solution. The protoplasts were separated from leaf debris by collecting them in a fresh 50 mL falcon tube using a mesh of 60 µM pore size. The falcon tube was centrifuged with a swing rotor system at 100 ×g for 2-3 min. All the centrifugation steps were done in this way, while the ascending and descending speed of the rotor was adjusted to the minimal level. The protoplast pellet was washed by dissolving in 10 mL of cold W5 solution (see 2.1.3) and mixed gently by holding the tube in a slanting manner. The protoplast solution was washed one more time with W5 solution and kept on ice for 4-5 hours. After incubation, the tube was centrifuged at 100 ×g for 2 min and the protoplast pellet was suspended in MMg solution (see 2.1.3) in a way that 1 mL of solution contained 1-2 × 10^5 cells. In a fresh 2 mL tube 15-20 µg of plasmid DNA was used for a single transformation, whereas in case of multiple transformations different plasmid DNAs (max. 40 µg) were mixed along with the control plasmid DNA. To each transformation reaction tube, 200 µL of protoplast solution and 220 µL of 40% PEG solution (see 2.1.3) was added and mixed gently by inverting the tubes several times. The PEG-mediated transfection of the protoplast was done by incubating the tubes for 30 min at RT. After the transfection, protoplasts were washed with 800 µL of W5 solution by centrifuging at 100 ×g for 1-2 min at RT. After the centrifugation W5 solution was removed completely and the pellet was dissolved in 1 mL of WI solution. The transformed protoplast solution was placed in a six-well-plate and incubated O/N in dark at 23ºC.

2.2.4 Nucleic acid methods

All nucleic acid methods were done as described in the handbook "Molecular Cloning, A Laboratory Manual" (Sambrook *et al.*, 1989), unless otherwise specified.

2.2.4.1 Isolation of plasmid DNA

Plasmid mini and maxi-prep kits were used to isolate plasmid DNA from DB3.1, DH10B and XL1Blue strains of *E.coli* that were transformed with different constructs

according to the manufacturer's instruction manual. The Invisorb Spin miniprep kit (Invitek, Berlin, Germany) utilized 2 mL of mid logarithmic *E. coli* cultures and yielded a maximum amount of 1-3 µg of plasmid DNA in 50 µL which was subsequently used for sub-cloning, sequencing or PCR. The Nucleobond Xtra Maxiprep kit (Macherey-Nagel, Düren, Germany) yielded around 200-300 µg of DNA from 100 mL culture volume. The quality and quantity of the isolated DNA was measured using a photometer and visualized by running a 1% (w/v) agarose gel containing ethidiumbromide.

2.2.4.2 Isolation of plasmid DNA from Agrobacterium

The plasmid DNA from *Agrobacterium* was isolated by using the Invisorb Spin miniprep kit (Invitek, Berlin, Germany) with slight modifications in the protocol. 2-4 mL of a two-days-old culture was transferred to microcentrifuge tubes and centrifuged for 1 min, 15,000 rpm, at RT. The pellet was resuspended by using 250 µL of solution A, supplied with the kit. To this 20 µL of lysozyme (from 20 mg/ml stock solution) was added and properly mixed. The tubes were incubated for 20 min at 37ºC, and subsequently 250 µL of solution B was added. The cells were mixed carefully by inverting the tubes 5-6 times and further incubated at 70ºC for 15 min. After the incubation, 250 µL of solution C was added and thoroughly mixed. The tubes were centrifuged for 5 min at maximum speed (12,000-16,000 rpm) and the supernatant was transferred onto the spin filters and incubated for 1 min. The spin filters were centrifuged for 1 min at 8,000 rpm, the filtrate was discarded and 750 µL of wash solution was added on to the column. After 1 min of incubation, the tubes were centrifuged for 1 min at 8,000 rpm, and further centrifuged at maximum speed for 3 min to remove the residual ethanol. The DNA was eluted into fresh microcentrifuge tubes by adding 30-50 µL of sterile ddH_2O onto the spin filters.

2.2.4.3 Agarose gels for DNA

For the separation and detection of molecular DNA, 0.8-2% agarose gels in 1x TAE (40 mM Tris-HCl; 1 mM EDTA, pH 8.0) buffer were prepared. For the detection of DNA under UV light 0.5 µg/mL of Ethidiumbromide was used in gel preparation. The samples were prepared by mixing with a suitable volume of 10x loading buffer (see 2.1.3). Depending on the percentage of the gel and the size of DNA fragments, running time of the gel was adjusted to 1-3 h at 70-110 V. The molecular weight marker *HyperLadderI*^{TM}

(Bioline, Luckenwalde, Germany) was used to determine the size of the DNA fragments. Visualization and pictures of gel were taken by using the SynGene Bioimaging system (Merck, Darmstadt, Germany).

2.2.4.4 Agarose gels for RNA

For the confirmation of quality after isolation and purification, 1 µg of RNA sample was mixed with appropriate volume of loading buffer and run with 0.8 to 2.5% agarose gels prepared with 1x TBE (see 2.1.3). For Northern blot analysis, a denaturing agarose-formaldehyde gel (1.2%) containing 10% of 10x MOPS (see 2.1.3) and 3% of 37% formaldehyde was prepared. A total of 20 µg RNA was separated in the gel containing 1x MOPS buffer for 3-4 h at 60-70 V.

2.2.4.5 Purification of PCR products

PCR amplicons ranging from 100 bp to 10 kb were purified by using the QIAquick PCR Purification Kit according to the manufacturer's (Qiagen, Hilden, Germany) instruction manual. The amplicons below 100 bp in size were purified by QIAquick Nucleotide Removal Kit using the manufacturer's (Qiagen, Hilden, Germany) instructions.

2.2.4.6 Isolation of DNA fragments from agarose gel

After agarose gel electrophoresis of PCR samples, the desired amplicon of the size ranges from 100 bp to 10 kb was cut from the gel and purified by using the QIAquick Gel Extraction Kit, according to the manufacturer's (Qiagen, Hilden, Germany) instruction manual.

2.2.4.7 Isolation of total RNA from **Arabidopsis**

For the isolation of total RNA from *Arabidopsis* and tomato plants, either 7-day-old seedlings or leaves were used. Please refer to part 2.2.7.2 for the method that was followed.

2.2.4.8 Northern blot analysis

Total RNA was extracted from three weeks old soil-grown plants or 7-days-old seedlings grown in 0.5x MS liquid medium as described (see 2.2.7.2). In case of cytokinin induction seedlings were treated with 5 µM 6-benzyladenine (BA) and incubated 0, 15, 30, and 120 min prior to harvesting. Preparation of the formaldehyde gel for Northern blot

analysis was performed as described (see 2.2.4.4). 20 µg of RNA was separated in a denaturing 1.2% agarose-formaldehyde gel (10 % of 10x MOPS and 3% of 37% formaldehyde) and transferred to a Hybond-N+ nylon membrane (Amersham, Little Chalfont, UK). The RNA was crosslinked to the membrane by using UV Stratalinker^{TM} 2400 (Stratagene Amsterdam, Netherlands). The probes were labeled with radioactive [α-^{32}P] dCTP by using the Prime-It^{TM} II Random Primer Labeling Kit according to the manufacturer's (Stratagene, Amsterdam, Netherlands) protocol. The membrane was hybridized with radioactive [α-^{32}P] dCTP labeled DNA. Hybridization was performed at 68ºC in a phosphate buffer containing 7% SDS and 1% BSA. Washing was done with 2x SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.2), and 0.2x SSC, 0.1% SDS at 65ºC. As a control for loading, blots were reprobed with an *Actin2* probe. The different probes were generated by amplifying the respective cDNAs (for used primers see Table 17 in Appendix).

2.2.5 Polymerase Chain Reaction (PCR) techniques

2.2.5.1 Standard PCR method

The standard PCR method was carried out as described (Mullis and Faloona, 1987). The desired amplicon was amplified from the respective plasmid DNA or cDNA by PCR using either a "low fidelity" *Thermus aquaticus* (*Taq*) polymerase or a "high fidelity" *Pyrococcus furious* (*Pfu*) polymerase. The following protocol and PCR program were used for amplification:

Table 7. The standard PCR components.

Components	Volume
Template DNA* (50-100 ng)	1.0 µL
10x Buffer	5.0 µL
dNTP-Mix (5 mM)	2.5 µL
Forward primer (20 µM)	1.0 µL
Reverse primer (20 µM)	1.0 µL
Taq or *Pfu* polymerase	1.0 µL
dd H_2O add to	50 µL

*The concentrations of template DNA and primers were varied depending on the conditions.

Table 8. The standard PCR program used.

Reaction	Temperature	Time	No. of cycles
Initial denaturation	95ºC	2 min	1
Denaturation	94ºC	30 sec	
Annealing*	52ºC-64ºC	30 sec	27-35
Elongation*	68ºC (*Pfu*) 72ºC (*Taq*)	1-4 min	
Final elongation	72ºC	8 min	1
Stop	16ºC	Hold	1

*The annealing temperature for the reactions depended on the melting temperature (T_m) of the primers that were used.

*The elongation time is 1 min in case of *Taq* or 2 min for *Pfu* polymerase for every 1 kb to be amplified.

2.2.5.2 Reverse transcriptase (RT)-PCR

Total RNA (1 µg) was used for the synthesis of the first strand cDNA, using the Qiagen One-step RT-PCR kit according to the manufacturer's (Qiagen, Hilden, Germany) protocol. Control reactions to check for the presence of mRNA were performed using the *Actin2* primer pair (see the Table 17 in Appendix). First, the RNA was transcribed into cDNA at 50ºC by the reverse transcriptase which was then denatured by incubation at 95ºC, this also activates the HotStarTaq DNA polymerase of the enzyme mix (Qiagen, Hilden, Germany). Then a conventional PCR was performed.

Table 9. The standard RT-PCR program.

Reaction	Temperature	Time	No. of cycles
Reverse transcription	50ºC	30 min	1
Destruction of reverse transcriptase	95ºC	15 min	1

Denaturation	94ºC	1 min	
Annealing	52ºC-64ºC	30 sec	30
Elongation	72ºC	1 min	
Final elongation	72ºC	8 min	1
Stop	16ºC	Hold	1

2.2.5.3 Real time PCR or quantitative RT-PCR (qRT-PCR)

PCR reactions were performed in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, USA), using SYBR™ Green (Sigma, München, Germany) to monitor dsDNA synthesis. Primers were designed according to a stringent set of criteria using the Genoplante™ Specific Primers and Amplification Design Software (SPADS) (http://urgi.versailles.inra.fr/spads/). In the SPADS program the Primer 3 algorithm was used to produce Gene specific Sequence Tags (GST) (Thareau *et al.*, 2003). The primers were designed with the following parameters: melting temperatures (Tm) of 60 ±2ºC, primer lengths of 20-24 nucleotides, guanine-cytosine (GC) content of 45-55%, and PCR amplicon lengths of 100-150 base pairs.

Total RNA was extracted and purified as described (see 2.2.7.2 and 2.2.7.3). 5 µg of cleaned total RNA was used for cDNA synthesis. The synthesis of cDNA was performed using SuperScript III Reverse Transcriptase according to the manufacture's (Invitrogen, Karlsruhe, Germany) protocol. The real time polymerase chain reactions were prepared in 20 µL volume containing 1 µL cDNA sample (100-150 ng), 1 µL of each primer (200 nM), 0.8 µL of $MgCl_2$ (2 mM), 2 µL 10x PCR buffer, 0.4 µL of dNTPs (5 mM each), 0.04 µL of ROX (1:1000 dilution from 2.5 mM stock, Sigma, München, Germany), 0.20 µL SYBR Green I (1:10,000 dilution in DMSO, Sigma München, Germany), 0.04 µL Immolase polymerase (5 U/µL, Bioline, Luckenwalde, Germany) and appropriate volume of distilled water. The following PCR program (Table 10) was used:

Table 10. The standard qRT-PCR program.

Reaction	Temperature	Time	No. of cycles
Initial denaturation	95ºC	15min	1
Denaturation	94ºC	10 sec	40
Annealing	55ºC	15 sec	
Elongation	72ºC	10 sec	
Stop	16ºC	Hold	1

Data were analyzed using the Applied Biosystems 7500 software v2.2 (Applied Biosystems). In each PCR reaction two housekeeping genes (*Ubiquitin10* and *AT3G25800*) were included along with a water control.

2.2.6 Cloning methods

2.2.6.1 Restriction digestion

All restriction enzymes were purchased from New England Biolabs (Frankfurt, Germany) and Fermentas (Leon-Rot, Germany) and used with the supplied buffers. In total 50 to 200 ng of plasmid DNA was used and 5 to 10 units of restriction enzyme were added for digestion. The digestion was carried out in 20 µL volume (for single digestion) or 50 µL (in case of double digestion) for 2-4 hours at the recommended temperature. In case of double digestion the appropriate buffer was used, if the appropriate buffer was not available, sequential digestion was followed using the manufacturer's (Fermentas, Leon-Rot, Germany) instructions.

2.2.6.2 DNA ligation

DNA fragment ligations were performed with T4-DNA Ligase (Fermentas, Leon-Rot, Germany) overnight at 16ºC. The DNA fragments were taken in a ratio of 1:1 up to 3:1 insert DNA to vector DNA. The ligase was heat denatured by incubation at 65ºC for 10 min.

2.2.6.3 Gateway™ recombination

The cloning of desired constructs in Gateway™ compatible vectors was done according to the manufacturer's (Invitrogen, Karlsruhe, Germany) protocol. For creating an Entry clone 100 ng of the vector (pDONR201, pDONR 221 or pDONR 207), 1 to 3 µL of

purified PCR product (300 ng) with Gateway™ compatible overhangs, 2 µL of 5x BP clonase reaction buffer and 0.5-1.0 µL of BP clonase mix was added. The reaction was carried out at 25ºC for O/N. The reaction was stopped by adding 1.0 µL of Proteinase K (2 µg/µL) and incubating at 37ºC for 10 min. Whereas the recombination to destination vectors was carried out by adding 1 µL (150-300 ng) of plasmid DNA of the entry vector, 2 µL 5x LR clonase reaction buffer and 1 µL of LR clonase mix to 100 ng of destination vector. The LR reaction was carried out O/N at 25ºC. The reaction was terminated by adding 1.0 µL Proteinase K (2 µg/µL) and incubating at 37ºC for 10 min. The reaction products (either BP or LR) were directly electroporated to bacterial host cells as described (2.2.2.3).

2.2.6.4 Cloning of the ARR1-SRDX *construct*

The cloning of ARR1-SRDX and transformation into *Arabidopsis* was done by Dr. Heyl. The gene was constructed in the following way: The protein coding region of the *ARR1* gene was amplified by PCR using a cDNA library from *Arabidopsis thaliana* C24 (Minet *et al.*, 1992) and inserted in the plasmid pDONR201 (Invitrogen, Carlsbad, USA) yielding pDONR201-ARR1 (Dortay *et al.*, 2006). The DNA fragment coding for the SRDX peptide (LDLDLELRLGFA) was synthesized with a TAA stop codon and a *BsrG*I restriction site at the 3' end and an in frame *Hha*I site at the 5´ end. The *ARR1* gene was isolated from the plasmid pDONR201-ARR1 by restriction digestion with *Bsr*GI and the resulting fragment was further digested with *Hha*I. The DNA fragments were ligated and recloned into pDONR201. During the digestion of the *ARR1* coding sequence with *Hha*I, the last 19 bp (5'-ACTTCTTAAGCAGGTTTGA-3') of the gene were removed. Later this part of the sequence was used to differentiate between *ARR1* and *ARR1-SRDX* specific probes designed for Northern blots and RT-PCR. The resulting *ARR1-SRDX* gene was shuttled into the destination vector pB2GW7 (Karimi *et al.*, 2002) for overexpression under the control of the 35S promoter.

2.2.6.5 Site-directed mutagenesis

The point mutations generated in the different regions of the *ARR6* promoter were done by using the QuikChange™ site-directed mutagenesis kit according to the manufacturer's (Stratagene, Amsterdam, Netherlands) protocol.

2.2.7 Transcription profiling on full genome chip

2.2.7.1 Growth conditions and harvesting of biological samples

The Complete Arabidopsis Transcriptome MicroArray (CATMA) v2.4 microarrays (ArrayExpress accession no. A-MEXP-274 at http://www.ebi.ac.uk/aerep) were used for transcription profiling. The CATMA v2.4 microarrays were spotted with a total of 26,173 Gene specific Sequence Tags (GSTs) for annotated genes (Sclep *et al.*, 2007). The experiment was conducted with four different genotypes: wild-type (Col-0), *35S:ARR1-SRDX*, *arr1* knockout and *35S:ARR1* overexpressor lines and at three different time points (0, 15 and 120 min) after cytokinin induction treatments. The seedlings of different genotypes were grown in sterile 0.5x MS liquid medium (1 g/L sucrose, 0.5 g/L MES, pH 5.7) in Petri dishes and cultured under conditions as described in 2.2.1.1. 5 DAG (days after germination) the seedlings were treated with either 5 µM BA dissolved in 1.13 mM KCl for 15 or 120 min (BA15 and BA120, respectively), or with 1.13 mM KCl solution for 120 min as a control (BA0). Timing of the treatment was such that the plants were harvested within an interval of 6 to 8 h after the onset of light. The content of two Petri dishes was pooled to yield one sample of biological material. Two biological samples were taken for each condition and each biological sample was hybridized onto two microarrays, resulting in a total of four hybridizations per experimental condition.

2.2.7.2 Extraction of total RNA

A total of 100-200 mg of *Arabidopsis* tissue (seedlings of 7 days old or leaf tissues) was ground under liquid nitrogen to a fine powder and put into 2 mL tubes. 1 mL TRIzol (see 2.1.3) was added and vigorously shaken to get a homogenous mixture. After 5 min of incubation at RT, the tubes were centrifuged at 16,000 ×g for 5 min at 4ºC. The supernatant was transferred into a fresh 2 mL tube and 400 µL of chloroform-isoamylalcohol 24:1 was added. The tissues were vigorously shaken until the mixture looked homogenous. The tubes were incubated for 5 min at RT, subsequently centrifuged at 16,000 ×g for 15 min at 4ºC. 700 µL of the upper phase was transferred to a fresh 1.5 mL tube, without taking anything of the two other phases. To this solution 350 µL of isopropanol and 350 µL of high salt solution (1.2 M sodium chloride, 800 mM sodium citrate) were added and mixed by repeated inversion until the mixture turned clear. After incubation for 10 min at RT, the samples were centrifuged at 12,000 ×g for 10 min at 4ºC and the supernatant was removed completely by

pipetting. The RNA was washed twice with 900 µL 75% Ethanol by centrifugation at 7,500 ×g for 5 min at 4ºC. The pellet was dried at 60ºC until it turned clear and subsequently dissolved in 30-40 µL of RNase-free water. Photometric measurement of the RNA was performed at 260 nm, 280 nm, and 230 nm to calculate the amount and quality of RNA. The RNA was considered clean if the ratio of R260/280 > 1.950, and R260/230 > 2.050.

2.2.7.3 Purification of total RNA

Purification of total RNA was done by using Qiagen RNeasy Kit, according to the manufacturer's (Qiagen, Hilden, Germany) protocol with slight modifications. The volume of the samples was adjusted to 100 µL with RNase-free water. 6 µL of 2-mercaptoethanol was added per 1 mL of buffer RLT. 350 µL RLT + 2-mercaptoethanol mixture was added to the RNA samples and mixed. 250 µL of ethanol was added to this solution and mixed well by pipetting. Later the whole mixture (700 µL) was transferred to a RNeasy mini column and incubated for 1 min at RT. The samples were centrifuged at 8,000 ×g for 15 s at RT and this step was repeated by applying the filtrate once again to the column. Subsequently on-column DNase digestion by using Qiagen RNase-Free DNase kit according to manufacturer's (Qiagen, Hilden, Germany) protocol was performed. After DNase digestion the columns were washed twice by using 500 µL of wash buffer RPE and centrifuged at 8,000 ×g for 15 s at RT. To remove residual ethanol after washing one final centrifugation step was done at 16,000 ×g, for 1 min, at RT. The filtrate and collection tube were discarded and the column was transferred to a fresh RNase-free 1.5 mL tube. The RNA was eluted by adding 35 µL of pre-warmed (at 65ºC) RNase-free water onto the column and incubated for 1 min and centrifuged at 8,000 ×g for 15 s at RT.

2.2.7.4 RNA amplification by in-vitro transcription

The protocol for RNA amplification was adapted from Puskás *et al.,* (Puskás *et al.*, 2002). RNA amplification was done in three stages: first strand synthesis, second strand synthesis and *in vitro* transcription.

(i) First strand synthesis:

For the first stand synthesis a total of 5 µg of cleaned RNA (2.2.7.2) was taken and 1 µL (40 U) of RNase inhibitor, RNaseOut^{TM} (Invitrogen, Karlsruhe, Germany) was added. The final volume was made up to 4 µL with RNase-free water and it was dried by using a

SpeedVac (Thermo Scientific, Leicestershire, UK). 1 µL (200 ng) of HT7T-Primer and 6 µL (10.2 µmol) of 1.7 M Trehalose were added. The tubes were incubated for 5 min at 75ºC, chilled on ice and spinned down by brief centrifugation. 4 µL of 5× first strand buffer (1/5 Vol.), 2 µL (200 nM) of 0.1 M DTT, 1 µL (10 nM) of 10 mM dNTP-Mix, 1 µL of (1.7 µM) 1.7 M Trehalose and 1 µL (200 U) of SuperScript II RT (Invitrogen, Karlsruhe, Germany) was added and mixed well. The total mixture was transferred to a PCR tube (0.2 ml tube) and incubated in a PCR machine with the following program listed in Table 11.

Table 11. The PCR program for *in vitro* transcription.

Step	Temperature	Time	No. of cycles
1	37ºC	5 min	1
2	45ºC	5 min	1
3	60ºC	2 min	9
4	55ºC	2 min	
5	65ºC	10 min	1
stop	4ºC	hold	1

(ii) Second strand synthesis:

To the first strand product, 32.4 µL of 5× second strand buffer (1/5 Volume, see 2.1.3), 3.4 µL (34 nM) of 10 mM dNTP-Mix, 1 µL of *E. coli* DNA-Ligase (10 U), 4 µL of *E. coli* DNA-Polymerase I (40 U), 1 µL of *E. coli* RNase H (2 U) and 103.8 µL of water was added. This mixture was mixed gently and incubated at 16ºC for 3 h. The enzymes were deactivated at 65ºC for 30 min. Finally the tubes were chilled on ice for 5-10 min. The product of second strand synthesis was purified *via* a column by using QIAquick PCR purification kit. For the purification, the manufacturer's (Qiagen, Hilden, Germany) protocol was followed with slight modifications. To the product of second strand synthesis 7 µL of 3 M sodium acetate of pH 5.0 and 5 Volumes of buffer PB was added, mixed well and incubated on ice for 2-3 min. This mixture was loaded onto a QIAquick column and centrifuged at 9,000 rpm for 15 s at RT. The filtrate was discarded, 500 µL of buffer PE was loaded on to the column, centrifuged at 9,000 rpm, and this step was repeated one more time. To elute the residual ethanol, the columns were centrifuged at maximum speed (16,000 rpm) for 1 min at RT, and placed into a fresh RNase-free 1.5 mL tube. The cDNA was eluted by adding 35 µL water onto the column. The cDNA was dried using a SpeedVac.

(iii) In vitro *transcription*:

The whole second strand product was used for *in vitro* transcription with the MEGAScript™ High Yield Transcription Kit (Ambion, Darmstadt, Germany). The dried cDNA was dissolved in 18 µL of the mixture containing 8 µL (150 nM) of 18.75 mM rNTP-Mix, 2 µL of (1/10 vol.) 10× T7 reaction buffer and 8 µL of water. To this 2 µL of MEGAScript™ T7 enzyme solution was added and mixed well. The tube was incubated for 3 h at 37ºC. The enzyme reaction was denatured by incubating at 65ºC for 10 min. The tube was chilled down to 4ºC by placing it on ice. The cRNA which was a product of *in-vitro* transcription was purified by using Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) columns. The procedure for purifying the cRNA was same like that was described in 2.2.7.3 but without DNase digestion step.

2.2.7.5 Labeling of cRNA

A total of 5 µg of cleaned cRNA was used for labeling and the whole procedure was done under dimmed light conditions since in the bright light Cy dyes degrade faster. To the cleaned cRNA 0.5 µL of RNaseOut™ (20 U) was added. The final volume was made up to 8 µL with RNase-free water and cRNA was dried out by using a SpeedVac. If the volume of cRNA exceeded 8 µL, 40 U (1 µL) of RNaseOut™ was added and dried. To this dried cRNA 2 µL of (2 µg) random nonamers N9 was added and incubated for 10 min at 70ºC. After this incubation tube was placed on ice for some time and spinned down. Subsequently a mixture of 9.5 µL containing 4 µL of 5× first strand buffer (1/5 Vol.), 2 µL (200 nM) of 0.1 M DTT, 1 µL of –C-dNTP (2 nM dCTP, 10 nM dDTP), 1.5 µL (1.5 nM) of Cy3-dCTP or Cy5-dCTP and 1 µL (200 U) of SuperScript II RT (Invitrogen, Karlsruhe, Germany) was added into this tube, mixed well and incubated for 2½ h at 42ºC. After that, the incubation tube was placed on ice for 5-10 min. Once the mixture was chilled down, 2 µL of 2.5 M NaOH was added and incubated exactly for 15 min at 37ºC. After the incubation 10 µL of 2 M MOPS was added. In the end the labeled cDNA was purified by using the QIAquick PCR purification kit. The amount of dye and cDNA was determined by using a NanoDrop™ photometer (Peqlab, Erlangen, Germany). The samples were stored at -20ºC in the dark until they were used for hybridization.

2.2.7.6 Hybridization

The hybridization strategy was followed as described in the European Compendium of *Arabidopsis* Gene Expression (CAGE) project (http://www.cagecompendium.org). A Cy3-labeled mixture of oligonucleotides complementary to the secondary primers for Gene Specific Tag-amplification was used as a common reference sample and Cy5-labeling for the biological samples. Prior to hybridization, 40 pmol dye of the Cy5-labeled sample and 10 pmol dye (1 µL from a 10 µM stock solution) of the Cy3-labeled oligo reference were mixed in a tube and dried by using a SpeedVac (SpeedVac was darkened with aluminium foil, for preventing bright light on Cy dyes). The dried sample of oligo mix was dissolved by adding 60 µL of the hybridization mixture (2 µL of of Poly-dT_{35}, 15 µL of Microarray hybridization buffer (Amersham, Little Chalfont, UK), 30 µL of 50% formamide and 13 µL of water). The tube was incubated for 3 min at 96ºC and then chilled on ice. The CATMA v2.4 microarrays (ArrayExpress accession no. A-MEXP-274 at http://www.ebi.ac.uk/aerep), spotted on Type 7* mirror slides (Amersham, Little Chalfont, UK) were used in this study. The microarrays slides were cross-linked at 50 mJ by using UV Stratalinker^{TM} 2400 (Stratagene Amsterdam, Netherlands) for immobilizing the probes on slides. The slides were incubated in 2× SSPE + 0.2% SDS for 30 min at RT. After incubation, the slides were washed by dipping in the distilled water 3-4 times and dried by using an air stream. The pre-chilled mixture was placed onto the microarray slides and a cover slip was placed carefully to avoid the formation of air bubbles. The hybridization was performed by placing the slides in hybridization chamber (Scienion, Berlin, Germany) in a water bath at 42ºC for 15 h.

2.2.7.7 Washing and scanning of microarrays slides

All washing steps were done in pre-warmed hybridization chambers (Scienion, Berlin, Germany) filled with different concentrations of SSC and SDS at different temperatures. After 15 h incubation at 42ºC, the cover slip of the slide was removed carefully by dipping in 1× SSC + 0.2% SDS solution. Later a series of washing steps were performed to remove unspecific binding as described below:

1. Washing for 10 min, 55ºC in 1× SSC + 0.2% SDS
2. Washing for 10 min, 55ºC in 0.1× SSC + 0.2% SDS
3. Washing for 10 min, 55ºC in 0.1× SSC + 0.2% SDS
4. Washing for 1 min, 37ºC in 0.1× SSC

After that, the slides were dipped into distilled water for five times at RT to get rid of the wash buffers. The slides were air dried by using an air stream and transferred to a light proof container until scanning. The microarray slides were scanned with a Fuji FLA-5000 scanner at 5 µm resolution and Photomultiplier tube (PMT) settings of 93 and 83 for Cy5 and Cy3 (red and green channels), respectively. The images were saved in TIFF format.

2.2.7.8 Analysis of raw data

The scanned images of the arrays were used to measure the signal intensities of the probes by using AIDA array metrix (http://www.raytest.com/index2.html) software. The raw data were submitted to the ArrayExpress database at the European Bioinformatics Institute (EBI) (www.ebi.ac.uk/aerep) and the data were normalized with a preprocessing pipeline (Allemeersch, 2006) written in R/Bioconductor (Gentleman *et al.*, 2004; Team, 2005). After normalization, general linear models were fitted for each gene (Kerr *et al.*, 2000; Wolfinger *et al.*, 2001). The resulting data were used to generate the interaction plots as well as a table of all expression values, ratios and p-values. FDR-corrected p-values (q-values) were also included. This table was exported into Microsoft Access, the filtering functions of which were used to identify significantly regulated genes and intersections between groups of genes. A gene was called significantly regulated if it had a q-value of ≤ 0.05, a fold change of ≥ 2.5, and at least half of the spots for the gene were above background.

2.2.8 Biological assay methods

2.2.8.1 Root growth assay

The *Arabidopsis* seeds were surface sterilized with hypochlorite solution as described (2.2.1.1). The seeds were placed on MS medium plates (see 2.1.1.1) containing different concentrations of BA ranging from 0.01 µM to 1.0 µM. 0.1% of dimethyl sulfoxide (DMSO) was included as a vehicle control. For equal germination of different genotypes, the stratification treatment was done by placing the plates in the dark for 3 days at 4ºC. After the treatment the plates were placed vertically in the growth chambers under standard conditions. The primary root lengths was determined 10 DAG, whereas the root elongation was measured between days four and nine after germination. Photographs were taken with a digital camera (Nikon Coolpix 8800) and root lengths were determined using Scion Image program version beta 4.0.2 (www.scioncorp.com). The number of lateral roots emerge from pericycle that had

grown through the epidermis of the primary root was counted under a microscope 10 DAG. For statistical analysis experiments were performed using three independent replicates and at least 15 seedlings in each replicate.

2.2.8.2 Chlorophyll retention assay

This assay was performed as described previously (Riefler *et al.*, 2006)**.** Seeds were surface sterilized and placed on MS-agar (see 2.1.1.1) plates. Then seedlings were grown at 22ºC under long day conditions (16 h light/8 h dark) at white light (~75-100 µE) for 24 days. Either the sixth or the seventh leaf was detached from 24-days-old *in vitro* grown seedlings and the fresh weight was measured. After taking the fresh weight one set of samples were frozen in liquid nitrogen, stored at -80ºC and used as zero time point control. The detached sixth or seventh leaf was floated on distilled water supplemented with 0; 0.01; 0.1; 0.5; or 1.0 µM BA dissolved in 0.1% DMSO in six well plates for 10 d in the dark at RT**.** After 10 days the chlorophyll was extracted by incubating the leaves for 24 h in the dark with methanol. The amount of chlorophyll was measured with a spectrophotometer, normalized to fresh weight, and the chlorophyll content was calculated as described (Porra *et al.*, 1989). The chlorophyll content of the fresh leaves was taken as a reference at 100%. For statistical analysis the experiment was performed in three replicates and each replicate consisted of leaves of 5 different plants of the same genotype.

2.2.8.3 Ethylene response assay

The affect of ethylene on dark hypocotyl elongation and root elongation was tested as described in the *Arabidopsis* Gauntlet project (http://thale.biol.wwu.edu/index.html). Seeds were surface-sterilized and placed on vertical plates containing sugar-free MS medium (2.1.1.1). Different concentrations of 1-aminocyclopropane-1-carboxylic acid (ACC) (0.1, 1.0, 10 or 30 µM) were included in the medium. The seeds were grown in the dark at room temperature. After four days the hypocotyl lengths were measured using the Scion Image program, version Beta 4.0.2 (www.scioncorp.com). The experiment was done in two replicates, each with more than 15 seedlings. The root elongation assay was performed as described by Beaudoin *et al.*, (Beaudoin *et al.*, 2000) using only one ACC concentration (10 µM). The root elongation was measured four days after germination. The experiment was done with 15 seedlings per genotype in two replicates.

2.2.8.4 Determination of seed size and weight

Determination of seed size and seed weight was carried out as described by Riefler *et al.,* and Werner *et al.,* respectively (Riefler *et al.,* 2006; Werner *et al.,* 2003). Seed size of wild type and *ARR1-SRDX* overexpressors was determined measuring the length and width of 60 seeds harvested from two different plants. The seeds were photographed by using a digital camera (Nikon Coolpix 8800) and the volume was estimated by calculating with the formula for a spheroid (volume = 4/3 π· length · width · depth). Biomass of the seed was weighed by using a fine balance (LE244S; Sartorius, Göttingen, Germany). The weight of one seed was calculated from the weight of pools of 200 seeds. The sample size for each genotype was ten.

2.2.8.5 Seed germination assay

Seeds of wild type and the *35S:ARR1-SRDX* plants used for germination experiments had been harvested and stored under the same conditions. Seed batches of approximately 100 seeds were sown in Petri dishes on four layers of filter paper and moistened with 5 mL of distilled water. Seeds were vernalized for three days at 4ºC in the dark followed by six hours at 35ºC to alleviate the seeds of hypersensitivity to red light (Fankhauser and Casal, 2004) and 30 min at 25ºC immediately prior to the irradiation. For the different light treatments seeds were exposed 5 min to either white light (30 µE), red light (660 nm, 6 µE), or far-red light (724 nm, 15 µE), or a sequential treatment of 5 min red light followed by 5 min far-red light. Seeds were stored at room temperature in a dark place for 7 days. Radical protrusion was employed as the criterion for germination.

2.2.8.6 Hormone assays of root growth

The affect of different hormones on the root growth of *35S:ARR1-SRDX* plants was tested using the conditions described in the *Arabidopsis* Gauntlet project (http://thale.biol.wwu.edu/index.html). The seeds were surface-sterilized and placed on ½ MS plates (2.15 g/L MS-salt mixture, 0.5 g/L MES, 0.5% sucrose; of pH 5.7 with 1 N KOH) containing different hormones tested. All hormones were added after autoclaving and when the media were at <55ºC. Different concentration of the methyl jasmonate (0.1 µM to 1.0 µM MJ), brassinolide (0.4 µM to 2.0 µM) and absicic acid (20 µM to 80 µM) were added. The seedlings were grown vertically under *in vitro* standard conditions for 9 days. Photographs

were taken with a digital camera (Nikon Coolpix 8800) and root lengths were determined using Scion Image program version beta 4.0.2 (www.scioncorp.com). The experiments were done with 15 seedlings per genotype in two replicates.

2.2.8.7 Salt stress assay

The salt stress assay was performed as described previously in the literature (Werner and Finkelstein, 1995; Wu *et al.*, 1996). Seeds of different genotypes were surface-sterilized and placed on ½ MS plates (2.15 g/L MS-salt mixture, 0.5 g/L MES, 0.5% Sucrose; of pH 5.7 with 1 N KOH). Different concentration of NaCl (50 nM to 200 nM) was added to the media after autoclaving and cooling to 55ºC. The rate of germination was analyzed 7 days after placing the plates in growth chambers. The presence of the radical was considered as criterion for germination. For hypocotyl length measurement of dark grown seedlings, seeds were placed in the dark at room temperature and after 4 days the lengths were measured. The length of the primary root was measured 9 days after germination. Photographs were taken with a digital camera (Nikon Coolpix 8800) and lengths of hypocotyls and roots were determined using the Scion Image program version beta 4.02 (www.scioncorp.com). The experiments were done with 15 seedlings per genotype in two replicates.

2.2.8.8 Protoplast transactivation assay

The protoplast transactivation assay was performed as described in Ehlert *et al.*, (Ehlert *et al.*, 2006). Protoplast isolation and transformation was performed according to the method described by Hwang and Sheen (Hwang and Sheen, 2001; see 2.2.3.4). For cytokinin treatment, protoplasts were incubated overnight with 500 nM *trans*-zeatin. For promoter deletion analysis, the reporter plasmid was generated by amplifying the 1 kb fragment directly upstream of the potential transcription start site of the *ARR6* (AT5G62920) gene (for used primers see Appendix Table 17). The resulting PCR product was digested with *Hind*III and *Xba*I and ligated into the pBT10-GUS vector (Sprenger-Haussels and Weisshaar, 2000). Different fragments of the *ARR6* promoter (350 bp, 300 bp, 279 bp, 241 bp, 220 bp, 193 bp, 173 bp and 145 bp fragments) were amplified using plasmid DNA of *ARR6 (1kb)*:*GUS* with appropriate primers (see appendix Table 17). Point mutations were introduced in different sites of the promoter by using site directed mutagenesis kit (Stratagene, Amsterdam, Netherlands) (see 2.2.6.5). For the transactivation assays, 9 μg of the *ARR6*:*GUS* reporter

plasmid and 14 μg of effector plasmid either *35S:ARR1*, *35S:ARR1-SRDX* or other B-type *ARRs* were used. For normalization, 3 μg of a plasmid harboring the *35S:NAN* a synthetic neuraminidase gene (Kirby and Kavanagh, 2002) was added. Both GUS and NAN enzyme assays were performed according to Kirby and Kavanagh (2002). The ratios of GUS and NAN activities were calculated as relative GUS/NAN activity units.

2.2.9 Microscopy

For the Bimolecular Fluorescence Complementation (BiFC) assay, nuclear staining was done according to Tanious *et al.,* (Tanious *et al.*, 1992). For visualization of nuclei, DAPI (4′, 6-diamidino-2-phenylindole dihydrochloride) solution (100 µg/mL of DAPI, 30 mM KCl and 10 mM MES-KOH of pH 6.5) was prepared. Epidermal peels of transformed tobacco leaves were incubated in DAPI solution for 10 min. Fluorescence signals of the specimens were observed under a Zeiss Axioskop (Zeiss, Jena, Germany) equipped with an epifluorescence UV-filter set and differential interference contrast (DIC). Different filters were used for specimens stained with DAPI (365 nm excitation, 420 nm emission spectra), and for YFP (Yellow fluorescent Protein) samples (490-500 nm excitation, 530 nm emission spectra). Images were captured and processed using the Zeiss AxioVision software release 4.6 (Zeiss, Jena, Germany).

2.2.10 Bioinformatics tools and computer analysis

2.2.10.1 Statistical data evaluation

All statistical analysis was carried out using Excel (Microsoft Office 2003). Significance of differences between means of data sets was determined using the Pairwise Student's t-test. Differences between data sets were regarded as significant when the probability of error was below 5% ($P < 0.05$). Significance limits were indicated by asterisks: No symbol, $p > 0.05$; *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $p < 0.001$.

2.2.10.2 Phylogenetic analysis

Sequence comparison and phylogenetic analysis of DNA binding domains of B-type ARRs was done by using publicly available software. Full-length protein sequences of the response regulators were obtained from Entrez Protein Database (National Centre for Biotechnology Information [http://www.ncbi.nlm.nih.gov/) and their DNA binding domain

sequences were identified by searching the Protein family database (Pfam, http://pfam.sanger.ac.uk/) (Finn *et al.*, 2006). Binding domain sequences were aligned using the ClustalW program (Thompson *et al.*, 1994) available on the European bioinformatics institute (EBI) server (http://www.ebi.ac.uk/clustalw/index.html). The conserved amino acids were highlighted using the Boxshade 3.21 program (www.ch.embnet.org/software/BOX_form.html). A phylogenetic tree of the DNA-binding domains of the B-type response regulators was constructed using the Neighbor joining algorithm (Saitou and Nei, 1987). Phylogenetic and molecular evolutionary analyses were conducted using the MEGA 3.1 and MEGA 4 programs (Kumar *et al.*, 2004; Tamura *et al.*, 2007).

2.2.10.3 Cis-*element data base search*

For finding known motifs and unknown potential binding sites, different databases available on the internet were used. Different databases used are listed in Table 12. The sliding window analysis was performed on a selected list of gene promoters by using the Microsoft Access programme (http://office.microsoft.com/). Promoters of selected genes were aligned for sequence comparison by using ClustalW algorithm (Thompson *et al.*, 1994).

Table 12. *Cis*-element databases.

