THE ROLE OF AUXIN RESISTANT 1 (AXR1) IN ARABIDOPSIS CYTOKININ SIGNALING

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THE ROLE OF AUXIN RESISTANT 1 (AXR1) IN
ARABIDOPSIS CYTOKININ SIGNALING

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture
at the University of Kentucky

By
Yan Li
Lexington, Kentucky

Director: Dr. Jan A. Smalle, Associate Professor of Plant Physiology
Lexington, Kentucky
2012
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The plant hormone cytokinin plays essential roles in many aspects of growth and development. The cytokinin signal is transmitted by a multistep phosphorelay to the members of two functionally antagonistic classes of Arabidopsis response regulators (ARRs): the type-B ARRs (response activators) and type-A ARRs (negative-feedback regulators). Previous studies have shown that mutations in AXR1, encoding a subunit of the E1 enzyme in the related to ubiquitin (RUB) modification pathway, leads to decreased cytokinin sensitivity. This research shows that the cytokinin resistance of axr1 seedlings is suppressed by loss-of-function of type-A ARRs and that the cytokinin resistance caused by ectopic expression of ARR5, a type-A ARR family member, is enhanced in axr1 background. Based on the established role of the RUB pathway in ubiquitin-dependent proteolysis, these data suggested that AXR1 promotes the cytokinin response by facilitating type-A ARR degradation. Indeed, both genetic (axr1 mutants) and chemical (MLN4924) suppression of RUB E1 increased ARR5 stability, suggesting that the ubiquitin ligase that promotes ARR5 proteolysis requires RUB modification for optimal activity. In addition, ARR1, a type-B ARR family member, also accumulated in the axr1 mutant background, suggesting that AXR1 regulates primary cytokinin signaling at multiple levels.

Key words: AXR1, cytokinin signaling, RUB pathway, 26S proteasome, Arabidopsis.
THE ROLE OF AUXIN RESISTANT 1 (AXR1) IN ARABIDOPSIS CYTOKININ SIGNALING

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Chapter 1: Introduction

1.1 Outline of this dissertation

The research described in this dissertation is about the ubiquitin/proteasome-dependent regulation of the stability of proteins involved in the primary cytokinin signaling pathway in *Arabidopsis thaliana*. Because both cytokinin signaling and proteasome-dependent regulation of protein stability are well-developed research fields, Section 1.2 summarizes the current knowledge of cytokinin function and signaling in Arabidopsis, emphasizing the signaling, and Section 1.3 focuses on the functional mechanisms of ubiquitin/proteasome-dependent regulation of protein stability.

Chapters 2 to 6 describe the isolation of a cytokinin resistant mutant *soi1*, the positional cloning of the gene defined by the *soi1* locus, and the elucidation of the function of the protein product encoded by this isolated gene, *AUXIN RESISTANT 1 (AXR1)*, in cytokinin signaling.

Chapter 7 summarizes the findings presented in previous chapters and presents the hypothesis concerning AXR1-dependent proteolytic control of cytokinin signaling.

1.2 Cytokinin: metabolism, signaling and functions

Cytokinins represent a class of N6-substituted adenine derivatives possessing either an isoprenoid or an aromatic side chain (Sakakibara, 2006). Kinetin, the first discovered cytokinin, was described in 1955 as a cell division promoting factor in tobacco tissue culture (Miller et al., 1955). The first isolated cytokinin trans-zeatin (tZ) was purified from maize endosperm (Miller, 1961). Following these initial discoveries, more than 200 natural and synthetic cytokinins have been described (Mok and Mok, 2001).

Since their initial description as cell division promoting factors, cytokinins have been reported to be involved in many aspects of plant growth and
development, such as seed germination (Riefler et al., 2006), shoot and root development (Werner et al., 2003), vascular differentiation (Mähönen et al., 2000), leaf senescence (Gan and Amasino, 1995; Riefler et al., 2006), transduction of nutrition signals (Takei et al., 2001b), nodulation (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007), circadian rhythms (Hanano et al., 2006), stress response (Rivero et al., 2007; Tran et al., 2007), pathogen resistance (Sakakibara et al., 2005; Igari et al., 2008) and increased crop productivity (Ashikari et al., 2005).

In spite of the importance of cytokinins in plant development and physiology, the basic molecular mechanisms of its biosynthesis and signal transduction have only been elucidated in recent years, facilitated by the genetic and molecular analysis of mutants and the identification of genes encoding enzymes and protein factors controlling key steps of cytokinin metabolism and signaling.

### 1.2.1 Cytokinin metabolism

A simplified model of cytokinin metabolism, which includes biosynthesis, activation and degradation, is shown in Figure 1.1.
Figure 1.1 Simplified model of cytokinin metabolism (adapted from Kudo et al., 2010).

Solid arrows indicate reactions catalyzed by known enzymes. Dashed arrows indicate reactions catalyzed by enzymes which remain unknown. CKX, cytokinin oxidase/dehydrogenase; cZ, cis-zeatin; cZR, cZ riboside; cZRMP, cZ riboside 5’-monophosphate; DMAPP, dimethylallyl pyrophosphate; iP, isopentenyladenine; iPR, iP riboside; iPRMP, iP riboside 5’-monophosphate; IPT, isopentenyltransferase; LOG, LONELY GUY; tRNA-IPT, tRNA-isopentenyltransferase; tZ, trans-zeatin; tZR, tZ riboside; tZRMP, tZ riboside 5’-monophosphate.

Cytokinin biosynthesis:

The rate limiting step of cytokinin biosynthesis is catalyzed by isopentenyltransferases (IPTs), which exist in two types: adenosine phosphate (ADP/ATP)-IPTs and tRNA-IPTs (Werner and Schmulling, 2009). IPTs catalyze the transfer of an isoprenoid moiety to adenine either in its nucleotide forms (ADP/ATP type) or bound to RNA (tRNA type) (Sakakibara, 2006; Frebort et al., 2011).
The first IPT gene was identified from Agrobacterium tumefaciens (Akiyoshi et al., 1984; Barry et al., 1984). The Tmr (tumor morphology root) gene located on the Ti (tumor-inducing) plasmid was shown to be able to induce tumorigenesis independent of other T-DNA located genes (Lichtenstein et al., 1984). The protein encoded by the Tmr gene was further shown to possess adenylate isopentenytransferase (IPT) activity (Akiyoshi et al., 1984; Barry et al., 1984).

Nine Arabidopsis IPT genes were identified through BLAST searches against bacterial IPT genes (Kakimoto, 2001; Takei et al., 2001a). Two of them encode putative tRNA-IPTs (AtIPT2 and AtIPT9), and the other seven encode ATP/ADP IPTs (AtIPT1, AtIPT3-8) (Kakimoto, 2001). The AtIPT6 gene is a pseudogene (at least in the WS ecotype) (Kakimoto, 2001). Expression pattern analysis of these genes suggests that IPTs catalyze cytokinin biosynthesis at different developmental stages and in different organs (Miyawaki et al., 2004). The expression of IPT genes was also shown to be regulated by phytohormones and nutrient status. For example, AtIPT1, AtIPT3, AtIPT5 and AtIPT7 are down-regulated by cytokinin, AtIPT5 and AtIPT7 are up-regulated by auxin, and AtIPT3 is up-regulated after the application of nitrate in mineral-starved Arabidopsis plants (Miyawaki et al., 2004).

The prenyl side chain of cytokinin could come from the methyldrythritol phosphate (MEP) pathway or the mevalonate (MVA) pathway. The MEP pathway is functional in bacteria and in the plastids of plants, which produces hydroxymethylbutenyl diphosphate (HMBDP), whereas the MVA pathway occurs in the cytosol of eukaryotes, which produces dimethylallyl pyrophosphate (DMAPP) (Kasahara et al., 2004).

Studies suggested that the majority of the prenyl side chain in isopentenyladenine (iP) and tZ comes from the MEP pathway in Arabidopsis (Kasahara et al., 2004). Four of the Arabidopsis IPTs (AtIPT1, AtIPT3, AtIPT5 and AtIPT8) were shown to be localized in plastids (Kasahara et al., 2004), consistent with the existence of the MEP pathway in plastids. Although HMBDP
is the major product of the MEP pathway, AtIPTs cannot use it directly as precursor (Sakakibara et al., 2005). Evidence suggested that the cytokinin biosynthesis in Arabidopsis plastids is achieved through the iP riboside 5’-monophosphate (iPRMP) -dependent pathway: HMBDP is first converted to DMAPP, and then used by AtIPTs to produce iPRMP, which is further converted to tZ riboside 5’-monophosphate (tZRMP) by cytochrome P450 monoxygenase (CYP735A1 and CYP735A2 in Arabidopsis) (Takei et al., 2004).

Ectopically expressed Tmr, the Agrobacterium IPT, was shown to be localized in the plastids in Arabidopsis seedlings, although lacking the typical plastid-targeting sequence (Sakakibara et al., 2005). Tmr is able to directly use HMBDP as the substrate to produce tZRMP in an iPRMP-independent manner without the P450 monoxygenase-mediated hydroxylation (Sakakibara et al., 2005). The expression of the CYP735A gene has been reported to be negatively regulated by auxin (Takei et al., 2004). Since tumorigenesis requires increased synthesis of both auxin and cytokinin, the direct usage of HMBDP as substrate for tZ biosynthesis was suggested as an approach which Agrobacterium uses to circumvent the hormonal homeostasis. Consistent with this character, engineered expression of the Agrobacterium IPT gene in plants increased the level of tZ-type cytokinin without a substantial increase in the production of iP-type cytokinins (Astot et al., 2000; Van der Graaff et al., 2001).

Cytokinin activation:

The isolation of the rice LONELY GUY (LOG) gene provides a new mechanism to regulate cytokinin function (Kurakawa et al., 2007). LOG encodes an enzyme with cytokinin-specific phosphoribohydrolase activity, which can convert inactive cytokinin nucleotides into active free-base forms. The LOG mRNA is localized in shoot meristem tips and its loss of function causes premature termination of the shoot meristem. Nine LOG genes (AtLOG1-9) were identified in the Arabidopsis genome, with two of them, AtLOG6 and AtLOG9, as probable pseudogenes (Kuroha et al., 2009). The AtLOG genes showed differential expression in various tissues during development and multiple mutants of AtLOGs showed a
lower sensitivity to iP riboside in terms of lateral root formation. The Arabidopsis LOG proteins prefer isoprenoid cytokinins as their substrates and the reactivity towards aromatic cytokinins is generally lower. Conditional overexpression of AtLOG genes increased the levels of iP and glucosides while reducing the levels of nucleotides.

Cytokinin degradation:

Cytokinin degradation is achieved through oxidative cleavage of the N⁶ side chain by cytokinin oxidase/dehydrogenases (CKXs). CKX is the only known enzyme shown to catalyze the irreversible inactivation of cytokinins. There are seven members (CKX1-7) in the Arabidopsis AtCKX gene family (Bilyeu et al., 2001; Schmulling et al., 2003). The AtCKX genes showed differential expression during plant development with the activity predominantly confined to zones of active growth, such as the vascular cylinder of lateral roots, the shoot apex, young shoot tissue, developing trichomes and stomata (Werner et al., 2003). The AtCKX proteins were shown to be localized to the vacuoles or the endoplasmic reticulum and possibly the extracellular space (Werner et al., 2003). AtCKXs are reported to have differential substrate specificity: AtCKX2, 4 and 6 preferred the free cytokinin base, while the others preferred glucosides or nucleotides as substrates (Galuszka et al., 2007). This substrate specificity is suggested to be related to either the specific difference of the enzyme’s three dimensional structure (Galuszka et al., 2007), or the enzyme’s subcellular localization (Kowalska et al., 2010).

1.2.2 Cytokinin signaling in Arabidopsis

The plant cytokinin signaling pathway is similar to two-component systems (TCS) known in prokaryotes and lower eukaryotes (Hwang and Sheen, 2001). The simplest form of TCS consists of two conserved proteins: a histidine kinase sensor and a response regulator protein (Hwang et al., 2002). The histidine kinase sensor perceives stimulus and autophosphorylates on a conserved His residue in its kinase domain and then transfers the phosphoryl group to a
conserved Asp residue in the receiver domain of the response regulator, which activates the downstream responses. More complex TCS involves a multi-step phosphorelay (His→Asp→His→Asp). The system is comprised of a hybrid histidine kinase sensor containing both a kinase and a receiver domain, an intermediate phosphotransfer protein containing a conserved His residue and a response regulator. Upon signal perception, the hybrid histidine kinase sensor autophosphorylates on a His residue in its kinase domain and transfers the phosphoryl group to a Asp residue in its receiver domain, then to the His residue in the histidine phosphotransfer protein and ultimately to the Asp residue in the response regulator (Hwang et al., 2002).

In Arabidopsis, the cytokinin signal is transmitted through a multi-step phosphorelay (Figure1.2). Cytokinins are first perceived by Arabidopsis histidine kinase receptors (AHKs), which were recently reported to be localized in the endoplasmic reticulum (ER) membrane (Inoue et al., 2001; Suzuki et al., 2001a; Yamada et al., 2001; Caesar et al., 2011; Wulfetange et al., 2011). Binding of cytokinin to a receptor triggers autophosphorylation on a conserved His residue, and the subsequent transfer of the phosphoryl group to a conserved Asp residue within the receptors' receiver domain. The phosphoryl group is then transferred to a conserved His residue in the Arabidopsis histidine phosphotransfer proteins (AHPs). Phosphorylated AHPs transfer the phosphoryl group to a conserved Asp residue in the Arabidopsis response regulators (ARRs) (Hwang and Sheen, 2001; Hutchison et al., 2006; Punwani et al., 2010). All the components of the Arabidopsis cytokinin signaling system are encoded by multigene families (To and Kieber, 2008).
Figure 1.2 The primary cytokinin signaling pathway in Arabidopsis

(adapted from Hwang et al., 2012).

H and D represent the conserved His and Asp residues, respectively. Solid blue arrows indicate the phosphotransfer within two-component elements. Dashed blue arrow indicates the dephosphorylation of AHPs by CRE1/AHK4 in the absence of cytokinin. Purple arrow indicates the active transport of AHPs between the cytosol and nucleus.