Database	Web link	Reference
Place	http://www.dna.affrc.go.jp/PLACE/	(Higo *et al.*, 1999)
AGRIS	http://Arabidopsis.med.ohio-state.edu/AtcisDB/	(Davuluri *et al.*, 2003)
Tocan2	http://homes.esat.kuleuven.be	(Aerts *et al.*, 2005)
ClustalW	http://www.ebi.ac.uk/clustalw/index.html	Thompson *et al.*, 1994

3.0 Results

3.1 Functional characterization of *35:ARR1-SRDX* plants

To investigate whether B-type response regulators are responsible for mediating the majority of cytokinin output traits, a dominant-negative variant of the B-type response regulator ARR1 was constructed by fusing the repressor motif SRDX to the C-terminus of ARR1. The resulting plants were studied for consequences on short-term and long-term responses to cytokinin at the morphological and the molecular level. The dominant negative plants showed a pleiotropic phenotype, including stunted shoot growth, increased root system and early flowering. In addition, an increased resistance to cytokinin in several bioassays, such as primary root elongation, lateral roots formation and detached leaf senescence was detected. Molecular analysis indicated attenuation of early transcriptional response to cytokinin. The role of B-type response regulators in mediating cross talk with other signalling pathways such as the PhyB-mediated inhibition of germination by far-red light, the ethylene signalling pathway and osmotic stress were presented.

3.1.1 The DNA-binding domains of B-type ARRs are highly conserved

A central part of this project is the finding of target genes of ARR1 and other B-type ARRs. For this purpose CRES-T (Chimeric repressor silencing technology) was used as a tool. The CRES-T aims to achieve the dominant repression of target gene expression of single or several related transcription factors (see 1.4.1). To effectively repress a whole transcription factor family by CRES-T, the DNA-binding domains and also probably the promoter target sequences of those transcription factors should be similar. Therefore, we first analysed the similarity of the DNA-binding domains of B-type ARRs. The respective domains were identified using the Pfam program (Finn *et al.*, 2006) and subsequently the identified sequences were aligned using the ClustalW algorithm (Thompson *et al.*, 1994) (Figure 5A). The DNA-binding domain of ARR1 shares the highest similarity with ARR2 and the lowest with ARR19 (96% and 47% identity, respectively). The high degree of conservation of amino acids is important for DNA recognition (Figure 5A) and the fact that several B-type ARRs have been shown to bind to the same or very similar sequence motifs (Riechmann *et al.*, 2000; Hosoda *et al.*, 2002), is consistent with a redundant function of B-type ARRs. In addition, nine amino acids, which were identified in ARR10 to be most likely

in direct contact with the DNA (Hosoda *et al*., 2002) are particularly well conserved (Figure 5A).

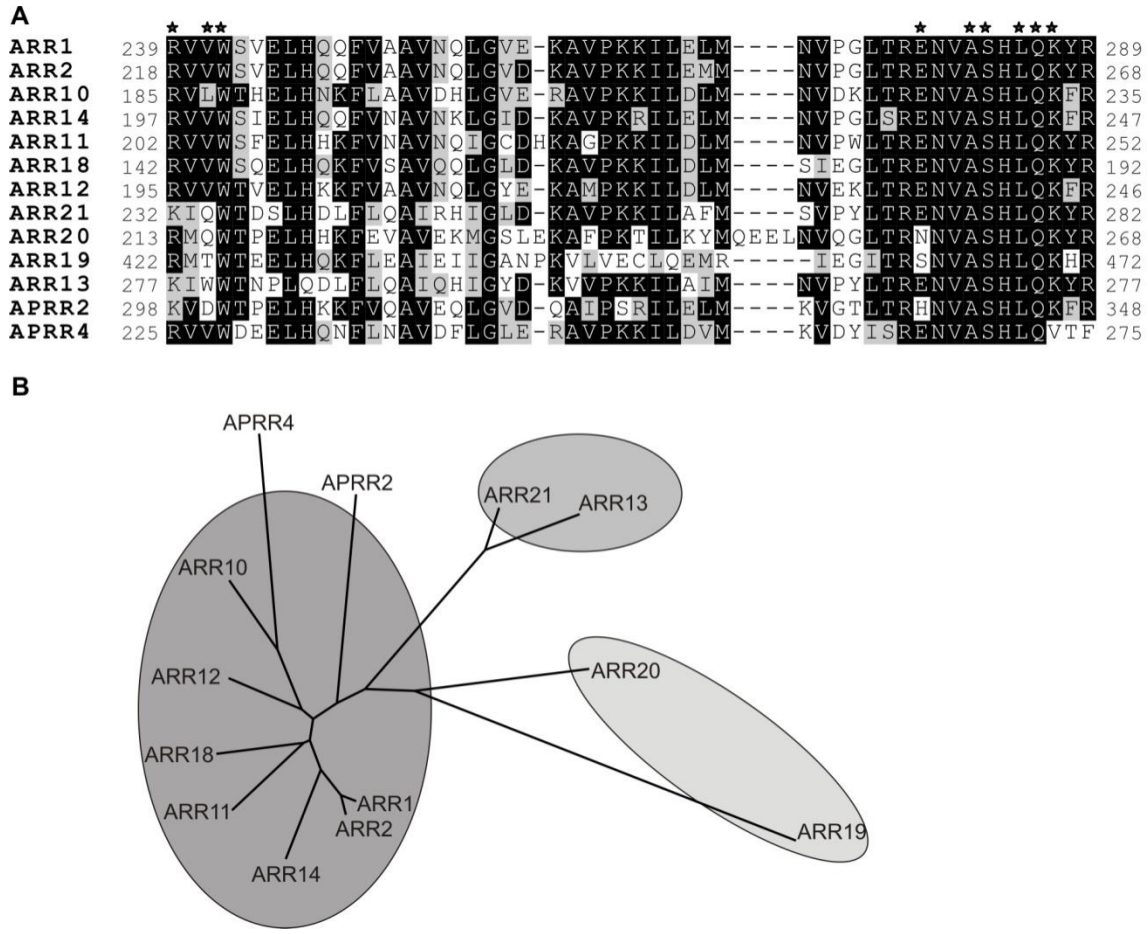

Figure 5: Sequence comparison and phylogenetic analysis of DNA-binding domains of B-type ARRs. A. Multiple sequence alignment of the DNA-binding domains (DBD) of B-type response regulators. Full-length protein sequences of the response regulators were obtained and their DNA-binding domain sequences were identified. The DBDs were aligned using the ClustalW program (Thompson *et al.*, 1994; Finn *et al.*, 2006). The conserved amino acids are highlighted. The stars indicate the amino acids which are in direct contact with the DNA (Hosada *et al.*, 2002). B. A phylogenetic tree of the DNA-binding domains of the B-type response regulators. The tree was constructed using the MEGA 3.0 program (Kumar *et al.*, 2004).

Phylogenetic analysis separates the B-type ARRs into three distinct subgroups, which is similar to the outcome of previous analyses using the complete protein sequences or response regulator domains for comparison rather than the DNA-binding domains (Figure 2; Figure 5B and Mason *et al.*, 2004; Tajima *et al.*, 2004). A large subgroup contains seven B-type ARRs. ARR19 and ARR20 as well as ARR21 and ARR13 form separate groups (Figure 5B). ARR13 was included in this alignment although Pfam did not detect a Myb domain in this protein using the default cut off of 1. Two of the *Arabidopsis* Pseudo-Response Regulators (APRRs) family members (APRR2 and APRR4) were also included in this study. These are

similar to B-type ARR proteins containing Myb DNA-binding region but lacking a conserved D-D-K motif in the receiver domain (Makino *et al.*, 2001).

3.1.2 A fusion of SRDX to the C-terminal of ARR1 suppresses its activity *in planta*

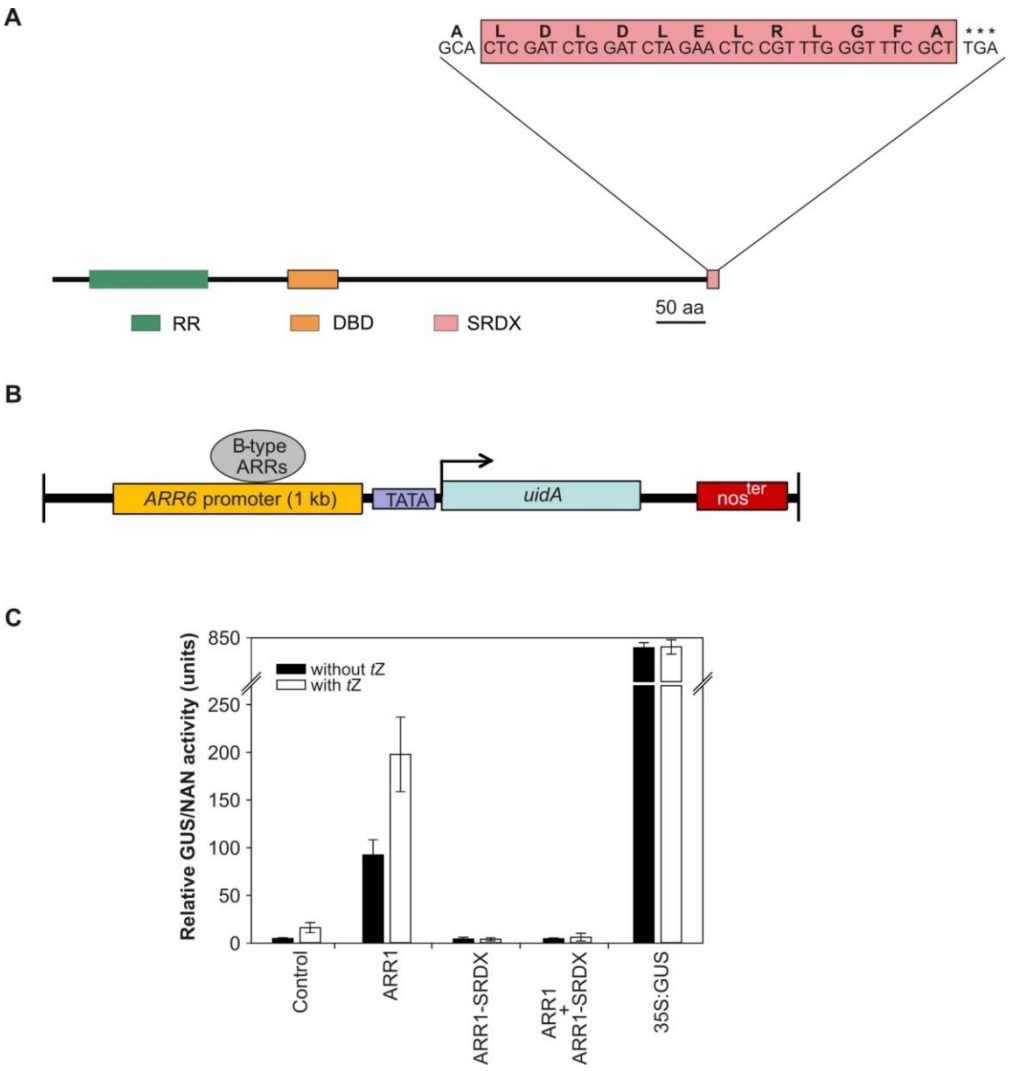

Figure 6: *35S:ARR1-SRDX* represses the transactivation capacity of ARR1 in a protoplast transactivation assay. A. Schematic representation of the ARR1-SRDX fusion protein. The figure shows a scheme of the ARR1-SRDX fusion protein with the junction between ARR1 and the SRDX peptide highlighted in the excerpt. (RR is response regulator domain; DBD is the DNA-binding domain, domains are drawn to scale). B. Schematic representation of the PTA system. 1 kb promoter region of *ARR6* gene was cloned into the pBT10:GUS vector and this reporter vector was transfected into *Arabidopsis* mesophyll protoplast along with effector plasmids **C.** *35S:ARR1-SRDX* represses the activation of a *ARR6:GUS* reporter gene by ARR1. The activation of the *ARR6:GUS* reporter gene was measured without and 16 h after the addition of 500 nM *trans*-zeatin. The *ARR6:GUS* reporter construct and a *35S:GUS* construct without any effector plasmid were used as controls. Variations in transformation efficiencies were normalized by using a *35S:NAN* reporter construct. The mean values and SD of four independent transfection assays were calculated and shown as relative GUS/NAN activity units. nos^{ter}, nopaline synthase terminator

To study the role of B-type ARRs in cytokinin signalling and other pathways, ARR1 was chosen as it is the best characterized B-type response regulator in *Arabidopsis* (Sakai *et al.,* 2000; Sakai *et al.,* 2001). A schematic representation of the construction of ARR1-SRDX is shown in Figure 6A. The construction of ARR1-SRDX was done as described in methods (see 2.2.6.4). The DNA fragment coding for the SRDX peptide (LDLDLELRLGFA, Hiratsu *et al.,* 2003) with a stop codon was ligated in frame to the 3' part of the *ARR1* gene (Figure 6A). The resulting *ARR1-SRDX* gene was shuttled into the vector pB2GW7 (Karimi *et al.,* 2002) for overexpression under the control of the 35S promoter. The ARR1-SRDX fusion construct was tested in a Protoplast Transactivation Assay (PTA) system (Figure 6B, Ehlert *et al.,* 2006), to analyze whether ARR1-SRDX can really suppress the activity of the *ARR1* transcription factor *in planta*. A schematic representation of the PTA system is shown (Figure 6B). The 1000 bp promoter fragment upstream of the potential transcriptional start of *ARR6,* a primary cytokinin response gene (Hwang and Sheen, 2001), was fused to the *GUS* reporter gene. Upon binding of the B-type ARRs to the promoter region of *ARR6,* the *GUS* gene is transcribed and the quantity of the GUS was measured by using enzymatic assays (Kirby and Kavanagh, 2002). The addition of cytokinin resulted in a more than threefold induction of the reporter gene expression compared to the non-induced condition (Figure 6C). Co-transfection with *ARR1* under the control of a 35S promoter led to a strong increase in GUS activity even in the absence of cytokinin. The addition of cytokinin caused a further, two-fold increase of the GUS activity, indicating that ARR1 mediates the cytokinin response in this assay. In contrast, the expression of ARR1-SRDX under the control of a 35S promoter effectively suppressed the cytokinin induction of GUS expression. Furthermore, ARR1-SRDX completely abolished the ARR1-mediated expression of the reporter gene in the absence and presence of cytokinin (Figure 6C). The expression of the *GUS* reporter gene under the control of a 35S promoter was not cytokinin inducible. The results indicate that the ARR1-SRDX fusion construct acts like a dominant repressor of the primary cytokinin response *in planta*.

3.1.3 ARR1-SRDX suppresses the activation capacity of other B-type ARRs in the PTA

The ARR1-SRDX fusion construct was tested further in the protoplast transactivation assay, to know whether ARR1-SRDX can also suppress the activity of other B-type ARRs. It has been shown previously that *ARR6* is a direct target gene of ARR2 in a similar protoplast

transactivation assay (Hwang and Sheen, 2001). So the same PTA system was used to test the activation of the *ARR6:GUS* reporter by other B-type ARRs and whether this activation could be suppressed by ARR1-SRDX. For these experiments five different B-type ARRs were chosen. ARR2 was chosen as this is the B-type ARR most closely related to ARR1 and *ARR6* was shown to be a direct target gene of ARR2 (Hwang and Sheen, 2001; Hass *et al.*, 2004). ARR10 and ARR12 were tested as both have been shown to be at least partly functionally redundant to ARR1 (Yokoyama *et al.*, 2007; Ishida *et al.*, 2008). Furthermore, ARR19 and ARR21 were included in the analysis as representatives of the two pairs of more distantly related B-type ARRs (Figure 5B). All of these B-type ARRs increased *ARR6:GUS* expression already without cytokinin in various degrees (Figure 7).

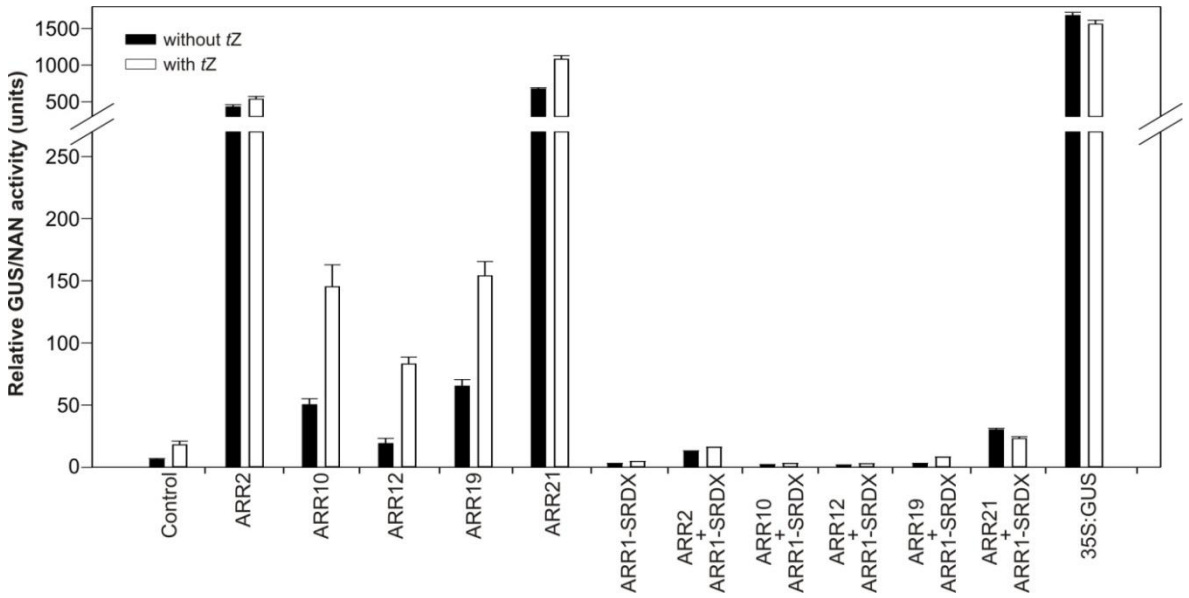

Figure 7: *35S:ARR1-SRDX* represses the activation of a *ARR6:GUS* reporter gene by several B-type ARRs. Protoplasts were co-transfected with the *ARR6:GUS* reporter and an effector plasmid expressing ARR1, ARR2, ARR10, ARR12, ARR19, ARR21, or ARR1-SRDX, respectively, or two effector plasmids expressing the respective B-type ARR and ARR1-SRDX. The activation of the *ARR6:GUS* reporter gene was measured without and 16 h after the addition of 500 nM *t*-zeatin. Variations in transformation efficiencies were normalized by using a *35S:NAN* reporter construct. The mean values and SD of four independent transfection assays were calculated and shown as relative GUS/NAN activity units.

For example ARR2 (428 ±30) and ARR21 (672 ±20) showed the strongest activation among the B-type ARRs tested. The addition of cytokinin increased the reporter gene activity further, however to a different extent. In case of ARR2 and ARR21 the activation was increased by 1.25- fold (536 ±37) and 1.6-fold (1080 ±50) respectively compared to without

cytokinin treatment (Figure 7). The addition of cytokinin increased the reporter gene activity in ARR10, ARR12 and ARR19 by 2.8 (145 ±18), 4.0 (83 ±6) and 2.5 (154 ±12) -folds respectively. The co-expression of ARR1-SRDX repressed effectively reporter gene activation by all of the B-type ARRs in the absence and presence of cytokinin completely (Figure 7). When ARR21 was co-expressed along with ARR1-SRDX, the activation was reduced to 4% (30 ±1.2) and 2% (23 ±1.5) with and without cytokinin treatment respectively compared to ARR21 alone (Figure 7). These results clearly demonstrate the potential of ARR1-SRDX as a dominant repressor of the primary transcriptional response to cytokinin *in planta.*

3.1.4 The ARR1-SRDX construct was transformed into *Arabidopsis*

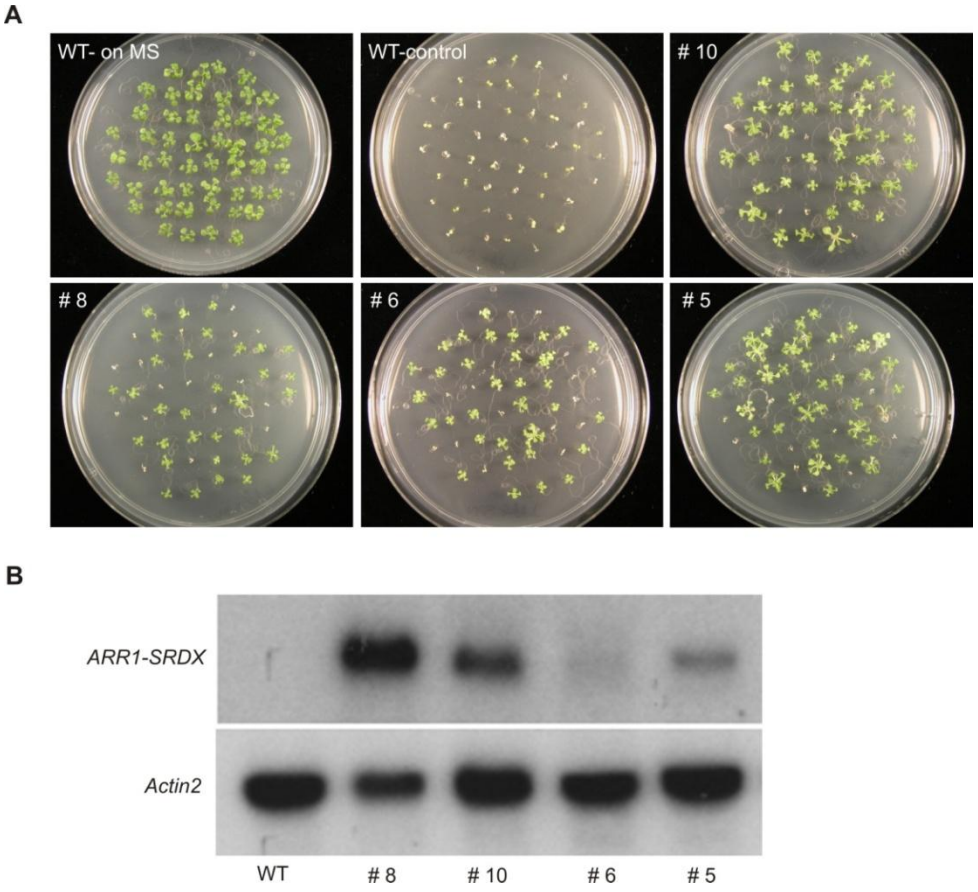

Figure 8: The *35S:ARR1-SRDX* gene was transformed into *Arabidopsis* plants. A. Genetic segregation analysis for number of insertion loci in transgenic plants. Seeds of transformed *Arabidopsis* plants (T_1) were analysed by selecting them on MS-PPT (selection media) plates. #10, 8, 5, 6 were different lines of *35S:ARR1-SRDX* transgenic plants. B. Analysis for transcript levels of the *35S:ARR1-SRDX* gene in different transgenic lines. Total RNA was isolated from three-weeks-old plants and semi-quantitative RT-PCR was performed to analyze the *ARR1-SRDX* transcript level. *Actin2* was used as loading control in the experiment.

After demonstrating the dominant repressor activity of the ARR1-SRDX construct *in planta* in the protoplast transactivation assay (see 3.1.3), the *35S:ARR1-SRDX* gene was transformed into *Arabidopsis* wild-type Col-0 plants. Since B-type ARR expression was detected virtually in all tissues investigated (Figure 3A; Heyl *et al.*, 2006) and because the present study aimed to study the effects of the repression of as many B-type ARRs as possible (not just ARR1 in the ARR1 expression domain), the 35S promoter was purposefully chosen to drive the gene expression. More than ten independent transgenic lines were selected after spraying the herbicide BASTA^{TM} which contains phosphinothricin, for selection of the transgenic plants. In the next generation the seeds of the transgenic plants were selected on MS-Phosphinothricin (MS-PPT) plates to analyze the segregation of the T-DNA encoded resistance gene (Figure 8A). The segregation analysis shown that out of four independent lines that were studied (line #10, 8, 6, and 5) the line #10 contains two independent insertions, whereas other three lines contain single insertions of the transgene *ARR1-SRDX* (Figure 8A). The selected four independent lines displayed a varying degree of a similar phenotype, which was distinct from the wild-type (data not shown). To know whether the different degrees of phenotype of the transgenic plants was due to different levels of expression of the transgene, the transgenic plants were further analysed for the transcript levels of *ARR1-SRDX* in the T_2 generation (Figure 8B). The signal strength of line #8 was highest among the tested lines and correlates with the strongest morphological phenotype shown by this line. Line #10 displayed an intermediate phenotype in comparison to the line #8 which correlated with a lower transcript level compared to the line #8 (Figure 8B). Two other lines, line #6 and line #5, showed the lowest transcript levels, which correlated with their weak morphological phenotypes. So the line #8 and the line #10 were chosen for further study and named ARR1-S-8 and ARR1-S-10 respectively. Later, six other independent homozygous lines which were similar in phenotype to ARR1-S-8 and ARR1-S-10 were identified (data not shown).

3.1.5 Transgenic *Arabidopsis* plants homozygous for a dominant ARR1-SRDX repressor

Two independent lines, ARR1-S-8 and ARR1-S-10, which showed a similar phenotype with different expressivity, were characterized in more detail. Experiments were carried out with homozygote and phenotypically uniform progeny (Figure 9A and B).

Northern blot analysis using a *SRDX*-specific probe showed the expression of the *ARR1-SRDX* transcript in both lines (Figure 9C). The steady state transcript levels were higher in ARR1-S-8 compared to ARR1-S-10 (Figure 9C). In order to check whether the transcript level of the *ARR1* gene itself was altered in the transgenic lines, its level was compared to wild-type using a 3'-part of the *ARR1* as a probe that specifically recognizes the wild-type *ARR1*, but not *ARR1-SRDX* transcripts. This analysis revealed an increased *ARR1* transcript level in both *35S:ARR1-SRDX* transgenic lines, indicating a feedback regulation of the *ARR1* gene (Figure 9D).

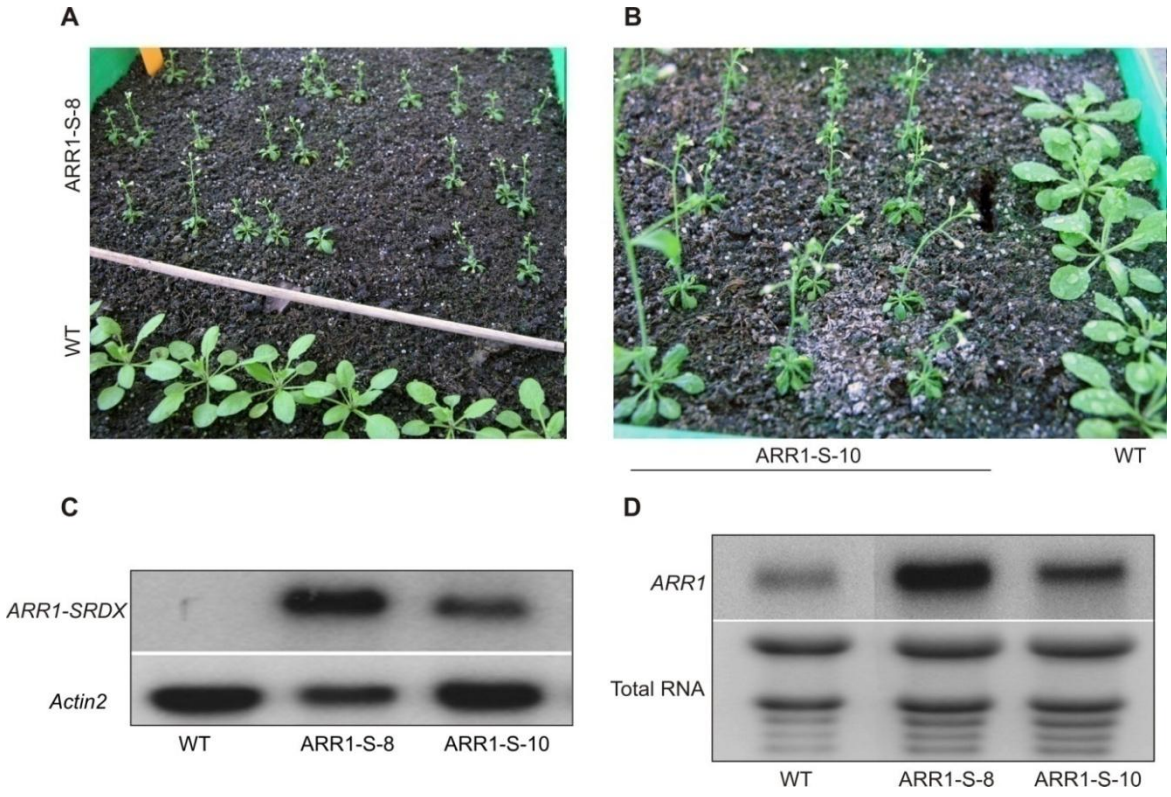

Figure 9: Phenotype of two independent homozygous *35S:ARR1-SRDX* lines. A&B. The two independent *35S:ARR1-SRDX* transgenic lines ARR1-S-8 and ARR1-S-10 are shown at 20 DAG and compared to wild-type plants (WT) of the same age. C. Expression of the *35S:ARR1-SRDX* gene fusion. Total RNA samples were isolated from three-weeks-old plants and the transcript was detected by Northern blot hybridization using a probe specific for the *ARR1-SRDX* gene fusion. The *Actin2* transcript was used as loading control in the experiments. D. Analysis of *ARR1* transcript levels. Total RNA was isolated from five-days-old wild type and *35S:ARR1-SRDX* transgenic seedlings. Northern blots were hybridized with a probe specific for that 3' part of the *ARR1*, which had been deleted during the construction of the *35S:ARR1-SRDX* gene and thus hybridizes only with the native *ARR1* and not with the *ARR1-SRDX* fusion transcript. Total RNA was used as loading control. WT, Wild-type Col-0; ARR1-S-8 and ARR1-S-10 are independent lines of *35S:ARR1-SRDX* transgenic plants

This increase in transcript level was specific for *ARR1* and did not affect four other B-type *ARR* genes, which were detectable in microarray experiments (see section 3.2 for the results of microarray) and showed similar transcript levels in the transgenic lines and the wild type.

3.1.6 The *35S:ARR1-SRDX* transgenic plants display a pleiotropic shoot phenotype

Transgenic plants expressing the *35S:ARR1-SRDX* construct displayed a strong pleiotropic shoot phenotype, with a higher expressivity in line ARR1-S-8 than in line ARR1-S-10 (Figure 10A). Plants were generally smaller and after flowering numerous individuals showed a varying degree of enhanced branching of the shoot. The leaves of the dominant repressors were strongly reduced in both size and number when compared to the wild-type plants.

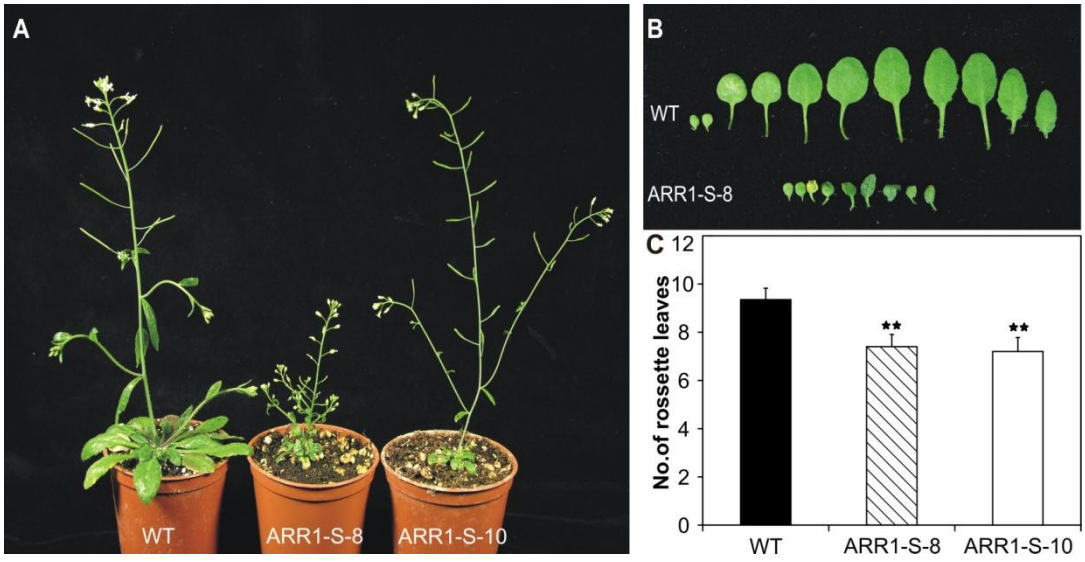

Figure 10: Shoot phenotype of *35S:ARR1-SRDX* transgenic plants. A. Shoot phenotype of transgenic *35S:ARR1-SRDX* plants, 35 DAG (days after germination) compared to wild-type. B. Leaf phenotype of line ARR1-S-8 and of a wild-type plant 20 DAG. The leftmost leaves are the cotyledons followed by the true leaves in order of their formation. C. *35S:ARR1-SRDX* transgenic plants have formed fewer leaves than wild-type at the onset of flowering. The total number of rosette leaves was counted 19 DAG. Error bars represent SD ($n \geq 20$); Pairwise Student´s t-test was used to compare values to the wild-type. **, $p < 0.01$. ARR1-S-8 and ARR1-S-10 are two independent lines of *35S:ARR1-SRDX* transgenic plants.

In the stronger expressing line ARR1-S-8 the true leaves were only about the size of the cotyledons of the wild type (Figure 10B). 20 DAG (Days after germination) the wild-type plants had on average nine rosette leaves, while the *ARR1-SRDX* expressing plants had only seven leaves at that time point (Figure 10C).

3.1.7 The *35S:ARR1-SRDX* plants develop an enlarged root system

In contrast to the reduced shoot size, *35S:ARR1-SRDX* transgenic plants showed a generally enhanced root system when compared to the wild-type (Figure 11A). While the ARR1-S-8 plants had 10 DAG only a slightly longer primary root than the wild-type, the primary root of line ARR1-S-10 was more than 30% longer (34.9 ±3.1 mm in wild-type compared to 51.9 ±6.6 mm in line ARR1-S-10; Figure 11B). The difference between the transgenic lines and the wild-type was also visually clear in the number of lateral roots. 10 DAG plants of line ARR1-S-8 had developed about twice as many lateral roots as the wild-type and line ARR1-S-10 had about three times more (13.2 ±1.9 lateral roots compared to 4.8 ±1.1 lateral roots in the wild-type; (Figure 11C).

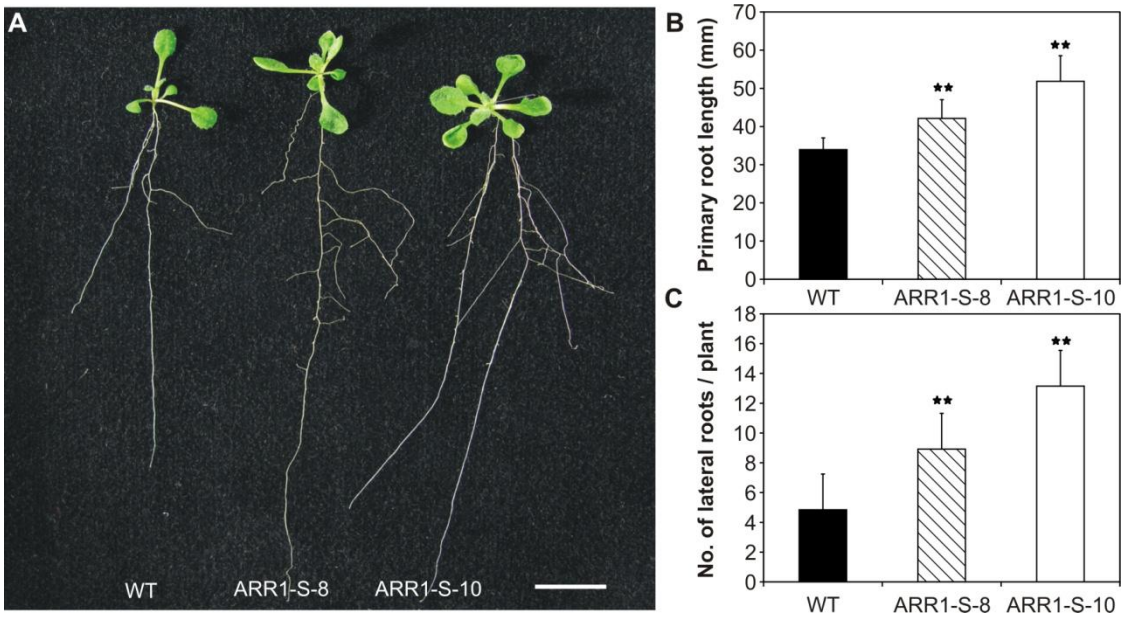

Figure 11: Root phenotype of *35S:ARR1-SRDX* transgenic plants. A. Transgenic plants show longer and more branched roots than wild type plants. Seedlings were grown vertically on MS media plates. The pictures were taken 10 DAG. Bar size is 1 cm. B. *35S:ARR1-SRDX* transgenic plants produce longer primary roots than wild type. The length of the primary root was determined 10 DAG. C. *35S:ARR1-SRDX* transgenic plants produce more lateral roots compared to wild type. The number of emerged lateral roots was determined 10 DAG. Results shown in B and C represent means from at least three independent replicates for each line. Error bars represent SD (n ≥15); Pairwise Student´s t-test was used to compare values to the wild-type; **, $p < 0.01$. ARR1-S-8 and ARR1-S-10 are two independent lines of *35S:ARR1-SRDX* transgenic plants.

3.1.8 The reproductive development of *35S:ARR1-SRDX* plants is altered

The *35S:ARR1-SRDX* transgenic plants flowered earlier than wild-type (Figure 12A). In the wild type the first flower buds opened 19 DAG. In contrast, both transgenic lines flowered at around 14 DAG (Figure 12B).

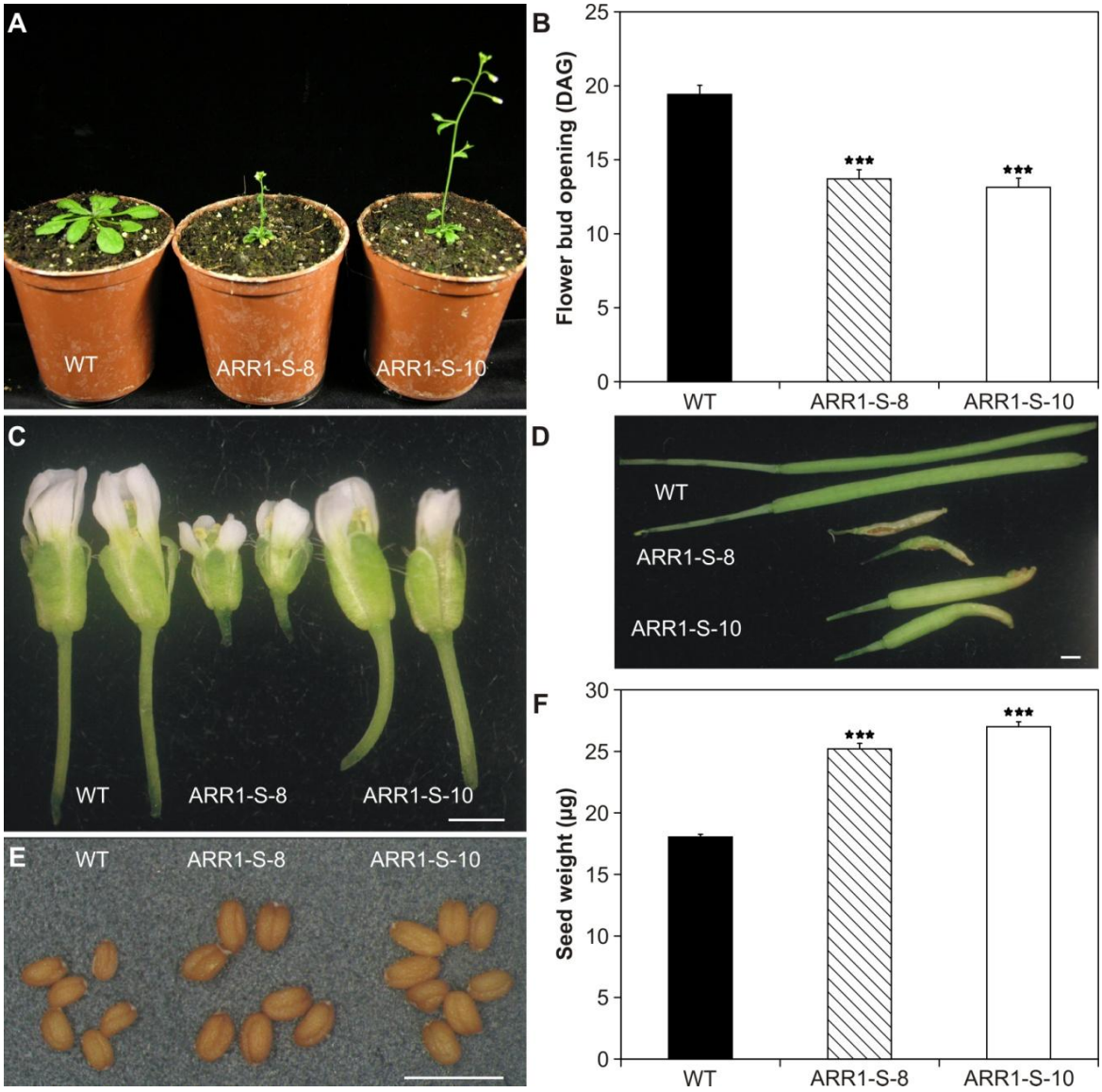

Figure 12: Reproductive development of *35S:ARR1-SRDX* transgenic plants. A. Flower induction occurs earlier in *35S:ARR1-SRDX* transgenic plants compared to wild type plants. The plants were grown under long day conditions and photographed 19 DAG. B. Quantitative analysis of the early flowering phenotype of *35S:ARR1-SRDX* transgenic plants. The graph shows the time point (DAG) of flower bud opening in transgenic plants compared to wild type. C. Flower morphology of *35S:ARR1-SRDX* transgenic plants compared to wild type. D. Siliques of *35S:ARR1-SRDX* transgenic plants compared to wild type. E. Seeds of *35S:ARR1-SRDX* transgenic lines compared to wild type. F. The seeds of *35S:ARR1-SRDX* transgenic plants have an increased weight. Seed biomass was determined by weighing ten pools of 200 seeds for each line. Error bars represent SD; Scale bar size in C-E is 1.0 mm; Pairwise Student's t-test was used to compare values to the wild-type. ***, $p < 0.001$. ARR1-S-8 and ARR1-S-10 are two independent lines of *35S:ARR1-SRDX* transgenic plants.