Abbreviations: AHK, Arabidopsis histidine kinase; AHP, Arabidopsis histidine phosphotransfer protein; ARR, Arabidopsis response regulator; CRE1, CYTOKININ RESISTANT 1; CRF, CYTOKININ RESPONSE FACTOR; ER, endoplasmic reticulum; PM, plasma membrane.
1.2.2.1 AHKs

The first identified cytokinin receptor was CRE1, which was isolated in a genetic screen for cytokinin insensitive mutants in tissue culture (Inoue et al., 2001). The cre1 mutant exhibited a reduced response to cytokinin in a cytokinin-induced callus formation assay. CRE1 overexpression confers a cytokinin dependent growth phenotype in a yeast mutant lacking the endogenous histidine kinase. CRE1 is identical to the WOODENLEG (WOL) gene, a previously identified gene required for the vascular morphogenesis of the Arabidopsis root (Mähönen et al., 2000). In parallel, functional characterization of histidine kinase gene AHK4, identified in silico, indicated that AHK4 acts as a cytokinin receptor and is identical to CRE1/WOL (Suzuki et al., 2001b; Ueguchi et al., 2001).

The AHK family has eight members, but only three of them function as cytokinin receptors: AHK2, AHK3 and CRE1/AHK4 (Hwang and Sheen, 2001). All three cytokinin receptors contain two to three transmembrane domains at the N-terminus, followed by a histidine kinase domain carrying the conserved His residue, a receiver-like domain, and a receiver domain carrying the conserved Asp residue (Figure 1.3, Heyl and Schmülling, 2003). The cytokinin binding region located in the N-terminus is called the CHASE domain (cyclases/histidine-kinase-associated sensory extracellular), which has a relatively low level of amino acid conservation among different receptors, but a highly homologous secondary structure (Heyl and Schmülling, 2003). The CHASE domain is present in a variety of functionally diverse membrane receptor proteins, but within the AHK family, it is specific for the three cytokinin receptors. Evidence suggested that different receptors have differential affinities for specific cytokinin molecules (Spichal et al., 2004; Romanov et al., 2006). The C-terminal receiver domain is required for signal transduction. CRE1/AHK4 proteins carrying a deletion of the receiver domain or a mutation of its conserved Asp residue were unable to complement the corresponding yeast TCS signal transduction mutants which lack the histidine kinase receptors (Hwang and Sheen, 2001; Inoue et al., 2001; Suzuki et al., 2001b).
The circled amino acids are conserved and participate in the phosphorelay. Abbreviations: AD, acidic domain; CHASE, cyclases/histidine kinase associated sensory extracellular; GARP, DNA-binding motif, so called after the founding members Golden2, ARR and Pst1; HK, histidine kinase; LB, putative ligand binding domain; NLS, nuclear localization signal; RD, receiver domain; RLD, receiver-like domain; TM, transmembrane domain; P/Q-rich domain: transactivating domain.

Mutant analyses indicated that AHK2, AHK3 and CRE1/AHK4 have overlapping functions in cytokinin responses (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). The inhibitory influence of cytokinin on root elongation is strongly reduced in the ahk4 mutant, but not in the ahk2, ahk3 or ahk2 ahk3 double mutant, which is consistent with the expression pattern of the AHK genes – AHK4 is most abundantly expressed in roots, whereas AHK2 and AHK3 are highly expressed in leaves (Higuchi et al., 2004; Nishimura et al., 2004). The ahk2, 3, 4 triple mutant is completely insensitive in various cytokinin response assays and the plant is small and infertile, with reduced meristem size and activity (Higuchi et al., 2004; Nishimura et al., 2004). Increased cytokinin content was detected in ahk mutants, indicating a homeostatic control of steady state cytokinin levels through signaling (Riefler et al., 2006).
CRE1/AHK4 was also reported to regulate cytokinin signaling via a bidirectional phosphorelay mechanism: in the presence of cytokinin, CRE1 acts as a kinase to phosphorylate AHPs and in the absence of cytokinin, CRE1 can act as a phosphatase to dephosphorylate AHPs (Mäöhonen et al., 2006a). This phosphatase activity may help to quickly shut off the signaling pathway when cytokinin is absent. AHK2 and AHK3 had either no or low phosphatase activity, suggesting that the bidirectional phosphorelay might be specific to CRE1/AHK4 (Mäöhonen et al., 2006a).

The initial cytokinin signaling model predicted signal perception at the plasma membrane (PM). However, recent data indicates that the majority of cytokinin receptors are localized to the ER, instead of the PM (Caesar et al., 2011; Wulfetange et al., 2011). Evidence comes from three different assays: cytokinin binding studies with membrane fractions, visualization of fluorescent-labeled cytokinin receptors, and immunoblotting on fractions of Arabidopsis plants expressing Myc-tagged receptors (Wulfetange et al., 2011). All these approaches supported the ER localization of cytokinin receptors. However, a minor, but functional, part of the cytokinin receptor pool might still be localized to the PM. The ER localization of cytokinin receptors could be a mechanism that plants use to control the intracellular cytokinin homeostasis in different subcellular compartments.

The other five members of the AHK family, AHK1, CKI1, CKI2, ETR1 and ERS1, all contain the conserved histidine kinase domain, but lack the CHASE domain (Müller and Sheen, 2007b). Two of them (ETR1 and ERS1) encode ethylene receptors (Hua et al., 1995; Qu and Schaller, 2004; Müller and Sheen, 2007a), AHK1 encodes an osmosensor (Urao et al., 1999; Wohlbach et al., 2008) and CKI1 confers constitutive histidine kinase activity when overexpressed (Kakimoto, 1996). CKI1 was the first identified two-component element in Arabidopsis and overexpression of CKI1 exhibited a cytokinin-independent cell division and greening phenotype in callus cultures (Kakimoto, 1996). Although CKI1 cannot act as cytokinin receptor because it lacks the CHASE domain, CKI1
interacts with AHP1 and AHP2 in yeast two-hybrid analyses (Urao et al., 2000). CKI1 knock-out plants showed elevated levels of cytokinin free base and riboside, suggesting that CKI1 might participate in the cytokinin signaling regulating the homeostasis of endogenous cytokinin levels (Glover et al., 2008). Loss-of-function mutations of CKI1 have been reported to cause female gametophytic lethality (Pischke et al., 2002). Further investigation revealed that CKI1, AHPs and type-B ARRs define a pathway needed for the regulation of female gametophyte development (Deng et al., 2010).

1.2.2.2 AHPs

The Arabidopsis genome encodes five authentic AHPs (AHP1-AHP5) and one pseudo AHP (AHP6) (To and Kieber, 2008). All five authentic AHPs contain the highly conserved XHXKGSSXS motif, carrying the conserved His (H) responsible for the phosphor transfer. The pseudo AHP, AHP6, carries a substitution in the conserved His residue, which impairs its function as a phosphotransfer protein (Figure 1.3, Heyl and Schmülling, 2003; Mähönen et al., 2006b).

The first evidence that AHPs function in the phosphorelay comes from findings that (1) AHPs can complement a corresponding yeast TCS mutant and that (2) AHPs can accept a phosphoryl group from an E. coli membrane preparation containing histidine kinase in the presence of ATP and transfer it to an Arabidopsis response regulator in vitro (Suzuki et al., 1998). Later, AHP2 overexpression was shown to cause cytokinin hypersensitivity (Imamura et al., 2001; Suzuki et al., 2002). AHPs have been detected to interact with all three cytokinin receptors and almost all ARRs in yeast two-hybrid analyses, suggesting that they act as signaling hubs of cytokinin responses (Urao et al., 2000; Dortay et al., 2006).

Analysis of loss-of-function T-DNA insertion mutants in Arabidopsis confirmed AHP positive action in primary cytokinin signal transduction and revealed the functional redundancy among AHPs (Hutchison et al., 2006).
Whereas single and double \textit{ahp} mutants showed no defects in cytokinin responses, triple and quadruple \textit{ahp} mutants showed reduced sensitivity to cytokinin in hypocotyls and root assays and the quintuple mutant \textit{ahp1,2,3,4,5} showed reduced cytokinin induction of type-A \textit{ARR} transcription (Hutchison et al., 2006). The quintuple mutant still exhibits residual cytokinin responses, which might be explained by the partial inactivation of some \textit{AHP} genes, and/or the involvement of other pathways in cytokinin responses (Hutchison et al., 2006).

The \textit{AHP} proteins were initially described as signaling shuttles which, upon phosphorylation, translocate from the cytoplasm into the nucleus where they transfer the phosphoryl group to the \textit{ARR}s (Hwang and Sheen, 2001; Yamada et al., 2004). This model is based on the observation that \textit{AHP}s move into the nucleus upon cytokinin treatment in Arabidopsis mesophyll protoplast (Hwang and Sheen, 2001) and in T87 Arabidopsis tissue culture cells (Yamada et al., 2004). This shuttle model was, however, recently questioned (Punwani et al., 2010). Punwani \textit{et al.} (2010) examined the subcellular localization of \textit{AHP}s in Arabidopsis roots and re-examined their localization in protoplasts, and found that \textit{AHP}s are distributed throughout the cytosol and nucleus. Importantly, the distribution pattern of \textit{AHP}s was not changed by cytokinin treatment or genetic disruption of the cytokinin signaling pathway. By analyzing the distribution of various \textit{AHP2} deletions fused with \textit{GFP}, the authors observed changed distribution patterns and proposed that the \textit{AHP} proteins are actively shuttled between the cytosol and nucleus and that their nuclear/cytosolic distribution pattern is maintained by constant cycling. The active transport of \textit{AHP}s may provide another node through which to regulate cytokinin signaling in plants.

The pseudo \textit{AHP}, \textit{AHP6}, is a negative regulator of cytokinin signaling (Mähonen et al., 2006b). The \textit{wol} mutant phenotype was partially suppressed by an \textit{ahp6} mutation, and \textit{ahp6} mutants are hypersensitive to cytokinin in root vascular differentiation (Mähonen et al., 2006b). The expression of \textit{AHP6} was also shown to be suppressed by cytokinin, indicating a positive feedback control in cytokinin signaling (Mähonen et al., 2006b). \textit{In vitro} assays indicated that
AHP6 cannot function as phosphotransfer protein, but can inhibit the phosphotransfer both from the histidine kinase domain to the receiver domain of a yeast HK, and from phosphorylated AHP1 to ARR1. These results suggested that AHP6 might inhibit cytokinin signaling by interacting with the phosphorelay machinery at multiple steps (Mähönen et al., 2006b). The existence of AHP6 provides an approach to limit the cell’s ability to respond to cytokinin and help to define the cell differentiation boundary.

1.2.2.3 ARRs

The Arabidopsis response regulator (ARR) gene family can be divided into four groups based on their structural similarity: 10 type-A ARRs (ARR3-ARR9, ARR15-ARR17), 11 type-B ARRs (ARR1, ARR2, ARR10-ARR14, ARR18-ARR21), 2 type-C ARRs (ARR22 and ARR24) and 9 ARABIDOPSIS PSEUDO RESPONSE REGULATORS (APRRs) (To and Kieber, 2008).

**Type-A ARRs** contain a receiver domain and a short C-terminal domain, and the corresponding genes are rapidly up-regulated by cytokinin treatments (Figure 1.3, Heyl and Schmülling, 2003; To and Kieber, 2008).

**Type-B ARRs** contain a receiver domain and a long C-terminal region, which is composed by a DNA-binding GARP (so called after the founding members Golden2, ARR and Pst1) domain and a transactivation domain. Type-B ARRs regulate the transcription of cytokinin-activated genes, including type-A ARRs (Figure 1.2, Heyl and Schmülling, 2003; To and Kieber, 2008).

**Type-C ARRs** do not contain a DNA-binding domain, are not transcriptionally regulated by cytokinin treatment and are more distantly related to type-A and type-B ARRs (To and Kieber, 2008). ARR22 has been reported to be exclusively expressed in the chalaza of developing seeds and the ARR22 protein is preferentially localized in the cytoplasm (Horak et al., 2008). Purified ARR22 could accept a phosphoryl group from AHP5 and overexpression of ARR22 caused a dwarf phenotype with a poorly developed root system similar to the wol mutant (Kiba et al., 2004). Overexpression of ARR22 also caused decreased
cytokinin responses, suggesting that ARR22 is a negative regulator of cytokinin signaling (Kiba et al., 2004). ARR24 is closely related to ARR22 (60% amino acid similarity), and its promoter activity is primarily restricted to pollen grains, indicating that ARR24 might have a different function from ARR22 (Gattolin et al., 2006).

**APRRs** lack a conserved Asp residue used to accept a phosphoryl group, thus APRRs do not function in a two-component system (To and Kieber, 2008). APRRs have been reported to modulate circadian rhythms in plants (McClung, 2006).

**Type-B ARRs**

As mentioned above, type-B ARRs contain a receiver domain, a GARP domain and a transactivation domain (Figure 1.3). The GARP domain is distantly related to the DNA binding domain of Myb transcription factors (Hosoda et al., 2002; Heyl and Schmülling, 2003). The consensus DNA-binding sequence for type-B ARRs (5'-(G/A)GAT(T/C)-3') is found in the promoters of many cytokinin primary response genes (Hosoda et al., 2002; Imamura et al., 2003; Rashotte et al., 2003; Ferreira and Kieber, 2005). The GARP domain of ARR10 has been shown to bind as a monomer based on structural analysis (Hosoda et al., 2002), but ARR18 was recently shown to form a homodimer *in planta* (Veerabagu et al., 2012). The C-terminal P/Q rich domain (Figure 1.3) has been demonstrated to function as a transactivation domain in ARR1, ARR2 and ARR11 (Sakai et al., 2000; Imamura et al., 2003). As expected, type-B ARRs also contain nuclear localization sequences (NLS) and are localized to the nucleus (Sakai et al., 2000; Hwang and Sheen, 2001; Hosoda et al., 2002; Mason et al., 2004).

Type-B ARRs are positive regulators of cytokinin signaling that activate the expression of numerous cytokinin induced target genes, including type-A ARRs. Overexpression of *ARR1, ARR2* or *ARR10* is sufficient to directly activate the transcription of several type-A ARRs in the absence of exogenous cytokinin in
Arabidopsis protoplasts (Hwang and Sheen, 2001; Sakai et al., 2001). Overexpression of truncated versions of ARR1 and ARR2 lacking the receiver domain led to a greater induction of cytokinin responses than that obtained by overexpression of the respective full-length proteins, suggesting that the receiver domain might negatively regulate the function of type-B ARR proteins (Sakai et al., 2000; Hwang and Sheen, 2001; Sakai et al., 2001). The negative regulatory function of the receiver domain has also been demonstrated for ARR11, ARR12, ARR18, ARR19 and ARR21, suggesting that this might be a common property of all type-B ARRs (Imamura et al., 2003; Liang et al., 2012).