In the *35S:ARR1-SRDX* transgenic plants all reproductive organs were reduced in size. This was more pronounced in line ARR1-S-8, where the flowers were only half the size of the wild type (Figure 12C). The smaller flowers of the *35S:ARR1-SRDX* plants gave rise to smaller siliques. The length of the siliques of line ARR1-S-8 was about 30% of the length of

the wild-type. The shape also differed from the wild-type as it was twisted and crooked (Figure 12D). The phenotype of line ARR1-S-10 was weaker as the reduction of the silique size was not as dramatic as in line ARR1-S-8 and the shape resembled more like the WT (Figure 12D). While the number of seeds obtained by selfing was considerably lower in the *35S:ARR1-SRDX* plants (data not shown), the seeds themselves showed increased size (Figure 12E). Their biomass was 40% and 50% higher in the lines ARR1-S-8 and ARR1-S-10, respectively (Figure 12F).

3.1.9 The cytokinin response of *35S:ARR1-SRDX* plants is altered in shoots and roots

As the phenotype of the *35S:ARR1-SRDX* plants is reminiscent of plants with reduced cytokinin signaling (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.*, 2006), several cytokinin sensitivity assays were carried out. Cytokinin is known to delay the onset of leaf senescence and to increase the chlorophyll retention in detached leaves incubated in the dark (Richmond and Lang, 1957; Riefler *et al.*, 2006). Detached wild-type leaves kept in the dark for 10 days lost more than 80% of their chlorophyll compared to fresh leaves (Figure 13A).The addition of increasing amounts of cytokinin reversed this effect. In wild-type leaves, 0.1 µM BA strongly increased chlorophyll retention and at 5 µM BA the chlorophyll level of dark-incubated detached leaves was similar to fresh leaves. In contrast, while in both *35S:ARR1-SRDX* lines the loss of chlorophyll under dark conditions was similar to wild type, the addition of cytokinin to the media caused only a minor increase in chlorophyll retention, even at the highest concentration (Figure 13A). This indicates that the cytokinin sensitivity in the leaves had been almost completely lost.

To investigate the cytokinin response in roots, seedlings of wild type and the *35S:ARR1-SRDX* lines were grown on media containing increasing amounts of cytokinin. On control media without cytokinin the transgenic seedlings developed a longer primary root compared to the wild type (Figure 13A). The relative difference in root length increased with increasing cytokinin concentrations (Figure 13B). Seedlings of line ARR1-S-8 displayed a higher resistance to cytokinin than line ARR1-S-10. A 50% inhibition of root elongation was achieved by an approximately fivefold higher cytokinin concentration. However, the sensitivity to cytokinin, while being clearly reduced, was not completely eliminated in either ARR1-SRDX lines (Figure 13B).

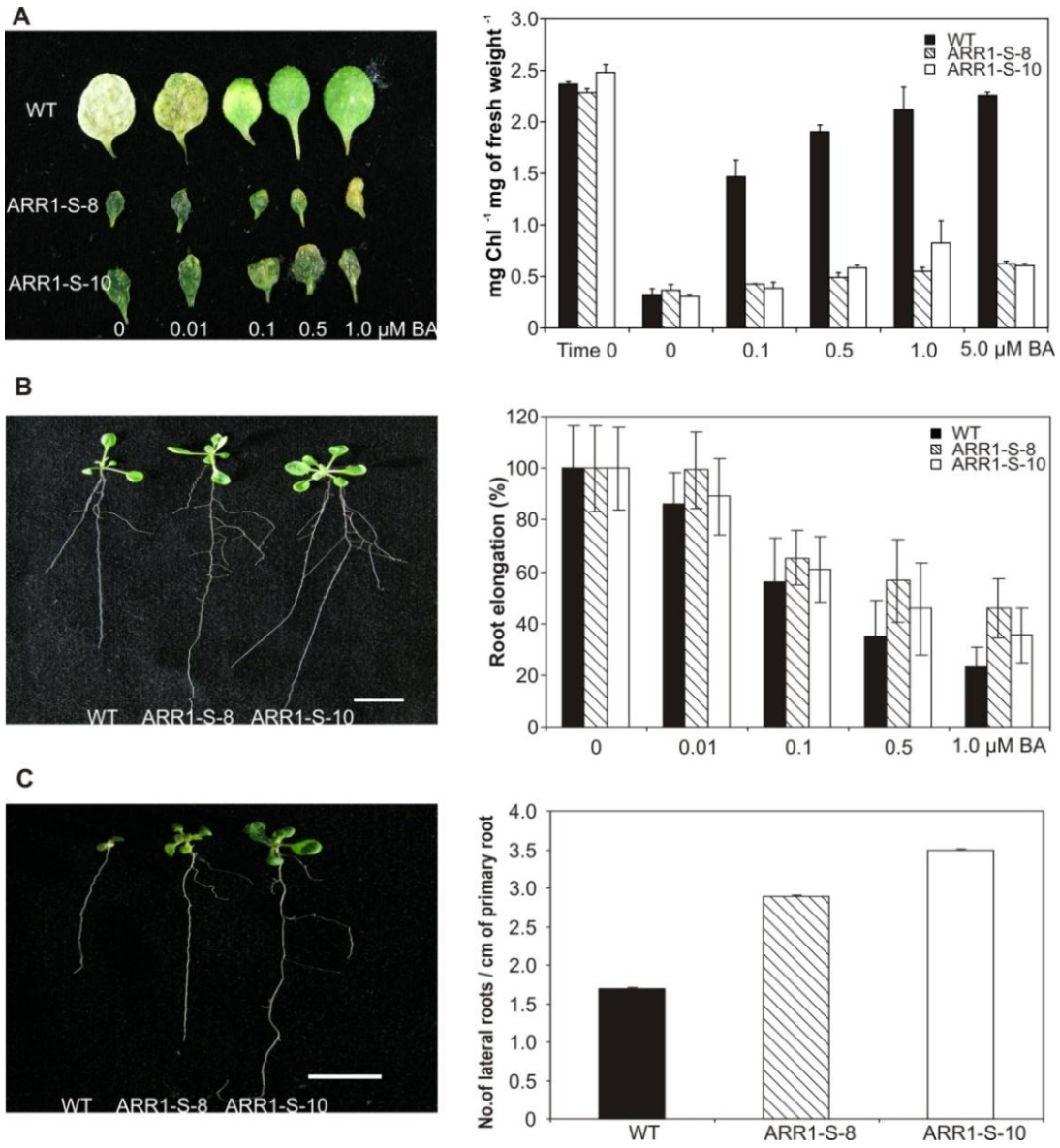

Figure 13: *35S:ARR1-SRDX* plants are less sensitive to cytokinin. A. Chlorophyll retention in dark-incubated leaves by cytokinin. Fully expanded leaves were excised from 24-days-old plants and floated for 10 d in the dark on water supplemented with various concentrations of cytokinin before the chlorophyll concentration was determined. The chlorophyll concentration at the beginning of the experiment was set to100%. B. Cytokinin sensitivity of primary root elongation. Root elongation was measured for each line between 4 and 9 DAG. The root elongation of each line is expressed as percentage of its DMSO control. The original data of the transgenic lines were significantly different from wild type with $0.001 < p < 0.01$ as calculated by pairwise Student´s t test. C. Cytokinin sensitivity of lateral root formation. The number of lateral roots was determined following growth on vertical plates 9 DAG. The number of lateral roots between wild type and the transgenic lines was significantly different with $p < 0.01$. Error bars represent SD ($n \geq 15$). ARR1-S-8 and ARR1-S-10 are two independent lines of *35S:ARR1-SRDX* transgenic plants.

Subsequently, to investigate whether the lateral root formation was altered in the presence of the hormone was tested. In all seedlings the addition of cytokinin to the media led to a dramatic decrease in the number of lateral roots (Figure 13C). But whereas the lateral

root formation of the wild type was almost totally repressed at 0.01 µM BA, lateral root formation of both *35S:ARR1-SRDX* lines was only halved at this cytokinin concentration. At 0.1 µM BA the wild type was unable to produce lateral roots, while the dominant repressor lines still showed a significant number (Figure 13C).

3.1.10 *35S:ARR1-SRDX* plants show a reduced induction of cytokinin response genes

The phenotypic changes and cytokinin bioassays described above report altered long-term responses to the hormone. To analyze whether rapid cytokinin responses were also altered in the *35S:ARR1-SRDX* transgenic lines, the expression of two known primary cytokinin response genes was tested. The transcript levels of *ARR5* and *ARR7*, which both encode A-type ARRs, are direct targets of the B-type ARRs and rapidly induced by cytokinin (D'Agostino *et al.*, 2000; Romanov *et al.*, 2002; To *et al.*, 2004). Both genes were rapidly and strongly induced in wild-type seedlings following the application of cytokinin. This induction was strongly diminished in both transgenic lines (Figure 14).

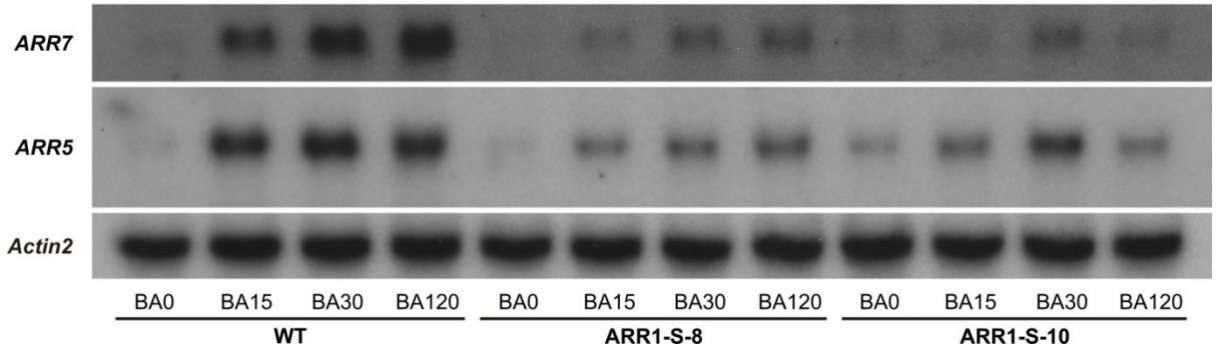

Figure 14: Northern blot analysis of *ARR5* and *ARR7* transcript levels. Total RNA was isolated from five-days-old seedlings before and after 15 min, 30 min and 120 min treatment with 5 µM BA. Northern blots were hybridized with a probe specific for the cytokinin response genes *ARR5* and *ARR7*. The *Actin2* gene was used as loading control.

3.1.11 *35S:ARR1-SRDX* plants are similar to other cytokinin-deficient plants

The phenotype of the *35S:ARR1-SRDX* transgenic plants resembled in various aspects the phenotype of cytokinin receptor triple mutants and cytokinin-deficient plants (Werner *et al.*, 2003; Yang *et al.*, 2003; Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.*, 2006). As in those mutants, the shoot growth and leaf size were strongly reduced in *35S:ARR1-SRDX* transgenic plants (Figure 15). The transgenic lines had also smaller flowers

and larger seeds like the triple receptor mutant (Riefler *et al.*, 2006). Furthermore, *35S:ARR1-SRDX* transgenic plants had longer primary roots, formed more lateral roots and had larger seeds which is similar to plants with a reduced cytokinin status (Figure 11; Figure 12).

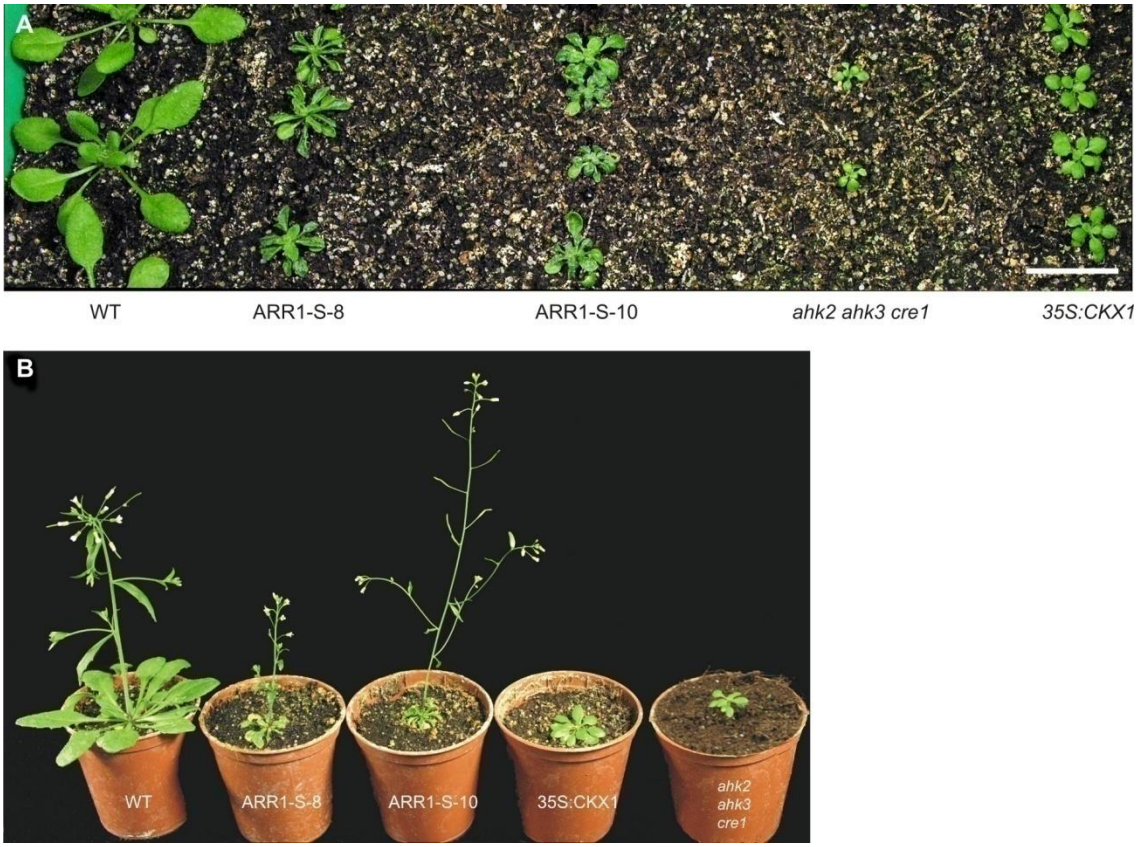

Figure 15: Comparison of phenotypes of *35S:ARR1-SRDX* plants with other cytokinin-related mutants. Wild type, the two *35S:ARR1-SRDX* lines, *35S:CKX1* and the *ahk2 ahk3 cre1* triple receptor mutant plants were grown on soil for A, 18 DAG and B, 28 DAG. Bar size in A is 3 cm.

An exception of the similarities between *35S:ARR1-SRDX* plants and plants with a reduced cytokinin status is the early flowering phenotype which is in contrasts with the retarded flowering reported for triple receptor mutants and cytokinin-deficient plants (Werner *et al.*, 2003; Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.*, 2006) (Figure 15).

3.1.12 Role of *35S:ARR1-SRDX* plants in other signaling pathways

3.1.12.1 The ethylene response of 35S:ARR1-SRDX *seedlings*

The role of cytokinin signaling components in other hormone signaling pathways has been studied. For example, an involvement of ARR2 in ethylene signaling has been discussed (Hass *et al.*, 2004; Mason *et al.*, 2005). To test the possible involvement of B-type

ARRs in mediating the ethylene response, the growth response of *35S:ARR1-SRDX* seedlings on medium containing the ethylene precursor ACC was compared to the wild-type seedlings. The hypocotyl length of the wild-type and *35S:ARR1-SRDX* transgenic seedlings decreased with the increasing concentrations of ACC in the medium (Figure 16A). In contrast to the wild type, in which the hypocotyl length decreased more with further increasing ACC concentrations, the *35S:ARR1-SRDX* seedlings showed resistance to ACC concentrations greater than 1 µM as their hypocotyl length did not decrease further (Figure 16A). However, root shortening of wild type and *35S:ARR1-SRDX* seedlings were similar at low and high ethylene concentrations (Figure 16B). Also the bending of the apical hook did not differ much between the genotypes (data not shown).

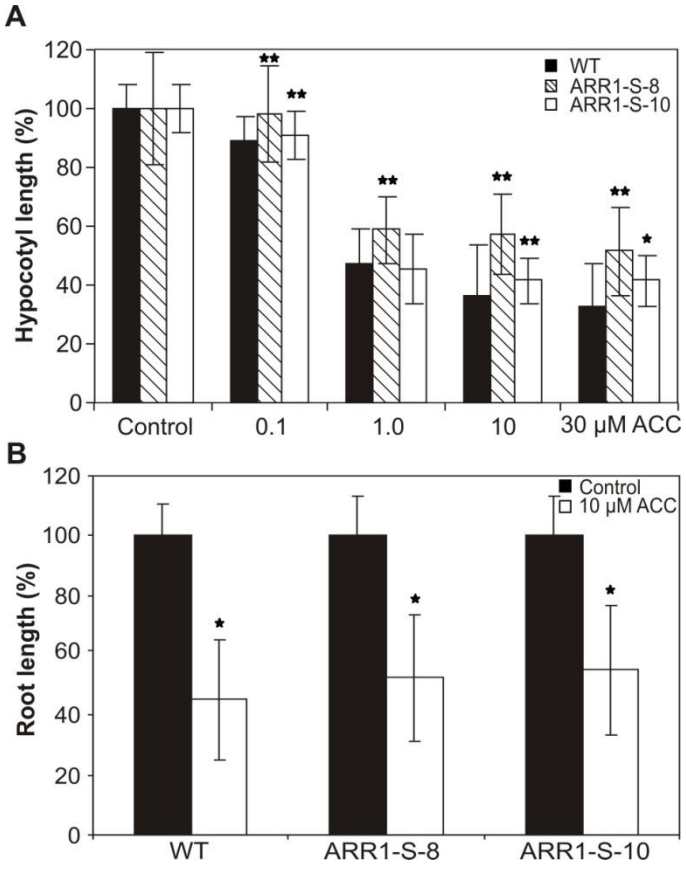

Figure 16: Hypocotyl and root elongation of *35S:ARR1-SRDX* transgenic seedlings in response to ethylene. A. Hypocotyl elongation of *35S:ARR1-SRDX* transgenic seedlings on ACC-containing medium. Seedlings were placed on vertical MS media plates containing 0.1, 1.0, 10 or 30 µM ACC. Hypocotyl lengths were measured after 4-days of dark incubation. B. Primary root elongation of *35S:ARR1-SRDX* transgenic plants on ACC containing medium. Seedlings were grown on medium containing 10 µM ACC for 4-days in the dark. More than 15 seedlings in two replicates were analysed for each genotype. Pairwise Student's t-test was used to compare values to the wild-type. **, $p < 0.01$; *, $p < 0.05$.

3.1.12.2 Germination of 35S:ARR1-SRDX *seeds is resistant to inhibition by far-red light*

Several experiments have indicated an involvement of the two-component signaling system in red light signaling (Sweere *et al.*, 2001; To *et al.*, 2004; Riefler *et al.*, 2006). We used a seed germination assay to explore the possible role of B-type ARRs in response to red light. Far-red light inhibits seed germination, a response that is, under our experimental condition, mediated by phytochrome B and is reversible by red light (Shinomura, 1997; Sullivan and Deng, 2003).

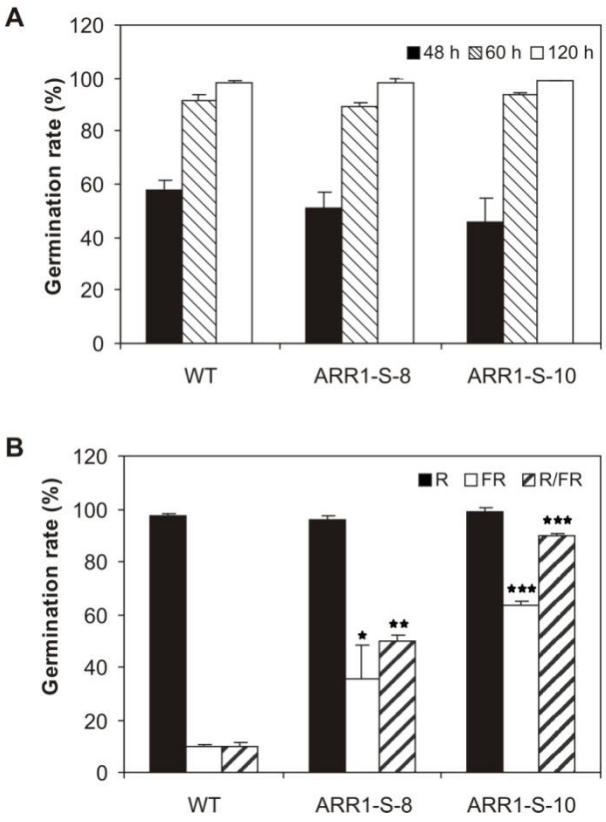

Figure 17: Germination behavior of *35S:ARR1-SRDX* seedlings under different light conditions. A. Germination rate after treatment by a white light pulse (5 min, 30 µE) and subsequent incubation in the dark. B. Germination rate after R, FR or R/FR treatment. Seedlings were treated for 5 min by the indicated light and kept subsequently in the dark. Germination frequency was determined from batches of approximately 100 seeds after 7-days of dark incubation (n=2). Pairwise Student´s *t* test was used to compare values to the wild-type. No symbol, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

The germination behavior of *35S:ARR1-SRDX* transgenic seeds and wild-type seeds in white light was similar. 48 h and 60 h after transfer from cold treatment to room temperature a similar percentage of the seeds of all genotypes had germinated and eventually reached about

100%, indicating that there was no marked difference in timing or capability of germination (Figure 17A). Similarly, about 100% of the seeds germinated after a white light pulse or a red light pulse of 5 min followed by a dark period of 7 days (Figure 17B). However, when wild type seeds were exposed for 5 min to a far-red light pulse prior to dark incubation only around 10% of the seeds were germinated (Figure 17B). In contrast, 35% and 60% of the seeds of lines ARR1-S-8 and ARR1-S-10, respectively, germinated following this treatment. A similar difference between wild type and the transgenic lines was seen when the far-red light treatment was preceded by a 5 min red light pulse (R/FR). Then the germination rate of the transgenic reached about 80%, while it remained around 10% for wild type seeds (Figure 17B).

3.1.12.3 The response of 35S:ARR1-SRDX *seedlings to salt stress*

The *Arabidopsis* Histidine Kinase 1 (AHK1) acts as an osmosensor in yeast *sln1* and *sho1* deletion mutants (Urao *et al.*, 1999).

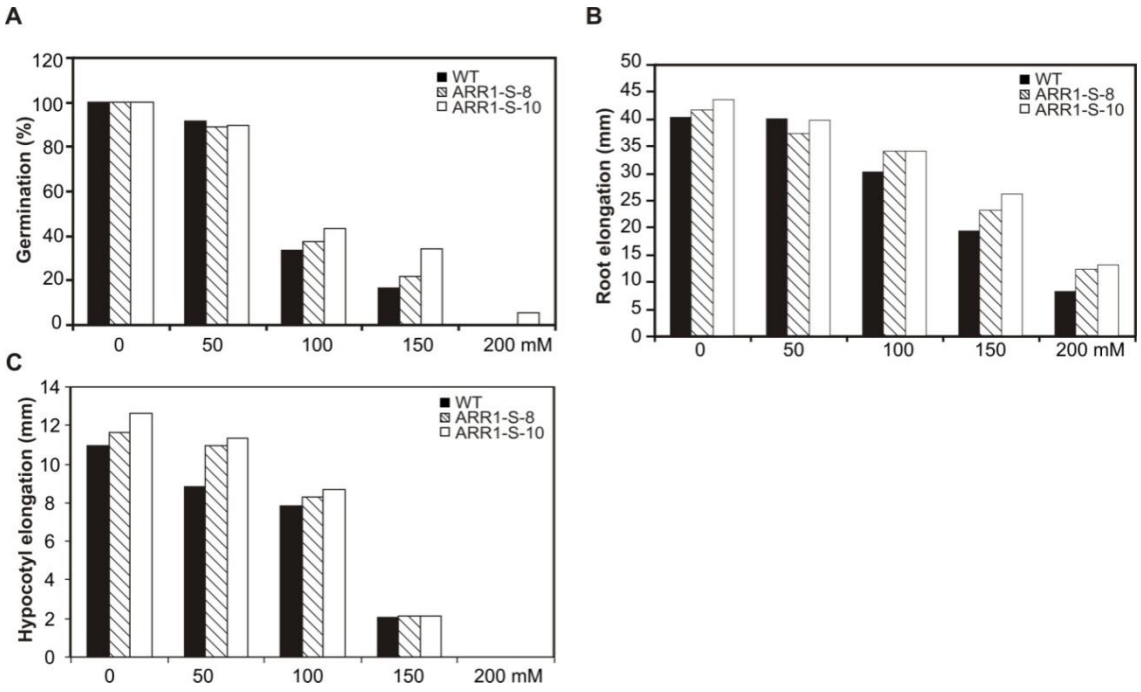

Figure 18: Analysis of the role of B-type ARRs in the osmotic stress response. A. Germination of *35S:ARR1-SRDX* plant seeds under salt stress. Seeds were surface-sterilized and germinated on MS-plates containing different concentration of NaCl for 7-days. The presence of the radicle was considered as criteria for germination. B. Root elongation of *35S:ARR1-SRDX* plants under different salt stress conditions. The length of the root was measured 9 DAG. C. Hypocotyl elongation of *35S:ARR1-SRDX* plants. The seedlings were grown four days in dark before the hypocotyl length was measured. All the experiments were done in three replicates with 15 seedlings in each replicate.

Later it has been shown that AHK1 can also act as positive regulator in osmotic signaling in *Arabidopsis* plants (Tran *et al.*, 2007). The *ahk2*, *ahk3* single, and *ahk2 ahk3* double mutants showed strong stress tolerance against both drought and high-salinity stresses (Tran *et al.*, 2007). So it was interesting to test whether B-type ARRs played any role in cytokinin mediated salt stress signalling. The germination rate of *35S:ARR1-SRDX* plants were not different from WT at lower concentrations of NaCl (Figure 18A). At 100 mM NaCl concentration the germination rate was reduced to 35-40% in all genotypes. At higher concentrations (150 mM) germination was reduced to 15%, 22% and 34% respectively in WT, ARR1-S-8 and ARR1-S-10 (Figure 18A). The slight resistant to salt stress was more evident in the case of line ARR1-S-10, as the germination rate was less reduced (34%) at 150 mM NaCl concentration. These seeds can even germinate (5%) on 200 mM NaCl, whereas WT seeds failed to germinate at this concentration.

In case of root elongation, transgenic plants differed slightly from WT only at higher concentrations. In WT the root lengths were halved at 150 mM NaCl and reduced to 20% at 200 mM NaCl compared to the control (Figure 18B). In comparison, the root elongation of transgenic plants was reduced to 60% and 30% at 150 mM and 200 mM NaCl, respectively (Figure 18B). The hypocotyl length of dark grown seedlings of transgenic plants behaves similar as WT on all tested NaCl concentrations. Together, the results indicate that the transgenic plants show slight resistance to salt stress which was mediated by B-type ARRs.

3.2 Microarray analysis

To identify the possible target genes of B-type ARRs by performing microarray analysis, the transcription profiles of *35S:ARR1-SRDX* plants were compared with those of WT plants. In order to identify eventually specific target genes of ARR1, an *ARR1* loss of function mutant (*arr1*) and a line overexpressing the *ARR1* gene constitutively (*35S:ARR1*) were characterised and their transcription profiles were analysed. Later, for the identification of *cis*-acting elements of B-type ARRs, a selected target gene promoter (*ARR6*) was analysed by GUS-reporter gene assays and promoter deletion analysis. In addition, towards understanding the regulation of B-type ARRs at the protein level, protein-protein interaction studies were also performed by taking ARR1 as representative of B-type ARRs.

3.2.1 Characterization of a T-DNA insertion allele of *ARR1* gene

The *ARR1* gene of *Arabidopsis* (AT3G16857) has a size of 3623 bp and is interrupted by five introns (Figure 19A). The coding sequence has a length of 2073 bp which codes for a protein with 690 amino acids. T-DNA insertion lines were ordered for *ARR1* from the NASC institute (http://arabidopsis.info).

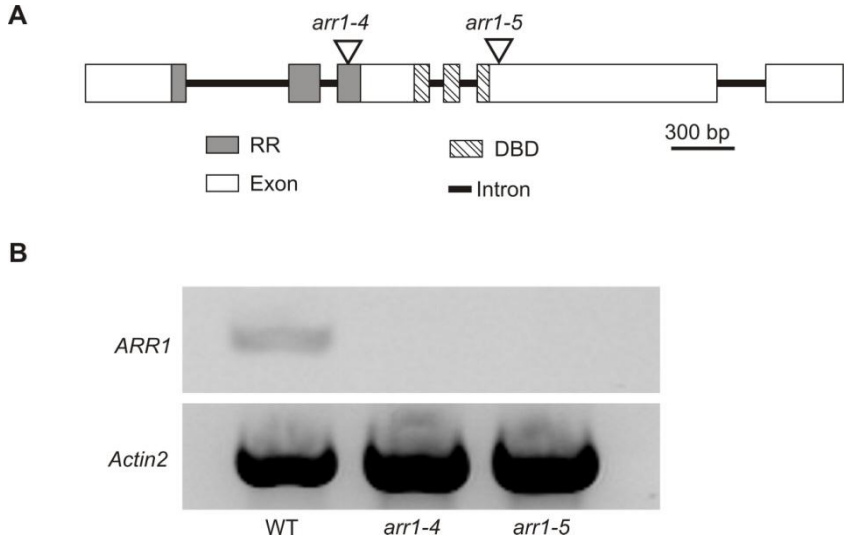

Figure 19: Characterization of *arr1* knockout lines. A. A schematic diagram of gene structure of *ARR1*. The T-DNA insertions are placed in the response regulator region (*arr1-4*) and in the beginning of the fifth exon region (*arr1-5*). B. Semi-quantitative RT-PCR analysis for *ARR1* transcript levels. Total RNA was isolated from the seven-day-old seedlings and RT-PCR was performed using *ARR1* specific primers. *Actin2* gene was used as loading control in the experiment.

Two different T-DNA insertion mutant lines (SALK_042196 and SALK_042196) were obtained and screened for homozygosity of the T-DNA insertion by sowing the seeds on MS plates containing kanamycin as selection marker. According to the TAIR data base (http://www.arabidopsis.org) it was predicted that the position of the T-DNA insertion in SALK_042196 is in the response regulator domain, whereas in case of SALK_001450 it is in the starting region of the 5^{th} exon. In the T_2 generation, *ARR1* expression of the selected independent lines was tested by using RT-PCR (Figure 19B). The RT-PCR was performed using gene specific primers for *ARR1* up to 35 cycles. The transcript of *ARR1* was completely absent in both knockout lines tested and a low abundance of *ARR1* was observed in WT (Figure 19B). The mutant line SALK_042196 was denoted as *arr1-4* and SALK_001450 mutant line as *arr1-5*. Different T-DNA insertion alleles of *arr1*, namely *arr1-1*, *arr1-2*, *arr1-3* and *arr1-4*, were characterized before in various studies (Sakai *et al.*, 2001; Mason *et al.*, 2005; Dello loio *et al.*, 2007; Ishida *et al.*, 2008). So in the present study only the *arr1* insertion allele *arr1-5* was chosen for further characterization. The *arr1-5* homozygous plants were carefully analyzed for visible phenotypes in the subsequent generation (see 3.2.3).

3.2.2 Generation of *35S:ARR1* transgenic plants

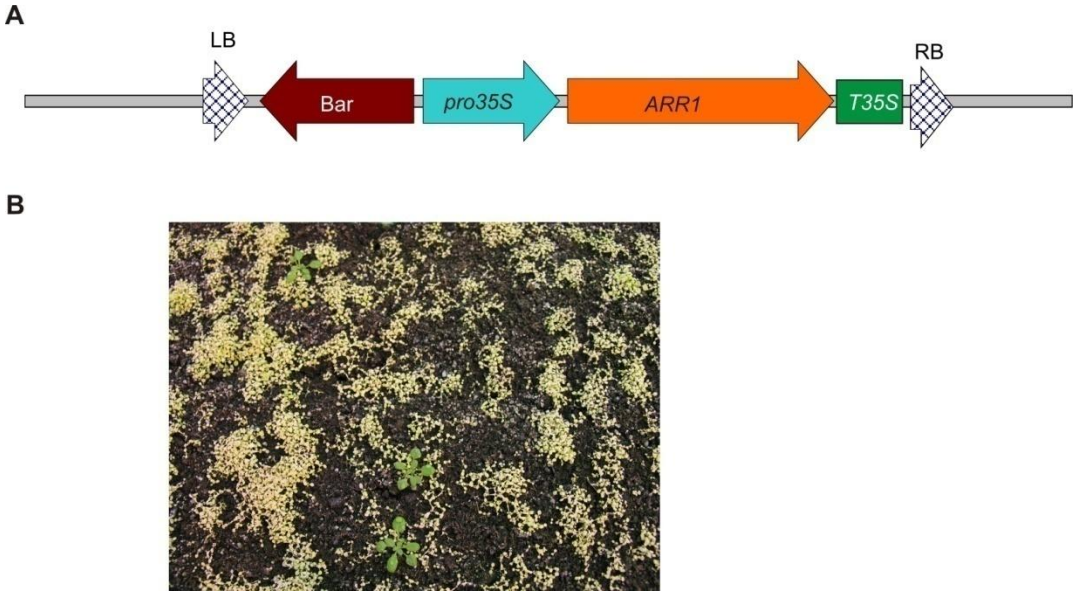

Figure 20: Transgenic plants overexpressing ARR1. A. Schematic presentation of the *35:ARR1* gene in the binary vector pB2GW7. B. Selection of transgenic plants. Plants that are overexpressing *ARR1* were selected by spaying BASTA. 0.1% BASTA was sprayed twice in a 4-days interval.

To study the specific role of ARR1 in plant development, transgenic plants that are overexpressing *ARR1* were generated and analysed along with the *arr1-5* knockout plants. Full length coding sequence of *ARR1* was cloned downstream of the 35S promoter in a binary vector carrying the *BAR* gene as selection marker and transformed into *Agrobacterium* strain GV3101 (Figure 20A). Plants overexpressing *ARR1* were generated by infiltration of *Agrobacterium* containing the *35S:ARR1* construct using the floral dip method (Clough and Bent, 1998). The seeds collected from the infiltrated plants (T_0) were sown on soil and the transformants were selected by spraying BASTA^{TM} (Figure 20B). Ten independent lines were selected after spraying and the seeds of these plants were collected. In the T_2 generation homozygous plant lines were selected by sowing the seeds of T_1 plants on MS-media containing phosphinothricin as selective agent. Three independent homozygous lines were obtained and one line was selected for further characterization.

3.2.3 The shoot phenotype of transgenic *arr1-5* and *35S:ARR1* plants is similar to WT

Homozygous *arr1-5* and *35S:ARR1* plants of the T_3 generation were characterized for their shoot phenotype. First, the seed germination rate on MS plates under standard long day conditions (16 h light/8 h dark regime at 22°C and 60% relative humidity) was analyzed after four days of vernalization. It was observed that both the *arr1-5* and *35S:ARR1* seeds were germinating similar to WT (Col-0) seeds (data not shown). Both transgenic lines were not differing from WT in the shoot phenotype too (Figure 21A). The size of the leaves and time of bolting was also similar to WT. Further the transcript levels of *ARR1* were analysed by performing Northern blot analysis using a *ARR1*-specific probe (Figure 21B). This analysis revealed an increased *ARR1* transcript level in *35S:ARR1* transgenic plants compared to WT and a complete absence of the transcript in *arr1-5* knockout plants (Figure 21B). It was also observed in previous studies that knockout line *arr1-1* and *ARR1* overexpressing plants do not have an obvious phenotype compared to WT (Sakai *et al.*, 2001).

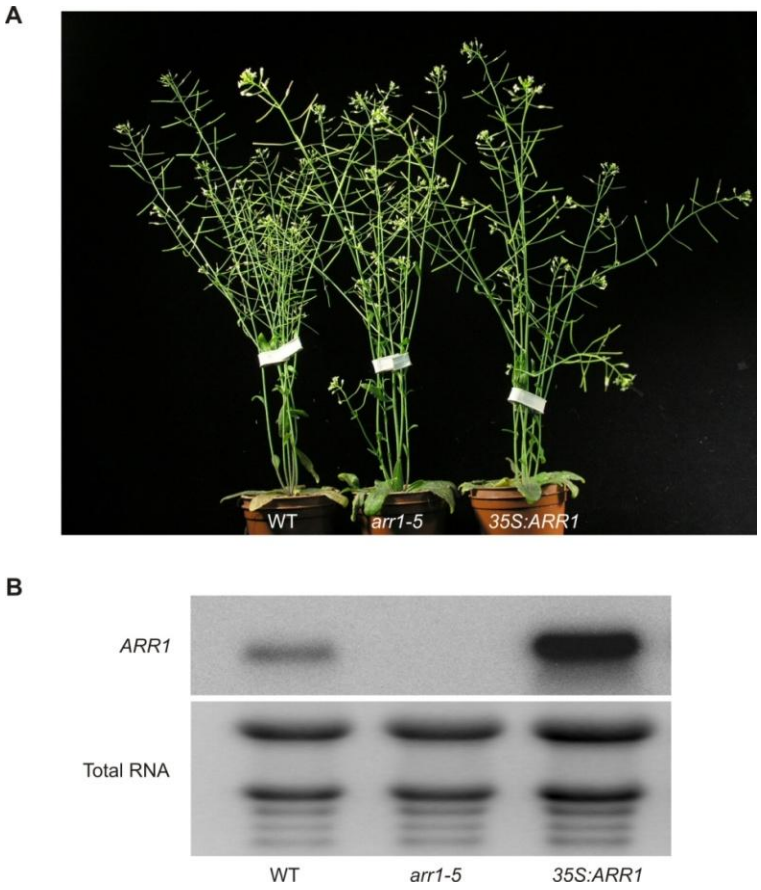

Figure 21: Characterization of *arr1-5* and *35S:ARR1* transgenic plants. A. Shoot phenotype of transgenic plants. Plants were grown under long-day conditions in the greenhouse and pictures were taken 45 DAG. B. Northern blot analysis of *ARR1* transcript levels. Total RNA was isolated from the leaves of WT, *arr1-5* and *35S:ARR1* transgenic plants. Northern blot was hybridized with a probe specific for *ARR1*. Total RNA was used as loading control.