Type-B ARRs display distinct, but often overlapping, expression patterns in Arabidopsis, suggesting functional redundancy (Mason et al., 2004; Tajima et al., 2004). ARR1, ARR2, ARR10, ARR11, ARR12 and ARR18 show particularly high expression in cells of the apical meristem region and in young leaves undergoing cell division. Some of them are also expressed near the root tip with the highest expression in the root elongation zone (Mason et al., 2004). T-DNA insertion mutant analysis of these genes showed that higher order mutants have progressively decreased sensitivity to cytokinin as determined by effects on root elongation, lateral root formation, callus induction and greening, and by induction of cytokinin primary response genes (Mason et al., 2005).

Among the eleven type-B ARRs, ARR1, ARR10 and ARR12 have been demonstrated to play major roles in cytokinin signal transduction. An arr1-4 arr10-5 arr12-1 triple mutant was reported to exhibit stunted growth and abnormality in vascular development (Yokoyama et al., 2007). This mutant was further shown to be severely defective in cytokinin responses and the observed cytokinin-associated phenotypes were highly analogous to those observed for certain ahk2 ahk2 ahk4 triple mutants (Ishida et al., 2008). The arr1-3 arr10-5 arr12-1 triple mutant showed reduced stature due to decreased cell division in the shoot, increased seed size, increased sensitivity to light, altered chlorophyll and anthocyanin concentrations and an aborted primary root with protoxylem but no metaxylem (Argyros et al., 2008). This triple loss-of-function mutant is almost
completely insensitive to cytokinin in many response assays (Mason et al., 2005; Argyros et al., 2008). Microarray analysis revealed that the expression of the majority of cytokinin regulated genes requires the function of ARR1, ARR10 and ARR12 (Argyros et al., 2008). These results suggest that cytokinins regulate a wide array of downstream responses though the action of ARR1, ARR10 and ARR12. The other type-B ARRs might play more specific roles spatially and temporally in the development of plants (Ishida et al., 2008).

Type-A ARRs

Type-A ARRs were originally identified as primary cytokinin response genes (Brandstatter and Kieber, 1998). ARR5 (IBC6, for induced by cytokinin) and ARR4 (IBC7) were identified by differential display as genes rapidly induced by cytokinins, but not by any other plant hormones. The ARR5 steady state transcript levels were elevated within 10 min by the exogenous application of cytokinin, and this induction did not require de novo protein synthesis. Subsequent analyses showed that the transcript levels of ARR6, ARR7, ARR15 and ARR16 were also up-regulated by cytokinin treatment (D'Agostino et al., 2000).

In parallel to the differential display analyses conducted in the Kieber lab, two other studies reported the identification of several type-A ARR genes based on homology with prokaryotic response regulators (Imamura et al., 1998; Urao et al., 1998). Imamura et al. identified five type-A ARR genes (ARR3, ARR4, ARR5, ARR6, ARR7) and showed that the encoded proteins can function as phosphoaccepting response regulators by employing the E. coli His-Asp phosphotransfer signaling system (Imamura et al., 1998). Urao et al. showed that the expression of ARR5 (ATRR2) is up-regulated by cold, dehydration and salt stress (Urao et al., 1998).

Type-A ARRs lack the DNA binding domain. Six of the 10 type-A ARR proteins (ARR3, ARR4, ARR7, ARR8, ARR9, ARR15) contain a receiver domain
followed by a short acidic C-terminal extension (<100 amino acids), whereas in the other four (ARR5, ARR6, ARR16, ARR17), the C-terminal domain is even shorter (<30 amino) (Figure 1.3, D’Agostino et al., 2000; Heyl and Schmülling, 2003). Both ARR6 and ARR7 were detected in the nucleus, whereas the C-terminally truncated version of these two proteins lost their ability to enter into the nucleus, suggesting that their C-terminal domains, although with different characters, both contain cryptic nuclear-localization signals (Imamura et al., 2001). The subcellular localizations of other type-A ARRs were also reported: ARR15 is found in the nucleus (Kiba et al., 2002), ARR16 mainly in the cytoplasm (Kiba et al., 2002) and ARR4 was detected in both the cytoplasm and nucleus (Sweere et al., 2001).

Functional analysis of type-A ARRs suggests that they are negative feedback regulators of the cytokinin signaling pathway. Transient overexpression of ARR4, ARR5, ARR6 or ARR7 all repressed ARR6-luciferase reporter activity in Arabidopsis mesophyll protoplast (Hwang and Sheen, 2001). Overexpression of ARR15 resulted in reduced cytokinin sensitivity detected by root elongation assay, green callus formation assay and molecular analyses of ARR4 and ARR7 expression levels (Kiba et al., 2003). Overexpression of ARR7 also reduced cytokinin sensitivity and showed distinctively repressive impacts on various groups of cytokinin regulated genes in a GeneChip analysis, including type-A ARR genes (Lee et al., 2007). However, stable overexpression of ARR4 and ARR8 in transgenic plants alters cytokinin responses differentially in a callus formation assay: ARR4 overexpression enhanced cytokinin responses, while ARR8 overexpression repressed cytokinin responses (Osakabe et al., 2002). These data suggest that some type-A ARRs may act as positive regulators in cytokinin signaling at least in a subset of cytokinin responses (Heyl and Schmülling, 2003).

The transcript levels of type-A ARR genes increase quickly upon cytokinin treatment, but as a result of the negative feedback regulation, their transcript levels decrease again after prolonged cytokinin treatment (D’Agostino et al.,
The mechanism underlying this negative feedback regulation still remains elusive. The interaction of type-A ARRs with AHPs, but not with type-B ARRs, suggests that this might be realized by interfering at the level of AHP protein mediated signaling (Dortay et al., 2006). For example, type-A ARRs could compete with type-B ARRs for the phosphryl group transfer from AHPs.

T-DNA insertion mutant analysis revealed functional redundancy among type-A ARRs (To et al., 2004). Whereas single type-A arr mutants are indistinguishable from the wild type in various cytokinin response assays, double and higher order arr mutants show progressively increasing sensitivity to cytokinin, indicating functional redundancy among type-A ARRs and their negative regulatory function in cytokinin signaling. The induction of primary cytokinin response genes is also amplified in the higher order mutants. These phenotypes are consistent with their overlapped spatial expression patterns in the presence of exogenous cytokinin, which is particularly clear in the root. Differential basal expression patterns are also observed, suggesting a degree of specificity during development (To et al., 2004).

Although the exact mechanisms of action of type-A ARRs is still unknown, two studies have reported that the phosphorylation of the conserved Asp residue in the receiver domain of type-A ARRs is required for their function as negative regulators in cytokinin signaling (To et al., 2007; Lee et al., 2008). Both groups explored the question by mutating the conserved Asp (D) phosphorylation target residue in the type-A ARR receiver domain. The first group showed that the phosphomimic mutation (ARR5^{D87E}) can partially rescue the cytokinin hypersensitive phenotype of the arr3,4,5,6 mutant and that the ARR5^{D87A} mutation which changed the Asp to an unphosphorylatable alanine (Ala, A) cannot, indicating that phosphorylation of Asp is required for ARR5 function (To et al., 2007). Similarly, ectopic expression of ARR7^{D85N}, an unphosphorylatable version, cannot generate the phenotypes caused by ectopic expression of wild-type ARR7, including decreased root growth inhibition and callus formation by cytokinin and decreased cytokinin-inducible gene expression (Lee et al., 2008).
In addition, cytokinin induced phosphorylation also stabilizes a subset of type-A ARRs (ARR5, ARR6 and ARR7), suggesting that type-A ARR proteins might be activated by phosphorylation in part through protein stabilization (To et al., 2007).

### 1.2.2.4 Other factors involved in cytokinin signaling

In addition to the two-component elements, the involvement of other factors in the Arabidopsis cytokinin signaling pathway has also been revealed.

**CRFs:** A small family of six genes, called *CYTOKIN RESPONSE FACTORS* (CRFs), was discovered to be involved in the cytokinin signaling pathway (Rashotte et al., 2006). These transcription factors belong to the greater *APETAL2*-like class of plant-specific transcription factors (To and Kieber, 2008). CRF genes were shown to be transcriptionally up-regulated by cytokinin through the TCS (Rashotte et al., 2006). CRF proteins migrate from the cytoplasm to the nucleus after perception of the cytokinin signal (Figure 1.2) and initiate the expression of a subset of cytokinin responsive genes in parallel with type-B ARRs. The relocalization of CRFs depends on AHKs and AHPs, but not ARRs, suggesting that CRFs define a parallel branch in the cytokinin signaling pathway (Rashotte et al., 2006). Loss-of-function mutation analysis revealed redundant functions of CRFs in the embryonic axis, cotyledon and leaf development (Rashotte et al., 2006).

**GeBP:** A subset of the plant-specific GeBP (GLABROUS1 enhancer-binding protein) transcription factor family (the founding member GeBP and GeBP-like proteins (GPL) 1, 2, and 3) are also reported to play redundant roles in cytokinin signaling pathway regulation (Chevalier et al., 2008). GeBP and GeBP-like proteins are noncanonical leucine-zipper transcription factors. A triple loss-of-function mutant of the three most closely related genes *gebp gpl1 gpl2* shows reduced sensitivity to exogenous cytokinins in a subset of cytokinin responses such as senescence and rosette growth. The transcript levels of type-A *ARR* genes are increased in this triple mutant. These results, together with their overlapping expression pattern with type-A ARRs, indicated that this subset of
GeBP/GPL proteins inhibit the type-A \textit{ARR} induction to antagonize the negative feedback regulation of cytokinin signaling (Chevalier et al., 2008).

\textbf{WUSCHEL (WUS)}, a homeodomain transcription factor, which is a positive regulator for proper shoot apical meristem function, has been reported to directly repress the transcription of several type-A \textit{ARR} genes (\textit{ARR5}, \textit{ARR6}, \textit{ARR7} and \textit{ARR15}) (Leibfried et al., 2005). These results suggest that type-A ARRs might negatively influence meristem size by decreasing cytokinin signal transduction and their repression by WUS might be necessary for proper meristem function.

\textbf{Chromatin remodeling factors:} Two cytokinin-hypersensitive mutants, \textit{ckh1} and \textit{ckh2}, exhibit increased cytokinin responses in callus formation and greening (Kubo and Kakimoto, 2000). \textit{CKH1} encodes a protein resembling TAF12 (TATA BOX BINDING PROTEIN ASSOCIATED FACTOR 12), which is a component of transcription factor IID (TFIID) and histone acetyltransferase containing complexes in yeast and animals (Kubo et al., 2011). The product of the \textit{CKH2} gene is PICKLE, a protein resembling the CHD3 class of SWI/SNF chromatin remodeling factors (Furuta et al., 2011). Microarray analysis revealed that a substantially greater number of genes respond to a low level of cytokinins in \textit{ckh1} and \textit{ckh2} mutants than in wild type plants, but the expression of cytokinin primary response genes was not affected (Furuta et al., 2011; Kubo et al., 2011). The \textit{ckh1 ckh2} double mutant showed synergistic phenotypes and yeast two-hybrid experiment showed protein interaction between CKH1/EER4/AtTAF12b and CKH2/PICKLE (Furuta et al., 2011). These two chromatin remodeling factors were suggested to regulate a set of genes involved in late cytokinin signaling responses, including cell proliferation and differentiation.

\textbf{1.2.3 Cytokinin responses}

Cytokinins are involved in many aspects of plant growth and development. Some plant responses initiated by cytokinin signaling are commonly used as diagnostic tools for changes in cytokinin sensitivity. Here I briefly describe some cytokinin response assays used in this study. The most commonly used is the
root growth assay: cytokinin inhibits root elongation and prompts root hair formation, thus a decreased response to cytokinin in root growth is used as an indicator of cytokinin insensitivity (Smalle et al., 2002; Tiryaki and Staswick, 2002; To et al., 2004). Shoot growth is also inhibited by cytokinin (Smalle et al., 2002). Exogenous cytokinin treatment leads to anthocyanin accumulation, which is caused by coordinate increases in transcript levels of four anthocyanin biosynthetic genes: phenylalanine ammonia lyase 1 (PAL1), chalcone synthase (CHS), chalcone isomerase (CHI) and dihydroflavonol reductase (DFR) (Deikman and Hammer, 1995). This response was also used as a marker for cytokinin responsiveness (Deikman and Ulrich, 1995). Cytokinin stimulates cell division, chloroplast development and greening of calli, which is used as a callus induction assay in tissue culture to test cytokinin responsiveness and resulted in the identification of the first cytokinin receptor, CRE1 (Inoue et al., 2001; Higuchi et al., 2004). At the molecular level, the cytokinin-induced type-A ARR gene expression is widely used as an indicator of a primary cytokinin response (D’Agostino et al., 2000; Hwang and Sheen, 2001; To et al., 2004).

1.3 Ubiquitin/26S proteasome system

One important mechanism that plants use to modulate protein levels is regulated protein degradation through the ubiquitin/26S proteasome system (UPS). In this system, ubiquitin (Ub), an evolutionarily conserved 76-amino acid protein, is covalently attached to a target protein through an isopeptide bond. Depending on the number of ubiquitin molecules attached and the position of their attachment, the consequence of this post-translational modification could be functional regulation (monoubiquitination) or protein degradation by the 26S proteasome (polyubiquitination). Genomic analysis indicated that 5~6% of the Arabidopsis proteome directly participates in the UPS (Vierstra, 2009; Santner and Estelle, 2010). The UPS has been indicated to influence nearly every aspect of plant growth and development, including hormone signaling, morphogenesis, environmental- and pathogen-responses (Vierstra, 2009).
1.3.1 Modification of proteins by ubiquitin

Ubiquitination, the modification of proteins by ubiquitin, is accomplished through the sequential action of E1, E2 and E3 enzymes (Figure 1.4 and Miura and Hasegawa, 2010; Santner and Estelle, 2010). Initially, ubiquitin is activated by an E1 ubiquitin-activating enzyme by using ATP as an energy source. The C-terminal glycine residue of ubiquitin is linked through a thiolester bond to the cysteine residue of the E1 active site. Activated ubiquitin is then transferred to a cysteine residue of the E2 ubiquitin-conjugating enzyme. Finally, with the help of E3 ligases, the ubiquitin is conjugated to the target protein through an isopeptide bond between the C-terminal glycine of ubiquitin and a lysine residue of the target protein. In most cases, the E3 ligase functions as a bridge to interact with both the E2 and the substrate, bringing them closer to facilitate the direct transfer of ubiquitin from the E2 to the substrate. For HECT type E3 ligases, the activated ubiquitin is first transferred to the E3, which then transfers it to the substrate (Santner and Estelle, 2010). When this process is repeated, a polyubiquitin chain is attached to the protein which turns it into a substrate for 26S proteasome-dependent degradation.

Figure 1.4 The ubiquitination reaction pathway.
In the UPS, it is the E3 that defines the substrate specificity of the pathway. In plants, four major types of E3s are known, which are classified based on their mechanism of action and subunit composition: HECT, RING, U-box and cullin-RING ligases (CRLs) (Vierstra, 2009). HECT, RING and U-box E3 ligases are all single polypeptides, defined by their respective signature HECT domain, RING or U-box motif. The CRL is multisubunit E3, which contains a cullin (CUL, acting as scaffold for the CRL assembly), a RING-box 1 (RBX1, binding to E2-ubiquitin), and a variable target recognition module (Figure 1.5). The CRLs are further divided into four subtypes based on their subunit composition. SCF-type E3 ligases contain the subunits S phase kinase-associated protein 1 (SKP1), cullin 1 (CUL1), F-box protein, and RBX1. They use the F-box protein for substrate recognition. Accordingly, F-box proteins form the largest gene family (>700 members) in Arabidopsis and SCF E3 ligases are extensively involved in numerous regulatory processes, including hormone signaling (Vierstra, 2009).

**Figure 1.5** Schematic representation of CRLs (adapted from Hotton and Callis, 2008).

Abbreviations: C, cysteine; CUL, cullin; K, lysine; RBX1, RING-box 1; RUB, related to ubiquitin; Ub, ubiquitin.

1.3.2 26S proteasome

The 26S proteasome is a large, multisubunit, ATP-dependent proteasome complex, which unfolds and degrades polyubiquitinated proteins. It contains a central core particle (CP) which has the protease active sites hidden inside the chamber. The CP is capped at either one or both ends by a 19S regulatory
particle (RP) that is composed of a base and a lid. The base sits over the CP and contains a ring of six related AAA-ATPases (RPT1-6) and three non-ATPase subunits (RPN1, 2 and 10). The lid contains the other non-ATPase subunits (RPN3, 5-9 and 11-12) (Smalle and Vierstra, 2004). The RP functions to recognize ubiquitinated substrates, remove the covalently bound ubiquitins, unfold the target, direct the target into the CP chamber and probably also in the release of breakdown products (Vierstra, 2009). Most, if not all, of the RP subunits are essential in plants.

1.3.3 The RUB modification pathway

In the past decade, a group of proteins called ubiquitin-like proteins (Ubls) have been recognized as post-translational modifiers to regulate protein function in yeast, plants and metazoans (Miura and Hasegawa, 2010). Ubls share a canonical three-dimensional structure called the Ub–fold and their conjugation to a substrate occurs through enzymatic pathways similar to ubiquitination, requiring Ubl-specific E1, E2 and E3 equivalents.

RUB (related to ubiquitin), one of these Ubls, is attached to its targets by an isopeptide bond between its carboxy-terminal glycine and a lysine of the target protein (Downes and Vierstra, 2005; Hotton and Callis, 2008). In Arabidopsis, the first step in this reaction is the activation of RUB through an ATP-dependent mechanism catalyzed by the heterodimeric E1 enzyme formed by AUXIN RESISTANT 1 (AXR1) and E1 C-TERMINAL RELATED 1 (ECR1) (del Pozo et al., 1998). Activated RUB is subsequently transferred from the E1 onto the E2 RUB-CONJUGATING ENZYME (RCE), and then to a target protein (Hotton and Callis, 2008). One prominent class of protein targets modified by RUB are the cullins (CUL) that function as scaffolds for the assembly of CRLs (del Pozo and Estelle, 1999; Hotton and Callis, 2008). The RBX1 subunit of the CRLs is suggested to function as an E3 to facilitate the conjugation of RUB to the target (Hotton and Callis, 2008). RUB modification of CULs is thought to increase CRL activity, thus accelerating the degradation of CRL-target proteins (Hotton and Callis, 2008).
1.4 Ubiquitin/proteasome system and cytokinin signaling

In contrast to the general function of the components in cytokinin response pathway, our understanding of the proteolytic control of cytokinin signaling has remained superficial. Evidence for the involvement of proteasome-dependent proteolysis in cytokinin signaling was initially obtained through the characterization of proteasome mutants (Smalle et al., 2002; Smalle et al., 2003). The *rpn12a-1* and *rpn10-1* mutants have defective subunits of the 26S proteasome regulatory particle, and as a result have lower degradation rates of polyubiquitinated proteins (Smalle et al., 2002; Smalle et al., 2003; Kurepa et al., 2008). Both proteasome mutants have decreased cytokinin sensitivity, suggesting that the activity of a cytokinin response inhibitor is controlled by proteasome-dependent proteolysis (Smalle et al., 2002; Smalle et al., 2003).

Later studies, involving ectopically expressed type-A ARRs, revealed that some family members (ARR5, ARR6, ARR7) are unstable proteins, and that their half-lives are extended by cytokinin treatments (To et al., 2007; Ren et al., 2009; Ryu et al., 2009). Subsequent proteasome inhibitor studies revealed that unstable type-A ARRs are degraded by the proteasome (Lee et al., 2008; Ren et al., 2009; Ryu et al., 2009).

Analyses of the function of the RUB modification pathway provided additional evidence for the involvement of ubiquitin-dependent proteolysis in the regulation of cytokinin signaling. Previous reports showed that *axr1* mutants have decreased sensitivity to auxin, but also to other hormones, including cytokinin (Lincoln et al., 1990; Abel et al., 1995; Timpte et al., 1995; Tiryaki and Staswick, 2002). To date, we know that the auxin resistance of *axr1* mutants is caused by stabilization of AUX/IAA proteins that repress the auxin response (Gray et al., 2001). In contrast, the underlying cause for the cytokinin insensitivity has remained elusive.

Recent evidence also shows that proteasome-dependent proteolysis is involved in the regulation of type-B ARRs. An AUXIN UP-REGULATED F-BOX
PROTEIN 1 (AUF1) has recently been reported to influence cytokinin signaling through ARR1 (Zheng et al., 2011). The auf1 mutant has increased ARR1 protein levels and is hypersensitive to cytokinin in root growth and type-A ARR expression assays (Zheng et al., 2011). ARR2, another type-B ARR, was also reported to be degraded via the proteasome pathway (Kim et al., 2012). A hemagglutinin (HA) tagged version of ARR2 is rapidly degraded by cytokinin treatment, and this degradation is inhibited by the proteasome inhibitor MG132 (Kim et al., 2012).

Thus, both the positive (type-B ARRs) and negative (type-A ARRs) regulators of cytokinin action are suggested to be regulated by ubiquitin/proteasome-dependent proteolysis.

1.5 Experimental objectives of this study

In contrast to other plant hormones, classic genetic screens did not identify any prominent cytokinin response mutants for decades. The gene activation tagging strategy led to the identification of the histidine kinase homolog CKI1 (Kakimoto, 1996) and essentially initiated the elucidation of the cytokinin signal transduction pathway. Most members of the current cytokinin signaling model were identified based on their sequence similarity with prokaryote TCS elements and reverse genetic characterization. The only two exceptions are CRE1/AHK4 and AHP6, which were identified by forward genetic screens and still fall into the category of TCS elements (Inoue et al., 2001; Mähönen et al., 2006b). The only members which are not TCS elements in primary cytokinin signaling are the CRFs. These factors were identified based on their up-regulated gene expression by cytokinin (Rashotte et al., 2006). Are there any other factors, different from TCS elements, functioning in primary cytokinin signaling? This study was undertaken to address this question.

A forward genetic approach was taken to investigate this question. A transgenic Arabidopsis line, ipt-161, which expresses the Agrobacterium IPT gene, was used as the starting material. The ipt-161 seeds were mutagenized
with ethyl methanesulfonate (EMS) and screened for mutations that suppress the phenotypes caused by the increased endogenous cytokinin level. This screen yielded a cytokinin resistant mutant, *suppressor of ipt-161 1* (*soi1*). The *soi1* mutation was mapped to a previously identified locus, *AUXIN RESISTENT 1* (*AXR1*). Further analysis reveals that AXR1 regulates primary cytokinin signaling by facilitating ARR5 (a type-A ARR) and ARR1 (a type-B ARR) proteolysis.
Chapter 2: Isolation of the *suppressor of ipt-161* 1 (*soI1*)

2.1 Introduction

In this study, a transgenic Arabidopsis line *ipt*-161 was used as starting material. This line expresses the *Agrobacterium IPT* gene under its natural full-length promoter in the C24 ecotype background (Van der Graaff et al., 2001). Two-week-old *ipt*-161 plants have been shown to have a 10-fold increase in the total cytokinin pool compared to C24. The largest increase was recorded for zeatin (Z) and zeatin riboside (ZR), followed by isopentenyladenine riboside (iPR). No changes in isopentenyladenine (iP) levels were detected (Van der Graaff et al., 2001). Consistent with the increased endogenous cytokinin levels, the *ipt*-161 plants have a range of phenotypes characteristic for increased cytokinin responses (e.g. smaller leaves, an underdeveloped root system and increased anthocyanin production in the hypocotyl area). These cytokinin phenotypes are severe during early development and after 3~4 weeks of growth on soil, these phenotypes attenuate and transgenic plants resemble the wild type. This phenotype reversion is thought to be caused by the decrease of *IPT*-promoter activity during development (Van der Graaff et al., 2001).

The goal of this study is to identify new factors involved in the control of cytokinin signaling in Arabidopsis. To do this, the *ipt*-161 seeds were mutagenized with ethyl methanesulfonate (EMS) and screened for mutations that could suppress the phenotypes caused by increased endogenous cytokinins. This chapter describes the isolation and basic characterization of one of these mutants, *suppressor of ipt-161* 1 (*soI1*).

2.2 Results

**EMS mutagenesis and suppressor screen**

The *ipt*-161 seeds were mutagenized with EMS as described in the materials and methods section of this chapter, and ~50,000 (1 g) M2 seeds were plated on
MS/2 medium to screen for cytokinin resistant mutants. Since the *Agrobacterium IPT* gene promoter is most active during the first 3 weeks of plant development and the phenotypes caused by cytokinin overproduction are most prominent during early developmental stages of *ipt*-161 plants, the suppressor mutants were screened soon after plated on MS/2 medium. Seven-day-old light-grown *ipt*-161 seedlings resemble the cytokinin-treated wild-type seedlings: they have short roots, thick hypocotyls and small cotyledons (Figure 2.1a and Van der Graaff et al., 2001). Seedlings with cotyledons larger than those of *ipt*-161 were selected as putative candidate suppressors.

Here the extragenic *ipt*-161 suppressor *soi1* is described. In addition to cotyledon size, the *soi1* mutation also reversed the short-root phenotype of *ipt*-161 (Figure 2.1a), and suppressed the anthocyanin accumulation in the upper hypocotyl region of *ipt*-161 seedlings (Figure 2.1b).

Although the *soi1* mutation reversed the phenotypes of *ipt*-161 to wild type level at the young seedling stage, the mature *ipt*-161 *soi1* plants differed from the wild type: overall rosette size was reduced, apical dominance was decreased, and plants were semi-sterile (Figure 2.1c).

Next, the effects of the *soi1* mutation on plant development were analyzed in the absence of the *ipt*-161 transgene. Cotyledons of seven-day-old *soi1* seedlings were similar in size to the wild type, but the roots were longer and resembled those of *ipt*-161 *soi1* (Figure 2.1a). Mature *soi1* plants were also similar to *ipt*-161 *soi1* plants: they had smaller rosettes, a short bushy inflorescence and severely reduced seed yield (Figure 2.1d).
Figure 2.1 The soi1 mutation suppresses the phenotypes of *ipt*-161.

(a) Morphology of wild-type (C24), *ipt*-161, *soi1* and *ipt*-161 *soi1* plants. Plants were grown for seven days on MS/2 medium under constant light. Scale bar = 5 mm.

(b) Anthocyanin accumulation in seven-day-old *ipt*-161 and *ipt*-161 *soi1* seedlings grown on MS/2 medium under constant light. Arrowhead points to the anthocyanin accumulation in the *ipt*-161 hypocotyl. Scale bar = 1 mm.

(c) Adult phenotype of *ipt*-161 *soi1* plants. Plants grown on MS/2 plates for 13 days were transferred to soil and photographed when they were 59 days old.

(d) Adult phenotype of a six-week-old *soi1* plant. Scale bar = 1 cm.

To explore the mechanism by which *soi1* suppresses the *ipt*-161 phenotype, I further tested if *soi1* affects the primary cytokinin response by comparing the expression level of the *ARR5* gene in wild type (C24), *soi1*, *ipt*-161 and *ipt*-161 *soi1* seedlings (Figure 2.2). *ARR5* is rapidly induced by cytokinin, and its kinetics
of induction serves as a diagnostic tool for cytokinin sensitivity (Brandstatter and Kieber, 1998). The ARR5 gene (and other type-A ARRs) is also constitutively up-regulated in plants overexpressing cytokinin biosynthesis genes (e.g., AtIPT4 and AtIPT8; Sun et al., 2003; Li et al., 2010) and down-regulated in Arabidopsis ipt T-DNA mutants (Nishiyama et al., 2011). Thus, type-A ARR expression levels can also serve as a marker for sustained changes in cytokinin action. Our RNA gel blot analyses showed that the ARR5 mRNA level was ~3-fold of the wild type in ipt-161 seedlings, but similar to the wild type in ipt-161 soi1 (90% wild-type levels), and ~60% of the wild type in soi1 (Figure 2.2). These results suggested that the soi1 mutation suppresses ipt-161 by either inhibiting the primary cytokinin response or by repressing cytokinin accumulation.

**Figure 2.2** The soi1 mutation decreases ARR5 expression of ipt-161.

RNA gel blot analyses of the steady state ARR5 transcript levels in C24, soi1, ipt-161 and ipt-161 soi1. Plants were grown in liquid Gamborg’s B5 medium for 21 days, and used for RNA extraction. Methylene blue-stained ribosomal RNA (rRNA) is shown as the loading control. Relative ARR5 levels (RL) are presented as mean ± SD (n=2).

**The soi1 mutant has decreased sensitivity to cytokinin**

To determine if soi1 affects cytokinin signaling, I first assessed the cytokinin sensitivity of the mutant seedlings by testing the effects of kinetin on root growth (Figure 2.3a and b). Whereas 0.1 µM kinetin inhibited root elongation in the wild
type by ~70%, this dose only inhibited the root growth by ~30% in soi1 seedlings. The cytokinin insensitivity of soi1 roots was also apparent at a higher kinetin concentration, suggesting a strong defect in cytokinin signaling (Figure 2.3a and b). Next, the cytokinin induction kinetics of the ARR5 gene in soi1 was analyzed (Figure 2.4). Wild-type and soi1 seedlings were treated with 5 μM benzyladenine (BA), and the ARR5 induction levels were found to be ~4-fold lower in the mutant (Figure 2.4). These results suggested that the soi1 mutation causes decreased cytokinin sensitivity by suppressing the primary cytokinin response.