3.2.4 Root elongation and lateral root formation of *arr1-5* and *35S:ARR1* plants

Cytokinin plays an antagonistic role in roots compared to the shoot. In roots, cytokinin inhibit primary root elongation and lateral root formation (Werner *et al.*, 2003). So the root phenotype of transgenic plants was analyzed. In case of *arr1-5* knockout plants, the primary root was longer (7%) compared to WT and *35S:ARR1* plants without cytokinin application (Figure 22A). At a concentration of 0.5 µM BA the root elongation of all genotypes was reduced to 50% of their control. The *arr1-5* knockout plants showed a marginal insensitivity to cytokinin at higher concentrations. At a BA concentration of 1.0 µM, primary root elongation of WT and *35S:ARR1* plants was reduced to 20% and 21.6% respectively, whereas it was only reduced to 34.1% in *arr1-5* plants (Figure 22A).

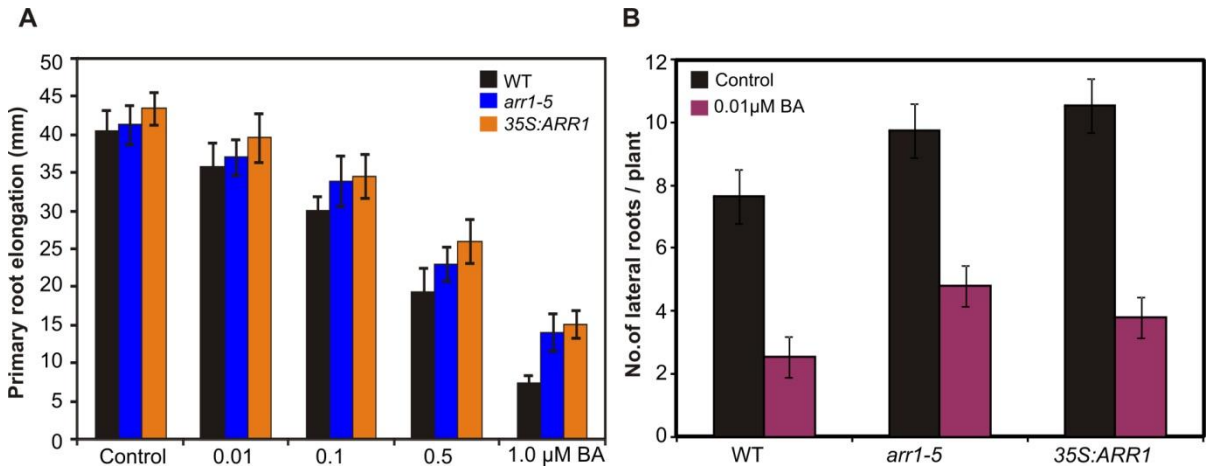

Figure 22: Root phenotype of *arr1-5* and *35S:ARR1* transgenic plants. A. Primary root elongation of transgenic plants. Seedlings were grown vertically on MS medium plates. The length of the primary root was measured 10 DAG. B. At 0.01 µM BA concentration, *arr1-5* knockout plants form more lateral roots compared to WT and *35S:ARR1* plants. The number of emerged lateral roots were determined 14 DAG. Results shown in A and B represents means of two independent replicates for each line. Error bars represent SD (n≥15).

In lateral root formation the *arr1-5* plants showed insensitivity to cytokinin on medium containing 0.01 µM BA. On 0.01 µM BA lateral root formation of the both WT and *35S:ARR1* plants was reduced to 33% and 36% respectively while it was 49% in case of *arr1-5* plants (Figure 22B). On 0.01 µM BA, the *arr1-5* plants produced 4.8 lateral roots per plant whereas it was 2.55 and 3.8 in WT and *35S:ARR1* plants, respectively (Figure 22B). This result indicates that the *arr1-5* knockout plants exhibit a slight insensitivity towards cytokinin and *35S:ARR1* plants were not differing with WT in their root phenotype.

3.2.5 Primary response of *arr1-5* and *35S:ARR1* plants to cytokinin

The transgenic plants were further tested at the molecular level for their primary response to cytokinin. The A-type ARR genes like *ARR5*, *ARR6* and *ARR7* are immediate early target genes of B-type ARRs (D`Agostino *et al.*, 2000; Hwang and Sheen, 2001; Romanov *et al.*, 2002). To analyze the rapid cytokinin response of *arr1-5* and *35S:ARR1* transgenic plants, the expression of *ARR5* and *ARR6* was tested by using quantitative RT-PCR (qRT-PCR) and Northern blot analysis (Figure 23). The transcript level of *ARR5* was induced rapidly 15 min after cytokinin treatment (~16-fold) and this induction was reduced to ~6-fold after 120 min in WT seedlings. The *arr1-5* plants also showed the same pattern like WT, but with a reduced *ARR5* transcript level (Figure 23A). In contrast to WT and knockout plants, the *35S:ARR1* seedlings showed a steadily increased level of *ARR5*

transcript with a longer induction period. The *ARR5* transcript was induced by 13-fold after 120 min cytokinin treatment (Figure 23A). In WT seedlings the *ARR6* transcript was also induced rapidly and reached to ~12-fold after 15 min of cytokinin treatment and the induction was reduced to ~9-fold after 120 min (Figure 23B). In contrast, in *arr1-5* and *35S:ARR1* transgenic plants the *ARR6* transcript was accumulated over time, the highest transcript level was observed after 120 min of cytokinin treatment. In *arr1*-5 plants the induction was ~8-fold after 15 min and reached 10-fold after 120 min of. In *35S:ARR1* plants the level of *ARR6* transcript was already more than ~2-fold compared to WT at time point zero condition and ~5-fold after 120 min of cytokinin induction (Figure 23B). Further Northern blot analysis was done for the *ARR5* transcript. In two independent biological samples of each genotype, the pattern of the *ARR5* induction was similar to the qRT-PCR data (Figure 23C).

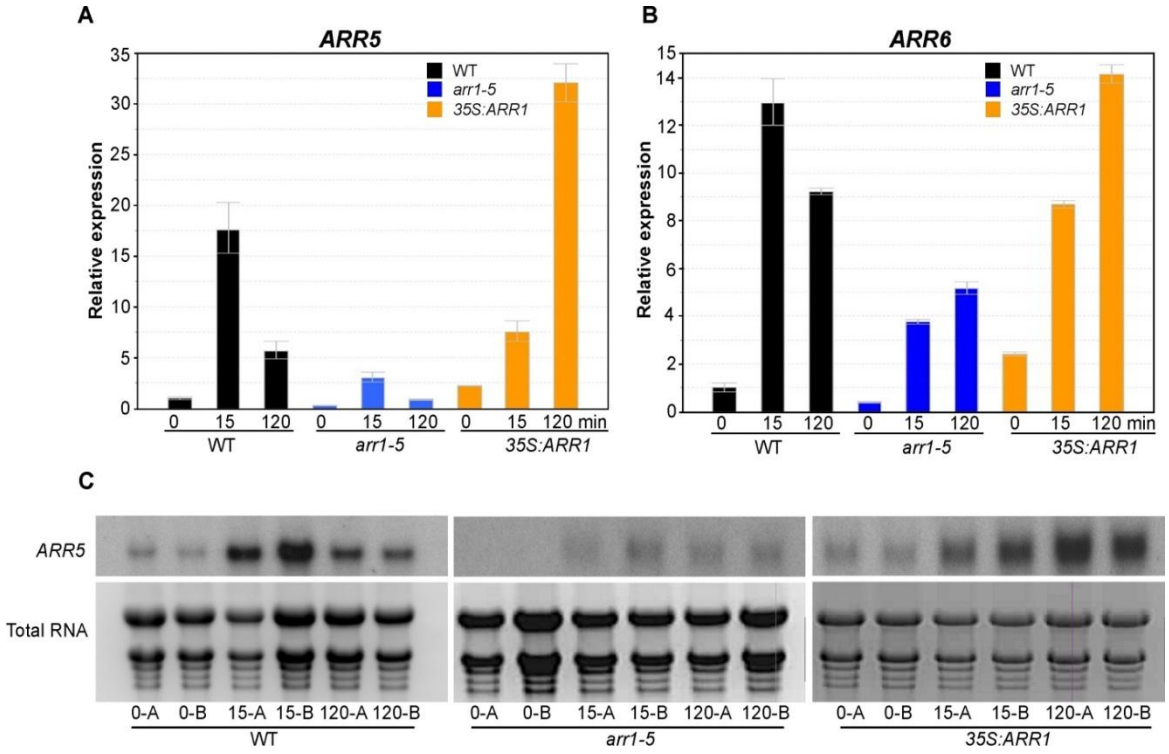

Figure 23: Primary cytokinin response of *arr1-5* and *35S:ARR1* transgenic plants. A&B. The relative expression levels of *ARR5* and *ARR6* transcripts. The qRT-PCR was performed by using the total RNA extracted from 5-day old seedlings. The *UBC10* gene was used as control in the analysis. C. The Northern-blot analysis of *ARR5* transcript levels in the transgenic plants. Total RNA was isolated from 5-day old seedlings before treatment and after 15 and 120 min with 5 µM BA. Northern blots were hybridized with a probe specific for the cytokinin response gene *ARR5*. The total RNA was used as loading control. The A and B along with different time points denote two independent biological samples of the same genotype.

3.2.6 Transcriptome analysis reveals complex changes in transgenic plants

After characterization of numerous morphological and physiological features of the *ARR1* mutant variants like the *35S:ARR1-SRDX*, *arr1* loss of function mutant (*arr1-5*), and a line overexpressing the *ARR1* gene constitutively (*35S:ARR1*), the expression profiles of these mutant lines were compared. More specifically, the immediate-early transcriptional cytokinin response of mutant lines was investigated by treating the seedlings with cytokinin for 15 min and 120 min. The changes occurring in the transcriptome of these mutant lines were recorded following cytokinin treatment of 5-d-old seedlings by using the CATMA v2.4 array. 5-d-old seedlings of the different genotypes showed only minor morphological differences. First the transcriptome of ARR1-S-8 plants was compared to the WT to eventually identify components downstream of B-type ARRs. Then the transcriptional profiles of *arr1-5* and *35S:ARR1* plants were compared to gain more information about the specific biological roles of ARR1. In all experiments genes of which the transcript level was changed ≥ 2.5-fold, the FDR-corrected p-value (q-value) was ≤ 0.05 and which had 50% or more spots above background in the higher expressed condition were considered as significantly regulated.

3.2.6.1 Transcriptome analysis of 35S:ARR1-SRDX *plants reveal target genes of B-type ARRs*

To identify putative immediate-early target genes of B-type response regulators the transcriptional profile of *35S:ARR1-SRDX* plants (line ARR1-S-8) was compared with WT. In total, 658 genes were found with a lower steady state mRNA level and 505 genes with a higher steady state mRNA level in seedlings of line ARR1-S-8 compared to the WT (data not shown). A total of 229 genes were up-regulated after 15 min in the wild type, of which 167 genes (60%) were no longer up-regulated in *35S:ARR1-SRDX* plants. After 120 min following cytokinin treatment, 144 genes were up-regulated in WT, 93 of those genes (65%) were no longer up-regulated in ARR1-S-8. In total 58% of the genes were no longer up-regulated in transgenic plants compared to WT (data not shown). Then the broader cytokinin-induced changes in the transcript abundance, which should reveal whether there is an overall dampening of the cytokinin response was studied. In *35S:ARR1-SRDX* transgenic seedlings the total number of genes up-regulated by cytokinin treatment was about half the number observed in wild type (data not shown). The induction of the ten strongest cytokinin-

regulated genes in the wild type was reduced in line ARR1-S-8 to 6% and 30% after 15 and 120 min, respectively (data not shown).

Table 13. Examples of down- and up-regulated genes at time point zero (BA0) in *35S:ARR1-SRDX* transgenic seedlings compared to WT.

Steady state mRNA levels of seedlings of wild type and line ARR1-S-8 were analyzed 5 DAG. The tables are sorted according to the ratio. The significantly regulated genes (>2.5-fold difference in transcript expression, q < 0.05) are tabulated. The q-value is the FDR corrected p-value and was calculated as described (Benjamini and Hochberg, 1995). This table was prepared by Dr. Brenner**.**

Down-regulated genes at time point zero (BA0)

AGI code	Expression in WT	Expression in ARR1-S-8	Ratio	q value	Gene description
AT1G37130	14.72	0.20	0.01	7.29E-08	Nitrate reductase 2 (*NR2*)
AT3G02050	0.48	0.03	0.07	3.38E-09	Potassium transporter (*KUP3*)
AT1G11260	14.59	1.03	0.07	1.18E-12	Glucose transporter (*STP1*)
AT4G35790	1.45	0.12	0.08	4.80E-10	Phospholipase Dd/PLDd (*PLDDELTA*)
AT5G24470	40.19	3.50	0.09	7.36E-11	Pseudo response regulator 5 (*APRR5*)
AT1G14920	0.75	0.07	0.09	1.16E-11	GA response modulator (*GAI; RGA2*)
AT4G26200	3.68	0.37	0.10	5.33E-10	1-Amino-cyclopropane-1-carboxylate synthase
AT2G01570	2.28	0.34	0.15	1.43E-03	GA response modulator (*RGA1*)
AT5G61520	2.68	0.43	0.16	2.11E-13	Hexose transporter, putative
AT3G19930	6.01	1.04	0.17	2.71E-10	Sugar transport protein (*STP4*)
AT5G57630	3.15	0.56	0.18	1.95E-14	CBL-interacting protein kinase 21, putative (*CIPL21*)
AT2G38290	4.21	0.79	0.19	1.04E-13	Ammonium transporter 2 (*AMT2*)
AT1G10760	1.47	0.28	0.19	1.16E-05	Starch excess protein (*SEX1*)
AT1g13790	0.45	0.09	0.20	3.67E-05	β-Fructosidase (*BFRUCT1*)
AT2G43790	1.65	0.36	0.22	7.04E-11	Mitogen-activated protein kinase, (*MPK6*)
AT1G69850	11.17	2.47	0.22	3.27E-10	Nitrate transporter (*NTL1*)
AT4G37450	3.69	0.91	0.25	2.08E-08	Lys-rich arabinogalactan protein (*AGP 18*)
AT3G15540	0.34	0.10	0.28	3.07E-09	Indoleacetic acid-induced protein (*IAA19*)
AT5G42210	1.07	0.31	0.28	4.04E-10	Ethylene response factor subfamily B-1 (*AtERF 9*)
AT3G54220	2.85	0.82	0.29	5.31E-03	Scarecrow transcription factor
AT5G65670	2.59	0.80	0.31	5.92E-09	Indoleacetic acid-inducible protein 9 (*IAA9*)
AT1G25540	2.80	0.86	0.31	4.17E-02	Phytochrome and flowering time regulatory protein (*PFT1*)
AT3G60390	0.30	0.09	0.31	5.95E-15	Homeobox-Leu zipper protein 3 (*HAT3*)
AT3G61470	68.70	22.74	0.33	1.27E-06	Chlorophyll a/b-binding protein (*LHCA2*)
AT3G46640	6.48	2.15	0.33	1.06E-09	Phytoclock 1 (*PCL1*)
AT5G60120	0.62	0.21	0.34	1.07E-05	AP2 domain-containing transcription factor
AT1G34210	2.19	0.81	0.37	9.91E-06	Somatic embryogenesis receptor-like kinase 2 (*SERK2*)
AT1G09530	16.80	6.28	0.37	1.01E-10	Phytochrome-interacting factor 3 (*PIF3*)
AT4G29740	0.15	0.05	0.38	4.11E-09	Cytokinin oxidase family protein (*CKX4*)
AT1G72010	3.11	1.21	0.39	1.87E-09	TCP family transcription factor, putative (*PCF2*)
AT5G02810	1.57	0.61	0.39	1.08E-11	Pseudo response regulator 7 (*APRR7*)

Up-regulated genes at time point zero (BA0)

AT3G22840	0.09	14.47	161.47	3.53E-14	Chlorophyll A-B binding family protein / early light-induced protein (*ELIP*)
AT5G08640	0.34	10.52	31.12	1.62E-16	Flavonol synthase 1 (*FLS1*)

AT1G65060	0.05	0.95	20.43	1.50E-11	Isoform of 4-coumarate:CoA ligase (*4CL3*)
AT2G27550	0.22	2.44	11.00	7.43E-11	Centroradialis protein, putative (*CEN*)
AT1G64780	1.34	11.66	8.67	1.38E-12	Ammonium transporter 1, member 2 (*AMT1.2*)
AT5G13930	19.01	135.85	7.14	1.58E-14	Chalcone synthase / naringenin-chalcone synthase
AT3G21240	0.15	0.84	5.74	1.66E-05	Encodes an isoform of 4-coumarate:CoA ligase (*4CL2*)
AT5G24850	3.30	17.66	5.36	1.83E-13	Cryptochrome dash (*CRYD*)
AT3G55120	1.93	9.94	5.14	2.24E-14	Chalcone-flavanone isomerase / chalcone isomerase (*CHI*)
AT2G32950	3.33	16.23	4.88	6.43E-08	COP1 regulatory protein, photomorphogenesis repressor / FUSCA protein (*FUS1*)
AT3G24290	0.11	0.53	4.62	1.68E-03	Ammonium transporter, putative
AT2G46340	4.58	20.90	4.57	1.99E-07	Phytochrome A suppressor (*SPA1*)
AT5G05410	1.18	5.30	4.47	2.22E-06	DREB subfamily A-2 of ERF/AP2 transcription factor family (*DREB2A*)
AT3G50630	0.16	0.56	3.46	3.14E-04	Kip-related protein 2 (KRP2) / cyclin-dependent kinase inhibitor 2 (*ICK2*)
AT2G27960	0.82	2.78	3.40	6.71E-07	Cyclin-dependent kinase / CDK (*CKS1*)
AT3G17510	5.06	16.65	3.29	1.27E-07	CBL-interacting protein kinase 1 (*CIPK1*)
AT1G75410	1.28	3.56	2.77	5.40E-07	BEL1-like homeodomain 3 protein (*BLH3*)
AT4G16280	0.17	0.47	2.77	5.25E-03	Flowering time control protein / FCA γ (*FCA*)
AT5G19040	0.32	0.81	2.54	8.27E-05	Adenylate isopentenyltransferase 5 / cytokinin synthase (*IPT5*)

The number of down-regulated genes was higher in transgenic plants compared to WT. After 15 min of cytokinin treatment 115 genes were down-regulated in ARR1-S-8 compared to 14 genes in WT. 50% of genes down-regulated in WT after 15 min were no longer regulated in ARR1-S-8. Similarly 120 min following BA treatment, 46 genes were down-regulated in WT, of which 76% of the genes were no longer down-regulated in ARR1-S-8. In Table 13, examples of down-and up-regulated genes that are significantly differed in expression compared to WT at time point zero (BA0) are listed. Analysis of the classes of genes and individual genes that are constitutively down-regulated in *35S:ARR1-SRDX* plants should yield indications about the direct or indirect regulatory functions of B-type ARRs.

There are numerous genes in line ARR1-S-8 that showed only a tenth or even less of their wild type transcript level indicating that they are in some way regulated by B-type ARRs. Prominent examples are the nitrate reductase gene *NR2* and the ammonium transporter gene *AMT2*, nitrate transporter gene *NTL1*, several sugar transporter genes (*STP1*, *STP4*) and the genes encoding the repressors of gibberellin responses RGA1 and RGA2 (Table 13). Among the individual genes that were very strongly up-regulated in ARR1-S-8 is a gene encoding an ELIP (early light-induced protein) of the chlorophyll A/B binding protein

family and the cyclin-dependent kinase inhibitor gene *ICK2*. Among the cytokinin-related genes *IPT5* was 2.5-fold up-regulated (Table 13). It was investigated further, whether genes that are down-regulated by cytokinin would show enhanced repression in *35S:ARR1-SRDX* seedlings. Analysis of a set of 56 early or late cytokinin-down-regulated genes (Brenner *et al.*, 2005) revealed only five examples which show a stronger downregulation following cytokinin treatment in the *35S:ARR1-SRDX* seedlings compared to the wild type. In addition, this concerned only genes that were down-regulated 2 h after cytokinin treatment but none of the genes that were rapidly (after 15 min) down-regulated. This result argues against the possibility that ARR1-SRDX enhances a putative repressor function of ARR1.

3.2.6.2 Cluster analysis reveals putative target genes of B-type ARRs

A clustering analysis was performed with the microarray data to know how similar the expression profile of genes that were regulated in WT and ARR1-S-8. The expression profiles of individual genes provide some clues to their function. This is because genes with similar function are likely to show similar expression patterns under various conditions (Hughes *et al.*, 2000; Szabo *et al.*, 2002). Clustering analysis was done using SOTA (Self Organising Tree Algorithm). First the genes of WT and ARR1-S-8 were clustered together by similarities in their expression profiling. In total, ten different expression profile clusters were constructed (Figure 24A). Out of these 10 clusters, cluster 3 and cluster 4 in particular gave valuable information regarding the genes that are immediate-early and late responding to cytokinin treatment, respectively. Cluster 3 contains 63 genes which are up-regulated after 15 min by cytokinin and again down-regulated after 2 h of cytokinin induction (Figure 24B). Whereas cluster 4 contains 46 genes which are up-regulated after 15 min, and their expression is further increased after 120 min of BA treatment (Figure 24B). In ARR1-S-8, all 63 genes of cluster 3 which are up-regulated in WT are no longer up-regulated. Whereas in cluster 4 many of the genes induced in WT are down-regulated after 15 min in ARR1-S-8 and very few of them responded lately at 120 min following BA treatment (Figure 24B). The list of the genes that are present in cluster 3 and cluster 4 were listed in a table (Table 18 in Appendix). For example, five out of the seven A-type *ARR* genes that are represented in the CATMA v2.4 array were present in cluster 4. The response to cytokinin was dampened and/or delayed in ARR1-S-8 plants (Figure 24C), which is consistent with the result of the Northern blot analysis (Figure 14).

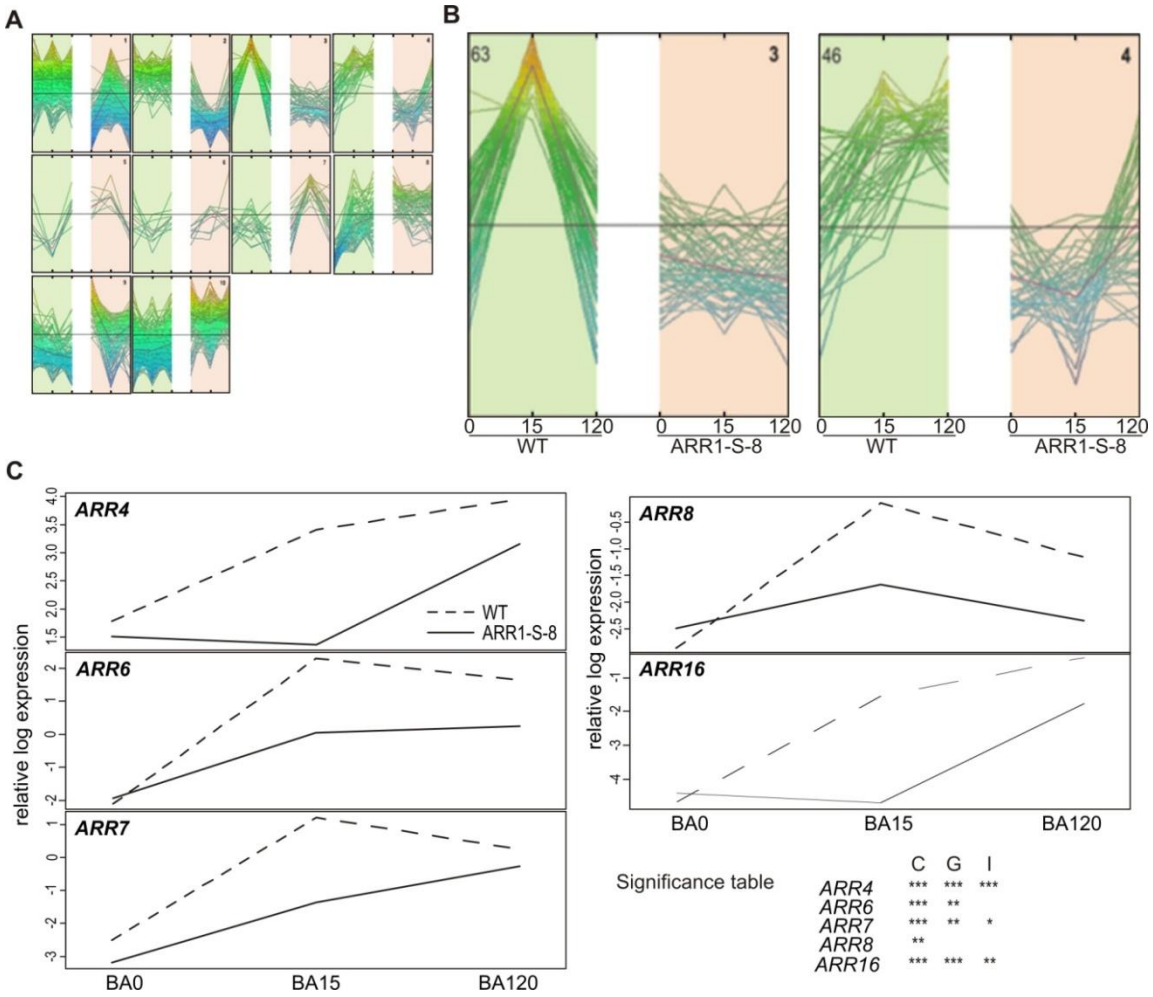

Figure 24: Clustering analysis reveals putative target genes of B-type ARRs. A. Clustering analysis was performed on all genes that are regulated in WT and ARR1-S-8 plants. B. Two of the clusters, cluster 3 and cluster 4 were highlighted. The genes present in cluster 3 are immediate response genes for cytokinin whereas the genes in cluster 4 are late response genes. C. Interaction plots showing the relative normalized expression values for five A-type ARR genes present in cluster 4 plotted against the time points of cytokinin treatment: BA0 (control), BA15 (15 minutes of BA treatment), and BA120 (120 minutes of BA treatment). The table in the lower right corner shows the significance of differences in gene expression. C (cytokinin effect) means the responsiveness of the respective gene to cytokinin treatment. G (genotype effect) denotes if the average expression of the respective gene in line ARR1-S-8 differs significantly from the wild type. The cytokinin response of genes with a significant I (interaction effect) is dependent of the genotype. The significance codes are based on the uncorrected p-value for each gene. Symbols used for p-values. no symbol, $p > 0.05$; *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $p < 0.001$.

However, the response curves show individual differences. For example, for *ARR4* and *ARR16* the early increase of transcript abundance seen in wild type is completely missing in the *35S:ARR1-SRDX* transgenic seedlings. Other genes like *ARR6* and *ARR7* show a weaker response at the early time point (Figure 24C). Some other important genes that are present in Cluster 3 and cluster 4 include genes encoding a zinc finger protein ZAT12 (AT5G59820) which plays a central role in reactive oxygen and abiotic stress signaling in *Arabidopsis*,

(Davletova *et al.*, 2005) a cytokinin response factor *CRF2* (AT4G23750), *CKX4*, *HAT22* and several ERF (ethylene response factor) transcription factors like *ERF102*, *ERF104*, and *ERF105*. For the full list of the genes see the table in the appendix (Table 18 in Appendix).

3.2.6.3 Several known target genes of the ARR1 were suppressed in ARR1-S-8

Next the data was analyzed whether the expression of known ARR1 target genes was altered in ARR1-S-8 transgenic plants. Eleven of the 23 ARR1 target genes published by Taniguchi *et al*., (Taniguchi *et al.*, 2007) were also on our array and could be detected. All of these were also induced by cytokinin in wild-type under our conditions, ten showed a diminished and/or delayed induction in the *35S:ARR1-SRDX* plants and seven of them showed a lower basal expression level in the *35S:ARR1-SRDX* plants compared to wild-type plants (Table 14). Together this shows a significant repressive influence of ARR1-SRDX on ARR1 target gene expression.

Table 14. Comparison of microarray results presented in this study with the results published by Taniguchi *et al*. (2007).

The results of the microarray study presented here were compared with the Northern blot results presented in Taniguchi *et al.*, (2007; Table 1). For genes with more than one target on the microarray the results for each target are listed separately. Genes for which no results could be obtained in the present study are grayed and the reason is indicated.
✓: positive result; ×: negative result. A p-value of ≤ 0.05 was regarded as significant. The cytokinin effect indicates if a transcript was regulated by cytokinin.

AGI code	Gene	Induction by cytokinin confirmed in WT	Lower expressed in ARR1-S-8	Induction by cytokinin diminished and/ or delayed in ARR1-S-8	p-value of cytokinin effect
AT1G59940	*ARR3*	✓	×	×	6.19E-10
AT1G10470	*ARR4*	✓	✓	✓	7.81E-13
AT5G62920	*ARR6*	✓	✓	✓	1.54E-19
AT1G19050	*ARR7*	✓	✓	✓	8.43E-12
AT2G41310	*ARR8*	✓	✓	✓	3.82E-07
AT3G57040	*ARR9*	✓	×	✓	3.02E-03
AT2G40670	*ARR16*	✓	✓	✓	8.74E-17
AT4G29740	*CKX4*	✓	✓	✓	9.98E-17
AT1G69530	Expansin (*EXP1*)	✓	×	✓	5.98E-08
AT2G40230	Putative transferase-family protein	✓	✓	✓	8.39E-06
AT3G62930	Putative glutaredoxin	✓	×	✓	3.09E-04

Genes that are not regulated or detected

AT3G48100	*ARR5*	not detected
AT1G74890	*ARR15*	not on array
AT3G56380	*ARR17*	not on array
AT1G67110	Cytokinin hydroxylase (*CYP735A2*)	not detected
AT4G11190	Putative disease resistance response protein	no CATMA GST
AT2G30540	Putative glutaredoxin	not on array
AT2G29490	Putative glutathione *S*-transferase	not on array
AT4G19030	Nodulin-like protein (*NIP1;1*) (*NLM1*)	not detected
AT4G11210	Putative disease resistance response protein	no CATMA GST
AT1G04240	AUX/IAA family protein (*IAA3*) (*SHY2*)	not detected
AT2G20520	Fascilin-like arabinogalactan protein (*FLA6*)	not detected
AT1G76410	RING finger protein	not on array

3.2.6.4 Putative specific target genes of ARR1 were revealed by comparing the expression profiles of arr1-5 *and* 35S:ARR1 *plants*

After identifying the putative direct target genes of B-type ARRs by comparing WT and ARR1-S-8 plants (Figure 24B and Table 18 in Appendix), the expression profiles of *arr1-5* and *35S:ARR1* transgenic plants were compared for identifying the putative specific target genes of ARR1.

Table 15. Putative target genes of ARR1.

The transcript levels of putative target genes in *35S:ARR1* and *arr1-5* seedlings were analyzed. The fold changes of transcripts before and after 15 min and 120 min of BA treatment are shown as ratios between BA15/BA0 and BA120/BA0. All significantly regulated genes (>2.0-fold difference in expression, $q < 0.05$) are listed. The q-value for the ratio is the FDR corrected p-value and was calculated as described (Benjamini and Hochberg, 1995).

AGI code	*35S:ARR1*		*arr1-5*		Fold change	q-value CK effect	Gene description
	BA15/ BA0	BA120/ BA0	BA15/ BA0	BA120/ BA0			
Up-regulated in *35S:ARR1* and down-regulated in *arr1-5* plants							
AT4G15530		3.6		0.1	20.5	0.0140	Pyruvate orthophosphate dikinase
AT2G36690		2.5		0.1	16.3	0.0020	Oxidoreductase, 2OG-Fe(II) oxygenase family protein
AT3G45050		2.3		0.2	11.1	0.0320	Expressed protein
AT4G21120		13.2		1.5	8.4	7.02E-13	Cationic amino acid transporter (*CAT1*)
AT1G28520		2.7		0.3	7.5	0.0410	Expressed protein
AT1G18900		7.0		1.1	6.1	0.0005	Pentatricopeptide (PPR) repeat-containing protein
AT4G40010		3.8		1.3	2.7	0.0002	Serine/threonine protein kinase

AT3G25900		3.6		1.6	2.2	2.33E-05	Homocysteine S-methyltransferase 1
AT2G40670	2.7		0.5		5.3	1.86E-13	*ARR16*
AT3G06070	6.4		1.5		4.2	0.0426	Expressed protein
AT2G41640	3.3		0.9		3.8	0.0300	Expressed protein,
AT3G21150	2.6		1.0		2.6	1.25E-06	Zinc finger (B-box type) family protein
Down-regulated in *35S:ARR1* and up-regulated in *arr1-5* plants							
AT5G44120		0.2		3.6	16.9	0.0018	12S seed storage protein
AT3G59140		1.2		7.1	5.7	0.0081	ABC transporter family protein,
AT1G26470		0.7		2.6	3.7	0.0327	Expressed protein
AT2G40460		1.2		3.7	2.9	0.0003	Proton-dependent oligopeptide transport (POT) family protein,
AT4G37410		1.5		4.1	2.8	7.59E-09	Cytochrome P450 family
AT4G25340		1.3		3.1	2.5	0.0044	Immunophilin-related protein
AT1G09100		0.2		2.3	9.0	0.0111	26S protease regulatory subunit 6A
AT5G49520	1.3		4.4		3.2	3.79E-06	WRKY family transcription factor
AT3G25600	1.7		4.5		2.6	1.87E-06	Calmodulin
AT3G49530	1.4		3.0		2.2	5.77E-06	No apical meristem (*NAM*) family protein
AT1G75000	1.2		2.6		2.1	2.33E-07	GNS1/SUR4 membrane family protein,
AT5G63180	0.9		2.7		3.0	0.0026	Pectate lyase family protein

The genes that are up-regulated with cytokinin treatment in WT and *35S:ARR1* plants but no longer up-regulated in *arr1-5* knockout plants are considered as putative specific target genes of ARR1 (Table 15). Similarly, another set of genes which are up-regulated with cytokinin treatment in *arr1-5* knockout plants but no longer up-regulated in *35S:ARR1* plants may also be considered as putative specific target genes of ARR1 (Table 15). Analysis of these mis-regulated genes should reveal the direct or indirect role of ARR1 in the regulation of those particular genes expression. Prominent examples are genes that are encoding Cationic Amino acid Transporter 1 (*CAT1*), an ABC transporter family protein and transcription factors belonging to the *WRKY*, *NAM* and *Zinc finger* families (Table 15). Interestingly, this analysis also indicated that the A-type *ARR*, *ARR16* might be a specific target gene for ARR1. Since it has been shown in previous studies that *ARR16* might be a direct target gene for ARR10 and ARR12 (Yokoyama *et al.*, 2007), further detailed analysis is needed to validate this result. Comparison of the expression profiles of *arr1-5* plants and WT showed that many of

the genes that were induced by cytokinin in WT were no longer regulated in *arr1-5* plants. Of 229 genes up-regulated after 15 min by cytokinin in wild-type, 138 genes (60%) were no longer up-regulated in *arr1-5* plants. After 120 min following BA treatment 144 genes were up-regulated in the wild-type, 93 of those genes (65%) were no longer up-regulated in *arr1-5* plants (data not shown). Similarly, 54% and 62% of the up-regulated genes by cytokinin in WT following 15 min and 120 min of BA treatment, respectively, were no longer up-regulated in *35S:ARR1* plants. These results indicate that ARR1 might be the main contributor among the B-type ARRs in mediating most of the cytokinin responses. However more detailed analysis is needed to ascertain the ARR1 contribution to cytokinin signalling.

3.2.6.7 qRT-PCR analysis validated a set of putative target genes of B-type ARRs

The regulation of putative target genes identified for B-type ARRs and ARR1 by microarray analysis was verified by performing quantitative RT-PCR (qRT-PCR). qRT-PCR is the commonly used validation tool for confirming gene expression results obtained from microarray analysis (Morey *et al.*, 2006). To identify the most reliable cytokinin response genes a meta-analysis was performed (done by Dr. Wolfram Brenner, data not shown). Meta-analysis is a set of classical statistical techniques to combine results from several independent studies and its applicability to microarray data was demonstrated previously (Rhodes *et al.*, 2002). The meta-analysis was performed using four different microarray sets that were available in-house for cytokinin action in different transgenic plant background. After comparing the data from these microarrays, a meta-list which contains the cytokinin-responsive genes was prepared (data not shown). A total of 20 genes, from SOTA cluster 3 (9 genes) and cluster 4 (11 genes) which are intersecting with the meta-list were identified and taken for qRT-PCR analysis. The results of the qRT-PCR are shown for six genes in a graphical presentation (Figure 25) and for the rest of the genes the data was summarized and listed in the following table (Table 16). For the *ARR6*, which is among the 20 genes tested, the data are shown in Figure 19B and Figure 23. The WT, *ARR1-S-8*, *arr1-5* and *35S:ARR1* seedlings (5-d-old) were treated with BA for 15 and 120 min and changes in the levels of the transcripts were examined by qRT-PCR (See Table 18 in Appendix for specific primers for putative target genes). The *UBC10* and *AT3G25800* genes were used as internal reference controls.

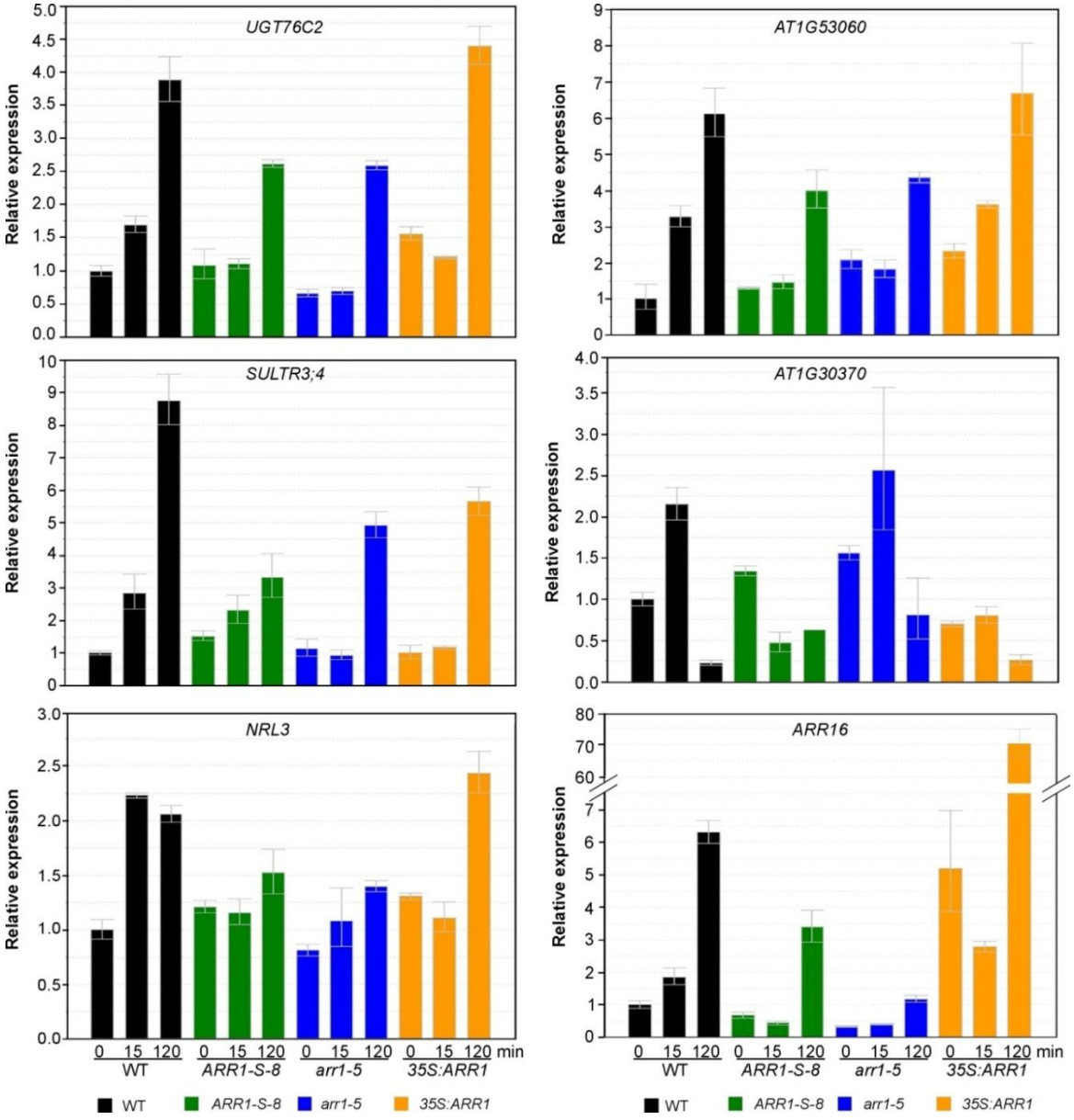

Figure 25: qRT-PCR analysis confirmed the putative target genes of B-type ARRs. The relative expression levels of target gene transcripts were compared. qRT-PCR was performed by using total RNA extracted from 5-day-old seedlings before and after 15 and 120 min treatment with 5 µM BA. The *UBC10* and *AT3G25800* genes were used as internal reference control. The mean relative expression levels from three replicates of the same genotype were plotted along with different time points. Error bars indicate the SD. The genes are considered as regulated if their transcript expression level increased by 2-fold after BA treatment.

The putative target genes were rapidly up-regulated in response to cytokinin in wild-type plants (Figure 25; Table 16). This response was severely attenuated for each target gene in the *ARR1-S-8* and in some cases in *arr1-5* plants. In most of the cases the *35S:ARR1* plants behave similar to the WT plants in putative target gene expression. With one exception, all cluster 4 genes that were tested in qRT-PCR confirmed the microarray data, whereas only

two out of the eight genes from cluster 3 were confirmed by qRT-PCR. In total, 60% of the selected putative target genes from microarray were confirmed by qRT-PCR.

Table 16. Summary of the qRT-PCR data for putative target genes of B-type ARRs.

The relative expression levels of target gene transcripts were compared before and after 15 min and 120 min of BA treatment. The fold change in expression of transcripts is shown as ratios between BA15/BA0 and BA120/BA0. The genes are considered as regulated, if their transcript expression level increased by ≥2-fold after BA treatment. The genes which are not confirmed by qRT-PCR were grayed.

AGI code	Description	WT		ARR1-S-8		*arr1-5*		*35S:ARR1*	
		BA15 /BA0	BA120 /BA0	BA15 /BA0	BA120 /BA0	BA15 /BA0	BA120/ BA0	BA15 /BA0	BA120/ BA0
Cluster 4									
AT4G23750	*CRF2*	6.1	6.4	1.6	3.2	0.9	2.9	1.3	3.1
AT4G29740	*CKX4*	46	126	2.8	4.6	1.7	4.3	1.2	4.5
AT3G63440	*CKX6*	4.8	6.8	1.2	2.2	1.6	1.3	1.1	1
AT1G19050	*ARR7*	110	36	16	7	12	3	2	8
AT3G29670	Transferase family protein	2.5	3.2	1.3	1.6	1	1.5	1.7	3.4
AT5G48000	Cytocrome P450 family protein	1.2	5.8	0.8	7	1.9	9	1.2	3.2
Cluster 3									
AT3G50800	Expressed protein	2	0.7	1.1	0.3	1.2	0.2	1	0.6
AT2G24600	Ankyrin repeat family protein	1.5	0.4	2	0.8	1	0.2	1.6	0.6
AT5G59820	*ZAT12*	1.2	0.6	0.4	0.3	0.5	0.2	0.4	0.6
AT4G23800	HMG Family potein	1.2	0.5	1.2	0.6	1.6	0.8	0.4	0.7
AT1G61340	F-box family protein	1.1	0.2	0.5	0.3	0.7	0.1	0.4	0.2
AT5G47230	*ERF5*	1.0	0.5	0.3	0.4	0.5	0.3	0.5	0.4
AT5G51190	*ERF105*	0.9	0.2	0.7	0.3	1.1	0.2	3	1.3

In wild-type plants after 120 min of BA treatment, the *ARR16* transcript level was increased by 6.3-fold compared to without BA treatment (Figure 22).Whereas in ARR1-S-8 and *arr1-5* plants, the cytokinin response was reduced by 20% and 48%, respectively, in terms of *ARR16* transcript level change. In *35S:ARR1* plants after 120 min of BA treatment,

the *ARR16* transcript level was increased by 14-fold compared to without BA treatment. This result supports the idea that *ARR16* might be a specific target gene for ARR1. Similarly, the *UGT76C2* gene which encodes a cytokinin-N-glucosyltransferase family protein, the sulfate transporter 3;4 (*SULTR3;4*) gene and a legume lectin family protein (*AT1G53060)* were regulated by cytokinin in WT plants. After 120 min of BA treatment the transcript levels of *UGT76C2*, *SULTR3;4* and lectin were up-regulated by 4-, 9- and 6-fold, respectively, in WT, whereas in ARR1-S-8 plants their response to cytokinin was reduced by 40%, 75% and 60%, respectively (Figure 22).

Recently it has been shown that the *CRF2* (cytokinin response factor 2) was induced by cytokinin in a B-type ARR-dependent manner (Rashotte *et al.,* 2006). Consistent with this, in the present study *CRF2* was also identified as a putative target gene of B-type ARRs (Table XX in Appendix) and this regulation by cytokinin was also confirmed by qRT-PCR. The *CRF2* transcript was increased by 6.1-fold in WT after 15 min BA treatment compared to untreated plants. In contrast, the transcript level in ARR1-S-8 plants was increased by only 1.6-fold—75% less than in WT (Table 16). Similarly, some other important putative target genes of B-type ARRs that were confirmed by qRT-PCR analysis are *CKX4*, *CKX6*, and a transferase family protein. In Table 16, qRT-PCR data for the tested putative target genes was summarized with their fold changes after 15 min and 120 min of cytokinin treatment.

3.2.7 The promoter deletion analysis for identification of binding motifs for ARR1 and/or B-type ARRs

After identifying potential B-type ARRs target genes by microarray analysis (Figure 24) and confirming some of the putative target genes by qRT-PCR (Figure 22), experiments were carried further to identify *cis*-acting elements for ARR1 and B-type ARRs using promoter deletion analysis. It has been shown previously that the DNA-binding domain of ARR1 binds to a specific DNA sequence containing the 5'-GAT(T/C)-3' motif *in vitro* (Sakai *et al.,* 2000). Since the identified sequence was too short and it appears once per 85.3 bp DNA region with a random sequence, Taniguchi et al. (Taniguchi *et al.*, 2007) investigated around the core binding sequence (5'-GAT(T/C)-3') and proposed an extended version of the binding motif for ARR1 (5'-AAGAT(T/C)TT-3'). So far the binding motifs identified for ARR1 and other B-type ARR were supported by *in vitro* binding experiments, where the ARR1 or B-type ARR DNA-binding domain was bound to binding sequences in the *ARR6*

promoter (Sakai *et al.*, 2001, Imamura *et al.*, 2003, Taniguchi *et al.*, 2007). The significance and function of this binding motif was not tested *in planta* till to date. In the present study, attempts were made to investigate the functionality of the known binding motif for ARR1 (5'-AAGAT(T/C)TT-3', from here on it was named „Taniguchi motif'' in this study) and also to identify the new motifs *in planta* by performing promoter deletion analysis using the PTA.

3.2.7.1 The promoter of ARR6 *was selected for promoter deletion analysis*

Among the target genes of B-type ARRs that were identified by microarry and qRT-PCR analysis, a suitable candidate gene promoter for the promoter deletion analysis was searched. It has been shown previously that *ARR6* is a direct target gene ARR1, ARR2 and other B-type ARRs (Sakai *et al.*, 2001; Hwang and Sheen, 2001, Hass *et al.*, 2004; Taniguchi *et al.*, 2007; Argyros *et al.*, 2008; Ishida *et al.*, 2008). In the present study, *ARR6* is present among the putative direct target genes of B-type ARRs (see Table 18 in Appendix). To confirm the microarry data qRT-PCR analysis was done for *ARR6* transcripts in WT and ARR1-S-8 plants. The *ARR6* gene transcript was significantly up-regulated in WT and ARR1-S-8 plants 15 min and 120 min after cytokinin treatment (Figure 23B; Figure 26).

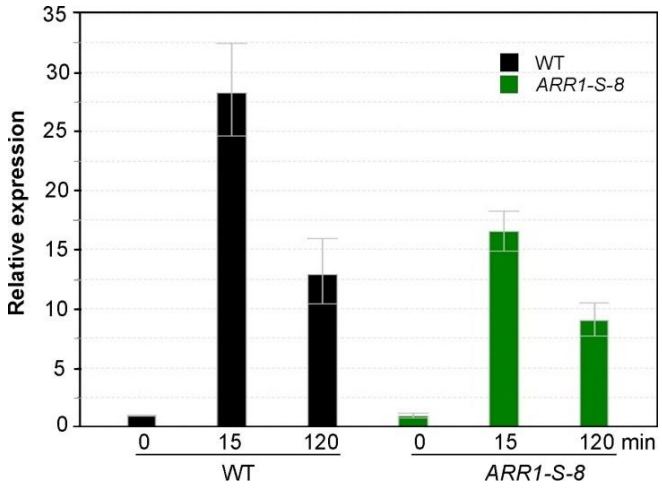

Figure 26: The *ARR6* gene is regulated by B-type ARRs. The relative expression levels of *ARR6* gene transcripts of *ARR1-S-8* plants were compared to WT. The qRT-PCR was performed by using the total RNA extracted from 5-day -old seedlings before and after 15 and after 120 min treatment with 5 µM BA. The *UBC10* and *AT3G25800* genes were used as internal reference control in analysis. Error bars indicate SD.

The *ARR6* transcript level was increased by ~28-fold 15 min after BA treatment in WT compared to untreated plants, whereas in ARR1-S-8 plants the increase in *ARR6* transcript level was only 16-fold (43% reduction in fold change) compared to WT. At the same time, it

has been shown in this study before that the *ARR6* promoter is activated by most of the B-type ARRs in the PTA (Figure 6 and 7). Therefore, the *ARR6* promoter was used for promoter deletion analysis to identify the binding motifs of ARR1.

3.2.7.2 Schematic representation of different deletion constructs used in promoter deletion analysis

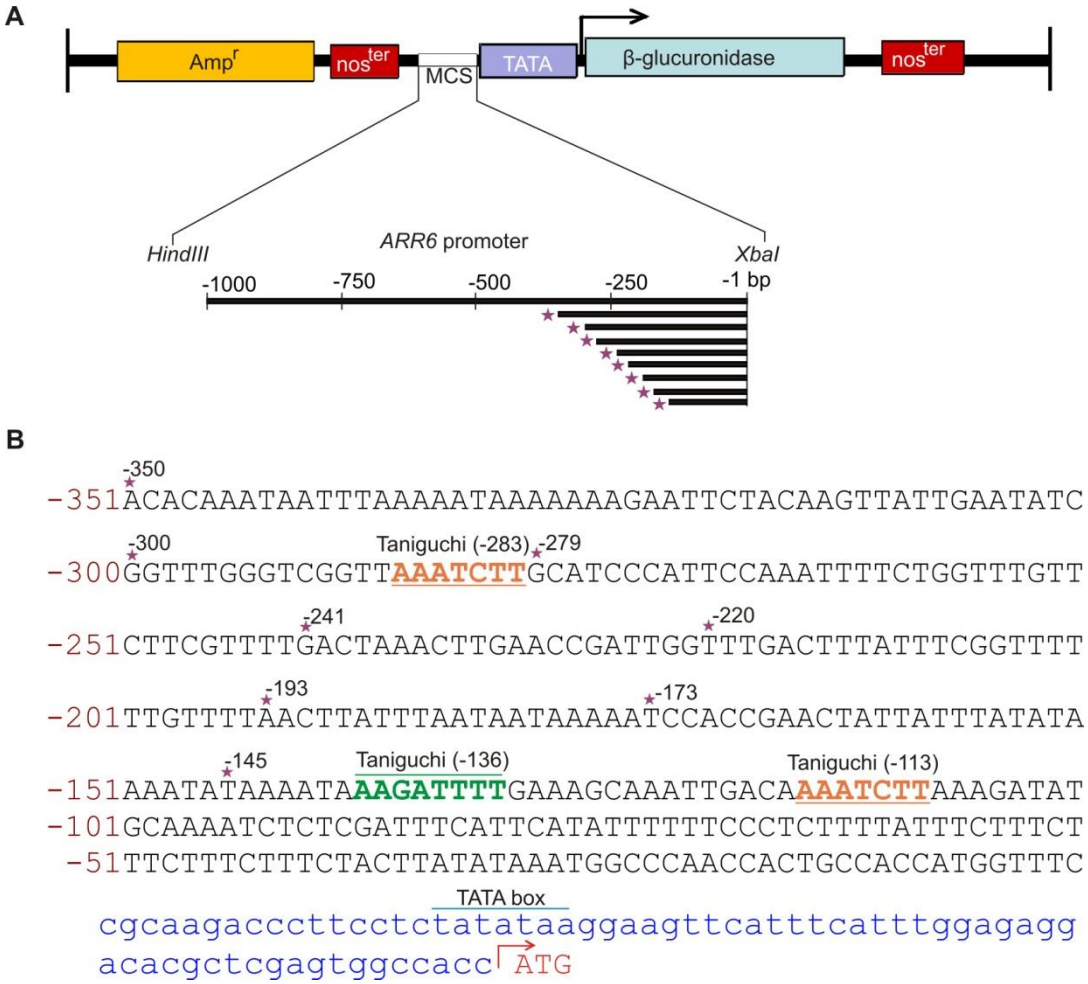

Figure 27: Schematic presentation of a series of the *ARR6* promoter:*GUS* reporter gene constructs used in PTA. A. The *ARR6* promoter fragments were amplified by PCR and verified by DNA sequencing. The fragments were subcloned into the binary vector pBT10:GUS (Sprenger-Haussels and Weisshaar, 2000) by using *HindIII* and *XbaI* restriction sites. Different sizes of the promoter fragments are indicated by ⋆. B. The nucleotide sequence of the *ARR6* promoter up to -350 bp upstream of transcriptional start site. The starting site of different deletion constructs are indicated by ⋆ with their position. The three copies of Taniguchi motifs for ARR1 were marked with coloured letters (orange motif in antisense, green motif in sense direction) and TATA box and start codon of the *GUS* gene translation are also included. MCS, multiple cloning site; Amp^r, ampicillin resistance gene; nos^{ter}, nopaline synthase terminator.

The -1000 bp promoter fragment upstream of the potential transcriptional start of *ARR6*, a direct target gene of B-type ARRs, was fused to the *GUS* reporter gene and cloned into the binary vector pBT10:GUS (Sprenger-Haussels and Weisshaar, 2000; Figure 27A). Upon binding of the B-type ARRs to the binding motif in the *ARR6* promoter region, the *ß-glucoronidase* (*GUS*) gene is transcribed and the quantity of GUS can be estimated by using an enzymatic assay (Kirby and Kavanagh, 2002). In total, nine different deletion constructs were generated and cloned into MCS (Multiple Cloning Site) of the binary vector pBT10:GUS (Figure 27A). The *ARR6* promoter contains three copies of the Taniguchi motif 5'-AAGAT(T/C)TT-3'. Out of three, two were in antisense direction and positioned at -283 bp and -113 bp and the motif in sense direction is positioned at -136 bp (Figure 27B). There is also a half-extended Taniguchi motif (5'-AGATTTT-3') (Taniguchi *et al.*, 2007) present in antisense direction at -95 bp. The PTA experiments were done as described before (Figure 6 and see 2.2.8.8).

3.2.7.3 Deletion analysis revealed that 241 bp fragment of the ARR6 *promoter is sufficient for transcriptional activation*

The promoter deletion analysis was started initially by using four different fragments of the *ARR6* promoter in the PTA. The -1000 bp, -350 bp, -173 bp and -145 bp of the *ARR6* promoter were taken and as an effector *35S:ARR1* was used in the PTA. When the effector plasmid was not used in the PTA, the activation capacity of the native ARR1 and other B-type ARRs on the *ARR6* promoter was very low. The relative GUS/NAN activity was 8 ±0.5 and 7.8 ±0.6 units respectively, when the -1000 bp and -350 bp promoter were used. But the activity was reduced to 50% (4 ±0.3) and 32% (2.5 ±0.5), respectively, when -173 bp and -145 bp promoter fragments were used (Figure 28A). When the *ARR1* effector plasmid was transformed along with the reporter gene construct in the PTA, the activation of the *ARR6* promoter was increased by ~38 fold. The activation capacity of the -1000 bp (262 ±11) was similar to the -350 bp (270 ±23) (Figure 28A), whereas when -173 bp and -145 bp of the promoter were used in the PTA, the activation capacity was reduced to 13.2% and 4.15% respectively compared to the -350 bp promoter (Figure 28A). This result indicates that there is a binding element present between the -350 bp and -173 bp region which is important for the activation of the *ARR6* promoter. Five more deletion constructs (-300 bp, -279 bp, -241

bp, -220 bp and -193 bp) between the -350 bp and -173 bp region of the *ARR6* promoter were made and tested in the PTA (Figure 28B).

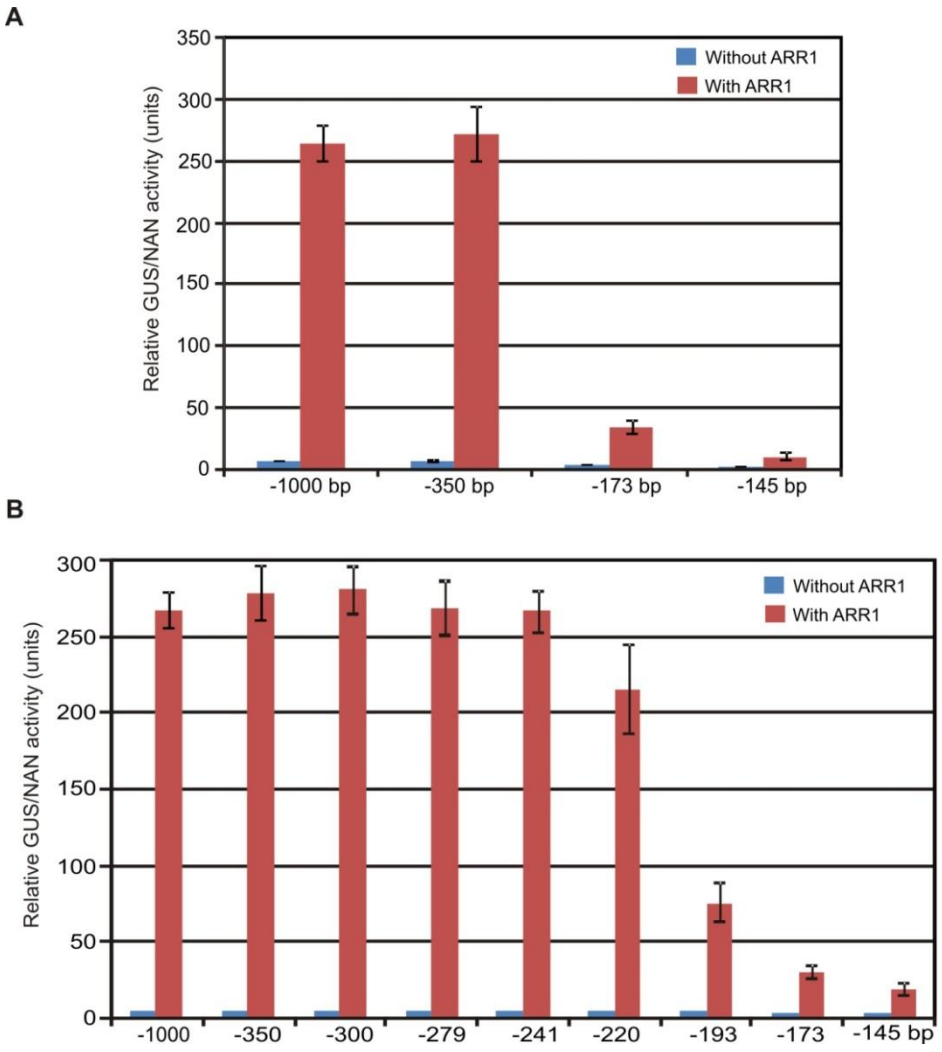

Figure 28: 240 bp of the *ARR6* promoter is sufficient for transcriptional activation. A. Initial deletion analysis revealed that -350 bp of the promoter is enough for total activation of *ARR6*. B. Further deletion analysis showed the importance of the promoter region between -220 bp and -193 bp. Protoplasts were co-transfected with the *ARR6:GUS* reporter and an effector plasmid expressing *ARR1*. The activation of the *ARR6:GUS* reporter gene was measured without and with addition of ARR1. Variations in transformation efficiencies were normalized by using a *35S:NAN* reporter construct. The mean values and SD of four independent transfection assays were calculated and shown as relative GUS/NAN activity units.

The -300 bp (282 ±22), -279 (272 ±21) bp and -241 bp (271 ±16) promoter fragments behaved similar to the -350 bp promoter in their activation capacity (Figure 28B). However, when the -220 bp and -193 bp promoters were used, the activation capacity was reduced to 82% (210 ±29) and 29% (75 ±10), respectively, compared to the -350 bp promoter (Figure

28B). From these results it was evident that 241 bp of the *ARR6* promoter is enough for transcriptional activation of the *ARR6* gene and the activation capacity was strongly diminished when only 193 bp or -173 bp of the promoter were used.

3.7.2.4 The Taniguchi motif alone is not sufficient for the complete transcriptional activation of ARR6

In order to study the importance of the Taniguchi motif (5'-AAGAT(T/C)TT-3') (Taniguchi *et al.*, 2007) in activation of the *ARR6* promoter, substitution mutation were done in this binding motif in a series of *ARR6* promoter fragments. It was shown previously that the core region (5'-GAT-3') of the Taniguchi motif is important for binding of ARR1 and any mutation within the core sequence abolished the DNA-protein interaction (Sakai *et al.*, 2000; Imamura *et al.*, 2003). So a mutant variant of known binding motif at -136 position of *ARR6* promoter was generated by mutating the core sequence in the motif (The nucleotide G and A in Taniguchi motif are substituted by C and T nucleotides, respectively, 5'-AACTTTTT-3, the mutated nucleotide positions are grayed).

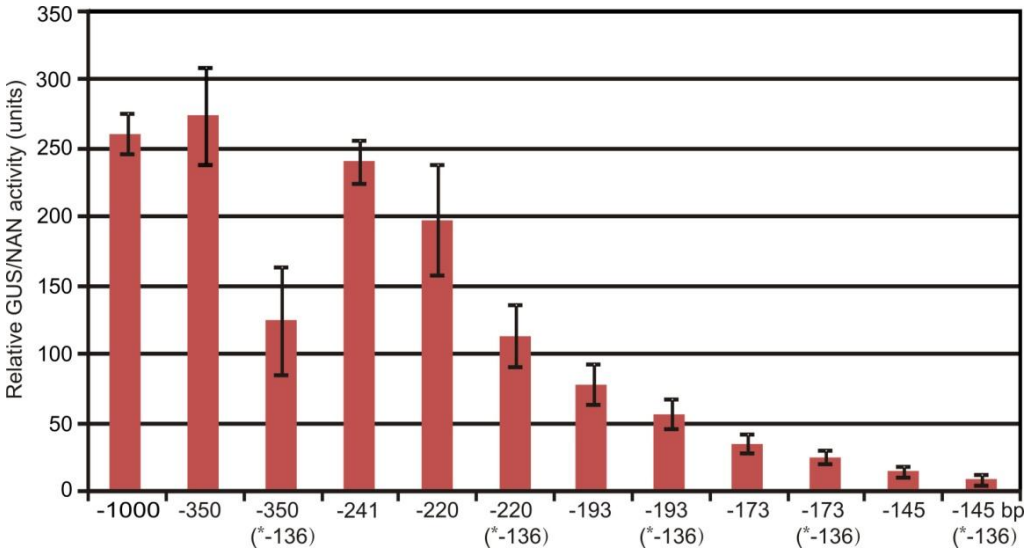

Figure 29: Mutation analysis of the *ARR6* promoter. The Taniguchi motif was mutated by site-directed mutagenesis and the mutated *ARR6* promoter fragments were verified by DNA sequencing. The mutated promoter fragments were indicated by *****. Protoplasts were co-transfected with the *ARR6:GUS* reporter and *ARR1* effector plasmid. Variations in transformation efficiencies were normalized by using a *35S:NAN* reporter construct. The mean values and SD of four independent transfection assays were calculated and shown as relative GUS/NAN activity units.

The binding motif was mutated in different *ARR6* promoter fragments -350 bp, -220 bp, -193 bp, -173 bp and -145 bp. The mutation in the Taniguchi motif reduced the activation of

different *ARR6* promoters by ARR1 to a varying degree. When the mutant variant of the -350 bp *ARR6* promoter was used in the PTA, the transcriptional activation capacity of ARR1 was reduced to ~50% (125 ±39) compared to the non-mutated -350 bp promoter (Figure 29). The reduction in transcriptional activation by ARR1 was also evident when the mutated -220 bp, -193 bp, -173 bp and -145 bp variants of the *ARR6* promoters were used in the PTA. The transcriptional activation capacity of ARR1 was reduced to 58% (114 ±23), 72% (57 ±11), 72% (25 ±4.5) and 60% (9 ±3), respectively, compared to the non-mutated promoters of these fragments (Figure 29). When the non-mutated -193 bp and the -173 bp promoters were used the activation capacity of the ARR1 was reduced to 30% and 13%, respectively, compared to the -350 bp promoter. The reduction of the transcriptional activation caused by the mutation in the Taniguchi motif at -136 bp is less (50%), compared to the reduction in non-mutated -193 bp (70%) and -173 bp (87%) promoters. So it is evident from this result that there might be other binding motifs contributing to the rest of the reduction that was observed in the -173 bp promoter.

3.2.7.5 An important region for the activation of the ARR6 *promoter was identified by deletion analysis*

The mutation in the Taniguchi motif positioned in sense orientation at position -136 bp is not completely abolishing the activation of the *ARR6* promoter by ARR1 (Figure 29). The mutation in the Taniguchi motif positioned in sense orientation at position -136 bp is not completely abolishing the activation of the *ARR6* promoter by ARR1 (Figure 29). Since the *ARR6* promoter contains three copies of the Taniguchi motif, it might be that the other two motifs which are presented in anti-sense orientation are important for the activation of *ARR6* promoter (see Figure 27B). So in this experiment, the core nucleotides of anti-sense Taniguchi motif 5'-AAATCTT-3 positioned at -283 bp of *ARR6* promoter was mutated to 5'-AAAAGTT-3 (the mutated nucleotides are grayed). When the anti-sense Taniguchi motif at position -283 bp was mutated, the transcriptional activation by ARR1 was reduced only by 8% (240 ±9) and 9% (255 ±20) in the -1000 bp and -350 bp promoters, respectively (Figure 30). This result indicates that the antisense motif at position -283 bp is not important for the activation of the *ARR6* promoter by ARR1. Further, the promoter regions between -241 bp and -193 bp (48 bp) and -220 bp and -193 bp (27 bp) were cloned into the pBT10:GUS

vector and tested in the PTA, to investigate whether these 48 bp and 27 bp regions alone are sufficient for the transcriptional activation of *ARR6*.

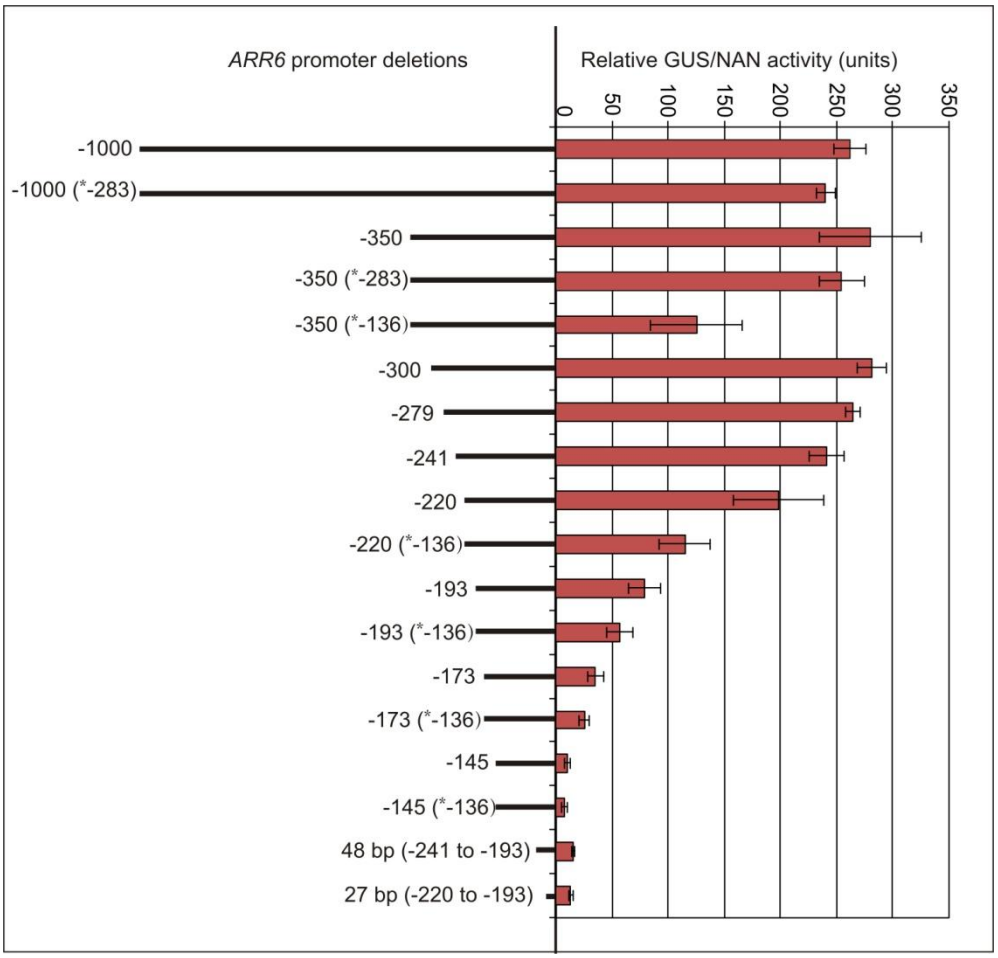

Figure 30: Promoter deletion analysis reveals a new binding region for the activation of *ARR6*. The antisense Taniguchi motif (at position -283 bp) was mutated using site-directed mutagenesis. The mutated promoter fragments were indicated by *****. The promoter regions between -241 bp and -193 bp (48 bp) and -220 bp and -193 bp (27 bp) were amplified by PCR and subcloned into the *pBT10:GUS* vector**.** The results from the previous experiments (Figures 26 and 27) were also plotted in the same graph. All the promoter fragments tested in the PTA were plotted along with their relative GUS/NAN activity units.

When the 48 bp and the 27 bp promoter regions were used in the PTA, ARR1 activated the *ARR6* promoter only by 6% (16 ±1) and 5% (14 ±1), respectively, compared to the total activation that was obtained with the -1000 bp promoter (Figure 30). This result indicates that it may not only be the region but also the position of this region relative to the transcriptional start site that is important for the transcriptional activation of *ARR6*.

3.2.7.6 The newly identified promoter region is also functional for other B-type ARRs

It has been shown before that the specificity of the Taniguchi motif varies to some extent for different B-type ARRs tested *in vitro*. In case of ARR1, the optimum binding was observed when the binding sequence is 5'-AGATT-3', whereas it is 5'-GGATT-3' in case of ARR11(Sakai *et al.,* 2000, Imamura *et al.,* 2003). So it was interesting to check whether the newly identified *ARR6* promoter region between -220 bp and -173 bp has any function in transcriptional activation by other B-type ARRs. To test this, ARR10, ARR19 and ARR21 were chosen as they are representing each of the three sub-groups of B-type ARRs (Figure 5B and 2B). Five different promoter fragments of *ARR6* were used in the PTA to test the transcriptional activation of reporter genes by ARR10, ARR19 and ARR21. The transcriptional activation of the reporter gene by ARR10 and ARR19 was lower compared to ARR1 and ARR21 (Figure 30 and Figure 31). For all B-type ARRs tested the transcriptional activation capacity was similar with the -1000 bp and -350 bp *ARR6* promoters and the degree of activation capacity varied when -220 bp, -193 bp and -173 bp promoters were used (Figure 31). For example, in case of ARR10 the activation of the *ARR6* promoter was reduced to 60% (16.2 ±1.9), 50% (13.3 ±1.6) and 20% (5.4 ±1) respectively, when the -220 bp, -193 bp and -173 bp promoters were used compared to the -1000 bp (26.5 ±2.1) promoter (Figure 31). For ARR10 the mutation in the Taniguchi motif at -136 was not strongly affecting its activation capacity. When the mutant variant of the -350 bp promoter was used, the activation capacity of ARR10 was reduced by 14% (21.5 ±2.7) compared to the non-mutated -350 bp promoter (25 ±3.2).

For ARR19, the activation capacity was reduced to 62% (24.2 ±2.1), 47% (18.3 ±2.5) and 26% (10.3 ±1.8) respectively, for -220 bp, -193 bp and -173 bp promoters compared to the -1000 bp (39 ±3.6) promoter used in the PTA. In addition, for ARR19 also the mutation in known binding motif at -136 position did not strongly reduce its activation capacity of *ARR6* promoter. When a mutated -350 bp promoter was used, the activation of *ARR6* promoter by ARR19 was reduced by 10% (34 ±2.9) compared to the non-mutated -350 bp promoter (36.7 ±3.8). The ARR21 was displayed the strongest activation of *ARR6* promoter compared to the ARR1, ARR10 and ARR19 (Figure 30; Figure 31B). Similar to other B-type ARRs tested, the activation capacity of the ARR21 was also reduced when different fragments of *ARR6* promoter were used.

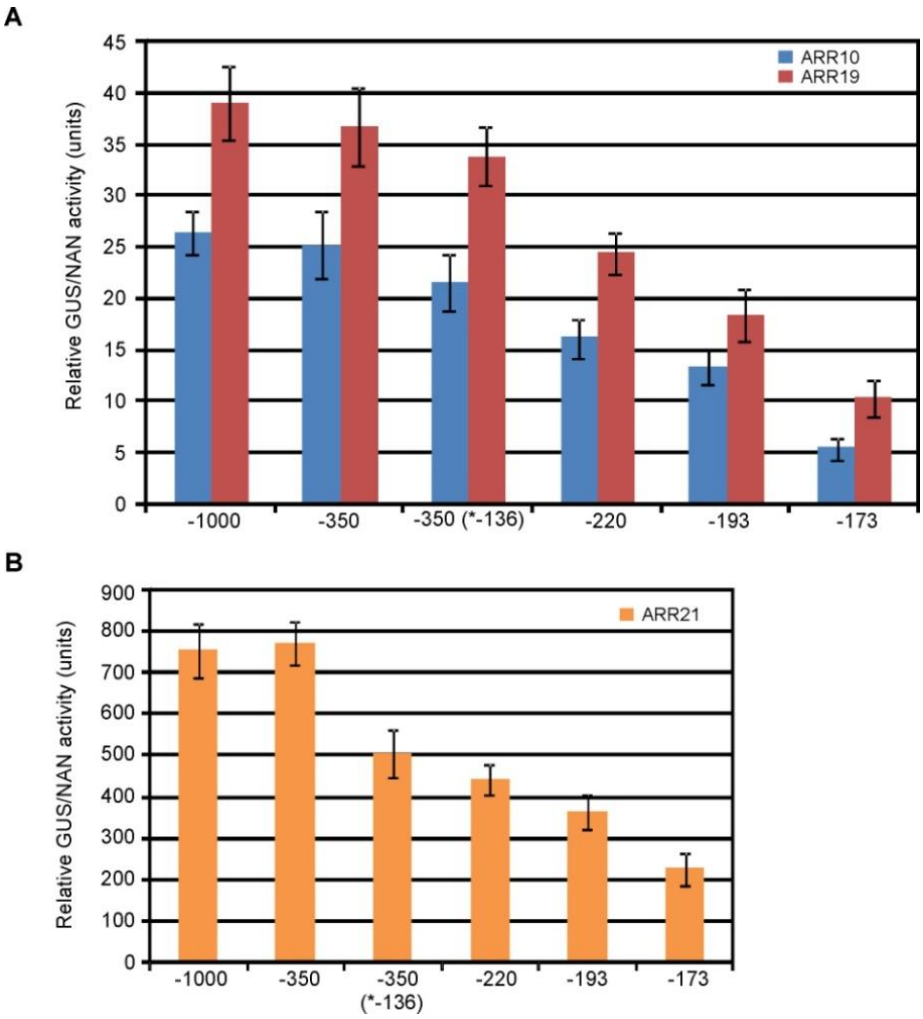

Figure 31: Transactivation capacities of different B-type ARRs. A, B. Protoplasts were co-transfected with the *ARR6:GUS* reporter and the *35S:ARR10, 35S:ARR19* and *35S:ARR21* effector plasmids. The mutated promoter fragments are indicated by *****. Variations in transformation efficiencies were normalized by using a *35S:NAN* reporter construct. The mean values and SD of four independent transfection assays were calculated and shown as relative GUS/NAN activity units.

The ARR21 activation capacity was reduced to 58% (440 ±37), 48% (362 ±41) and 30% (225 ±39), respectively, when the -220 bp, -193 bp and -173 bp promoters used compared to the -1000 bp (752 ±65) promoter. The mutation in the known binding motif reduced its activation capacity by 35 % (502 ±56) compared to when the non-mutated -350 bp promoter was used (770 ±53). In the end these results indicate that the newly identified 27 bp promoter region of *ARR6* is important for transcriptional activation of *ARR6* by ARR1 and other B-type ARRs tested. However the region seems to be more important in case of ARR1 than other B-type ARRs tested. As the the reduction in transcriptional activation from -220 bp to -193 bp is nearly 34% in case of ARR1 it was only 18%, 25%, 18%, respectively, in ARR10,

ARR19 and ARR21 (Figures 30 and 31). So it might be the region for other B-type ARRs might be wider than the identified 27 bp region.

3.2.7.7 Promoters alignment shows conserved positions within the newly identified 27 bp region of the ARR6 *promoter*

Next, the putative target gene promoters of B-type ARRs were investigated to see whether the 27 bp promoter region (between -220 to -193) is conserved. The -1000 bp promoter sequence upstream of A-type *ARR* genes and other target genes of B-type ARRs (Figure 25; Table 16) were aligned by using the Align X program of Vector NTI Advance™. The Align X program aligns the promoter sequences using the ClustalW algorithm (Thompson *et al.,* 1994; Figure 32). In total 18 promoter sequences were aligned to the *ARR6* promoter. For convenience, the alignment around the 27 bp region between -220 bp and -193 bp of the *ARR6* promoter is shown in Figure 32 but not the entire alignment. Some nucleotide sequences within the 27 bp region are very well conserved among the A-type *ARR*s and putative target gene promoters. For example the nucleotide A at position -216 bp of the *ARR6* promoter is identical in every A-type *ARR* promoters (Figure 32A). Similarly, the nucleotide T at the -201 bp,-203 bp, -204 bp and 210 positions is conserved and present in 80-90% of all A-type *ARR* promoters (Figure 32A). Similar to A-type ARR promoters, the nucleotide T at the -200 bp,-201 bp, -204 bp and 210 positions is conserved and present in 70-80% of all promoters of putative target genes (Figure 32B). It is also interesting to note that the the 27 bp regions of the all tested target gene promoters were aligned more or less in similar distance from from the starting of the promoter. This result indicates that the position of the 27 bp regions among the promoters is also conserved (Figure 32). To conclude, preliminary analysis of the multiple promoter alignment revealed that they are some nucleotide positions are conserved among the promoters of the different target genes of B-type ARRs, but no specific binding site consensus was concluded.