Figure 2.3 The soi1 mutant exhibits decreased cytokinin sensitivity in root elongation assay.

(a) Root elongation assay. Four-day-old seedlings germinated on MS/2 medium were transferred to plates with medium containing the indicated concentrations of kinetin, and were grown vertically for another 6 days. At day 10, representative seedling were rearranged on a new plate and photographed. Arrows point to the root tips. Scale bar = 5 mm.

(b) Quantification of the kinetin effect on root length. Four-day-old seedlings grown on MS/2 medium were transferred to plates with kinetin, and were grown vertically for another 6 days. The length of the root grown during 6 days of treatment was measured. Data are presented as mean ± SD (n=5). ***, $P < 0.001$; ****, $P < 0.0001$ for C24 vs. soi1 (two-way ANOVA with Bonferroni multiple comparisons post-test).
Figure 2.4 The soi1 mutant exhibits decreased cytokinin sensitivity at the molecular level.

RNA gel blot analyses of the ARR5 levels. C24 and soi1 plants were grown in liquid Gamborg’s B5 medium for 21 days, and were then treated for the indicated times with BA. Methylene blue-stained rRNA is shown as the loading control. Relative levels (RL) were calculated as the ratio between the signal strength in C24 vs. soi1 for the same treatment time.

2.3 Materials and methods

Plant materials and growth conditions

The ipt-161 (C24) line was previously described (Van der Graaff et al., 2001). For all Arabidopsis lines, seeds were surface-sterilized, sown on half-strength Murashige and Skoog medium (pH 5.7) with 1% sucrose, 0.8% PhytoAgar (MS/2 medium), and chilled for 4 days at 4°C before being grown at 22°C under continuous light (80 µmol m⁻²s⁻¹). For growth on soil, plants were transferred to 1:1 mix of Miracle Grow potting soil and vermiculite and grown at 22°C under continuous light (200 µmol m⁻²s⁻¹).

EMS mutagenesis and suppressor screen

Ethyl methanesulphonate (EMS) mutagenesis was performed as previously described (Kim et al., 2006b). In brief, ipt-161 (C24) seeds were imbibed in 0.1% (w/v) KCl for 12 hours, mutagenized with 80 mM EMS (Sigma) for 3 hours,
washed twice for 15 minutes in 100 mM sodium thiosulphate, rinsed in water, and sown on soil. Progeny M2 seeds were harvested, sterilized, plated on MS/2 media at low density, chilled for 4 days at 4°C, and transferred to a growth chamber. Lines with suppression phenotypes were transferred to soil and allowed to self-pollinate. The suppressor of ipt-161 (soi) mutant lines were outcrossed three times to wild type C24 before further analysis.

**Root elongation assay**

For the root elongation assay, 4-day-old seedlings grown on MS/2 media were transferred to fresh MS/2 plates containing either DMSO (solvent control) or kinetin. Kinetin (Sigma) was prepared as a stock solution in DMSO. Root tips were marked at the moment of transfer to test plates, the plates were positioned vertically in a growth chamber, and the plants were photographed after 6 days of growth. The root length was measured from the transfer mark using Image J software (National Institute of Health, Bethesda, MD).

**RNA isolation and gel blot analyses**

Total RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA) from Arabidopsis seedlings grown with continuous shaking (20 rpm) in liquid Gamborg’s B5 medium supplemented with 1% sucrose (pH 5.7). The RNA gel blot analyses were done as described (Smalle et al., 2002). The *ARR5* antisense probes were prepared from the ABRC plasmids 103N10 linearized with KpnI+PstI using SP6 RNA polymerase. The hybridization signal was visualized by Bio-Rad Pharos FX™ Plus Molecular Imager system (Bio-Rad Laboratories, Inc., Hercules, CA).
Chapter 3: The soi1 mutation locates in the AXR1 gene

3.1 Introduction

This chapter describes the identification of the soi1 locus through map-based cloning. The soi1 mutation was identified as a point mutation of a previously described gene AXR1.

Two previously identified axr1 alleles, axr1-3 and axr1-12, were also tested for their cytokinin sensitivity in various assays. The results of these tests show that both axr1 alleles have decreased cytokinin sensitivity and can suppress the phenotypes of ipt-161 in the Col-0 background.

3.2 Results

Map-based cloning of the soi1 locus

The phenotypic and molecular analyses (Figures 2.3 and 2.4) indicated that the soi1 mutation affects a gene encoding a component involved in the primary cytokinin response pathway. To isolate the soi1 locus, the soi1 (C24) mutant was crossed with the Col-0 ecotype, and the F1 plants were allowed for self-pollination to produce F2 seeds. The cytokinin-resistant root growth phenotype of the soi1 mutant seedlings was used to identify the mapping population. First, the cytokinin sensitivity of both C24 and Col-0 wild type plants was tested (Figure 3.1a). The result suggested that for both accessions, 10 µM kinetin can completely inhibit their root growth. Then, F2 seeds of the mapping cross were plated on 10 µM kinetin containing medium to screen for cytokinin resistant seedlings. A total of 106 F2 plants were identified and used as the mapping population (Figure 3.1b).
Figure 3.1 Identification of cytokinin resistant seedlings in the soi1 (C24) x Col-0 F2 mapping cross.

(a) Differential sensitivity of the C24 and Col-0 accessions to kinetin. Three-day-old seedlings grown on MS/2 plates were transferred to vertical MS/2 plates with 0.1% DMSO (control), 1 µM, 5 µM or 10 µM kinetin. Root length was measured for seven days from the moment of transfer (day 3). Data are presented as mean ± SD (n=10), and they show that for both accessions, 10 µM kinetin can be used for the identification of cytokinin-resistant plants.

(b) Mapping population. Three-day-old seedlings grown on MS/2 plates were transferred to vertical MS/2 plates with 10 µM kinetin, and grown for another 7 days before phenotyping. Seedlings with the long root (red arrowhead) were selected and used for DNA extraction and mapping. Scale bar = 5 mm.
The *soi1* mutation was mapped to the top of chromosome 1 between 1362041 and 1603470 markers (Figure 3.2a). One of the genes in this region is *AXR1* (At1g05180). Loss of *AXR1* function causes an increase in primary root elongation, decreases in shoot apical dominance and fertility, and leads to root insensitivity to multiple hormones, including cytokinin (Lincoln et al., 1990). These characteristics are consistent with the developmental phenotypes of the *soi1* mutant (Figure 2.1 and 2.3). Thus, I compared the *AXR1* sequence from the *soi1* mutant with that of the C24 wild type, and found a missense mutation G3179A in *soi1*'s *AXR1* gene (Figure 3.2b).

This mutation resulted in a glutamic acid (Glu)-to-lysine (Lys) substitution in the C-terminal region of the *AXR1* protein (Figure 3.3). *AXR1* is homologous to the human NEDD8-activating enzyme E1 regulatory subunit 1 called amyloid precursor protein binding protein-1 (APPBP1) (del Pozo et al., 1998; Welchman et al., 2005). The crystal structure of APPBP1 has been resolved (Walden et al., 2003b; Walden et al., 2003a). Protein sequence alignment between *AXR1* and APPBP1 suggested that the Glu487 of *AXR1* is a conserved residue and its homologous residue in APPBP1, Glu481, is located in the 4 helix bundle domain, which is required for the formation of NEDD8-activating enzyme E1 heterodimer (Walden et al., 2003a).
Figure 3.2 Identification of the soi1 mutation.

(a) The soi1 mutation was mapped between markers 1362041 and 1603470 on chromosome 1 (marker names indicate their position on the chromosome) which encompass 4 BAC/YAC clones. One of the genes in the region is AXR1. Because growth phenotypes of axr1 and soi1 mutants were virtually identical, the AXR1 sequence was analyze first.

(b) The soi1 mutation is a missense G-to-A mutation at position 3179 (exon 13). Introns are shown as lines and exons as black boxes. The three differentially spliced forms and the mutations in the axr1-3 and axr1-12 lines (Leyser et al., 1993) are also presented.
AXR1.1 protein is shown as well as the N-, or N- and C-terminal regions absent in AXR1.2, and AXR1.3 splice variants, respectively (grey boxes). The relative positions of the three mutations are labeled. Protein sequence alignment between AXR1.1 and its homologous human NEDD8-activating enzyme E1 regulatory subunit 1 called amyloid precursor protein binding protein 1 (APPBP1) revealed the locations of the three mutations’ homologous residues in the APPBP1 protein structure (Walden et al., 2003a).

AXR1 is a subunit of the activating enzyme in the RUB/NEDD8 pathway of protein modification. The RUB protein is conjugated to the Cullin subunit of the large class of ubiquitin CUL-RING E3 ligases (CRLs), and this modification is important for CRL activity. Accordingly, loss of AXR1 function is believed to negatively affect the activities of numerous CRLs resulting in a pleiotropic phenotype that includes altered responses to different hormones (Tiryaki and Staswick, 2002).

Since the soi1 mutant was isolated based on its decreased cytokinin sensitivity, to further test if soi1 indeed affects AXR1 function, I tested its responses to the hormone auxin and the precursor of the gaseous hormone ethylene – ACC, through root elongation assay. Indeed, soi1 showed decreased sensitivity to both IAA and ACC (Figure 3.4a and c). The auxin response of soi1 mutant was also examined at the molecular level and the results showed that the auxin-induced IAA3 gene expression was impaired, confirming the decreased auxin sensitivity (Figure 3.4b).
Figure 3.4 The *soi1* mutant has decreased sensitivity to auxin and ACC.

(a) Four-day-old seedlings germinated on MS/2 medium without IAA were transferred to plates with IAA, and were grown vertically for another 5 days. At day 9, seedlings were rearranged on a new plate for photography. Scale bar = 5 mm.

(b) Seedlings grown in Gamborg’s B5 medium for 18 days were treated with 20 μM 2,4-D (2,4-dichlorophenoxyacetic acid, dissolved in DMSO) for one hour prior to the isolation of total RNA. Riboprobe for the RNA gel blot analyses was prepared using KpnI+PstI-linearized DKLAT1G04240 plasmid (ABRC) and SP6 RNA polymerase. Methylene blue-stained *rRNA* is shown as the loading control.

(c) Four-day-old seedlings germinated on MS/2 medium without ACC were transferred to plates with ACC, and grown vertically for another 5 days. Scale bar = 5 mm.
The *axr1-3* and *axr1-12* mutants have decreased cytokinin sensitivity

Earlier studies showed that *axr1* mutants are less sensitive to the effects of BA in a root elongation response assay (Timpte et al., 1995; Tiryaki and Staswick, 2002). Here I first confirmed the decreased cytokinin sensitivity in root elongation assay with another cytokinin, kinetin (Figure 3.5a). I also observed decreased root hair formation, relative to wild type Col-0, in both *axr1-3* and *axr1-12* mutants after 0.5 µM kinetin treatment (Figure 3.5b). The *axr1-12* mutation has a stronger effect on the root hair formation phenotype than the *axr1-3* mutation (Figure 3.5b). However, reduced cytokinin sensitivity in roots can be a secondary effect of for example decreased ethylene sensitivity (Cary et al., 1995; Van der Graaff et al., 2001; Chae et al., 2003). To clarify the cause of the cytokinin insensitivity, I tested the effects of cytokinin on (1) rosette growth, (2) anthocyanin accumulation, and (3) type-A *ARR* expression in the weak *axr1-3* and the strong *axr1-12* mutant.

Consistent with the difference in strength of the mutations (Lincoln et al., 1990), a higher kinetin dose was required to affect the shoot growth of *axr1-12* mutant plants compared to *axr1-3* or the wild type (Figure 3.6a). Both the *axr1-3* and *axr1-12* mutants were less responsive to BA in an anthocyanin accumulation assay (Figure 3.6b). Whereas wild-type seedlings grown on 0.2 µM BA accumulated ~6-fold more anthocyanin compared to the untreated control, the anthocyanin level increase mediated by 0.2 µM BA treatment were ~4-fold and ~3-fold in *axr1-3* and *axr1-12*, respectively (Figure 3.6b).

Finally, real time RT-PCR analyses showed that the cytokinin-induction of *ARR5* expression was attenuated in both *axr1-3* and *axr1-12* seedlings (Figure 3.7). Thus, similar to the *soi1* mutation (Figures 1.4), *axr1-3* and *axr1-12* mutations also cause decreased cytokinin sensitivity by suppressing the primary cytokinin response.
Figure 3.5 Cytokinin responses of axr1 mutants in root assays.

(a) Root elongation assay. Four-day-old seedlings grown on MS/2 medium were transferred to plates containing the denoted concentrations of kinetin and grown for another 5 days. Data are presented as mean ± SD. Difference in root length between Col-0 and axr1 mutants was statistically significant for all tested doses (n≥10, P < 0.0001; Bonferroni multiple comparisons post-test). Difference in the root length of untreated seedlings was statistically significant only for Col-0 compared to axr1-12 (n≥10, P < 0.05; Bonferroni multiple comparisons post-test). The response of the weaker axr1-3 allele was significantly different from axr1-12 only for the root length of seedlings treated with 0.1 μM kinetin.

(b) Root hair growth. Four-day-old seedlings germinated and grown on MS/2 medium were transferred to medium containing 0.5 μM kinetin or DMSO (control), and were grown vertically for another five days before photograph.
Figure 3.6 Cytokinin responses of axr1 mutants in rosette and anthocyanin assays.

(a) The rosette growth of the axr1-12 plants was less sensitive to cytokinin effects. Seeds were germinated and grown for 4 days on MS/2 medium, and then transferred to medium containing the indicated kinetin concentrations. Photos show representative 22-day-old plants. Scale bar = 1 cm.

(b) Decrease in cytokinin-induced anthocyanin biosynthesis in axr1 mutants. Four-day-old seedlings germinated on MS/2 medium were transferred to medium containing BA, and were grown for another 8 days. Data are presented as mean A_{520nm}/FW (fresh weight) ± SD of three independent pools of 6 seedlings each. The anthocyanin levels of Col-0 were significantly different from that of axr1-3 and axr1-12 (***, P < 0.0001, two-way ANOVA with Bonferroni multiple comparison post-test).
Figure 3.7 Cytokinin responses of axr1 mutants at the molecular level.