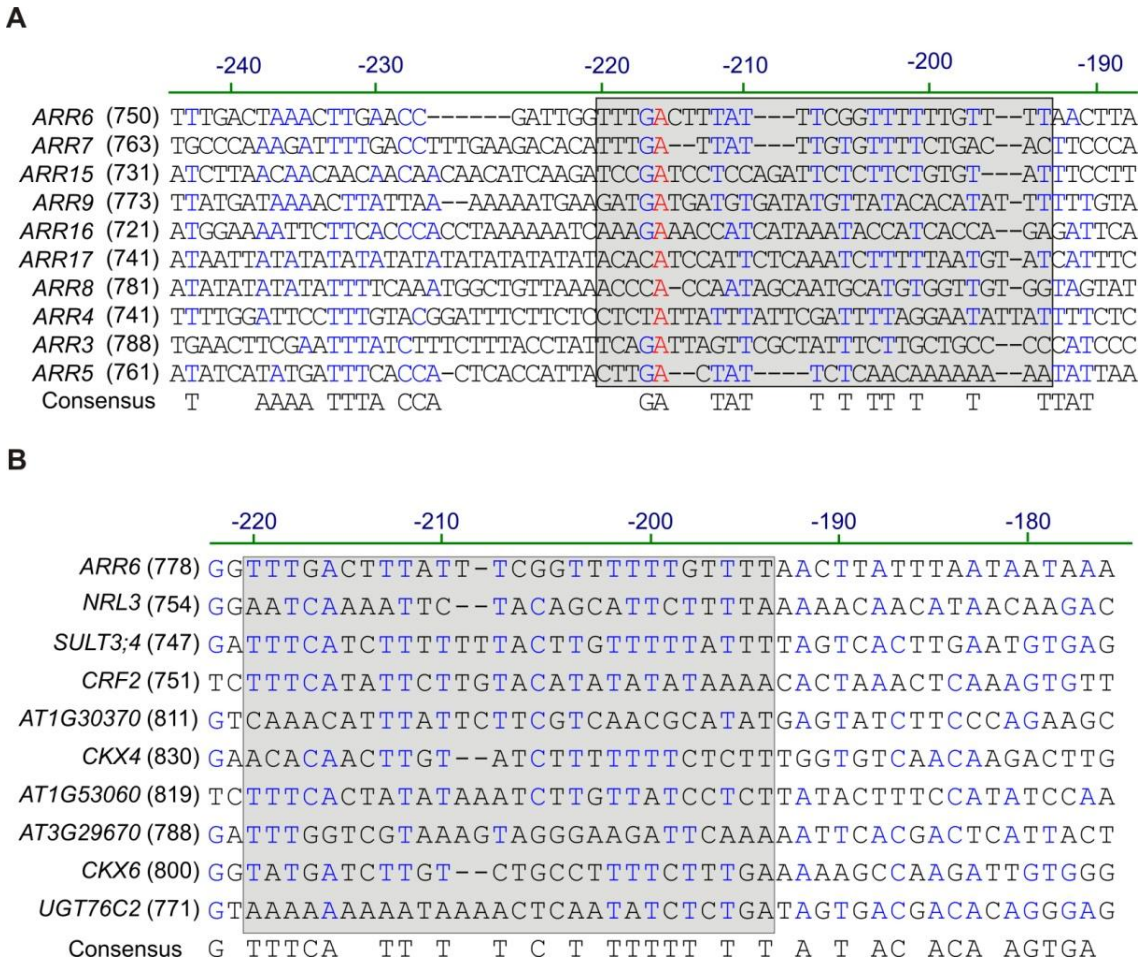

Figure 32: The 27 bp region of *ARR6* promoter share similar nucleotides among the B-type ARRs target gene promoters. A. The multiple alignment of A-type *ARR* promoters. B. The multiple alignment of newly identified B-type ARR putative target gene promoter alignment with the *ARR6* promoter. The -1000 bp promoters up stream of target genes were obtained from TAIR (http://www.arabidopsis.org) and the promoters were aligned by using Align X, of the Vector NTI Advance™ program (http://www.invitrogen.com/) which uses the ClustalW algorithm for multiple sequence alignment. The 27 bp region of the *ARR6* promoter was grayed. The identical nucleotides among the promoters denoted by red coloured letters, and the strong similarity in nucleotides were indicated by blue coloured letters. The position of nucleotide sequences of *ARR6* promoter was numbered on top of the alignment. For every promoter the postion of the first nucleotide in a 1000 bp promoter sequence of the respective gene in the alignment is numbered in parenthesis.

3.2.7.8 The promoters of some A-type RRs from poplar and rice show conserved positions within the 27 bp region of the ARR6 *promoter*

Functionally relevant DNA sequences are conserved between genes within species and among homologous genes across the species. In particular, DNA elements recognized by the same or similar proteins are well conserved in orthologous regulatory regions (Stormo, 2000). So the analysis of cross-species sequence conservation is an effective way for improving selectivity of detection of functional elements in DNA sequences (Sandelin *et al.*,

2004). Since the alignment of putative target gene promoters of B-type ARRs from *Arabidopsis* showed some conservation in the newly identified 27 bp region of *ARR6* promoter (Figure 32), it was interesting to analyze how much conservation was observed among orthologous *ARR6* gene promoters. Poplar (*Populus trichocarpa)* and rice (*Oryza sativa* L. ssp. *Japonica*) representing dicots and monocots, were chosen for comparison. In poplar, the components of the cytokinin signalling pathway were identified and seven A-type *RR*s (Response regulators) were shown as rapidly induced by exogenous cytokinins (Tuskan *et al.*, 2006; Ramírez-Carvajal *et al.*, 2008). Similarly, in rice also the cytokinin signalling components were identified and A-type *RR*s were shown to be immediate response genes to cytokinins (Jain *et al.*, 2006; Du *et al.*, 2007).

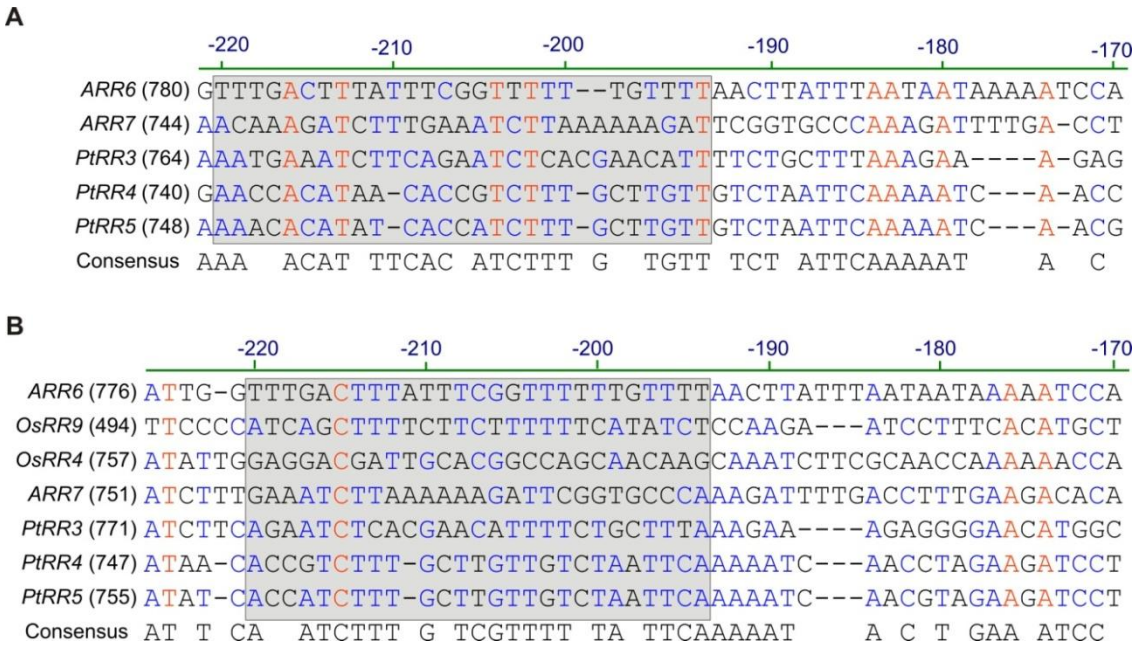

Figure 33: Some nucleotide positions within the 27 bp region of *ARR6* promoter is conserved among the A-type *RR* genes of poplar and rice. A. Promoter alignment of some A-type *RR* genes of *Arabidopsis* and poplar. B. Promoter alignment of *ARR6* promoter with A-type *RR*s of poplar and rice. The 1000 bp promoters upstream of the *RR* genes were obtained from respective genome data bases of *Arabidopsis*, poplar and rice and the promoters were aligned by using Align X of the Vector NTI Advance ™ program (http://www.invitrogen.com/). The 27 bp region of the *ARR6* promoter was grayed. The identical nucleotides among the promoters are indicated by red letters, and a strong similarity in nucleotides is indicated by blue letters. The position of nucleotide sequences of the *ARR6* promoter numbered on top of the alignment for the convenience in understanding. For every promoter the postion of the first nucleotide in the alignment is numbered in parenthesis.

The ARR6 protein sequence was blasted against protein data bases of rice and poplar genomes and the closest orthologs of ARR6 were selected for this study. Since *Arabidopsis* and *Populus* are dicots and more closely related to each other compared to rice which is a

monocot, the promoter alignment of *Arabidopsis* and *Populus* promoters alone may give more information. So the promoters of *ARR6* and *ARR7* were aligned initially with three A-type *RR* genes of poplar, namely *PtRR3*, *PtRR4* and *PtRR5* (Figure 33A). The nucleotide positions at -194, -202 bp, -204 bp -213 bp and -216 bp within the 27 bp regions of the *ARR6* promoter were identical among the three promoters of poplar (Figure 33A). Similarly, the nucleotides at positions -200 bp, -202 bp, 203 bp -212 and 214 bp were 90% similar among the promoters in the alignment (Figure 33A). Subsequently the *ARR6* and *ARR7* promoters were aligned with the closely related orthologs of A-type *RR* genes of poplar and rice. The conservation among the promoters is not as good as it was observed between *Arabidopsis* and *Populus* alone (Figure 33A). The nucleotide C at position -215 bp was identical in all the promoters in the alignment, and the nucleotide T at positions -212 bp, -213 bp, -214 bp, -204 bp and -202 bp were 80-90% similar among the promoters of *Arabidopsis*, *Populus* and rice (Figure 33B). The result indicates that six nucleotide positions within the 27 bp regions of the *ARR6* and *ARR7* promoters of *Arabidopsis* and *ARR6* ortholog gene promoters from poplar and rice are conserved.

3.2.7.9 Distribution of putative new binding motif among the known target genes of B-type ARRs and A-type RR *genes of poplar and rice*

The multiple alignment of B-type ARR target gene promoters and *ARR6* orthologous gene promoters from poplar and rice showed that the conservation within the 27 bp region of the *ARR6* promoter but no particular binding site of hexamer or hectamer size was conserved. A sliding window analysis was done among the -1000 bp promoters of all A-type *ARR*s, by taking the 27 bp region of *ARR6* as a search reference (the sliding window analysis was done by Dr. Wolfram Brenner, data not shown). The sliding window analysis indicated that a hexamer 5'- TTTATT-3 and a heptamer 5'-TTTTGTT-3 are present within the 27 bp region and in the promoters of all A-type *ARR*s. In the putative heptamer binding motif 5'-TTTTGTT-3 which is spanning the positions -202 bp to -196 bp of the *ARR6* promoter, the second and fourth T of the heptamer were identical and very well conserved among the orthologous gene promoters (Figure 33A and B). Considering this information from sliding window analysis and promoter alignments, target gene promoters of the B-type ARRs were searched for a consensus motif by using the TOUCAN 2 program (http://www.esat.kuleuven.ac.be/~saerts/software/toucan.php; Aerts *et al.,* 2005). The

TOUCAN 2 programme allows searching for a consensus motif among the given promoters and also identifies *de novo* motifs.

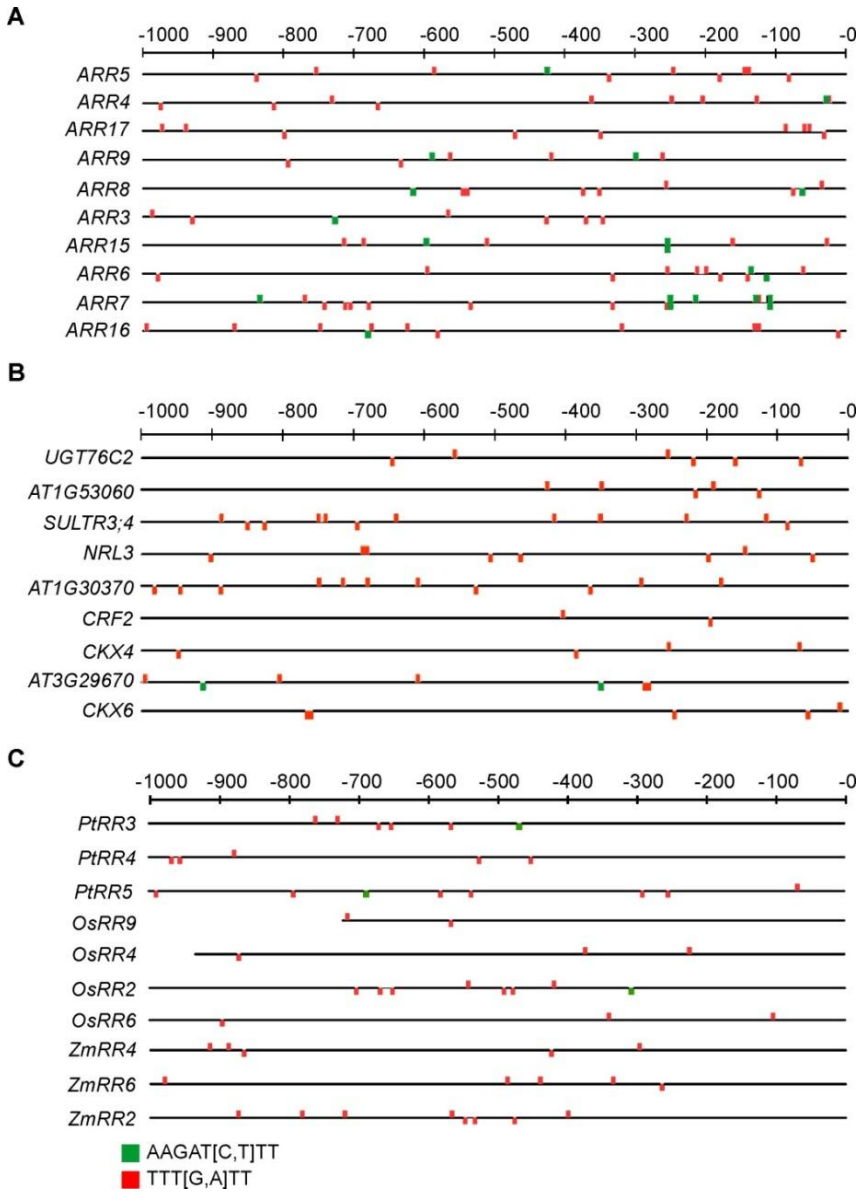

Figure 34: The distribution of putative new binding motifs among the promoters of B-type ARR target genes. The Taniguchi and the putative new binding motif distribution in the promoters of A) A-type *ARR* genes of *Arabidopsis*, B) newly identified putative target genes of B-type ARRs and C) known A-type *RR* genes of poplar, rice and maize. The distribution of motifs was done by using TOUCAN 2 program (http://www.esat.kuleuven.ac.be/~saerts/software/toucan.php; Aerts *et al.*, 2005). The 1000 bp promoter was shown as a single dark line and the binding motif in sense orientation is placed on the top of the line and anti-sense orientation motif placed on the bottom of the line. The Taniguchi and putative new binding motifs were marked with green and red colour respectively.

The promoters of A-type *ARR* genes, newly identified B-type ARRs putative target genes and A-type *RR*s from poplar, rice and maize were searched for the presence of Taniguchi

motif (5'-AAGAT(T/C)TT-3') and a hexamer consensus sequence (5'-TTT(G/A)TT-3'). This hexamer consensus sequence was derived from the identified hexamer and heptamer from the sliding window analysis, which were present in all A-type *ARR* genes promoters. The new hexamer consensus sequence was named as putative new binding motif of B-type ARRs (5'-TTT(G/A)TT-3') (Figure 34). In all A-type *ARR*s except in *ARR17* both Taniguchi motif of ARR1 and the putative new binding motif were present (Figure 34A). The putative hexamer binding motif occurs more often than the known octamer binding motif within the -1000 bp promoters. The reason for this is that the statistical probability for appearance of a hexamer compared to an octamer is more in a 1000 bp sequence. In case of B-type ARR putative target gene promoters the putative new binding motif was present in all promoters, whereas Taniguchi motif was present only in one gene (*AT3G29670,* which encodes a transferase family protein) out of the nine genes under study (Figure 34B). The *ARR6* ortholog gene promoters from poplar, rice and maize also contain the putative new binding motif frequently compared to the Taniguchi motif (Figure 34C). The Taniguchi motif is present in two A-type *RR* genes of poplar (*PtRR3* and *PtRR5*) and in one response regulator gene of rice (*OsRR2*) but not present in any of the A-type RR genes from maize under study (Figure 34C). A detailed analysis is needed to check for the overrepresentation of the putative binding motif among the putative target gene promoters and random promoters. The functionality of the putative new binding motif in gene activation needs also to be tested *in planta* using the PTA

3.2.8 Elucidating the functional context of ARR1 by protein–protein interaction studies

To get more insights into cytokinin signalling at protein level, protein-protein interactions studies were performed between the components of cytokinin pathway and other signalling pathways (Dortay *et al.*, 2006, 2008). In the present study, attempts were made to get potentially relevant information on the regulation of ARR1 activity by characterizing the proteins that were identified to interact with ARR1 (Dortay *et al.*, 2008).

3.2.8.1 Confirmation of identified ARR1 interactors by yeast two-hybrid analysis

Several interacting partners for ARR1, ARR2, ARR10 and ARR14 were identified by yeast two-hybrid screens and were reported previously (Dortay *et al.*, 2006, 2008). Some of the interesting interacting partners of ARR1 include VIP1 (VirE2 Interacting Protein 1,

AT1G43700), ERF8 (Ethylene Responsive element binding Factor 8, AT1G53170), RPN9 (a 26S proteasome regulatory subunit, AT5G45620), and the IAA30 (Indole-3-Acetic Acid inducible 30, AT3G62100) protein (unpublished data from Dortay *et al.*,). In the present thesis VIP1 and ERF8 were chosen for further study. VIP1 is identified as a basic region/leucine zipper (bZIP)-like putative transcription factor and plays a vital role in the transportation of *Agrobacterium* T-DNA into the nucleus of the host cell (Tzfira *et al.*, 2002). In the transportation of T-DNA, VIP1 functions as an adopter between VirE2, a Vir (virulence) protein encoded by *Agrobacterium* and the nuclear-import machinery of the host-cell (Tzfira *et al.*, 2002; Li *et al.*, 2005). ERF8 is a member of ERF/AP2 transcription factor family whose function is not characterized so far. Since VIP1 and ERF8 were identified as interacting partners of ARR1 in a yeast two-hybrid screens, to reconfirm the interaction *in vivo* the yeast two-hybrid system was used. The full length cDNA clones of *ARR1* and *VIP1* and *ERF8* were cloned into prey and bait vectors, respectively. The interactions were tested using two different reporter genes (*HIS* and *URA*), and growth was evaluated by transferring the cell suspension of the respective clones on SDII and SDIV media in a series of dilution steps (Figure 35). The growth of the cell suspension on SDIV media supplemented with 5 mM 3AT confirmed the interactions of ARR1 with VIP1 and ERF8 (Figure 35).

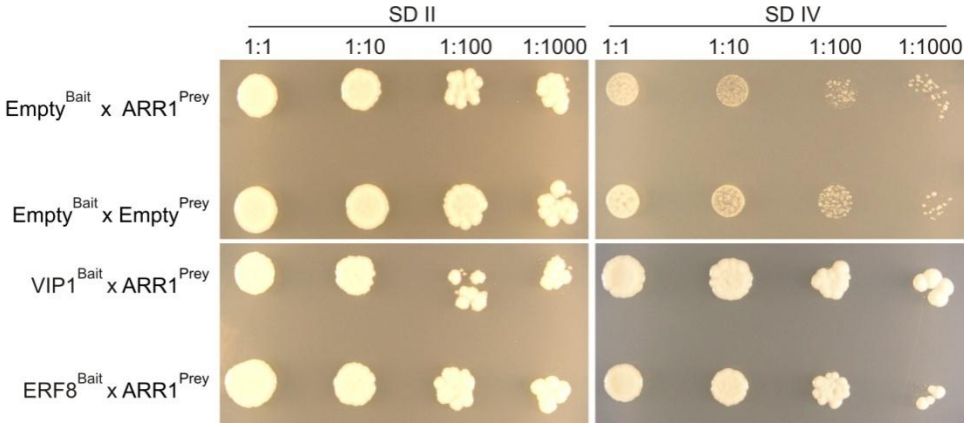

Figure 35: Confirmation of interaction of ARR1 with VIP1 and ERF8. Yeast two-hybrid analysis was performed for putative interacting partners of ARR1. ARR1 and its putative interacting partners ERF8 and VIP1 were transformed in prey and bait vectors respectively. A cell suspension culture in sterile water with OD_{600} of 0.2 was prepared for each clone and 10 µl of each cell suspension was transferred on SDII control (+his, +ura) and SDIV interaction (-his, -ura) media supplemented with 5 mM 3AT. Series of dilutions (1:10, 1:100 and 1:1000) of each cell suspension were prepared and transferred on SDII and SDIV interaction medium.

The interaction of ARR1 and VIP1 was confirmed in both possible bait-prey combinations. The interaction with ERF8 was only possible in one direction, because ERF8 showed strong

autoactivating activity when it was cloned in the prey plasmid (data not shown). The specificity of these interactions among the B-type ARRs needs to be tested.

3.2.8.2 BiFC experiments confirmed the interaction of ARR1 with VIP1 and ERF8 in planta

In the next step, the *in vivo* confirmed interaction of ARR1 with VIP1 and ERF8 were tested for their interaction *in planta* by using BiFC (Bimolecular Fluorescent Complementation). In BiFC, the visualization of protein–protein interaction is possible in living plant cells and the usefulness of BiFC in detecting protein-protein interaction in plant cells was described in several studies (Bracha-Drori *et al.*, 2004; Walter *et al.*, 2004; Citovsky *et al.*, 2006).

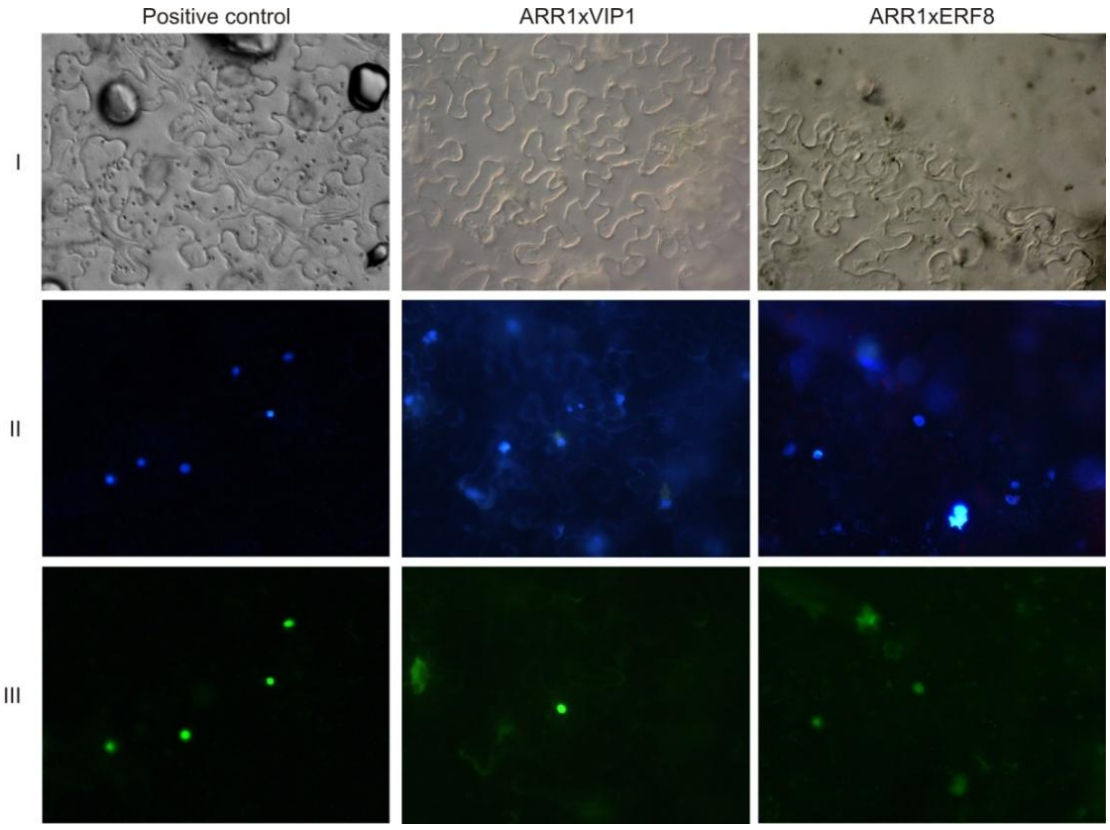

Figure 36: Visualization of ARR1 interaction with VIP1 and ERF8 in living plant cells of tobacco leaves. I) Bright field, II) DAPI filter and III), epifluorescence images of tobacco epidermal leaf cells. The tobacco leaves were infiltrated with a mixture of *Agrobacterium* suspensions harboring constructs encoding pSPYCE-ARR1, and pSPYCNE-VIP1 and pSPYCNE-ERF8 fusion proteins. For technical details of infiltration and microscopy see section 2.2.3.3 and 2.2.9 of Materials and Methods. For visualization of nuclei DAPI staining was performed.

To test the *in planta* interactions of ARR1, the cDNAs of *ARR1*, *VIP1* and *ERF8* were cloned into the BiFC binary vectors pSPYCE-35S and pSPYNE-35S, respectively (Walter *et*

al., 2004). As a positive control for the experiment, bZIP63 protein (AT5G28770), which is a member of the *Arabidopsis* bZIP factor family, was used. It was shown before that the bZIP63 protein has the ability to form homodimers and is localized to the nucleus of the plant cells (Walter *et al.,* 2004). The different constructs were delivered into epidermal leaf cells of tobacco (*Nicotiana benthamiana*) by *Agrobacterium* infiltration (Walter *et al.*, 2004; Witte *et al.*, 2004). Four days after the infiltration, the epidermal layer of the tobacco leaves were peeled and stained with DAPI for visualization of the nuclei. The fluorescence signals of the specimens were observed using the epifluorescence microscope**.** As expected, in the positive control 80% of the epidermal cells which had been infiltrated with *Agrobacteria* carrying pSPYNE-bZIP63 and pSPYCE-bZIP63 showed BiFC-induced fluorescence in the nucleus (Figure 36). The ARR1 interaction with VIP1 and ERF8 was confirmed *in planta* as BiFC-induced fluorescence was observed. In both cases the interaction was localized to the nucleus (Figure 36). In summary, the ARR1 interaction with VIP1 and ERF8 was confirmed both in yeast and *in planta*. The biological significance of these interactions needs to be tested by genetic studies.

3.3 CRES-technology is a valuable tool to study the functional role of gene families

The genomic sequence information from several plant species revealed that plants have similar sets of transcription factors and their function is conserved across the species (Maizel *et al.,* 2005). In *Arabidopsis* several genetic studies revealed functional redundancy among the *RR*s of the cytokinin signalling pathway (Sakai *et al.,* 2001; Hass *et al.,* 2004; Mason *et al.,* 2005). The CRES-T used in the present study to overcome the functional redundancy of B-type ARR family can be useful as a tool to understand the role of transcription factors wherein /if the functional redundancy is a problem. Attempts were made in the present study in order to prove that the CRES-T, can effectively knockdown many of the genes within the functional redundant gene family. At the same time to test the effectiveness of the CRES-T *in planta* across the different species the *35S:ARR1-SRDX* transgenic tomato plants were generated and their morphological phenotype was partly characterized.

3.3.1 *35S:SRDX-ARR1* transgenic plants show a similar phenotype as *35S:ARR1-SRDX* plants

It was already shown in the present study that the ARR1-SRDX protein can suppress the transcriptional activation capacity of several B-type ARRs in the PTA (Figure 7).

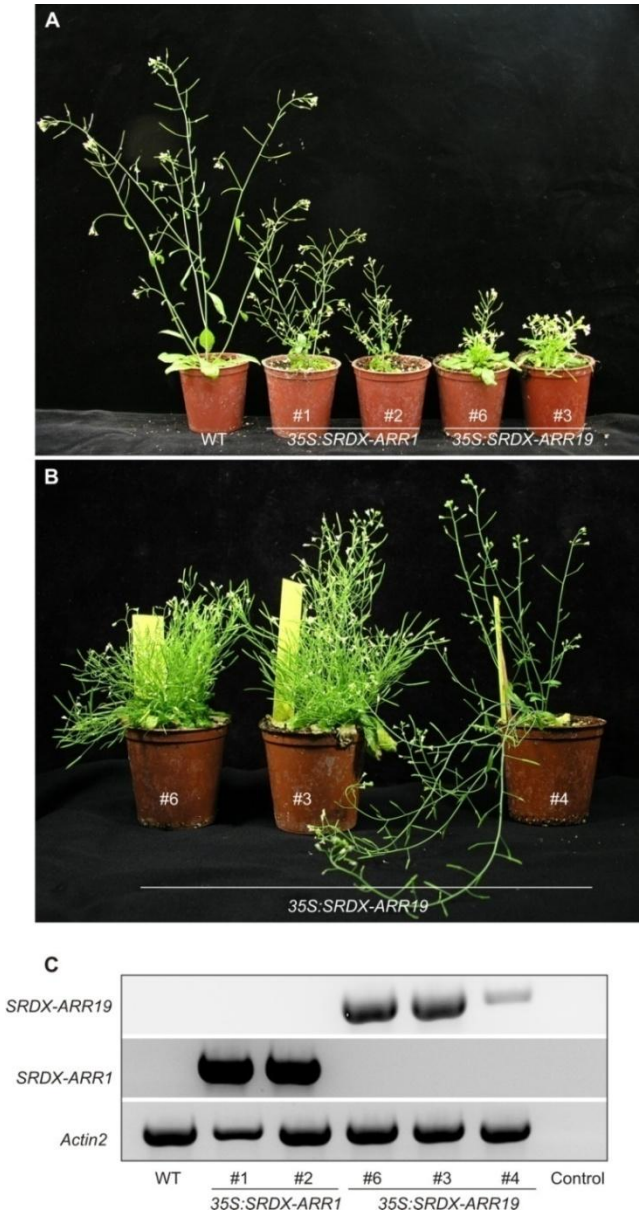

Figure 37: Characterization of *35S:SRDX-ARR1* and *35S:SRDX-ARR19* transgenic plants. A. The shoot phenotypes of the N-terminal SRDX-ARR1 and SRDX-ARR19 plants were compared with WT plants. Photographs were taken 30 DAG. B. Shoot phenotype of different independent homozygous lines of *35S:SRDX-ARR19* plants. Photographs were taken 45 DAG. C. Analysis for transcript levels of *35S:SRDX-ARR1* and *35S:SRDX-ARR19* in different transgenic lines. Total RNA was isolated from 3-4-week-old transgenic plants and RT-PCR was performed using specific primers to analyze the transgene transcript levels. *Actin2* was used as loading control in the experiment.

To test whether the observed phenotype of *35S:ARR1-SRDX* transgenic plants is representing the compound knockout phenotype of whole B-type ARRs or some of B-type ARRs, the *35S:SRDX-ARR1* and *35S:SRDX-ARR19 Arabidopsis* transgenic plants were generated and compared with the *35S:ARR1-SRDX* plants for their morphological phenotypes. The ARR19 was selected for this study, because it is distantly related to ARR1 and belongs to a separate subgroup within the B-type ARRs family (see Figures 2B and 5B). A Gateway™ compatible N-terminal SRDX vector was prepared by Dr. Wolfgang Dröge-Laser group from Göttingen University, Germany. This vector facilitates easy shuttling of any transcription factor of interest and is helpful in functional studies of the transcription factor.

First, the N-terminal SRDX-ARR1 plants were compared with the C-terminal ARR1-SRDX plants, to determine whether the position of SRDX domain influence the ARR1 function. The transgenic *35S:SRDX-ARR1* plants look similar to the *35S:ARR1-SRDX* plants with respect to their shoot phenotype (data not shown). The phenotype of line #4 of the *35S:SRDX-ARR19* plants was also similar to the phenotype exhibited by the *35S:ARR1-S-10* plants, whereas the strongest phenotype of the *35S:SRDX-ARR19* plants differed in some aspects compared to the *35S:SRDX-ARR1* transgenic plants (Figure 37B). In the strongest phenotype *35S:SRDX-ARR19* plants, the apical dominance was completely lost and a higher number of lateral branches was observed (Figure 37B). The seed size of the *35S:SRDX-ARR19* plants was bigger than the WT seeds as in 35S:ARR1-SRDX plants (date not shown). Further, the selected independent homozygous transgenic lines of *35S:SRDX-ARR1* and *35S:SRDX-ARR19* displayed a varying degree of a similar phenotype, which was distinct from the wild type (data not shown). To know whether the different degrees of phenotype of the transgenic plants was due to different levels of expression of the transgene, the transgenic plants were analysed for the levels of the *SRDX-ARR1* and *SRDX-ARR19* transcripts, in the T_2 generation (Figure 38C). The signal strength of line #1 and #2 of the *35S:SRDX-ARR1* transgenic plants correlated with the stronger morphological phenotypes displayed by them (Figure 38C). Likewise, the signal strength of line #6 and line #3 were higher compared to line #4 of the *35S:SRDX-ARR19* plants and correlated with the stronger morphological phenotypes that were displayed by #6 and #3 (Figure 38C).

3.3.2 Generation of transgenic 35S:ARR1-SRDX tomato plants

To test whether the targets of the B-type RRs are conserved across the species, apart from *Arabidopsis*, the *35S:ARR1-SRDX* transgenic tomato plants were generated. During this process, the CRES-T is used as a tool to study the reduced cytokinin status/signalling in other plant species.

Figure 38: Characterization of *35:ARR1-SRDX* and *35S:ARR1* transgenic tomato plants. Shoot phenotype of transgenic plants. A. *35:ARR1-SRDX* and *35S:ARR1* tomato transgenic plants were compared to WT with respect to their shoot phenotype. B. Two independent lines of *35:ARR1-SRDX* transgenic tomato plants showed similar phenotype with each other compared to the WT. Photographs was taken 38 DAG. C. Expression analysis for *ARR1-SR*DX and *ARR*1 transcript levels. Total RNA samples were isolated from six-weeks-old plant leaves and the transcripts were amplified by using specific primers for *ARR1-SRDX* and *ARR1*genes in a RT-PCR. *Actin2* was used as loading control in analysis.

The tomato (*Solanum lycopersicum*) plants were used a model system for this purpose as it possess some important traits such as fleshy fruit, sympodial shoots and compound leaves, which *Arabidopsis* does not have. The tomato cultivar Micro-Tom was used in this study as it has been widely used in several functional genomics studies of tomato (Meissner *et al.*, 1997; Mathews *et al.*, 2003; Dan *et al.*, 2006). The *35S:ARR1-SRDX* and *35S:ARR1* constructs, that were used before in this study were transferred to the binary vector pK2GW7 which contains kanamycin as selection marker. The stable transformation of tomato was done according to Sun *et al.* (Sun *et al.*, 2006; and see section 2.2.3.2 for detailed description). The cotyledons of 8-9-day-old seedlings were used as explants and transformed with *Agrobacterium* suspension mixture containing *35S:ARR1-SRDX* as well as *35S:ARR1* constructs. Several independent calli were observed for both constructs and shoots regenerated from these calli were transferred to soil and grown in a green house.

In the next generation, three independent *35S: ARR1-SRDX* plant lines were characterized. Similarly, two independent *35S:ARR1* plant lines were characterized (Figure 38). Similar to *Arabidopsis*, the *35S:ARR1-SRDX* transgenic tomato plants displayed stunted shoot growth and the leaf size was reduced compared to WT and *35S:ARR1* transgenic tomato plants (Figure 38A and B). The phenotype that was observed in these transgenic tomato plants suggests that the dominant-negative ARR1-SRDX suppresses the B-type RR mediated cytokinin signalling pathway in tomato. Next the transcript levels of the *ARR1-SRDX* and *ARR1* genes were analysed in the transgenic tomato plants (Figure 38C). The signal strength of line #1 and line #2 was highest among the tested lines of *35S:ARR1-SRDX* transgenic plants and correlated with the strong morphological phenotypes they displayed. Similarly, two independent lines of *35:ARR1* tomato plants, line #3 and line #5 showed higher levels of *ARR1* transcript levels and both displayed similar phenotype with each other (Figure 38A and C).

3.3.3 The *35S:ARR1-SRDX* tomato plants leads to a parthenocarpic fruit growth

The fruit setting is an important step in the development of all sexually reproducing higher plants. In general, the fruit set is induced after pollination and successful fertilization of the egg cells in the ovules (Gillaspy *et al.*, 1993). In tomato plants, the fruit originates from the ovary and auxin is known as a key regulator of ovary growth derepression during fruit set (Goetz *et al.*, 2006; Pandolfini *et al.*, 2007). The phytohormone gibberellin (GA)

also plays an important role in fruit set as it had been shown that the application of GA to tomato flowers leads to parthenocarpic fruit growth (Fos *et al.*, 2000).

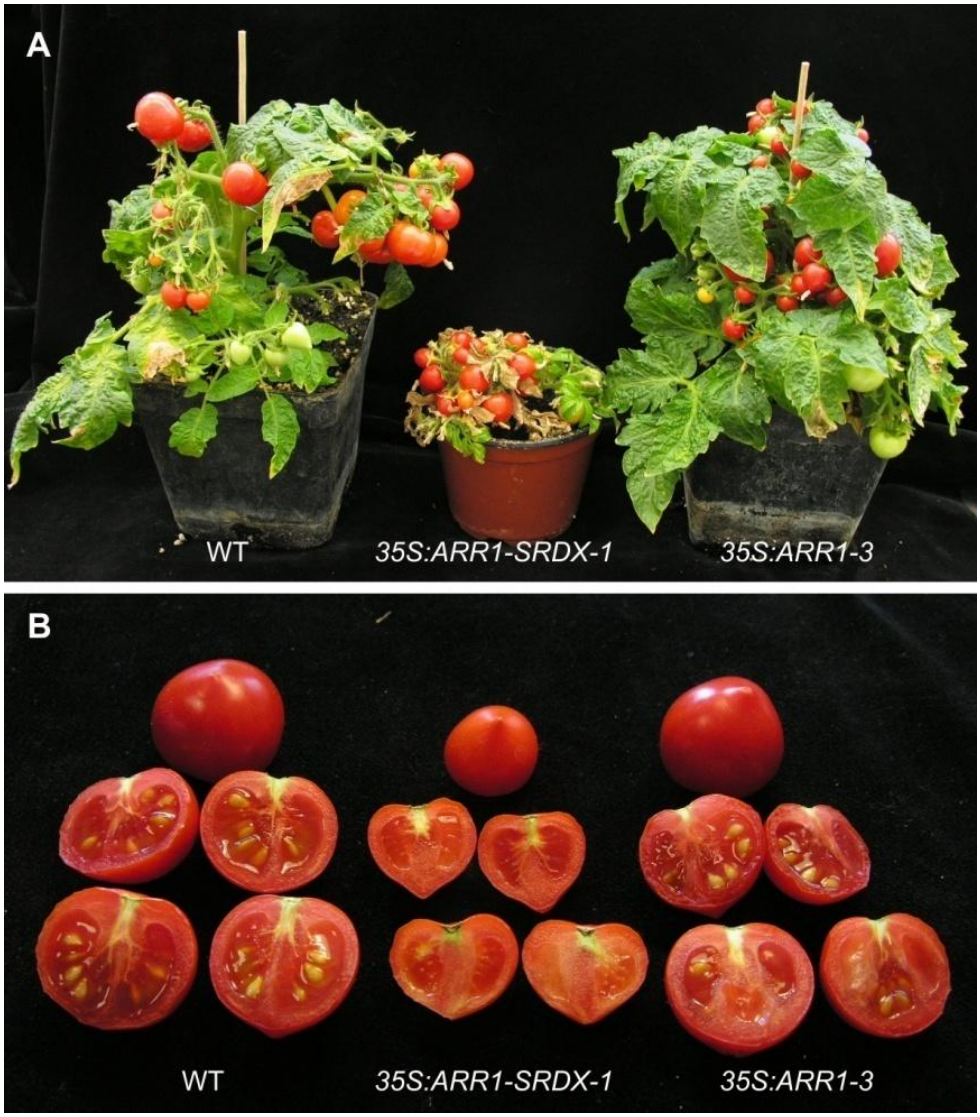

Figure 39: The transgenic *35:ARR1-SRDX* tomato plants produce seedless tomato fruits. A. The fruit setting and development in transgenic plants. Photographs were taken 80 DAG. B. The wild-type and transgenic plant fruits were dissected and compare for fruit size and seed number. The *35:ARR1-SRDX* plants produce small and seedless fruits compared to WT and *35S:ARR1* plant bigger and seeded fruits.