Real-time RT-PCR analyses of the cytokinin-induced ARR5 expression in Col-0, axr1-3, axr1-12. Eight-day-old seedlings grown in Gamborg’s B5 liquid media were treated with 5 μM BA for 30 min and used for RNA isolation. The ARR5 expression levels were normalized against GAPDH (At1g13440), and the average relative transcript level (n=3) for untreated (control, C) Col-0 was assigned the value of 1. The transcript levels in all other samples were normalized to that of the untreated wild-type plants. ****, P< 0.0001 for Col-0 vs. axr1 mutants (two-way ANOVA with Bonferroni multiple comparisons post-test).

The axr1-3 and axr1-12 mutations suppress the phenotypes of ipt-161(Col-0)

To test if the axr1-3 and axr1-12 mutations also suppress the ipt-161 phenotype, the ipt-161 transgene was first introgressed into Col-0 through crossing ipt-161 (C24) with Col-0 and six rounds of backcrossing, and then the double homozygous ipt-161 axr1-3 and ipt-161 axr1-12 line were generated by crossing ipt-161 (Col-0) with axr1 mutants.

For root growth, both axr1-3 and axr1-12 alleles restored the short root phenotype of ipt-161 to wild-type level (Figure 3.8a and b). The suppression effect of the axr1-3 mutation seems to be stronger than axr1-12 for root elongation (Figure 3.8b). The root of the ipt-161 plant was densely covered with root hairs and this phenotype was also reversed by both axr1 mutations (Figure 3.8c). In this case, the effect of axr1-12 mutation was stronger than the effect of axr1-3: the root hair formation on the ipt-161 axr1-12 root was not detected.
Figure 3.8 The axr1 mutations suppress the root phenotypes of ipt-161 (Col-0).

(a) Representative Col-0, ipt-161, ipt-161 axr1-3 and ipt-161 axr1-12 seedlings. Seedlings were grown vertically on MS/2 medium for nine days. Scale bar = 5 mm.

(b) Suppression of the short-root phenotype of ipt-161. Seeds were germinated and grown on MS/2 medium for nine days. At day four, the root tips were marked. The root length between day four and day nine was measured, and presented as mean ± SD (n=10). The root length of Col-0, ipt-161 axr1-3 and ipt-161 axr1-12 seedlings were the same (one-way ANOVA with Bonferroni multiple comparisons post-test; $P > 0.05$), but the difference between ipt-161 axr1-3 and ipt-161 axr1-12 was statistically significant. **, $P < 0.01$.

(c) Root tips were photographed after 9 days of growth on vertically positioned MS/2 plate. Scale bar = 1 mm.
Both *axr1-3* and *axr1-12* alleles restored the shoot size of *ipt-161* seedlings back to the wild-type level, with no visible difference in the strength of the suppression phenotypes (Figure 3.9a).

The anthocyanin content, which was ~7-fold higher in *ipt-161* (Col-0) compared to the wild type, was also restored to the wild-type level in both *ipt-161 axr1-3* and *ipt-161 axr1-12* (Figure 3.9b).

The callus induction response of the *ipt-161 axr1* lines was also analyzed. The simultaneous treatment of explants with a specific ratio of cytokinin and auxin is known to promote the formation of white calli. By gradually increasing the dose of cytokinin, explants will develop first green calli, and then shoots (Valvekens et al., 1988; Thorpe, 2006). Accordingly a cytokinin overproducing line, such as *ipt-161*, might be able to form green calli on the medium that contains only auxin. Indeed, after 40 days of incubation on 0.5 μM NAA, *ipt-161* hypocotyl explants developed green calli, whereas the hypocotyls from wild-type Col-0 plants did not (Figure 3.9c). The *ipt-161 axr1-3* and *ipt-161 axr1-12* hypocotyl explants also did not develop calli on the auxin-supplemented medium, confirming that the effect of the increased cytokinin content in *ipt-161* was attenuated by both *axr1* mutations (Figure 3.9c). Some of the hypocotyl explants from *ipt-161 axr1-3* and *ipt161 axr1-12* plants even formed roots, suggesting the cytokinin insensitivity caused by the *axr1* mutation decreased the apparent cytokinin-to-auxin ratio.
Figure 3.9 The axr1 mutations suppress ipt-161 (Col-0) phenotypes.

(a) Suppression of the shoot phenotype in seven-day-old seedlings grown on MS/2 medium. Arrowhead points to the anthocyanin accumulation in the ipt-161 seedling. Scale bar = 1 mm.

(b) Anthocyanin levels in six-day-old seedlings. The fresh weight (FW) of 9-20 seedlings per line was measured prior to the extraction of anthocyanin and spectrophotometry (A_{520 nm}, AU, absorbance units). Data are presented as mean A_{520 nm}/FW ± SD of five independent samples. The anthocyanin levels of Col-0, ipt-161 axr1-3 and ipt-161 axr1-12 seedlings did not differ significantly (one-way ANOVA with Bonferroni multiple comparison post-test; P > 0.05).

(c) Cytokinin-independent callus induction assay. Hypocotyl segments of six-day-old seedlings were incubated for 40 days on full-strength MS medium containing 2% sucrose and 0.5 μM NAA. Ten hypocotyl segments per line are shown. Scale bar = 5 mm.
Next, the type-A ARR steady state gene expression levels in Col-0, ipt-161, ipt-161 axr1-3 and ipt-161 axr1-12 plants were compared (Figures 3.10). RNA gel blot analyses showed that, similar to ipt-161 (C24) and the ipt-161 soi1 lines (Figure 2.2), the ARR5 steady-state level in ipt-161 (Col-0) plants was higher than in the wild type and was suppressed below ipt-161 amounts by the axr1-3 and axr1-12 mutations (Figure 3.10a). Real time RT-PCR analyses confirmed these results and also revealed that the other two type-A ARR genes, ARR4 and ARR6, had similar expression patterns to that of ARR5 in these four lines (Figure 3.10b). Here these genes’ steady state expression levels in Col-0, axr1-3 and axr1-12 plants were also compared. Similar to soi1, the steady-state ARR5 levels in the axr1 single mutants were lower compared to the wild type (Figure 3.11). Similar accumulation trends were also detected for ARR4- (in axr1-12) and ARR6- (in both axr1 mutants) transcripts (Figure 3.11).
Figure 3.10 The axr1 mutations suppress ipt-161 (Col-0) phenotypes at the molecular level.

(a) RNA gel blot analyses of ARR5 transcript levels. Plants grown in liquid Gamborg’s B5 medium for eight days were used for RNA extraction. Methylene blue-stained rRNA is shown as the loading control.

(b) Eight-day-old seedlings grown in Gamborg’s B5 were used for RNA isolation. Relative transcript levels (determined by real-time RT-PCR) in Col-0 were assigned a value of 1, and the relative transcript levels in the mutants were normalized to Col-0.
Figure 3.11 Steady state type-A ARR transcript levels in Col-0 and axr1 mutants.

Real-time RT-PCR analyses of type-A ARR expression in Col-0, axr1-3, axr1-12. Eight-day-old seedlings grown in Gamborg’s B5 medium were used for RNA isolation. Relative transcripts levels in Col-0 were assigned a value of 1, and the relative transcript levels in the mutants were normalized to Col-0. ****, \( P < 0.0001 \) for Col-0 vs. axr1 mutants (one-way ANOVA with Bonferroni multiple comparisons post-test; \( n=3 \)).
Cytokinin has been shown to be involved in light-controlled developmental processes. Whereas seedlings grown in light have short hypocotyls and expanded cotyledons, seedlings grown in dark are etiolated having long hypocotyls, unopened cotyledons and apical hooks (Von Arnim and Deng, 1996). Cytokinin can mimic the effect of light: dark-grown seedlings exposed to cytokinin have short hypocotyls and expanded cotyledons, which are called de-etiolated phenotypes (Chory et al., 1994). The effect of cytokinin and light on etiolation has been shown to be independent and additive (Su and Howell, 1995). To examine their de-etiolated phenotypes, seeds of Col-0, ipt-161, ipt-161 axr1-3, and ipt-161 axr1-12 plants were germinated and grown for four days in dark. The wild-type plants developed long hypocotyls, apical hooks and unopened, unexpanded cotyledons (Figure 3.12). The cytokinin overproducer ipt-161 plants had shorter hypocotyls, but at odds with the de-etiolation responses, an even more pronounced apical hook than the wild type (Figure 3.12a and b). Both of these phenotypes of ipt-161 were reversed by the axr1 mutations, and in the ipt-161 axr1-3 and ipt-161 axr1-12 plants, no apical hooks were formed and the cotyledons were partially opened and partially expanded in dark (Figure 3.12a). Similar to the root growth (Figure 3.8b), the axr1-3 mutation seems to be more efficient than axr1-12 for the reversion of hypocotyl length to wild type level (Figure 3.12c).
Figure 3.12 The axr1 mutations suppress the dark-grown phenotypes of ipt-161 (Col-0).

(a) Apical hook formation in 4-day-old etiolated seedlings. Scale bar = 500 µm.
(b) Representative seedlings grown in the dark for 4 days. Scale bar = 5 mm.
(c) Hypocotyl length of etiolated seedlings. Seeds were germinated and grown in the dark for 4 days, photographed, and the hypocotyl length was measured using Image J. Data are presented as mean ± SD (n=15). The hypocotyl length of Col-0, ipt-161 axr1-3 and ipt-161 axr1-12 seedlings were the same (P > 0.05), but the difference between ipt-161 axr1-3 and ipt-161 axr1-12 was statistically significant (one-way ANOVA with Bonferroni multiple comparisons post-test; **, P < 0.01).

Taken together, results of these analyses indicated that the cytokinin insensitivity of soi1 and axr1 mutants is caused by a defect in primary cytokinin signaling, and that this defect affects hormone sensitivity throughout plant development.
3.3 Discussion

The AXR1 gene encodes a protein which has significant sequence similarity to the ubiquitin-activating enzyme E1, but lacks the key cysteine residue that is essential for E1 activity (Leyser et al., 1993). AXR1 is suggested to function together with E1 C-TERMINAL RELATED 1 (ECR1), to activate members of the RUB/NE DD8 [related-to-ubiquitin (RUB) in plants and budding yeast (Saccharomyces cerevisiae), and neuronal precursor cell-expressed developmentally down-regulated 8 (NEDD8) in animals and fission yeast (Schizosaccharomyces pombe)] family of ubiquitin-like proteins (del Pozo et al., 1998; Hotton and Callis, 2008). ECR1 contains the key cysteine residue and forms a heterodimer with AXR1 to act as activating enzyme for the ubiquitin-like protein RUB1, both in vitro and in vivo (del Pozo et al., 1998; del Pozo et al., 2002).

In humans, the homolog of RUB is called NEDD8 and is activated by a heterodimeric E1 enzyme formed by amyloid beta precursor protein-binding protein 1 (APPBP1) and Ubiquitin-like modifier activating enzyme 3 (UBA3), which are homologous to AXR1 and ECR1, respectively. Similar as the AXR1-ECR1 complex, UBA3 contains the catalytic cysteine residue (del Pozo et al., 1998). The structure of APPBP1-UBA3 heterodimer has been resolved, with an adenylation domain as a base and the catalytic cysteine-containing domain and ubiquitin-like domain as the walls (Walden et al., 2003a). The amino acid sequence of APPBP1 could be divided into four parts based on their positions in the heterodimer structure: N-terminal and C-terminal adenylation domains (A), catalytic cysteine domain (CC) and 4-helix bundle (4HB) (Figure 3.3, Walden et al., 2003a). Glu487 is a conserved residue between AXR1 and APPBP1, and its homologous Glu481 residue is located at the C-terminal region of the 4HB, which contains most of the highly conserved residues on the contacting interface between APPBP1 and UBA3 subunits (Walden et al., 2003a). The amino acid change from acidic residue Glu to basic residue Lys in the soi1 mutant probably changed the electrical environment at the interface, thus influencing the
formation of a functional AXR1-ECR1 complex. The previously reported axr1-12 mutation introduces a stop codon at residue Gln416 and its homologous residue in APPBP1 is localized at the N-terminal region of the 4HB (Figure 3.3), suggesting that the whole C-terminal adenylation domain and most of the 4HB are deleted in this AXR1 protein. On the other hand, the axr1-3 mutation only introduced a single amino acid change in the N-terminal adenylation domain (Figure 3.3), which probably affects AXR1 function without preventing the assembly of the E1 heterodimer. This might explain why the axr1-12 mutant has more severe phenotypes than the axr1-3 mutant (Figure 3.5, Figure 3.6 and Figure 3.7), yet axr1-3 represses ipt-161 more efficiently for some phenotypes (Figure 3.8b and Figure 3.12c).

The RUB protein is post-translationally conjugated to the scaffold subunit Cullin of the multisubunit ubiquitin CUL-RING E3 ligases (CRLs) and this modification is important for the CRL E3 ligase activity (del Pozo and Estelle, 1999; Hotton and Callis, 2008). A viable and weak allele of CUL1, called cul1-6, has been reported to have decreased cytokinin sensitivity in the root elongation assay and this cul1-6 mutant has an increased unmodified CUL1 protein level (Moon et al., 2007). This result is consistent with our observation and suggests that CUL1 might be a component of the CRL E3 ligase specific to cytokinin signaling.

Because the exaggerated curvature of apical hook is part of the triple responses caused by ethylene, this phenotype observed in ipt-161 seedlings (Figure 3.12a) might be a result of increased ethylene levels caused by increased endogenous cytokinin overproduction (Guzman and Ecker, 1990).Ethylene production has been reported to be increased by exogenous cytokinin treatment and to account, in part, for the cytokinin-caused phenotypes in dark (Cary et al., 1995). The axr1-12 mutant has been reported as a “hookless” mutant which shows no differential growth in the apical region of the hypocotyl when grown in dark, but it forms an apical hook upon ethylene treatment (Lehman et al., 1996). These reports suggest that the ethylene insensitivity
caused by \textit{axr1} mutations might only partially contribute to the suppression of apical hook formation in \textit{ipt-161 axr1} plants. The cytokinin insensitivity caused by \textit{axr1} mutations must also contribute to this phenotype by decreasing the endogenous ethylene production. Thus, the observed suppression of apical hook formation in \textit{ipt-161 axr1} double mutant seedlings (Figure 3.12a) is a combined effect of these mechanisms.

For both the root and hypocotyl growth, the \textit{axr1}-3 allele showed a stronger suppression effect on \textit{ipt-161} phenotypes than the \textit{axr1}-12 allele (Figure 3.8b and Figure 3.12c). This seems to be contradictory to the notion that \textit{axr1}-12 is a stronger mutant than \textit{axr1}-3. The steady state transcript levels of \textit{ARR4}, \textit{ARR5} and \textit{ARR6} were also observed to be lower in \textit{ipt-161 axr1}-3 than in \textit{ipt-161 axr1}-12 plants (Figure 3.10), consistent with the root and hypocotyl phenotypes. Interestingly, these phenotypes were only observed in the \textit{ipt-161} background. In Col-0 wild type background, \textit{axr1}-12 mutant showed stronger phenotypes in most tested assays (Figure 3.5, Figure 3.6 and Figure 3.7). The mechanism underlying these observations is still unclear. One possibility is that this is not only about mutation strength but also involves interactions between cytokinin and other hormones, or maybe other signaling pathways that are controlled by RUB modification.

3.4 Materials and methods

Map-based cloning

In order to map the \textit{soi1} locus, F2 progenies from a cross between \textit{soi1} (C24) and Col-0 were used. F2 seeds were sterilized, sown on MS/2 plates, and after 3 days growth, transferred to MS/2 plates containing 10 μM kinetin. Plates were positioned vertically, and plants were grown for another 7 days. DNA was isolated from 106 kinetin-resistant seedlings, and mapping was done using microsatellite markers (Bell and Ecker, 1994), INDEL markers (Jander et al., 2002), and markers for single nucleotide polymorphisms between Col-0 and C24 sequences (Appendix A). The candidate gene was amplified from both the \textit{soi1}
mutant and the C24 wild type, and sequenced using Genome Lab™ Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, Inc., Fullerton, CA). Primers used to sequence the AXR1 gene are listed in Appendix B.

**Hormone response assays**

To test the soi1 mutant’s sensitivity to auxin and ethylene, four-day-old seedlings grown on MS/2 medium were transferred to MS/2 plates containing DMSO (solvent control), indole-3-acetic acid (IAA; Sigma) or 1-aminocyclopropane-1-carboxylic acid (ACC; Sigma). The root tips were marked at the moment of transfer, and the seedlings were photographed at day nine. The root length was measured from the transfer mark using Image J software (National Institute of Health, Bethesda, MD).

To test the cytokinin sensitivity of axr1-3 and axr1-12 mutants, four-day-old seedlings were transferred to fresh MS/2 plates containing either DMSO (solvent control) or cytokinin (kinetin or benzyladenine (BA; Sigma)). For root length analyses, root tips were marked at the moment of transfer to test plates, the plates were positioned vertically in a growth chamber, and the plants were photographed after 5 days of growth. The root length was measured from the transfer mark using Image J. For rosette growth analysis, plants were photographed and analyzed after 18 days of growth. For anthocyanin quantification, seedlings were collected after eight days of growth on test plates, weighed, and used for isolation of total flavonoids as described (Kubasek et al., 1992). The anthocyanin content was measured at 520 nm using DTX 880 Multimode Detector (Beckman Coulter). A detailed description of this procedure is included in the characterization of ipt-161 axr1-3 and ipt-161 axr1-12 plants section of this chapter.

**Characterization of ipt-161 axr1-3 and ipt-161 axr1-12 plants**

The ipt-161 (Col-0) was generated by introgressing the transgene from ipt-161 (C24) into Col-0, and used for genetic and biochemical analyses after six rounds
of backcrossing. The \textit{axr1-3} and \textit{axr1-12} mutants (Lincoln et al., 1990), were in the Col-0 background, and were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University). To construct \textit{ipt-161 axr1-3} and \textit{ipt-161 axr1-12} plants, stigmas of emasculated flowers on the \textit{axr1-12} and \textit{axr1-3} plants were pollinated with pollen from \textit{ipt-161(Col-0)} plants, and F1 individuals with wild type phenotypes were selected for self-pollination. To isolate homozygous \textit{ipt-161 axr1-3}, \textit{ipt-161 axr1-12} mutants, F2 individuals were tested by PCR genotyping, followed by sequencing of the amplified fragments for \textit{axr1-3} and \textit{axr1-12}. Primers used for genotyping are listed in Appendix B and Appendix C.

For root phenotypes, seeds were germinated and grown vertically on MS/2 medium for nine days. At day four, the root tips were marked. At day nine seedlings were photographed and the root length between day four and day nine was measured using Image J. The root hair phenotypes were photographed at day nine with an Olympus SZX12 microscope.

For shoot phenotypes, seven-day-old seedlings germinated and grown on MS/2 medium were photographed with an Olympus B061 light microscope.

For anthocyanin quantification, six-day-old seedlings, grown on MS/2 medium, were grouped and their fresh weight (FW) was measured. Anthocyanin from each seedling sample was extracted overnight with 300μl 1% HCl/methanol in dark at 4°C with continuous shaking (~20rpm). On the second day, 200μl H2O and 500μl chloroform was added to each sample and samples were centrifuged at 14000rpm for 3 minutes at room temperature. The supernatant was used for measurements. The anthocyanin content was measured at 520nm using DTX 880 Multimode Detector (Beckman Coulter). The anthocyanin level for each sample was presented as absorbance (absolute units, AU)/FW(mg).

For callus induction assay, seeds were sterilized and sown on MS/2 plates, chilled for 4 days at 4°C, exposed to light at room temperature for 3 hours, and then grown in dark for 4 days. The plates were then transferred to a growth
chamber and grown for another 2 days under constant light. Hypocotyls were excised and transferred to full strength MS media containing 0.5 μM 1-naphthaleneacetic acid (NAA, Sigma, prepared as stock solutions in DMSO) and 2% sucrose. Plates were kept in a growth chamber at 22°C for 40 days. Representative callus were selected, arranged, and photographed.

For the de-etiolation tests, sterile and chilled Arabidopsis seeds sown on MS/2 medium were exposed to light at room temperature for 3 hours, and returned to darkness. After 4 days of growth, seedlings were arranged onto a new plate and photographed. The hypocotyl length was measured using Image J. Seedlings were photographed with an Olympus B061 light microscope.

**RNA isolation, gel blot analyses and quantitative RT-PCR**

RNA isolation and gel blot analyses were done as described in Chapter 2.

For quantitative RT-PCR, TURBO DNase (Ambion)-treated RNA was reverse transcribed with RNA to cDNA EcoDry™ Premix (Clontech, Mountain View, CA). The cDNA equivalent of 20 ng of total RNA was used in a 10-μl reaction with the DyNAmo Flash SYBR Green qPCR kit (Finnzymes) on the StepOne real-time PCR system (Applied Biosystems). The primers used are listed in Appendix C. For each experiment, At3g18780 (ACT2, ACTIN 2), At1g13440 (GAPDH, GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C-2), At3g53090 (UBIQUITIN TRANSFERASE) and At4g33380 (EXPRESSED PROTEIN) were also amplified using previously described primers (Czechowski et al., 2005), and the best reference gene was selected using geNorm (Vandesompele et al., 2002). PCR efficiency was estimated by LinReg PCR software (Ramakers et al., 2003; Ruijter et al., 2009), and the average efficiency of each amplicon group was used for calculation. Three biological replicates of each sample were tested and reactions were carried out with two technical replicates.

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Chapter 4: ARR5 proteolysis is facilitated by the RUB modification pathway

4.1 Introduction

Previous studies have shown that the AXR1-ECR1 heterodimer is inactive in strong axr1 mutants, which prevents RUB modification of the CUL subunit of CRL-type ubiquitin ligases and leads to a decrease in ligase activity (del Pozo et al., 1998; Hotton and Callis, 2008). This, applied to our case, suggested that the decrease in cytokinin sensitivity in axr1 mutants is caused by accumulation of a cytokinin response repressor that is normally targeted to the proteasome by a CRL-type E3. Because several type-A ARRs (e.g., ARR5, ARR6 and ARR7) were shown to be unstable proteins that accumulate in response to treatments with a proteasome inhibitor or cytokinins (To et al., 2007; Lee et al., 2008; Ren et al., 2009; Ryu et al., 2009), the effects of RUB pathway inhibition on type-A ARR stability were then analyzed. Here ARR5 was selected as a type-A ARR representative because the overexpression of ARR5 typically leads to a stronger cytokinin resistance than the overexpression of other tested type-A ARRs (To et al., 2007; Ren et al., 2009).

In this chapter, a newly identified inhibitor of the RUB E1 enzyme, MLN4924 was used to independently test the requirement for AXR1 in cytokinin signaling and to examine the relationship between ARR5 stability and RUB modification pathway. MLN4924 was initially identified as an inhibitor of NEDD8-activating enzyme (NAE1) in humans (Soucy et al., 2009; Bennett et al., 2010; Brownell et al., 2010). MLN4924 is suggested to inhibit NAE1 through a substrate-assisted mechanism: NAE1 catalyzes the formation of a covalent NEDD8-MLN4924 adduct, which resembles NEDD8 adenylate, the first intermediate of the NAE reaction cycle, but cannot be utilized in subsequent intraenzyme reactions. The NEDD8-MLN4924 adduct stays in the NAE1 active site and blocks enzyme activity, thereby accounting for the potent inhibition of NEDD8 pathway by MLN4924 (Brownell et al., 2010). MLN4924 has also been shown to effectively and specifically inhibit the RUB modification of cullins in Arabidopsis and can
thus be used to mimic the phenotypes of mutants with a partial defect in RUB modification (Hakenjos et al., 2011).

This chapter first describes the successful suppression of developmental and molecular phenotypes of *ipt-161* by MLN4924 and then the generation of transgenic plants ectopically overexpressing N-terminally tagged FLAG-ARR5 (*FLAG-ARR5ox*) and their use for analyses of the effect of genetic and chemical inhibition of RUB modification pathway on FLAG-ARR5 stability.

### 4.2 Results

**Chemical inhibition of the RUB modification pathway suppresses *ipt-161***

To independently test the requirement for AXR1 in cytokinin signaling, the effects of MLN4924, the inhibitor of the RUB E1 enzyme, on the *ipt-161* developmental and molecular phenotypes were tested (Soucy et al., 2009; Bennett et al., 2010; Hakenjos et al., 2011). To that end, the *ipt-161* (Col-0) seedlings were germinated and grown on medium containing MLN4924. The seedling morphology and the expression of type-A *ARR* genes were analyzed (Figure 4.1). The *ipt-161* phenotype was partially suppressed by MLN4924: whereas a lower dose of the inhibitor (10 µM) led to a near-complete restoration of cotyledon size and a partial suppression of the short-root phenotype, a higher dose (25 µM) appeared to be toxic and particularly effected root growth (Figure 4.1a). Real-time RT-PCR analyses revealed that MLN4924 also suppressed the constitutive up-regulation of type-A *ARR* genes *ARR4*, *ARR5* and *ARR6* in *ipt-161* (Figure 4.1b). Thus, both genetic studies and pharmacological assays showed that the RUB pathway is required for primary cytokinin signaling. MLN4924 also provided an alternative approach to mimic the effect of *axr1* mutation for protein stability analysis.
Figure 4.1 Pharmacological inhibition of the RUB modification pathway partially suppresses the ipt-161 (Col-0) phenotypes.

(a) Morphology of seven-day-old ipt-161 seedlings grown on MS/2 medium supplemented with the Rub modification pathway inhibitor MLN4924 (MLN) or DMSO (control). Wild-type (Col-0) seedlings grown on control media are shown. Scale bar = 5 mm.

(b) Real-time RT-PCR analyses of the ARR4, ARR5 and ARR6 steady-state levels. Plants shown in (a) were used for analyses. Type-A ARR expression levels were normalized against GAPDH (At1g13440), and the average relative transcript level (n=3) for each gene in Col-0 was assigned the value of 1 (shaded). The transcript levels of type-A ARRs in control and MLN4924-treated ipt-161 were normalized to Col-0. The statistical significance of the difference between transcript levels in treated and non-treated ipt-161 is marked (****, P < 0.0001; ANOVA with Bonferroni multiple comparison post-test).
Generation and basic characterization of FLAG-ARR5 overexpression lines

In order to analyze the endogenous ARR5 levels, the anti-ARR5 antisera was generated through genomic antibody technology, which generates antibodies by directly immunizing the host animal with DNA. I tried to detect the endogenous ARR5 levels in untreated and BA-treated Col-0 and arr5-1 seedlings, but was unable to detect a protein of the correct size (~21 kDa) which was supposed to be present in the wild type but not in the mutant (arr5-1). A possible explanation is that the endogenous ARR5 level, even after BA treatment, is too low to be detected.

To improve the chances to detect ARR5, plants ectopically expressing an N-terminally FLAG-tagged ARR5 driven by CaMV 35S promoter were generated and two overexpression lines (in Col-0 background) were isolated: FLAG-ARR5ox #1 and #2. Both of them had increased ARR5 protein levels (Figure 4.2). In accordance with previous studies (To et al., 2007; Ren et al., 2009), the FLAG-ARR5 overexpression decreased the plants’ cytokinin sensitivity in the root elongation assay (Figure 4.3). The FLAG-ARR5 levels increased after BA and MG132 treatments, and decreased after cycloheximide (CHX) treatment, which is also consistent with previous analyses describing the stability of Myc-tagged ARR5 (Figure 4.4 and To et al., 2007; Ren et al., 2009).

**Figure 4.2** Generation of the FLAG-ARR5 overexpression lines.

Immunoblotting analyses of two homozygous lines overexpressing N-terminal FLAG-tagged ARR5 (FLAG-ARR5ox). Seven-day-old seedlings grown on MS/2 medium were collected, weighed and used for protein isolation. Ponceau S stained LSU is shown as a loading control.
Figure 4.3 Overexpression of *FLAG-ARR5* decreased cytokinin sensitivity in Col-0.

(a) Four-day-old seedlings grown on MS/2 medium were transferred to plates containing the denoted concentrations of BA and grown for another five days. Plates were photographed, and the root length was measured using Image J. Data are presented as mean ± SD (n=7).

(b) Representative plates showing the increased resistance of *FLAG-ARR5ox* line #2 to 0.2 μM BA.
Figure 4.4 Basic characterization of the FLAG-ARR5 protein.

(a) Immunoblotting analyses of BA-induced accumulation of FLAG-ARR5 protein. Col-0 and FLAG-ARR5ox #2 seedlings were grown on MS/2 media for 7 days, and were then treated with 5 µM BA for the indicated time. Endogenous ARR5 was not detected in Col-0 plants after BA treatments.

(b) Immunoblotting analyses of FLAG-ARR5 stability. The FLAG-ARR5ox #2 seedlings were grown on MS/2 media for 7 days, and were then treated with 200 µM cycloheximide (CHX), 5 µM BA, 100 µM MG132 alone or their combinations for one hour. Ponceau S stained LSU is shown as the loading control.