It has been shown before that other phytohormones such as cytokinin, ethylene and brassinosteroids either affect and/or are involved in fruit initiation and development (Nitsch, 1970; Vriezen *et al.*, 2008). However the precise actions and interaction of cytokinins with other hormones in regulating normal fruit development is not well understood. So the *35S:ARR1-SRDX* transgenic tomato plants can be useful to study the role of cytokinins

during the fruit development. Even though the *35S:ARR1-SRDX* tomato plants have stunted shoots, their fruit set was not disturbed and the number of fruits per branch was similar compared to the *35S:ARR1* transgenic plants and WT plants (Figure 39A). The fruits of the *35S:ARR1-SRDX* tomato plants ripened faster compared to *35S:ARR1* transgenic plants and WT plants (data not shown). The *35S:ARR1-SRDX* tomato plants produced smaller size fruits compared to the WT and *35S:ARR1* tomato plants. When the fruits were dissected there was no seed set observed in the *35S:ARR1-SRDX* plant fruits, where as seed set was present in *35S:ARR1* plant fruits similar to the WT plant fruits (Figure 39B). This result indicates that the *ARR1-SRDX* mediated suppression of the B-type RRs in tomato, leads to the development of seedless fruits.

4.0 Discussion

4.1 The dominant negative *35S:ARR1-SRDX* plants are valuable tools for investigating B-type ARR functions

Several members of the B-type ARRs are involved in mediating cytokinin signalling (Imamura *et al.,* 2003; Tajima *et al.,* 2004; Mason *et al.,* 2005; Yokoyama *et al.,* 2007). However, because of a high degree of functional redundancy, the extent of their contribution was unclear. In the present study also the knockout and overexpression of a single B-type ARR (*ARR1*) did not show any symptoms of the cytokinin deficiency syndrome (Figure 21A). Therefore, the CRES-T was used as a tool to overcome the functional redundancy among the B-type ARRs by generating a dominant repressor form of a B-type ARR, ARR1-SRDX. The dominant negative ARR1-SRDX causes a strong cytokinin-deficiency phenotype, which is most likely due to the concerted repression of the activities of ARR1 and related transcription factors. This suggests that the activity of B-type ARRs is involved in most, if not all, of the cytokinin-regulated processes.

The results of the protoplast transactivation assay clearly demonstrated that the dominant negative effect of the ARR1-SRDX protein on the ARR1-dependent activation of a reporter gene as well as its induction by cytokinin (Figure 6C). This dominant negative activity also suppressed the activity of all other B-type ARRs tested (Figure 7), thus further validating the efficiency of CRES-T. The transcript level of the native *ARR1* gene was actually enhanced in the *35S:ARR1-SRDX* transgenic plants, indicating that the observed changes of gene expression and plant morphology were not due to co-suppression (Figure 9D). The phenotypic changes described for *35S:ARR1-SRDX* plants were not observed in *35S:ARR1* overexpressing plants (Sakai *et al.,* 2001; Figure 21 in the present study), making it unlikely that non-specific negative effects such as transcriptional squelching (Kornberg, 2005) contribute to the phenotype.

To investigate whether the resulting phenotype of the *35S:ARR1-SRDX* plants represented the knockout of whole B-type ARRs or a subgroup of the family, the *35S:SRDX-ARR19* transgenic plants were generated, since it is distantly related to ARR1 and belongs to a separate subgroup within the B-type ARRs family (see Figures 2A and 5B). As a control for C-terminal SRDX expression the *35S:SRDX-ARR1* gene was also generated. The morphological phenotype of the *35S:SRDX-ARR1* plants was similar to that of the

35S:ARR1-SRDX plants, indicating that the position of the SRDX fusion to the *ARR1* did not affect its repressive action on *ARR1* and related transcription factors. So far, there is no study on *arr19* knockouts available, but the knockout of *arr21*, which is the closest homologue of *ARR19*, did not show any morphological phenotype when compared to the wild-type (Horák *et al.,* 2003). The result that the phenotype of line #4 of the *35S:SRDX-ARR19* transgenic plants is similar to the *35S:SRDX-ARR1* plants further confirms the strength of CRES-T in studying the functionally redundant transcription factors. However, the strongest shoot phenotype of the *35S:SRDX-ARR19* differed from that of the strongest *35S:SRDX-ARR1* plants. This result indicates that both ARR1 and ARR19 might share some common set of target genes and in addition ARR19 might have some specific set of target genes. Unlike *ARR1*, which is expressed in all vegetative parts of the *Arabidopsis*, the *ARR19* and *ARR21* were specifically expressed in siliques containing seeds (Figure 3; Tajima *et al.,* 2004; Tiwari *et al.,* 2006). The expression of *SRDX-ARR19* gene in vegetative parts of the *Arabidopsis* plants might be the cause for the severe phenotype of the *35S:SRDX-ARR19* in the strongest lines. However a detailed analysis is needed to confirm this hypothesis. Further, a transgenic *SRDX-ARR19* gene driven by its own promoter might give valuable information regarding the role of cytokinin in reproductive development.

One can not completely exclude that part of the changes in phenotype and gene regulations caused by ARR1-SRDX are off target effects due to interference with other related transcription factors. Candidates are the MYB-domain-containing pseudo-response regulators APRR2 (*Arabidopsis* pseudo-response regulator 2) and APRR4 (*Arabidopsis* pseudo-response regulator 4), which show in their DNA-binding domains similarity to B-type ARRs. The function of these APRRs *in planta* is not known yet (Makino *et al.*, 2000). However, recent experiments and also the present study indicates that the DNA motif necessary for ARR1 binding is actually longer than previously reported (Taniguchi *et al.,* 2007), and there is also a possibility of new binding motif for B-type ARRs. This makes it less likely that the effect of the ARR1-SRDX dominant repressor spreads far beyond the B-type ARRs. The phenotypic characteristics of the *35S:ARR1-SRDX* plants clearly demonstrate that the CRES-technology is useful to study larger transcription factor families and can be applied to study other regulatory pathways involving transcriptional regulators hampered by redundancy (Mitsuda *et al.*, 2005; Koyama *et al.*, 2007; Mitsuda *et al.*, 2007). However, one has to be aware that not all B-type ARRs have the same functions, so some of

the phenotypes seen in the *35S:ARR1-SRDX* plants might be due to the repression of specific ARRs and are not representative for all members of this protein family.

4.2 The phenotype of *35S:ARR1-SRDX* plants is consistent with a broad function of B-type ARRs in cytokinin signaling

The phenotype of the *35S:ARR1-SRDX* transgenic plants resembled in various aspects the phenotype of cytokinin receptor triple mutants and cytokinin-deficient plants (Werner *et al.,* 2003; Yang *et al.,* 2003; Higuchi *et al.,* 2004; Nishimura *et al.,* 2004; Riefler *et al.,* 2006; see Figure 15). As in those mutants, the shoot growth and in particular leaf size was strongly reduced, while seed size and the root system were enlarged. An exception of the similarities between *35S:ARR1-SRDX* plants and plants with a reduced cytokinin status is the early flowering phenotype which contrasts with the retarded flowering reported for triple receptor mutants and cytokinin-deficient plants (Werner *et al.,* 2003; Higuchi *et al.,* 2004; Nishimura *et al.,* 2004; Riefler *et al.,* 2006). Earlier flowering in *35S:ARR1-SRDX* plants was developmentally regulated as less leaves had formed at the onset of flowering. This indicates that this function may be cytokinin-independent as cytokinin-deficiency causes only minor changes of leaf number at the onset of flower formation (Werner *et al.,* 2003). A cytokinin-independent role in flowering has been suggested for the rice B-type *RR* gene *Edh1* (Doi *et al.,* 2004). Interestingly, *arr2* mutant plants also flower earlier than wild type, indicating a negative regulatory function of ARR2 in the induction of flowering (Hass *et al.,* 2004). It could be that the early flowering phenotype of the *35S:ARR1-SRDX* plants is at least partially due to the suppression of ARR2 function, which was demonstrated in the PTA (Figure 7).

A decreased cytokinin sensitivity of the *35S:ARR1-SRDX* transgenic plants was detected in several bioassays. *35S:ARR1-SRDX* plants showed a complete resistance to cytokinin in the chlorophyll retention assay, which is similar to the phenotype of *ahk2 ahk3* receptor double mutants (Riefler *et al.,* 2006). This indicates that the cytokinin activities in regulating chlorophyll retention are entirely dependent on B-type ARRs. In particular ARR2, the closest homologue of ARR1, has been shown to play a role in mediating cytokinin-dependent chlorophyll retention (Kim *et al.,* 2006) and may thus be suppressed in leaves of *35S:ARR1-SRDX* plants. The reduction of cytokinin sensitivity was less pronounced in roots of *35S:ARR1-SRDX* plants. One possibility to explain this distinction is that different sets of B-type ARRs act in shoots and roots and that the B-type ARRs in the roots are less affected

by the dominant repressor activity of ARR1-SRDX. Alternatively, the affected B-type ARRs could play antagonistic roles in the root. The latter possibility is supported by the observation that the inclusion of an *arr2* knockout allele in *arr* mutant combinations causes a reduction of the strength of the root phenotype, indicating that ARR2 might have an antagonistic function to the other B-type ARRs in regulating root elongation and root branching (Mason *et al.,* 2005). Therefore, reduced activity of ARR2 in the dominant repression plants could explain the somewhat weaker root phenotype in the *35S:ARR1-SRDX* transgenic plants.

The expression and inducibility of A-type *ARR* genes and other early cytokinin response genes, which have been shown to be direct target genes of B-type ARRs in the past (Hwang and Sheen, 2001; Taniguchi *et al.,* 2007), was dampened in *35S:ARR1-SRDX* transgenic seedlings (Figure 24C; Table 14; Table 18 in Appendix). This clearly shows that the ARR1-SRDX protein suppresses the activation of direct target genes of endogenous B-type ARRs. Interestingly, the response of the majority of the response genes was generally reduced but each to a different extent. These differences may reflect the different contribution of ARR1 and related B-type ARRs to the cytokinin-dependent regulation of the respective genes. It is also interesting to note that the activation capacities of different B-type ARRs varies in the PTA system, strengthening the idea that different B-type ARRs contribute to a different degree to the regulation of cytokinin responsive genes. Furthermore, recent work has shown that transcription of A-type ARRs is also regulated via a B-type ARR-independent pathway. The target genes of the cytokinin response factors (CRFs) of the ERF family of transcription factors partially overlap with those of the B-type ARRs (Rashotte *et al.,* 2006).

4.3 Putative target genes of B-type ARRs suggests path of cytokinin action

In the present study a large set of genes that show significant changes of their transcript levels in association with the cytokinin-deficiency syndrome of the *35S:ARR1-SRDX* plants were indentified. The genes that are present in cluster 3 and cluster 4 might serve as important clues to understand the function of B-type ARRs in plant development (see section 3.2.6.2 and Table 18 in Appendix). The fact that the known cytokinin-responsive genes identified in previous studies were largely present in these two clusters, further confirms the notion that the genes in these clusters might be down-stream target genes for B-type ARRs (Table 18 in Appendix). The qRT-PCR data validate the induction of the genes

belonging to these clusters by cytokinin in WT, and consistently the induction was attenuated in ARR1-SRDX plants (Figure 25; Table 16). In total, 60% of the genes that were tested by qRT-PCR confirmed the similar expression profiles of the target genes that were observed in the microarray experiment. The 60% confirmation might be due to the background noise that occurs in array experiments; also the sensitivities in detection of transcript levels vary between qRT-PCR and microarray experiments. The function of the deregulated genes (see section 3.2.6.2 and Table 13) can in many cases be linked to known functions of cytokinin in plant physiology and development, such as nitrogen metabolism (Sakakibara *et al.,* 2006) and gibberellin (GA) action (Brenner *et al.,* 2005; Jasinski *et al.,* 2005). The fact that the transcript level of genes related to these processes (e.g. *NR2*, *AMT2*, *NTL1*, *RGA1*, *RGA2*; see Table 13) changes in plants with repressed B-type ARR functions is consistent with data that cytokinin plays an important role in the control of these processes and suggest that the B-type ARRs are at least partly involved in this regulation.

The present study also indicates the role of cytokinins in different signalling pathways of the phytohormones and abiotic signalling, apart from the known interactions. For example, the role of cytokinins in regulating the sulfate uptake and transport from root to shoot was shown previously (Maruyama-Nakashita *et al.,* 2004). The exogenous application of cytokinin down-regulates the expression of the high-affinity sulfate transporter genes *SULTR1;1*, and *SULTR1;2* and this regulation is mediated by the cytokinin receptor CRE1 (Maruyama-Nakashita *et al.,* 2004). In the present study, the *SULT 3;4* gene which is also a member of sulfate transporters family, was identified as a putative target gene of B-type ARRs. The expression profile of *SULT 3;4* gene in response to cytokinin was also confirmed by qRT-PCR (Figure 25). The function of SULT3;4 is not known yet, but the closest homologue SULT3;5 was shown to act synergistically with SULT2;1 in facilitating sulfate transport from root to shoot (Kataoka *et al.,* 2004). So it could be that cytokinins via B-type ARRs play a role in sulfur uptake and transport from root to shoot. The present study also gave hints for the role of cytokinins in abiotic stress signalling. A recent study, pointed out that cytokinins might be involved in abiotic stress signalling in both ABA-dependent and independent pathways (Wohlbach *et al.,* 2008). In *Arabidopsis*, *ERF4* was shown to act as a negative regulator in the expression of ethylene, jasmonate, and ABA responsive genes (McGrath *et al.,* 2005; Yang *et al.,* 2005). Interestingly, the microarray data from the present study revealed that *ERF4* is also responsive to cytokinins and might be a putative target gene

of B-type ARRs. The transcript level of *ERF4* was up-regulated 4-fold in WT after 15 min of cytokinin treatment and this induction by cytokinin was absent in ARR1-SRDX plants (see Table 18 in Appendix). However, the expression profile of the *ERF4* transcript in response to cytokinin needs to be confirmed by qRT-PCR. So, the microarray data sets from the present study will be an important source to identify and study processes downstream of B-type ARRs.

In the present study an attempt was made to identify specific target genes of ARR1 by characterizing the *arr1-5* and *35S:ARR1* plants and comparing their expression profiles. The morphological phenotype of homozygous plants of *arr1-5* and *35S:ARR1* did not differ from WT. The *arr1-5* plants exhibited a slight insensitivity towards cytokinins, *35S:ARR1* plants did not differ from WT in their response to cytokinins in primary root elongation or lateral root formation (Figures 22). A set of 24 genes was identified as putative specific target genes of ARR1 by comparing the expression profiles of *arr1-5* with *35S:ARR1* seedlings (Table 15). Preliminary analysis indicates that as many as 60% of the genes are no longer up-regulated in *arr1-5* plants compared to WT. Based on this data it is tempting to speculate that ARR1 might be the main contributor among the B-type ARRs in mediating cytokinin responses, at least in seedlings. The result from multiple knockouts of B-type ARRs also confirms this notion. Whenever the *arr1* was added to the single and higher order mutant like *arr10* and *arr12* single mutants or *arr10 arr12* mutant, the resultant mutant combinations containing the *arr1* allele generally showed less sensitivity to exogenous cytokinin than those combinations containing a functional *ARR1* allele (Mason *et al.,* 2005; Yokoyama *et al.,* 2007; Ishida *et al.,* 2008). In future, a more refined analysis of the data sets that were generated in this study will helpful to understand the specific functions of ARR1 in plant development.

4.4 B-type ARRs are possible nodes for integration of different signalling pathways

As developmental programs are subject to fine-tuning by different factors, integration of different signalling pathways is necessary. Evidence for a function for B-type ARRs in crosstalk with phytochrome signalling came from the red light experiments of the present study (Figure 17). Red light is a key regulator of seed germination and its effect is mediated by phytochromes (Shinomura, 1997; Sullivan and Deng, 2003). Red light stimulates seed

germination, while far-red light acts inhibitory. The response under the tested light regime in the experiments is mediated by PhyB and reversibility of red-light stimulated germination by far-red light is a hallmark of its activity (Shinomura, 1997). This reversibility was almost completely lost in *35S:ARR1-SRDX* transgenic seeds (Figure 17B). This result is consistent with the previously described reduced far-red light sensitivity of cytokinin receptor mutant seeds (Riefler *et al.,* 2006) and seeds of cytokinin-deficient plants (data not shown). A plausible explanation is that cytokinin in an unknown way alters the Pfr/Pr ratio of PhyB, e.g. by stabilization of the active Pfr form. This may be mediated directly by downstream factors of cytokinin signalling such as A-type ARRs as it was proposed for red-light dependent regulation of hypocotyl elongation (Sweere *et al.,* 2001; Mira-Rodado *et al.,* 2007). The dominant repressor effect of ARR1-SRDX could block the cytokinin signal involved in regulating this process. Alternatively, B-type ARRs may be involved in regulating red light responses independent of cytokinin, e.g. by acting downstream of phytochromes. In any case, there is now considerable evidence that the cytokinin status has a strong influence on the reaction to red light.

Similarly, the evidence for the role of cytokinin in abiotic stress signalling pathways is increasing with recent studies. The *Arabidopsis* Histidine Kinase 1 (AHK1) acts as positive regulator in osmotic signaling in *Arabidopsis* plants (Tran *et al.,* 2007). It was proposed that AHK1 also plays a unique role in sensing or regulating the vegetative water stress and desiccation of seeds through regulation of ABA synthesis (Wohlbach *et al.,* 2008). A role of cytokinin in osmotic stress signalling pathway is proposed as the *ahk2, ahk3* single, and *ahk2 ahk3* double mutants showed strong stress tolerance against both drought and high-salinity stresses (Tran *et al.,* 2007). At the same time it was also shown that AHK1 mediated osmotic stress regulation is carried out by the complex function of A-type *ARR*s. It was suggested that *ARR3, ARR4* and *ARR8, ARR9* play positive and negative roles, respectively, in the AHK1 mediated stress response (Wohlbach *et al.,* 2008). Preliminary data from the present study showed a slight resistance to salt stress which is mediated by B-type ARRs (Figure 18). The slight resistance that was observed might be due to the repression of both positive (*ARR3, ARR4*) and negative (*ARR8, ARR9*) regulators of this pathway at the same time by ARR1-SRDX. However, a detailed analysis is needed to ascertain the role of B-type ARRs in drought and osmotic stress response.

4.5 A new promoter region that is important for cytokinin-responsive gene regulation has been identified

The identification of functional *cis*-acting DNA regulatory elements is a crucial step towards understanding gene regulation. In *Arabidopsis*, the 11 B-type ARRs are broadly expressed, mostly in overlapping domains, with the exception of ARR19 and ARR21 which are specifically expressed in siliques containing seeds (Tajima *et al.*, 2004; Tiwari *et al.*, 2006; Day *et al.*, 2007). Similar to their overlapping expression, B-type ARRs tested so far optimally binds *in vitro* to the core DNA sequence 5'-(A/G)GAT(T/C)-3' (Sakai *et al.*, 2001; Hosoda *et al.*, 2002; Imamura *et al.*, 2003). Later it was shown that ARR1 requires extensions an either side of the core sequence for optimal binding *in vitro* (Taniguchi *et al.*, 2007). So far, the importance of this *in vitro* binding sequence for the B-type ARRs has not been tested *in planta*. The present study is the first of its kind to confirm the functionality of this known binding motif *in planta* (see section 3.2.7.1). Moreover, it also identified another promoter region that is important for the activation of the *ARR6* gene by B-type ARRs. The results from this study indicate that the known binding element is only responsible for about half of the activation and the 27 bp region between -220 bp to -193 bp of *ARR6* promoter is necessary for a complete transcriptional activation of *ARR6* by B-type ARRs. Thus, it is likely that the indentified 27 bp promoter region contains some other B-type ARR *cis*-element(s) and that these putative binding element(s) function co-operatively with the known *cis*-element to enhance the transcriptional activation of *ARR6*. To elucidate the mechanism of transcriptional activation by B-type ARRs, further identification of the putative binding element(s) present in the 27 bp region of the *ARR6* promoter is necessary. Further gel shift assays with the DNA-binding domain of ARR1 and the *ARR6* promoter in which all three copies of the Taniguchi motif were mutated will yield the potential information regarding the presence of new binding elements in the *ARR6* promoter.

Gene regulation can be conserved in related species and functional DNA elements evolve at a slower rate than non-functional elements. Therefore, these functional elements are well conserved in orthologous regulatory regions of species (Moses *et al.*, 2003). Preliminary analysis indicates that in the identified 27 bp region of *ARR6* promoter some nucleotide positions are showing high conservation among the orthologous gene promoters of *ARR6* in poplar and rice further supports the significance of this 27 bp region for the transcriptional activation of *ARR6* (Figure 33). It is a common phenomenon in plant gene

regulation that expression of the downstream genes for different stimuli is regulated by distinct regulatory regions that control tissue specificity and levels of transcription in a combinatorial manner (Singh, 1998). The gene regulation requires multiple copies of a single *cis*-element in some cases, whereas in some other cases *cis*-elements must work in concert with another distinct motif or several other motifs in order to form specific structures within the promoter for the transcriptional activation (Yamaguchi-Shinozaki and Shinozaki, 2005). For example, the ABA responsive element (ABRE) is a major *cis*-acting element in ABA-responsive gene expression. Either an additional copy of ABRE or other *cis*-elements called coupling elements (CE) like CE1 and CE3 which constitute an ABA-responsive complex, are necessary for ABA-responsive gene expression (Shen *et al.*, 1996; Uno *et al.*, 2000). In cereals, during seed germination gibberellins are synthesized and induce the expression of hydrolytic enzymes such as α-amylases and proteinases in the aleurone-layer cells. The gibberellic acid (GA)-induced activation of these genes requires two pairs of different *cis*-elements called GARE (GA-response element) and CARE (CAACTC regulatory element) (Gubler and Jacobsen, 1992; Sutoh and Yamauchi, 2003). Similar to these phytohormones, five different types of *cis*-elements have been implicated in sucrose-regulated gene expression (Rolland *et al.*, 2002). The G-box, the B-box (Grierson *et al.*, 1994; Zourelidou *et al.*, 2002), the SURE (Grierson *et al.*, 1994), the SP8 element (Ishiguro and Nakamura, 1994) and the TGGACGG sequence (Maeo *et al.*, 2001) are the main elements in regulation of the genes involved in sugar signalling of plants. So it is likely that for the cytokinin induced gene expression also there might be different *cis*-elements involved. One of the elements is the Taniguchi motif and other might be present in the 27 bp promoter region of *ARR6*.

In a recent study, a synthetic cytokinin reporter was generated to visualize the cytokinin output *in vivo*. This synthetic reporter harboured the concatemerized B-type ARR binding motifs and a minimal 35S promoter binding motif (Müller and Sheen, 2008). At the same time, the finding of the enriched common-binding motif 5'-(A/G)GAT(T/C)-3' of the B-type ARRs within 1 kb upstream of the translational start site of *AHP* genes is further strengthening the idea that the known binding motif is responsible for general responsiveness to cytokinins (Hradilová *et al.*, 2007). In cucumber, cytokinins and light activate the transcription of the NADPH-protochlorophyllide reductase (*POR*) gene. POR functions in the chloroplast development at the onset of greening and plays a key role in maintaining the chlorophyll biosynthesis in mature plants (Kuroda *et al.*, 2001). The promoter of the *POR*

gene contains a novel *cis*-element exhibiting cytokinin-depended protein binding *in vitro*. However, the identified binding sequence 5'-TATTAG-3' does not have the sole ability to mediate cytokinin-responsive transcriptional activation but the sequence is shown as critical for cytokinin-enhanced binding to the *POR* promoter in a gel shift assay (Fusada *et al.*, 2005). Based on these findings mentioned above and the data from the present study, it can be speculated that the Taniguchi motif might be responsible for a general cytokinin response, whereas the newly identified region might contain the motif/motifs that is/are acting as coupling elements similar to the ABA responsive gene regulation or it might recruit some other protein which might be important for the activation.

So in order to prove this hypothesis, the specific motif(s) present in the 27 bp region should be identified. The search for a new *cis*-element in the data bases like Place and AGRIS revealed that they are no known *cis*-elements present within the 27 bp region (Higo *et al.,* 1999; Davuluri *et al.,* 2003). Therefore, a sliding window analysis using the 27 bp promoter region was done for the identification of a new binding motif(s). The analysis identified a putative hexamer 5'-TTT(A/G)TT-3' binding element is present in all known cytokinin responsive gene promoters. Moreover, the multiple alignment of orthologous gene promoters of *ARR6* in poplar and rice indicates a high degree of conservation among the second and fourth nucleotide position (T) within the putative hexamer binding motif (Figures 32 and 33). The preliminary analysis indicates that the putative binding motif is distributed among the known cytokinin responsive genes of *Arabidopsis*, poplar and rice. However, a detailed analysis needs to be done for statistical overrepresentation of this motif among the cytokinin response gene promoters compared to random gene promoters. It is also worth, to test the significance of this putative binding motif *in planta*. The mutational analysis by substituting the second and fourth T positions within the hexamer will reveal the functionality of this putative motif and its contribution to the transcriptional activation of the genes. Another approach to identify specific motif(s) present in the 27 bp region is linker-scanning method (Biery *et al.,* 2000). It would also be interesting to indentify the interacting proteins of the 27 bp binding region by using the yeast one-hybrid system.

4.6 Two potential interaction partners of ARR1 were identified

The study of protein interactions has been vital to the understanding of how proteins function within the cell. Previous studies were done towards elucidating the interactome of the *Arabidopsis* TCS, based on the yeast two-hybrid screens using the proteins of the cytokinin signalling pathway. The studies indicated that these proteins have a specific set of interaction partners among the TCS components and with various proteins belonging to different categories (Dortay *et al.,* 2006, 2008). In the present study, an attempt was made to study putative interacting partners of ARR1, VIP1 and ERF8 *in planta*. The results from this study confirmed the interaction of ARR1 with VIP1 and ERF8 in a heterologus system and *in planta* by using a yeast two-hybrid and BiFC analysis, respectively (Figure 35 and Figure 36). The *ERF8* gene of *Arabidopsis* is one of the eight members that belongs to the group VIIIa of *ERF* gene family and its function has not described so far. On the other hand, VIP1 has been identified as a basic region/leucine zipper (bZIP)-like putative transcription factor and its function in the transport of *Agrobacterium* T-DNA into the nucleus of the host cell was well studied (Tzfira *et al.,* 2002). In the transportation of T-DNA, VIP1 functions as an adapter between VirE2, a Vir (virulence) protein encoded by *Agrobacterium*, and the nuclear-import machinery of the host-cell (Tzfira *et al.,* 2002; Li *et al.,* 2005). The fact that, ARR1 and VIP1 co-expressed and co-localized to the same subcellular compartment, i.e. the nucleus, of the cell, further supporting the potential biological relevance of this interaction. In order to understand the biological significance of these protein interactions the knockout lines of *vip1* and *erf8* were obtained and double mutant lines of *arr1 vip1* and *arr1 erf8* were generated (data not shown). The characterization of these double mutant lines will possibly reveal the functional context and relevance of the interaction of these proteins. Based on the known functions of the ARR1 and VIP1 the biological significance of these interactions can be hypothesized in the following ways. However, the possibilities that are mentioned below are only assumptions more detailed studies need to be done in order to understand the biological significance of these protein interactions.

i) It was postulated that the acquisition or maintenance of pluripotence in *Arabidopsis* protoplasts is brought about by changes in methylation pattern and reorganization of specific chromosomal domains (Avivi *et al.,* 2004). The changes in chromosomal domains cause the activation of silent genes such as NAC-domain genes (*NAM*/*ATAF1*/*CUC2*) and *VIP1*. It has been reported previously that the NAC-domain genes are implicated in shoot meristem

function (Duval *et al.*, 2002) and a VIP1 function in maintenance of shoot meristem was shown recently as well (Avivi *et al.*, 2004). VIP1 has been reported to be capable of suppressing differentiation and shoot formation in transgenic tobacco plants (Avivi *et al.*, 2004). On the other hand, cytokinins are known to act as positive regulators and are necessary in maintaining shoot meristem activity in *Arabidopsis* (Werner *et al.*, 2003; Leibfried *et al.*, 2005). A higher cytokinin status in the shoot apical meristem is for example achieved by biosynthesis of cytokinins activated by the *STM* gene or by negative regulation of A-type *ARR*s by the *WUS* gene (Leibfried *et al.*, 2005; Yanai *et al.*, 2005). It was shown that cytokinins control root meristem cell differentiation specifically at the transition zone of the root via AHK3/ARR1 and ARR12 (Dello Ioio *et al.*, 2007; Dello Ioio *et al.*, 2008). The role of B-type ARRs in the shoot apical meristem is not yet known. It might be possible that the VIP1 mediated suppression of cell differentiation and inhibition of shoot growth is achieved by regulation of the ARR1 mediated cytokinin signalling. VIP1 has been shown to be interacting with H2A *in planta*, and thereby participates in chromatin regulation (Loyter *et al.*, 2005). It is known that chromatin regulation by chromatin assembly factors and histone modification factors is involved in regulating the meristem function in *Arabidopsis* (reviewed in Guyomarc`h *et al.*, 2005). VIP1 might be involved by a yet unidentified mechanism in positive regulation of cytokinin signalling and maintenance of the shoot apical meristem.

ii) All the eight members of the VIIIa subgroup of the *ERF* gene family to which *ERF8* belongs, contain the characteristic ERF-associated amphiphilic repression (EAR) motif, which has been shown to function as a repression domain (Fujimoto *et al.*, 2000; Ohta *et al.*, 2001). The function of ERF8 in *Arabidopsis* is not known yet. But the nearest homologues of *ERF8*, *ERF4* and *ERF7* were well studied and characterized. ERF4 has been shown to be a negative regulator of the expression of ethylene, jasmonate, and ABA-responsive genes, whereas the ERF7 has been shown to play an important role in the ABA and drought response (McGrath *et al.*, 2005; Yang *et al.*, 2005; Song *et al.*, 2005) Since the role of cytokinin in different abiotic stresses like osmotic stress, salt stress and drought stress has been reported (Rivero *et al.*, 2007; Tran *et al.*, 2007; Wohlbach *et al.*, 2008), it is possible that the interaction of ARR1-ERF8 might plays a role in abiotic stress signalling.

4.7 The *35S:ARR1-SRDX* transgenic tomato plants are a valuable tool to study the role of cytokinin in parthenocarpic fruit development

Essential gene functions are generally considered to be carried out by orthologs, which have remained conserved in sequence and function in evolutionarily related species (Mushegian, 1999). For example, the *ABC* genes which belong to the *MADS-box* transcription factors are conserved among the flowering plants like *Antirrhinum*, *Petunia* and *Chrysanthemum* and the ABC model of flowering can be applied to these plants (Bradley *et al.*, 1993; Honma and Goto, 2001; Ferrario *et al.*, 2003; Shchennikova *et al.*, 2004). Similarly, between *Arabidopsis* and tomato essential homologous gene functions of iron uptake are conserved (Bauer *et al.*, 2004). Moreover, it has been proposed in previous studies that *Arabidopsis* chimeric repressors may be effective in a wide variety of plant species, as transcription factors are conserved across the different species (Mitsuda *et al.*, 2006, 2007; Shikata and Ohme-Takagi, 2008). Also B-type *RR*s are conserved in their sequence and function across the higher plant species like *Arabidopsis*, *Populus* and rice (Ito *et al.*, 2006; Jain *et al.*, 2006; Ramirez-Carvajal *et al.*, 2008). Therefore, the effectiveness of CRES-T to act across different species was investigated by generating *35S:ARR1-SRDX* transgenic tomato plants. The morphological phenotype of the *35S:ARR1-SRDX* tomato plants is likely to be due to the suppression of B-type *RR* mediated cytokinin signalling. The *35S:ARR1-SRDX* transgenic tomato plants are partly similar to plants showing the compound phenotype of the cytokinin deficiency syndrome. Some of the main features of this syndrome like stunted shoots with small leaves and reduced apical dominance were observed in these transgenic plants (Figure 35). The cytokinin deficiency leads to bigger seed size in *Arabidopsis* (Werner *et al.*, 2003; Riefler *et al.*, 2006; Hutchison *et al.*, 2006 and present study), whereas in tomato plants it leads to the production of seedless fruits (Figure 36B). Previous studies on parthenocarpic tomato fruits revealed that the seedless fruits contain 3-fold and 4-fold higher levels of auxin-like and GA-like substances, respectively, when compared to the normal fruit. In contrast, cytokinin-like substances are twenty times lower than in the normal fruit (Mapelli, 1981). This is consistent with the finding that reduced cytokinin signalling in *35S:ARR1-SRDX* tomato plants cause parthenocarpic fruit development. The phytohormone auxin and gibberellin are known to play a key role during the early stage of fruit development and artificial parthenocarpy can be induced by exogenous application of these hormones (Fos *et al.*, 2000; Serrani *et al.*, 2007; de Jong *et*

al., 2009). The role of cytokinins during fruit development has not yet been studied in detail. The cytokinin-deficient *35S:ARR1-SRDX* tomato plants generated in this study might be helpful in understanding the role of cytokinins in tomato plant growth and development and also give more insight into the function of cytokinins in parthenocarpic fruit development.

4.8 Future outlook of CRES-technology in cytokinin mediated crop improvement

Cytokinins are key regulators in plant growth and development. Since last decade cytokinin research progressed very rapidly and resulted in an increase in our knowledge regarding the role of cytokinins in control of numerous processes associated with plant growth and development (Mok and Mok 2001; Heyl and Schmülling, 2003, 2006; Müller and Sheen 2007; Argueso *et al.*, 2009). As a result, the regulation of the cytokinin status in the transgenic plants has become feasible opening up the possibility of its application to agriculture. So far, the transgenic plants that were studied in relation to agriculture mainly concentrated on the production of cytokinins, both spatially and temporally controlled by using specific promoters (Gan and Amisano, 1995; Rivero *et al.*, 2007). For example, cytokinins are key regulators of leaf senescence (He *et al.*, 2005b; Kim *et al.*, 2006). The ability to increase cytokinin was used extensively in different transgenic crop plants to confer delayed leaf senescence, which is a useful trait in agriculture. Transgenic tobacco plants expressing a senescence-specific *pSAG12:IPT* gene had increased biomass and an enhanced postharvest longevity of leaves due to the inhibition of leaf senescence by auto-regulated production of cytokinins (Gan and Amasino, 1995). Later, a similar approach was successfully applied to delay senescence in a variety of crop plants like maize, rice, lettuce and tomato (McCabe *et al.*, 2001; Robson *et al.*, 2004). Genetic engineering of cytokinin status also helped in the production of drought-tolerant crops by expressing a *pSARK:IPT* gene in tobacco plants (Rivero *et al.*, 2007). Similarly, fruit specific expression of the isopentenyl transferase (*IPT*) gene has been achieved in tomato by using the promoter of the ovary-specific gene *2A11* of tomato. The fruits of these transgenic tomato plants had a significantly higher content of total and soluble solids and yet did not differ in terms of yield compared to the non-transformed control plants (Martineau *et al.*, 1995).

In contrast to the increased cytokinin status, reduced cytokinin status may also be important for agriculture. The symptoms of the cytokinin deficiency syndrome include

reduced apical dominance with increased branching, stunted shoots with small leaves, an enhanced root system and increased seed biomass and size, which are valuable crop traits. In higher plants, one of the methods to achieve the cytokinin deficiency syndrome is to suppress the B-type RR-mediated cytokinin signaling. The present study shows the efficiency of the CRES-T to achieve this goal in *Arabidopsis* and tomato plants. Transgenic crops with spatially and temporal reduced levels of cytokinin have an exciting potential in agriculture to improve crop productivity. For example, reduced apical dominance with increased branching will be a useful trait in ornamental and tree plants. The enlarged root system will be helpful for efficient up-take of water and nutrients from the soil and thereby may reduce the consumption of fertilizers in agriculture. The larger seed size is a very important trait in all cereals and legume crops to increase the crop yield. At the same time, cytokinin-mediated parthenocarpic fruit growth in tomato is also an important aspect in horticulture and food industry. It is very helpful for tomato ketchup industries where often separation of the seeds from the tomato pulp is a laborious process. So in future, the spatial and temporal reduction of the cytokinin status by using specific promoters combined with CRES-T will not only yield valuable information regarding the role of cytokinins in different stages of plant growth and development but also contribute to crop improvement.

5.0 Summary

Cytokinins are essential for the regulation of many developmental processes in plants. In *Arabidopsis* the signal transduction of cytokinins is mediated by a multi-step His-to-Asp phospho-relay system. The B-type response regulators are one component of this phospho-relay system. The B-type response regulators are transcription factors that at least partially mediate the response to cytokinin. *In planta* functional analysis of this protein family is hampered by the high level of functional redundancy of its eleven members.

In order to explore the functions of the B-type response regulators and to overcome their functional redundancy, the chimeric repressor silencing technology (CRES-T) was employed by generating a dominant-negative version of the *Arabidopsis* response regulator ARR1 (ARR1-SRDX). The *35S:ARR1-SRDX* transgenic *Arabidopsis* plants showed phenotypic changes reminiscent of plants with a reduced cytokinin status, such as a strongly reduced leaf size, an enhanced root system and larger seeds. Several bioassays showed that *35S:ARR1-SRDX* plants have an increased resistance towards cytokinin. Molecular analysis indicated attenuation of the early transcriptional response to cytokinin. In addition, a role for B-type ARRs in mediating crosstalk with other pathways was supported by the resistance of *35S:ARR1-SRDX* seeds to phyB-mediated inhibition of germination by far-red light. Components downstream of the B-type ARRs were identified by performing expression profiling using CATMA arrays. The rapid induction of a large part of cytokinin response genes was dampened. The transcript levels of more than 500 genes were >2.5-fold reduced in *35S:ARR1-SRDX* transgenic seedlings suggesting a broad function of the B-type ARRs. A total of 106 genes were identified as putative target genes of the B-type ARRs, and altered expression profiles of some of these genes were confirmed by qRT-PCR.

In order to identify specific target genes of ARR1, *arr1* and *35S:ARR1* transgenic plants were characterised, and their transcription profiles were analysed. A set of 24 genes was identified as putative specific target genes of ARR1. After finding these potential target genes by microarray analysis, the promoter of one target gene (*ARR6*) was analysed to identify *cis*-acting elements by promoter deletion analysis. The result of this analysis confirmed for the first time the *in planta* function of a known ARR1 binding motif. Also, a new promoter region important for the activation of the *ARR6* gene was identified. The newly found 27 bp promoter region will be useful for further studies to pinpoint the binding

motif of ARR1 and/or other B-type ARRs. In addition, attempts were made to obtain potentially relevant information for the regulation of ARR1 activity by studying proteins that were recognised as ARR1 interactors.

In order to investigate whether the target genes of the B-type ARRs are conserved across species and to study the efficiency of the CRES-T in other plant species, *35S:ARR1-SRDX* transgenic tomato plants were generated and characterized. These transgenic tomato plants also showed the cytokinin deficiency syndrome similar to *Arabidopsis*, suggesting the conservation of the target genes and target gene sequences of these transcription factors across species. Further, these transgenic tomato plants produced seedless tomato fruits thereby indicating a role for cytokinin in parthenocarpic fruit development.

In conclusion, the suppression of pleiotropic cytokinin activities by a dominant-negative version of a B-type ARR indicates that this protein family is involved in mediating most, if not all, of the cytokinin activities in *Arabidopsis*. The *35S:ARR1-SRDX Arabidopsis* and tomato transgenic plants and the microarray data sets comprising the putative target genes of B-type ARRs and ARR1 are valuable tools for investigating these functions.

6.0 Zusammenfassung

Cytokinine sind entscheidend an der Regulation diverser pflanzlicher Entwicklungsprozesse beteiligt. In *Arabodopsis thaliana* wird die Signaltranduktion dieses Hormons über eine mehrstufige Phosphatkaskade vermittelt. Die so genannten *Arabidopsis Response Regulatoren* (ARRs) des B-Typs sind als Transkriptionsfaktorproteine Bestandteil dieses Signalsystems und vermitteln als Transaktivatoren die Cytokininantwort auf molekularer Ebene. Eine hohe funktionelle Redundanz unter den 11 Mitgliedern dieser Genfamilie erschwert jedoch funktionelle Analysen *in planta*.

Um die Funktion der B-Typ ARRs zu ergründen und die Redundanz innerhalb dieser Genfamilie zu überbrücken, wurde die *Chimeric Repressor Silencing* Technologie (kurz CRES-T) angewandt, durch die eine dominant-negative Repressorvariante von ARR1 generiert wurde (ARR1-SRDX). Die erzeugten transgenen *35S:ARR1-SRDX Arabidopsis*-Pflanzen wiesen phänotypische Veränderungen auf, die denen von Pflanzen mit reduziertem Cytokiningehalt ähnelten. Dazu gehörten eine reduzierte Blattgröße, ein verstärktes Wurzelsystem und größere Samen. Diverse Cytokininsensitivitätstests zeigten auf eine verstärkte Cytokininresistenz der *35S:ARR1-SRDX* transgenen Pflanzen. Molekulare Analysen konnten zudem eine Abschwächung der frühen Antwort auf Cytokinin auf transkriptioneller Ebene zeigen. Darüber hinaus wiesen *35S:ARR1-SRDX* transgene Samen eine Resistenz gegenüber der phyB-vermittelten Inhibierung der Keimung durch dunkelrotes Licht auf, was auf eine B-Typ ARR-vermittelte Interaktion zwischen der Cytokininsignaltransduktion und anderen Signalwegen hindeutet. Des weiteren wurden durch Erstellung von Expressionsprofilen mittels CATMA-*Arrays* den B-Typ ARRs nachgeschaltete Komponenten im Signalweg identifiziert. Die schnelle Induktion einer Vielzahl an Cytokininantwortgenen war hierbei abgeschwächt. Insgesamt war in *35S:ARR1-SRDX* transgenen Keimlingen die relative Transkriptabundanz von mehr als 500 Genen um mehr als 2,5- fach reduziert. Dies deutet auf ein weites Funktionsspektrum der B-Typ ARRs hin. 106 Gene wurden als mögliche Zielgene der B-Typ ARRs identifiziert und für einige von ihnen wurde die veränderte Expression mittels quantitativer PCR verifiziert.

Um ARR1-spezifische Zielgene zu identifizieren, wurden neben einer molekularen und phänotypischen Charakterisierung der Mutante *arr1* und von *35S:ARR1* transgenen

Pflanzen die Transkriptionsprofile beider Genotypen mittels *Microarrays* analysiert. Dabei wurden insgesamt 24 Gene als potentielle spezifische Zielgene von ARR1, u. a. *ARR6*, identifiziert. Der Promotor von *ARR6* wurde für Promotordeletionsanalysen verwendet, um *cis*-aktive Elemente zu identifizieren. Durch diese Analysen konnte erstmals die Funktion eines zuvor bereits bekannten ARR1 Bindemotivs *in planta* gezeigt werden. Des weiteren wurde eine neue für die Aktivierung von *ARR6* notwendige Region im Promotor ermittelt. Diese 27 bp lange Region wird für weitergehende Analysen zur Identifizierung des Bindemotivs von ARR1 und/oder weiterer B-Typ ARRs benutzt werden. Darüber hinaus wurden mit ARR1 interagierende Proteine untersucht, um möglicherweise relevante Informationen zur Regulation der ARR1 Aktivität zu erhalten.

Um zu überprüfen, ob die ARR1-Zielgene über die Speziesgrenze konserviert sind und um die Effizienz der CRES-Technologie auch in anderen Pflanzenspezies zu zeigen, wurden *35S:ARR1-SRDX* transgene Tomatenpflanzen generiert und charakterisiert. Diese Pflanzen zeigten das Cytokinindefizienzsyndrom ähnlich dem in *Arabidopsis*, was auf die Konservierung der Zielgene und Zielsequenzen dieser Transkriptionsfaktoren über die Speziesgrenze hinaus hinweist. Das Fehlen von Samen in diesen transgenen Pflanzen weist darüber hinaus auf eine Rolle von Cytokinin bei der parthenocarpischen Fruchtentwicklung hin.

Zusammenfassend zeigen die erzielten Resultate, dass die pleiotropen Cytokininaktivitäten, die in der vorliegenden Arbeit durch Verwendung einer dominanten Repressorvariante eines B-Typ ARR unterdrückt worden waren, im wesentlichen, wenn nicht sogar vollständig, auf die Proteinfamilie der B-Typ ARRs zurückzuführen sind. Die *35S:ARR1-SRDX* transgenen *Arabidopsis*-Pflanzen, die transgenen Tomatenpflanzen und die erzeugten *Microarray*-Daten, die die möglichen Zielgene von ARR1 und anderer B-Typ ARRs umfassen, sind wertvolle Hilfsmittel, um diese Funktionen weiter zu untersuchen.

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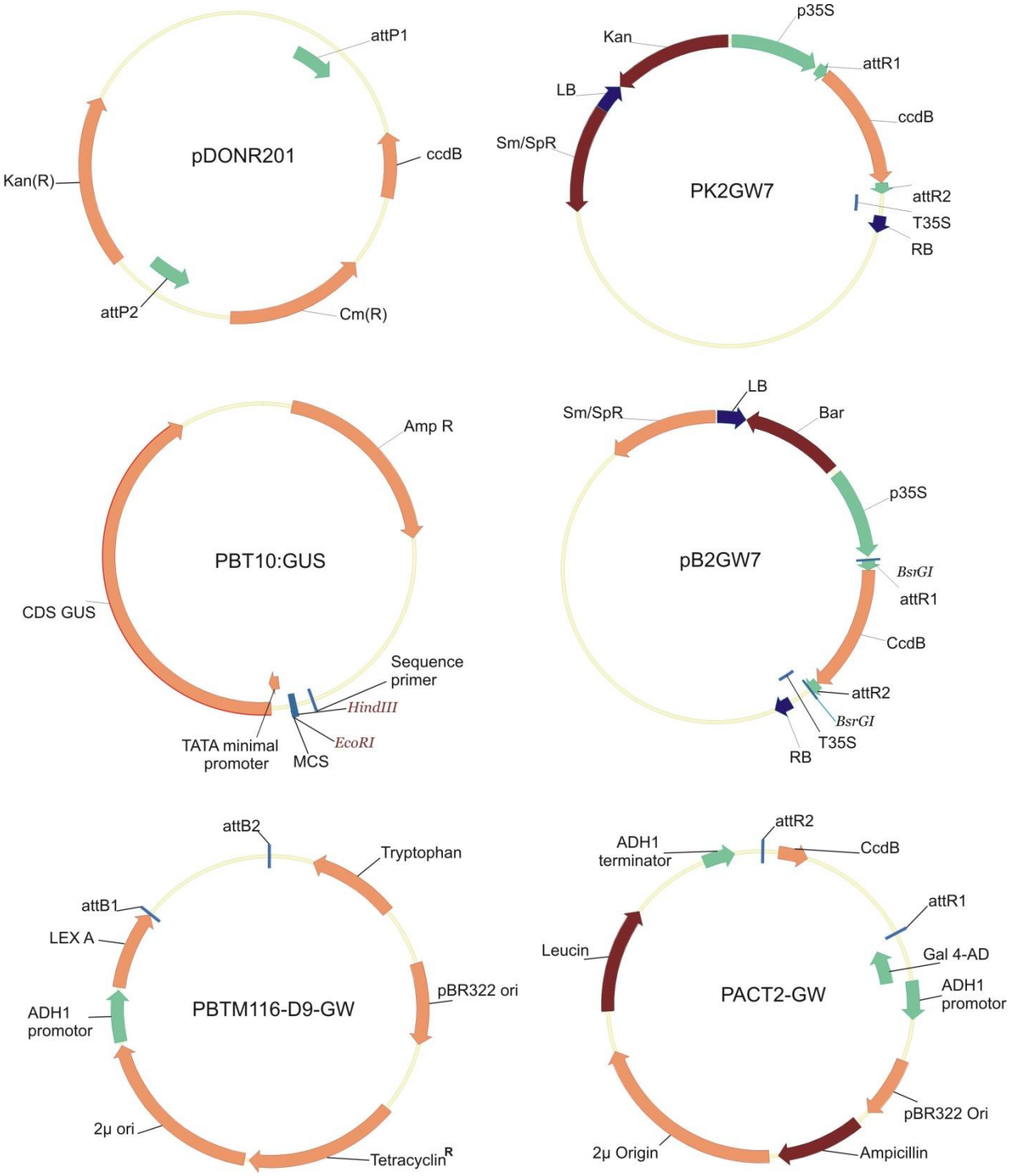
9.0 Publications

Heyl, A*., Ramireddy, E*., Brenner, W.G., Riefler, M., Allemeersch, J. and Schmülling, T. (2008) The transcriptional repressor ARR1-SRDX suppresses pleiotropic cytokinin activities in *Arabidopsis*. *Plant Physiology*, **147**, 1380-1395.

*****These authors contributed equally to the article.

10 Appendix

10.1 Vector maps



10.2 Abbreviations

AHK	*Arabidopsis* histidin kinase
AHP	*Arabidopsis* histidin-phosphotransmitter
ARR	*Arabidopsis* response regulator
bp	Base pair
DAG	Days after germination
DBD	DNA-binding domain
dNTPs	Desoxy-nucleotide triphosphate
GUS	*E. coli* β-glucuronidase gene
H_2O	Water
L	Liter
LB	Luria-Broth
M	Molar
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
MOPS	3-(N-morpholino) propanesulfonic acid
MS	Murashige-Skoog
nM	Nanomolar
OD_{600}	Optical density (600 nm)
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
SSC	Saline sodium citrate buffer
TAE	Tris-acetate-EDTA
TBA	Thiobarbituric acid
Tris	Tris-(hydroxymethyl)-aminomethane
V	Volt
w/v	Weight per volume
°C	Degree celsius
µg	Microgram
µL	Microliter
3-AT	3-Amino-1, 2, 4-Triazole

10.3 Tables

Table 17. Primers used in the present study.

Name	Sense primer	Antisense primer
Primers used in qRT-PCR		
ARR16	CCTTTGAAGCTTGCTGATGT	GAAAACGTTTCATGGTTAGGG
ARR6	GAGCTCTCCGATGCAAAT	GAAAAAGGCCATAGGGGT
ARR7	CTTGGAACCAATCTGCTCTC	ATCATCGACGGCAAGAAC
ARR5	CTACTCGCAGCTAAAACGC	GCCGAAAGAATCAGGACA
CKX4	TCTCAACCTCCGTCTCGT	CATCAATGCCCTCATCTG
CKX6	CAATACGCACCAACCAAG	CCTATTGGGCCTGAAAGA
CRF2	TCCTCCATCAGCAGAAGC	TCTTCCTTGACTACAACGGC
SULT3;4	ATCCTGTGGGGACTGTGA	TTCAAGGCTGGCCATTAG
NRL3	TTAGCCGAGAGGTTGAAGAC	GGGGTGCAAATGAGATTG
AT1G30370	CTTCCACCGCAAGAAATC	CAGACCCTTATGTGCCACT
AT1G53060	ACGGTATCTAAGAAGGCCG	GACTGTCACTTTCCCGTTCT
AT3G50800	AGTCACGAGTCAACTCAACG	CTGGTAATATCAGCTTCGCC
UGT76C2	GGAGATTGCGTGTGGTTT	CTACTAACCAGCCCTTCAGAGA
AT5G48000	TCCGAGACTTTTCGGTTG	ATCCCAAAGTCTTGGGTCTC
AT4G23800	CAACGCTACTGTTACTGCTCTC	CTGGTAGTAGCCGCAGACTT
AT1G61340	TTCGCGTATAGTACACCTCG	GACGGTACCTTTTCTGCAAC
ERF5	GAAACGGAAGAGAGACGATG	GAAACCCCGTTAAATCCC
ERF105	ACGCCATCAAGTTGGAAG	TCCAGTTTGACCCTCTGAAG
AT3G29670	CTGTATGATTCCGGTGGG	CCTTCCACGAAAGTGTCC
AT2G24600	GACCGGCACAAGATTTACTT	AGAGATAACATAGGTGAGACCAGTT
ZAT12	GGAGATCAAGTCGACGGT	TAAGGCTTGGAACGAATGA
UBC10	CCATGGGCTAAATGGAAA	TTCATTTGGTCCTGTCTTCAG
AT3G25800	CCATTAGATCTTGTCTCTCTGCT	GACAAAACCCGTACCGAG
Primers used in the *ARR6* promoter deletion analysis		
-1000 bp	GCAAGCTTCAATCACAACAGCTCATGAACAAAATC	GCTCTAGAGAAACCATGGTGGCAGTGGTTGGGC
-350 bp	GCAAGCTTCACAAATAATTTAAAAATAAAAAAAGAATTC	GCTCTAGAGAAACCATGGTGGCAGTGGTTGGGC
-300 bp	GCAAGCTTGGTTTGGGTCGGTTAAATCTT	GCTCTAGAGAAACCATGGTGGCAGTGGTTGGGC
-279 bp	GCAAGCTTGCATCCCATTCCAAATTTTC	GCTCTAGAGAAACCATGGTGGCAGTGGTTGGGC
-241 bp	GCAAGCTTGACTAAACTTGAACCGATTG	GCTCTAGAGAAACCATGGTGGCAGTGGTTGGGC
-220 bp	GCAAGCTTTTTCGGTTTTTTGTTTT	GCTCTAGAGAAACCATGGTGGCAGTGGTTGGGC
-193 bp	GCAAGCTTAACTTATTTAATAATAAAAA	GCTCTAGAGAAACCATGGTGGCAGTGGTTGGGC
-173 bp	GCAAGCTTTCCACCGAACTATTATTTATAT	GCTCTAGAGAAACCATGGTGGCAGTGGTTGGGC
-145 bp	GCAAGCTTTAAAATAAAGATTTTGAAAGCA	GCTCTAGAGAAACCATGGTGGCAGTGGTTGGGC
48 bp (-241 to -	GCAAGCTTGACTAAACTTGAAC	GCTCTAGAAAAACAAAAAACC

193)	CGATTG	
27 bp (-220 to -193)	GCCAAGCTTTTTGACTTTATTT	GCTCTAGAAAAACAAAAAACC
Primers used in site-directed mutagenesis		
-283 bp	GGTTTGGGTCGGTTAAAAGTTGCATCCCATTCCAAA	TTTGGAATGGGATGCAACTTTTAACCGACCCAAACC
-136 bp	ATATAAAATATAAAATAAACTTTTTGAAAGCAAATT	AATTTGCTTTCAAAAAGTTTATTTTATATTTTATAT
Primers used in RT-PCR and Northern blot analysis		
ARR1-SRDX	CTATGCTAACCTCTTCGCAATCATCCATCA	AACCCAAACGGAGTTCTAGATCCAGATCGA
ARR1	GAACAATATCCCCGAGAGCA	TCTGGTGGTGAACATGTCGT
ARR5	GGAAGTTCATCGAGCGGTT	ATCCAGTCATCCCAGGCATA
ARR7	GAGGAATGAAGCTGAGGAATGC	CATTCGTTTTGAACATGAAGAGTCC
SRDX-ARR1	CTCTCGATCTGGATCTAGAACTCC	TCCGAGTCCTCTTCCGTGAC
SRDX-ARR19	CTCTCGATCTGGATCTAGAACTCC	GAAGTCCATCAATTCCAGGC
Actin2	TACAACGAGCTTCGTGTTGC	GATTGATCCTCCGATCCAGA
Primers used in the PTA		
ARR2 genomic clone	CTTCTTCTCCTTCTCTGATCGTTCG	GTTTTAGAGTGCACTGTATTCGAGT
ARR10 cDNA	CACCATGACTATGGAGCAAGAAATTG	TCAAGCTGACAAAGAAAAGGG
ARR12 cDNA	AAAAAGCAGGCTTCATGACTGTTGAACAAAATTT	AGAAAGCTGGGTCTGTCACACCTGCTTCATCGT
ARR19 cDNA	AAAAAGCAGGCTTCATGTTGGTGGGAAAGATAA	AGAAAGCTGGGTCAGTGAGTTCAAGATCTGTCG
ARR21 cDNA	AAAAAGCAGGCTTCATGGCTTCTGCTCAATCTTTCT	AGAAAGCTGGGTCTCAATTGTGACCAATCTGATCGAAA
Primers used in yeast two-hybrid and BiFC analysis		
ERF8	AAAAAGCAGGCTTGATGCCCACATCACCATG	AGAAAGCTGGGTACTATTCCGCCGGAGGAG
VIP1	AAAAAGCAGGCTTGATGGAAGGAGGAGGAAGA	AGAAAGCTGGGTATCAGCCTCTCTTGGTGAA

Table 18: Putative target genes of B-type ARRs. Clustering analysis was performed on all genes that are regulated in WT and ARR1-S-8 plants. The genes that are present in Cluster 3 and cluster 4 are representing immediate-early and late responding genes, respectively (for details see 3.2.6.2). The significantly regulated genes (>2.5-fold difference in transcript expression, q< 0.05) are tabulated. The q value for cytokinin effect and cytokinin and genotype interaction effect were calculated as described (Benjamini and Hochberg, 1995). This table was modified after Dr. Brenner.

Cluster 3

	No. of spots above back ground	Ratio Col-0		Ratio ARR1SRDX		FDR-adjusted *p*-value			
CATMA ID		BA15 vs. BA0	BA120 vs. BA0	BA15 vs. BA0	BA120 vs. BA0	Cytokinin effect	interaction effect	AGI	Name or Description
CATMA1a25965	44	13.33	0.81	2.13	1.58	5.99E-04	1.85E-03	AT1G27730	Salt tolerance zinc finger protein responsive to chitin oligomers.
CATMA2a22930	44	10.99	1.69	1.29	1.60	5.25E-02	1.02E-06	AT2G24600	ankyrin repeat family protein, contains ankyrin repeats, Pfam:PF00023
CATMA2a37060	41	3.71	0.68	1.83	1.16	1.33E-03	2.11E-02	AT2G38790	expressed protein
CATMA3a18300	48	10.09	3.26	1.06	0.83	5.17E-03	4.50E-03	AT3G18680	aspartate/glutamate/uridylate kinase family protein, similar to UMP-kinase GB:CAB38122 gi:4468612 from (*Lactococcus lactis*)
CATMA3a37170	42	13.04	1.58	1.05	0.98	1.22E-01	1.04E-04	AT3G44260	CCR4-NOT transcription complex protein, putative, similar to SWISS-PROT:Q9UFF9 CCR4-NOT transcription complex, subunit 8 (CAF1-like protein, CALIFp) (*Homo sapiens*)
CATMA3a43085	48	5.59	0.94	3.71	1.64	2.11E-07	1.81E-04	AT3G50060	myb family transcription factor, contains Pfam profile: PF00249 myb-like DNA-binding domain; identical to cDNA MYB-related protein (1107 bp) GI:1263096
CATMA4a25620	45	8.75	0.93	2.13	1.61	2.97E-06	6.13E-01	AT4G23800	high mobility group (HMG1/2) family protein, similar to HMG2B (*Homo sapiens*) GI:32335; contains Pfam profile PF00505: HMG (high mobility group) box
CATMA4a25630	45	7.62	0.97	1.94	1.32	2.43E-06	3.40E-01	AT4G23800	high mobility group (HMG1/2) family protein, similar to HMG2B (*Homo sapiens*) GI:32335; contains Pfam profile PF00505: HMG (high mobility group) box
CATMA5a31810	51	3.40	2.14	1.57	1.68	1.53E-02	1.78E-03	AT5G36250	protein phosphatase 2C, putative / PP2C, putative
CATMA5a43215	60	8.94	0.33	0.70	0.86	2.37E-01	4.99E-01	AT5G47230	AtERF-5. a member of the ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family. The protein contains one AP2 domain.
CATMA5a47120	45	74.84	0.99	2.38	0.74	3.38E-04	4.49E-03	AT5G51190	encodes a member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family.
CATMA5a57200	58	5.05	0.55	0.87	0.42	4.61E-02	2.54E-01	AT5G61600	encodes a member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family. The protein contains one AP2 domain.
CATMA1a26550	9	28.35	0.62	0.72	0.47	3.56E-07	2.11E-01	AT1G28370	encodes a member of the ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 15 members in this subfamily including ATERF-3, ATERF-4, ATERF-7, and leafy petiole.
CATMA1a61750	23	14.07	0.49	0.70	1.12	2.94E-03	7.36E-01	AT1G72520	lipoxygenase, putative, similar to lipoxygenase gi:1495804 (*Solanum tuberosum*), gi:1654140 (*Lycopersicon esculentum*), GB:CAB56692 (*Arabidopsis thaliana*)
CATMA2a44770	8	12.05	0.37	0.77	0.68	1.51E-03	6.72E-03	AT2G46400	WRKY family transcription factor
CATMA1a50390	43	11.86	1.00	1.66	0.92	6.05E-08	7.74E-03	AT1G61340	F-box family protein, contains Pfam PF00646: F-box domain; similar to LEA protein GI:1350540 from (*Picea glauca*)
CATMA1a50390	43	11.86	1.00	1.66	0.92	6.05E-08	7.74E-03	AT1G61340	F-box family protein, contains Pfam PF00646: F-box domain; similar LEA protein GI:1350540 from (*Picea glauca*)
CATMA4a18523	47	11.26	0.87	0.95	0.57	4.07E-03	4.98E-04	AT4G17490	encodes a member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family (ATERF-6). The protein contains one AP2 domain. There are 18 members in this subfamily including ATERF-1, ATERF-2, AND ATERF-5.

CATMA3a43800	52	11.10	0.60	1.89	1.19	6.71E-06	3.48E-01	AT3G50800	expressed protein
CATMA3a09980	46	9.80	1.01	0.88	0.71	1.98E-03	2.88E-01	AT3G10930	expressed protein
CATMA1a62160	41	9.61	1.35	0.91	0.94	1.45E-04	3.82E-01	AT1G72940	disease resistance protein (TIR-NBS class), putative, domain signature TIR-NBS exists, suggestive of a disease resistance protein.
CATMA1a28380	38	9.08	1.39	1.21	0.57	1.73E-04	7.58E-01	AT1G30370	lipase class 3 family protein, similar to DEFECTIVE IN ANTHER DEHISCENCE1 (*Arabidopsis thaliana*)
CATMA5a55595	57	8.86	0.96	1.05	1.22	2.74E-06	1.75E-01	AT5G59820	zinc finger (C2H2 type) family protein (ZAT12), identical to zinc finger protein ZAT12 (*Arabidopsis thaliana*)
CATMA4a31980	33	8.52	0.82	1.26	0.97	1.33E-05	4.05E-02	AT4G30370	zinc finger (C3HC4-type RING finger) family protein, contains Pfam profile: PF00097 zinc finger,
CATMA4a25830	52	8.33	1.23	1.63	0.99	5.28E-05	5.88E-01	AT4G24110	expressed protein
CATMA2a24120	36	7.94	3.21	1.18	0.68	1.19E-04	9.44E-01	AT2G25735	expressed protein
CATMA1a16150	48	7.88	1.22	1.76	0.74	9.53E-08	5.12E-04	NA	NA
CATMA2a33890	11	7.05	0.86	1.75	0.87	1.45E-01	8.37E-01	AT2G35710	glycogenin glucosyltransferase (glycogenin)-related, low similarity to glycogenin-2 from *Homo sapiens* (SP|O15488)
CATMA2a18610	5	6.77	1.20	2.32	0.80	4.24E-03	6.56E-01	AT2G20142	expressed protein
CATMA1a18050	58	6.46	1.62	1.49	1.05	1.56E-08	5.28E-02	AT1G19020	expressed protein
CATMA3a49870	56	6.41	1.70	0.98	0.89	1.06E-03	9.62E-02	AT3G56880	VQ motif-containing protein, contains PF05678: VQ motif
CATMA1a11510	34	6.38	3.07	1.13	1.40	4.66E-01	2.38E-01	AT1G12500	phosphate translocator-related, low similarity to glucose-6-phosphate/phosphate-translocator precursor (Zea mays) GI:2997589, phosphoenolpyruvate/phosphate translocator precursor (Mesembryanthemum crystallinum)
CATMA1a70060	19	6.37	0.63	0.68	0.44	4.80E-03	7.83E-03	AT1G80840	WRKY family transcription factor, similar to WRKY transcription factor GB:BAA87058 GI:6472585 from (Nicotiana tabacum)
CATMA4a28860	60	6.34	0.41	1.57	0.98	2.67E-04	2.99E-01	AT4G27280	calcium-binding EF hand family protein, similar to EF-hand Ca2+-binding protein CCD1 (*Triticum aestivum*)
CATMA2a37900	33	6.15	0.79	1.30	1.65	5.64E-06	3.46E-05	AT2G39650	expressed protein, contains Pfam profile PF04720: Protein of unknown function (DUF506)
CATMA2a24855	55	5.65	0.23	0.86	0.58	8.92E-03	4.25E-01	AT2G26530	mRNA for AR781, complete cds
CATMA4a11410	18	5.58	0.16	1.83	0.90	7.06E-07	8.72E-01	AT4G11280	encodes a a member of the 1-aminocyclopropane-1-carboxylate (ACC) synthase (S-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14) gene family
CATMA1a17615	19	5.48	0.67	2.37	0.71	9.03E-06	5.64E-04	AT1G18570	myb family transcription factor (MYB51), contains PFAM profile: PF00249
CATMA4a16796	21	5.47	0.46	2.75	1.58	1.31E-03	8.60E-01	AT4G15975	zinc finger (C3HC4-type RING finger) family protein, contains Pfam domain, PF00097: Zinc finger,
CATMA3a15920	57	5.25	1.00	1.02	0.89	1.87E-05	8.43E-01	AT3G16510	C2 domain-containing protein, contains similarity to shock protein SRC2 (*Glycine max*) gi|2055230|dbj|BAA19769 ; contains Pfam profile PF00168:C2 domain
CATMA5a39280	40	4.78	1.52	1.03	1.37	4.61E-04	8.37E-01	AT5G43420	zinc finger (C3HC4-type RING finger) family protein, low similarity to RING-H2 zinc finger protein ATL4 (*Arabidopsis thaliana*),
CATMA2a39370	28	4.54	0.73	3.02	1.38	1.74E-07	8.03E-01	AT2G41010	VQ motif-containing protein, contains PF05678: VQ motif
CATMA1a27610	60	4.46	1.63	2.68	1.33	8.97E-09	1.90E-03	AT1G29690	Encodes a protein containing a domain with significant homology to the MACPF (membrane attack complex and perforin) domain of complements and perforin proteins that are involved in innate immunity in animals.
CATMA3a54340	55	4.24	0.59	1.24	1.01	1.76E-04	4.04E-02	AT3G61190	BON1-associated protein 1 (BAP1), identical to BON1-associated protein 1 (*Arabidopsis thaliana*) GI:15487384; contains Pfam profile PF00168: C2 domain; supporting cDNA
CATMA3a16130	60	3.92	0.84	1.24	0.79	9.49E-02	2.22E-01	AT3G16720	zinc finger (C3HC4-type RING finger) family protein, contains Pfam domain, PF00097:
CATMA1a18220	51	3.84	0.51	1.28	1.14	7.85E-06	3.76E-04	AT1G19180	expressed protein
CATMA3a25380	41	3.79	0.95	1.34	0.80	1.87E-06	4.18E-03	AT3G25600	calmodulin, putative, similar to calmodulin GI:239841 from (*Paramecium tetraurelia*)

CATMA2a38380	59	3.77	0.54	0.98	0.78	1.34E-03	8.19E-02	AT2G40140	zinc finger (CCCH-type) family protein, contains Pfam domain, PF00642: Zinc finger C-x8-C-x5-C-x3-H type (and similar) and Pfam domain, PF00023: Ankyrin repeat
CATMA2a34120	50	3.69	0.57	1.19	1.17	1.29E-03	6.51E-02	AT2G35930	U-box domain-containing protein, similar to immediate-early fungal elicitor protein CMPG1 (*Petroselinum crispum*)
CATMA5a52970	20	3.68	0.90	2.67	1.87	4.67E-05	4.07E-01	AT5G57220	cytochrome P450, putative, similar to Cytochrome P450 (SP:O65790) (*Arabidopsis thaliana*); Cytochrome P450 (GI:7415996) (*Lotus japonicus*)
CATMA1a52950	50	3.66	1.00	1.33	1.02	1.97E-05	3.12E-02	AT1G63720	expressed protein, similar to putative protein GB:CAA18164 (*Arabidopsis thaliana*)
CATMA5a39830	52	3.45	1.33	1.46	1.27	1.37E-05	7.95E-02	AT5G44060	expressed protein, similar to unknown protein (gb|AAD10670.1)
CATMA5a45500	19	3.40	0.83	0.86	0.22	3.79E-06	1.01E-02	AT5G49520	WRKY family transcription factor, contains Pfam profile: PF03106 WRKY DNA -binding domain
CATMA3a50050	45	3.28	1.45	1.79	1.14	3.02E-02	5.48E-02	AT3G57070	glutaredoxin family protein, contains Pfam profile PF00462: Glutaredoxin
CATMA3a50050	45	3.28	1.45	1.79	1.14	3.02E-02	5.48E-02	AT3G57070	glutaredoxin family protein, contains Pfam profile PF00462: Glutaredoxin
CATMA1a23010	9	3.23	0.70	1.26	1.37	5.20E-03	1.23E-01	AT1G24145	expressed protein
CATMA1a60030	51	3.18	1.17	1.95	1.31	6.89E-06	1.65E-01	AT1G70740	protein kinase family protein, contains protein kinase domain, Pfam:PF00069
CATMA1a13385	60	3.08	1.19	0.92	1.19	1.85E-06	1.11E-01	AT1G14370	APK2a mRNA for protein kinase, complete cds
CATMA5a57490	47	2.89	1.04	1.06	0.77	2.56E-04	4.08E-01	AT5G61900	copine BONZAI1 (BON1), nearly identical to BONZAI1 (*Arabidopsis thaliana*) GI:15487382; contains Pfam profile PF00168: C2 domain
CATMA3a42560	60	2.82	1.00	1.28	1.05	5.77E-06	5.24E-04	AT3G49530	no apical meristem (NAM) family protein, contains Pfam PF02365: No apical meristem (NAM) domain; similar to NAC2 - *Arabidopsis thaliana*, EMBL:AF201456
CATMA3a43300	60	2.59	0.76	0.98	0.73	2.81E-05	6.71E-01	AT3G50260	encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family. The protein contains one AP2 domain.
CATMA1a31220	58	2.54	0.90	0.98	1.03	8.95E-02	1.38E-02	NA	NA
CATMA5a02916	55	0.93	0.33	1.31	1.47	8.85E-01	1.38E-03	AT5G03740	zinc finger (C2H2 type) family protein, contains Pfam domain, PF00096:
CATMA5a43410	58	1.15	0.24	1.14	0.52	8.35E-13	9.49E-11	AT5G47450	major intrinsic family protein / MIP family protein, contains Pfam profile: MIP PF00230

Cluster 4

CATMA2a24060	58	0.78	1.36	0.57	0.90	1.26E-07	1.19E-12	AT2G25680	expressed protein, ; expression supported by MPSS
CATMA4a31370	29	32.55	15.95	2.59	3.17	3.28E-07	0.57914	AT4G29740	FAD-binding domain-containing protein / cytokinin oxidase family protein, similar to cytokinin oxidase, *Zea mays* (gi:3882018) (gi:3441978)
CATMA4a40560	59	2.98	2.69	2.05	2.25	0.004874	0.09291	AT4G39070	zinc finger (B-box type) family protein, salt-tolerance protein - *Arabidopsis thaliana*,
CATMA5a04390	35	5.44	1.57	5.57	4.48	1.47E-06	0.119359	AT5G05190	expressed protein, similar to unknown protein (emb|CAB88044.1)
CATMA1a41350	12	23.70	32.00	1.09	1.44	0.039554	0.074622	AT1G50280	phototropic-responsive NPH3 family protein, contains NPH3 family domain, Pfam:PF03000
CATMA1a18090	38	21.16	10.81	3.57	7.63	1.07E-05	0.537151	AT1G19050	ARR7 mRNA for response regulator 7
CATMA1a03190	18	12.20	4.35	3.26	2.08	3.35E-05	0.332441	AT1G04360	zinc finger (C3HC4-type RING finger) family protein, contains Pfam profile: PF00097 zinc finger, C3HC4 type (RING finger)
CATMA5a58475	11	10.67	6.84	3.96	4.53	3.42E-09	0.00072	AT5G62920	ARR6 mRNA for response regulator 6
CATMA2a31135	8	8.23	4.84	2.82	2.50	0.005811	0.88824	AT2G32930	zinc finger (CCCH-type) family protein, contains Pfam domain, PF00642: Zinc finger C-x8-C-x5-C-x3-H type (and similar)
CATMA5a35240	13	5.84	1.44	1.36	1.33	0.00213	0.000649	AT5G39670	calcium-binding EF hand family protein, contains INTERPRO:IPR002048 calcium-binding EF-hand domain

CATMA2a38980	18	5.82	12.64	0.81	6.21	7.39E-09	0.001534	AT2G40670	response regulator 16
CATMA4a25570	55	4.59	1.90	1.56	2.84	4.87E-09	7.08E-08	AT4G23750	*CRF2*, cytokinin response factor2, subfamily B-5 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 7 members in this subfamily.
CATMA3a56645	30	4.18	4.34	1.44	1.89	0.001288	0.371512	AT3G63440	CKX6, cytokinin oxidase family protein *Arabidopsis thaliana*
CATMA5a43950	81	4.12	20.52	1.79	11.50	0.000214	0.785307	AT5G47980	transferase family protein, similar to alcohol acyltransferase (Fragaria x ananassa)(GI:10121328)(PMID:10810141), deacetylvindoline 4-O-acetyltransferase (*Catharanthus roseus*)
CATMA2a06710	16	3.71	5.81	2.84	2.59	0.036522	0.342756	NA	NA
CATMA1a11430	26	3.56	3.67	1.43	1.74	1.04E-06	0.155239	AT1G12420	ACR8; functions in amino acid binding; Involved in: response to abscisic acid stimulus; located in: cellular_component unknown;
CATMA4a24390	37	3.51	6.83	0.85	1.51	3.28E-05	0.957813	NA	NA
CATMA2a39655	26	3.47	1.70	1.77	1.11	0.045911	0.075814	AT2G41310	two-component responsive regulator / response reactor 3 (RR3), identical to response reactor 3 GI:3273200 from (*Arabidopsis thaliana*); contains Pfam profile: PF00072 response regulator receiver domain
CATMA3a14930	55	3.46	1.54	1.10	1.18	0.000767	0.002143	AT3G15520	peptidyl-prolyl cis-trans isomerase TLP38, chloroplast / thylakoid lumen PPIase of 38 kDa / cyclophilin / rotamase, cyclophylin-type; identical to SP|P82869 Thylakoid lumenal 38 kDa protein, chloroplast precursor (P38) {*Arabidopsis thaliana*}
CATMA2a22070	49	3.37	2.29	0.55	0.58	0.027001	0.000108	AT2G23680	stress-responsive protein, putative, similar to cold acclimation WCOR413-like protein gamma form (*Hordeum vulgare*) gi|18449100|gb|AAL69988; similar to stress-regulated protein SAP1 (*Xerophyta viscosa*)
CATMA3a43440	24	3.23	3.45	0.88	0.70	0.038152	0.114084	AT3G50380	expressed protein
CATMA4a22800	49	3.21	3.24	1.96	1.12	0.008596	0.225011	AT4G21210	expressed protein, contains Pfam domain PF03618: Domain of unknown function (DUF299)
CATMA3a29930	36	3.12	2.20	1.87	2.36	5.52E-05	0.020486	AT3G29670	transferase family protein, similar to anthocyanin 5-aromatic acyltransferase from Gentiana triflora GI:4185599, malonyl CoA:anthocyanin 5-O-glucoside-6'''-O-malonyltransferase from *Perilla frutescens*
CATMA1b14565	33	2.87	2.26	1.48	2.26	1.24E-06	0.076647	AT1G15550	gibberellin 3-beta-dioxygenase / gibberellin 3 beta-hydroxylase (GA4), identical to gibberellin 3 beta-hydroxylase (GI:2160454)
CATMA2a21630	36	2.87	2.36	2.03	1.58	0.001881	0.006106	AT2G23130	AGP17 is a lysine-rich arabinogalactan-protein (AGP) and part of a multi-gene family of glycoproteins with approx. 50 members. It falls into one subclass with AGP18 and AGP19, other lysine-rich AGPs.
CATMA4a39295	36	2.83	2.52	1.74	0.92	0.001208	0.000909	AT4G37790	homeobox-leucine zipper protein 22 (HAT22) / HD-ZIP protein 22, identical to homeobox-leucine zipper protein HAT22 (HD-ZIP protein 22) (SP:P46604) (*Arabidopsis thaliana*)
CATMA4a09010	57	2.57	1.55	1.55	1.34	8.77E-05	7.66E-05	AT4G09030	arabinogalactan protein (AGP10) mRNA, complete cds
CATMA1a09322	60	2.07	3.02	0.91	3.13	1.38E-09	2.65E-05	AT1G10470	ARR4 mRNA for response regulator 4 and two-component response regulator homolog (IBC7)
CATMA3a15410	11	4.03	6.66	0.85	3.55	2.5E-05	0.153883	AT3G15990	sulfate transporter, putative, similar to sulfate transporter (*Arabidopsis thaliana*) GI:2285885; contains Pfam profiles PF00916: Sulfate transporter family, PF01740: STAS domain
CATMA3a29720	60	1.83	2.59	0.89	1.88	2.35E-05	0.651252	AT3G29575	expressed protein
CATMA3a54760	59	1.81	3.22	0.93	1.19	1.96E-06	0.102064	AT3G61630	AP2 domain-containing transcription factor, putative, transcription factor Pti6-*Lycopersicon esculentum*, PIR:T07728
CATMA5a05050	47	1.79	4.10	1.38	2.73	4.78E-06	0.032819	AT5G05860	UDP-glucoronosyl/UDP-glucosyl transferase family protein, contains Pfam profile: PF00201 UDP-glucoronosyl and UDP-glucosyl transferase
CATMA5a19270	23	1.91	3.06	1.38	2.71	0.000104	0.057081	AT5G20740	invertase/pectin methylesterase inhibitor family protein, low similarity to pectinesterase from *Arabidopsis thaliana* SP|Q42534,
CATMA5a43970	75	1.27	5.65	1.00	6.90	0.00011	0.101481	AT5G48000	cytochrome P450 family protein, similar to steroid 22-alpha-hydroxylase; DWF4; CYP90B1 (GI:2935342) (*Arabidopsis thaliana*)
CATMA5a47710	56	1.73	3.56	0.98	3.08	1.96E-07	0.001154	AT5G51780	basix helix-loop-helix (bHLH) family protein, contains Pfam profile: PF00010 helix-loop-helix

									DNA-binding domain
CATMA5a56490	9	1.44	2.97	0.96	1.19	0.001126	0.002378	AT5G60760	2-phosphoglycerate kinase-related, contains weak similarity to 2-phosphoglycerate kinase (GI:467751) (*Methanothermus fervidus*)
CATMA5a62970	8	2.39	3.38	0.77	1.72	0.008991	0.559083	AT5G67520	adenylylsulfate kinase, putative, similar to adenylylsulfate kinase, chloroplast precursor (APS kinase, Adenosine-5'phosphosulfate kinase, ATP adenosine-5'- phosphosulfate 3'-phosphotransferase) (*Catharanthus roseus*)
CATMA2a24280	13	1.18	0.90	3.74	2.17	0.016014	0.000181	AT2G25890	glycine-rich protein / oleosin
CATMA2a33425	22	1.47	1.26	3.68	2.45	0.00022	4.2E-07	AT2G35300	late embryogenesis abundant group 1 domain-containing protein
CATMA2a40780	44	2.36	1.16	2.67	1.34	1.73E-05	0.000222	AT2G42380	bZIP transcription factor family protein
CATMA3a21260	8	1.08	1.06	3.09	2.87	0.108864	0.005973	AT3G21380	similar to jacalin lectin family protein [*Arabidopsis thaliana*] (TAIR:At1g52040.1); similar to myrosinase binding protein [*Brassica napus]* (GB:CAA70587.1)
CATMA5a40370	27	0.95	1.00	2.87	2.76	0.048447	0.003877	AT5G44570	expressed protein
CATMA1a44090	52	1.75	1.95	1.35	2.83	6.28E-05	0.216003	AT1G53060	legume lectin family protein
CATMA2a34180	11	0.92	0.67	0.61	5.14	0.026737	0.018091	AT2G35980	harpin-induced family protein (YLS9) / HIN1 family protein / harpin-responsive family protein, similar to harpin-induced protein hin1 (GI:1619321) (*Nicotiana tabacum*); identical to cDNA YLS9 mRNA for hin1 homolog GI:13122295
CATMA4a29590	33	1.67	1.24	1.43	2.51	4.18E-05	0.000178	AT4G27970	C4-dicarboxylate transporter/malic acid transport family protein, contains Pfam profile PF03595: C4-dicarboxylate transporter/malic acid transport protein
CATMA5a03540	39	1.56	1.67	0.92	3.15	0.017453	0.766181	AT5G04330	cytochrome P450, putative / ferulate-5-hydroxylase, putative, Similar to Cytochrome P450 84A1 Ferulate-5-hydroxylase)(SP:Q42600)(*Arabidopsis thaliana*);