ARR5 is stabilized by MLN4924

Next, I tested the stability of FLAG-ARR5 in response to the RUB pathway inhibitor treatments. Immunoblotting analyses showed that treatment with MLN4924 promoted a gradual increase in FLAG-ARR5 with FLAG-ARR5 levels being ~2 fold of the control after 4-hour treatment (Figure 4.5a). Cycloheximide (CHX) chase experiments (Kurepa and Smalle, 2011) further revealed that the MLN4924-induced accumulation of FLAG-ARR5 was a result of a decrease of its degradation rate (Figure 4.5b).
Figure 4.5 The FLAG-ARR5 protein is stabilized by MLN4924.

Plants overexpressing N-terminally FLAG-tagged ARR5 (FLAG-ARR5ox line #2) were grown on MS/2 medium for five days prior to treatment with 100 µM MLN4924 (a) or 200 µM CHX and 100 µM MLN4924 (b) for the indicated times. Immunoblotting analyses were done with anti-ARR5 antisera. LSU is shown as a loading control. Quantifications of the signal strength is presented on the right-hand side as mean ± SEM (n=2).

To test the relative contributions of the RUB modification pathway and cytokinin signaling on ARR5 stability, the combined effects of MG132, BA and MLN4924 on FLAG-ARR5 proteolysis were analyzed (Figure 4.6). After a 4-hour-long treatment, MLN4924 and BA alone increased the steady-state levels of FLAG-ARR5 (3.0±0.2 and 2.3±0.1, respectively). The combined treatments of BA+MLN4924 and MG132+MLN4924 had additive and supra-additive effects (4.6±0.4 and 7.5±0.4), respectively.

Thus, our data suggested that when the RUB modification pathway is interrupted by MLN4924, the ARR5 protein is stabilized. Stabilized ARR5 could give a stronger repression of the cytokinin signaling pathway, which might be the underlying reason for the decreased cytokinin sensitivity of axr1 mutants.
Figure 4.6 Additive effects of MLN4924 and BA or MG132 treatments.

Five-day-old seedlings were treated with different combinations of 200 µM CHX, 100 µM MLN4924 (MLN), 100 µM MG132, and 5 µM BA or 0.1% DMSO (control) for 4 hours, and used for isolation of total proteins. Immunoblotting analyses were done with anti-ARR5 antisera. LSU is shown as a loading control. Quantifications of the signal strength is presented on the right-hand side as mean ± SEM (n=2).

ARR5 is stabilized in the axr1 background

To test if FLAG-ARR5 also accumulates in response to genetic suppression of the RUB pathway, the FLAG-ARR5 transgene was introgressed into the axr1-3 background by crossing FLAG-ARR5 overexpression lines with axr1-3 mutant. For the stronger, FLAG-ARR5ox #2, the double homozygous FLAG-ARR5ox axr1-3 plants could not be isolated successfully. One possible explanation is that the transgene might be inserted into a position linked with the AXR1 locus. Finally, by introgressing the weaker FLAG-ARR5 transgene (FLAG-ARR5ox #1) into the axr1-3 background, the double homozygous FLAG-ARR5ox axr1-3 plants were isolated and FLAG-ARR5 protein was found to accumulate in response to genetic suppression of the RUB pathway (Figure 4.7a). Moreover, root growth response assays confirmed that the cytokinin resistance of FLAG-ARR5ox seedlings was indeed enhanced in the axr1-3 background (Figure 4.7b).
Figure 4.7 Increased FLAG-ARR5 accumulation in axr1-3 background caused enhanced cytokinin resistance.

(a) Stabilization of FLAG-ARR5 in the axr1-3 background. Five-day-old plants were used for the isolation of total proteins and immunoblotting with anti-ARR5 antisera. Relative signal level (RL) is presented as mean ± SD of four biological replicates.

(b) Root growth assay. Four-day-old seedlings were transferred from MS/2 medium to medium with BA. Root growth between day four (transfer) and day nine was measured (n=15). The difference between root lengths of FLAG-ARR5ox in Col-0 and axr1-3 backgrounds is statistically significant (****, P < 0.0001; ANOVA with Bonferroni multiple comparison post-test).

Taken together, these results show that ARR5 degradation by the 26S proteasome requires a functional RUB modification pathway. In addition, the increased accumulation of at least one cytokinin response repressor potentially provided an explanation for the decreased cytokinin sensitivity of axr1 mutants.

4.3 Discussion

The increased accumulation of ARR5 protein in the axr1 mutant suggested that the decreased cytokinin sensitivity of axr1 mutants might be caused by increased endogenous ARR5 protein levels, which give a stronger suppression on the primary cytokinin signaling pathway.
On the other hand, the \textit{ARR5} transcript abundance was previously observed to be decreased in the \textit{axr1} mutant compared to the wild type (Figure 2.2, 2.4 and 3.11). These two observations seem to be paradoxical: the \textit{axr1} mutants have decreased \textit{ARR5} mRNA level but increased \textit{ARR5} protein level. However, the transcript abundance is not the only determinant for protein abundance. The intracellular protein level is determined by a series of processes, including transcription, processing and degradation of mRNA, translation and protein degradation. The steady-state transcript abundance can only partially predict the corresponding protein abundance. Studies from both mammalian cells and bacteria suggested that the correlation between transcript and protein abundance is quite low for many genes especially when they encode unstable regulatory proteins and only \(\sim 40\%\) of the variation in protein concentration can be explained by knowing mRNA abundance (Vogel and Marcotte, 2012). In fact, a low correlation can also be inferred from the cytokinin induction profile of the \textit{ARR5} gene. Cytokinin induction of \textit{ARR5} typically leads to an induction peak which is followed by a gradual decrease in transcript level later on, and it is generally believed that the decline in \textit{ARR5} transcription reflects the increased abundance of the \textit{ARR5} protein (D'Agostino et al., 2000). Accordingly, the lower \textit{ARR5} transcript abundance in both the untreated and cytokinin-treated \textit{axr1} mutant is not in disagreement with an increased \textit{ARR5} protein level.

The identity of the \textit{ARR5} specific CRL E3 ligase still remains unknown. The hypothetical CRL probably contains the \textit{CUL1} isoform, since the loss of function of the corresponding gene was reported to cause cytokinin insensitivity in the root (Moon et al., 2007). Two approaches can be used to search for the subunit factors composing the \textit{ARR5} specific CRL E3 ligase: one is to screen for \textit{ARR5} interacting proteins, for example by yeast-two hybrid assays and the other is to search for genes co-expressed with \textit{ARR5} from the gene expression database.
4.4 Materials and methods

Pharmacological inhibition of ipt-161 phenotypes

The ipt-161 seeds were germinated on MS/2 medium supplemented with either the RUB modification pathway inhibitor MLN4924 (MLN) or DMSO (control, dissolving reagent), and grown for seven days before they were photographed. Wild-type (Col-0) seedlings were grown on control medium. RNA isolation and quantitative RT-PCR analyses were done as described in Chapter 3.

Generation of FLAG-ARR5 overexpressors in Col-0 and axr1-3

To generate overexpressor lines, a full-length ARR5 cDNA was amplified for Gateway cloning (Life Technologies) with gene-specific primers containing the attB sites (Appendix C). The cDNA was recombined into pDONR221 (the BP reaction), and transferred into pEarlyGate202 (the LR reaction) which carries the L-phosphinotricine (Basta) resistance gene, and allows the 35S promoter-driven expression of N-terminally FLAG-tagged proteins (Earley et al., 2006). Arabidopsis Col-0 plants were transformed by the floral dip method (Clough and Bent, 1998). Two lines were selected for further analyses. After screening F2 seedlings of a cross between axr1-3 and the overexpressing FLAG-ARR5ox line #2, no double mutants were isolated and it was concluded that the T-DNA insertion carrying the overexpression construct is located close to axr1-3. Further analyses were conducted on a homozygous double mutant generated from crossing between axr1-3 and FLAG-ARR5ox line #1. Root growth assay was done as described in Chapter 3.

Protein isolation, antibody generation and immunoblotting analysis

Protein extraction and immunoblotting analyses were done as previously described (Kurepa and Smalle, 2011). Anti-ARR5 antibodies were generated in rabbits against the C-terminal amino acids 85 to 184, and were affinity purified before use (SDIX, Brown et al., 2011). For immunoblotting analyses, membranes were first incubated with the anti-ARR5 antibody, and then with HRP-conjugated
anti-rabbit IgG goat antibodies. The signal was developed with SuperSignal West Femto substrate (Thermo-Pierce) using Bio-Rad Molecular Imager ChemiDoc™ XRS (Bio-Rad Laboratories, Inc). The signal intensities were measured using QuantityOne software (Bio-Rad) as described (Kurepa and Smalle, 2011).
Chapter 5: Cytokinin insensitivity of \textit{axr1} requires type-A ARR function

5.1 Introduction

In order to test whether stabilized ARR5 and other type-A ARRs indeed cause cytokinin insensitivity of \textit{axr1} mutants, I generated double and higher order mutants of type-A ARRs and \textit{axr1}. Two previously described T-DNA mutants, \textit{arr5-1} and \textit{arr3,4,5,6}, were used for crossing with the \textit{axr1-3} allele. The insertion in \textit{arr5-1} is located in exon 4 and predicted to disrupt the receiver domain of the \textit{ARR5} gene. RNA gel blot analysis shows a shift in transcript size and a decrease in transcript levels (To et al., 2004). Besides the insertion \textit{in arr5-1}, the \textit{arr3,4,5,6} mutant contains three more insertions in \textit{ARR3}, \textit{ARR4} and \textit{ARR6} genes, respectively. The T-DNA insertion in \textit{ARR3} abolishes the expression of \textit{ARR3} transcript, while the insertions in \textit{ARR4} and \textit{ARR6} substantially reduced the corresponding transcript levels (To et al., 2004). Because of functional redundancy, the single mutant is indistinguishable from the wild type in various cytokinin assays, while the quadruple mutant shows increased sensitivity to cytokinin (To et al., 2004).

In this chapter, the isolation and analyses of the homozygous \textit{arr5-1 axr1-3} and the pentuple mutant \textit{arr3,4,5,6 axr1-3} are described. The results suggest that although the stabilization of \textit{ARR3}, 4 and/or 6 also contribute to the cytokinin insensitivity of the \textit{axr1-3} mutant, the stabilization of \textit{ARR5} is the major contributor to the cytokinin insensitive phenotype.

5.2 Results

To further investigate the role of type-A ARRs in the cytokinin resistance of \textit{axr1} mutants, a homozygous \textit{arr5-1 axr1-3} line was generated, and the effects of cytokinins on type-A \textit{ARR} expression, root length, root hair formation and anthocyanin accumulation in this line were tested. The cytokinin induction of type-A \textit{ARR} genes in the single- and double-mutant backgrounds was first analyzed (Figure 5.1). Consistent with the induction of the \textit{ARR5} gene (Figures
2.4 and 3.7), *ARR4* and *ARR6* inductions were suppressed in *axr1-3* plants treated for 30-minutes with 5 µM BA. Although this molecular phenotype was reversed in *arr5-1 axr1-3* plants, the reversion was only partial (Figure 5.1).

![Graph showing cytokinin induction in different conditions.](image)

**Figure 5.1** Loss-of-function of *ARR5* partially reverses the cytokinin insensitivity caused by *axr1*.

Cytokinin-induced *ARR4* and *ARR6* expression in Col-0, *arr5-1*, *axr1-3* and *arr5-1 axr1-3* analyzed by real-time RT-PCR. Eight-day-old seedlings grown in liquid Gamborg’s B5 medium were treated with 5 µM BA for 30 min. The reference gene was *GAPDH*. The untreated samples (controls) were assigned the value of 1. The data represent average relative quantity (RQ) values of three replicates, and the bars denote the RQ$_{\text{Min}}$ to RQ$_{\text{Max}}$.

Next, the cytokinin growth responses were analyzed. Compared to the *axr1-3* mutant, *arr5-1 axr1-3* seedlings showed statistically significant increases in cytokinin sensitivity for the root elongation ($P < 0.001$), root hair growth ($P < 0.0001$), and anthocyanin accumulation ($P < 0.001$) responses (Figure 5.2 and Figure 5.3).
However, the addition of *arr5-1* again only partially suppressed the cytokinin insensitivity of *axr1-3* suggesting that the accumulation of other type-A ARR s also contributes to this phenotype. To test this hypothesis, I generated the pentuple mutant *arr3,4,5,6 axr1-3* and compared its cytokinin-induced growth responses to those of *arr5-1 axr1-3* (Figure 5.2 and 5.3). With the exception of root hair growth (Figure 5.2b and c), the additional removal of *ARR3, ARR4* and *ARR6* led to a further, albeit very mild, increase in *axr1-3* suppression which was statistically significant only at higher cytokinin doses (Figure 5.2a and 5.3). Thus, comparison of the overall reversion effects of the *arr5* single versus the *arr3,4,5,6* quadruple mutations suggested that whereas stabilization of multiple type-A ARR s contribute to the cytokinin insensitivity of *axr1-3*, the stabilization of ARR5 is its major cause.
Figure 5.2 Loss-of-function of type-A ARRs reverses cytokinin insensitivity caused by axr1: root assays.

(a) Root growth assay. Four-day-old seedlings were transferred from MS/2 media to media with BA and grown for another six days. Root growth between day four and day ten was measured (n=15). The difference between axr1-3 and arr5-1 axr1-3 or arr3,4,5,6 axr1-3 is statistically significant except for the highest BA dose (P < 0.01; ANOVA with Bonferroni multiple comparison post-test).

(b) The promotion of root hair formation and elongation by kinetin. Four-day-old seedlings germinated on MS/2 media were transferred to media with kinetin, and roots were photographed six days after transfer. Root hair length was measured from photographs using Image J. Data are presented as mean ± SD (n=20). The difference between average length of root hairs in axr1-3 and arr5-1 axr1-3 or arr3,4,5,6 axr1-3 is statistically significant for all treated samples (P < 0.001; ANOVA with Bonferroni multiple comparison post-test). Legend is the same as in (a).

(c) Representative images of root tips and root hairs of Col-0, axr1-3 and axr1-3 arr3,4,5,6. Scale bar = 1 mm.
Figure 5.3 Loss-of-function of type-A ARR\(_s\) reverses cytokinin insensitivity caused by \(axr1\): cytokinin-induced anthocyanin accumulation.

Four-day-old seedlings germinated on MS/2 media were transferred to media with BA and grown for another six days. Data are presented as mean \(A_{520\ nm}/FW \pm SD\) of three independent samples (5 seedlings per sample). The difference between \(axr1\)-3 and \(arr5\)-1 \(axr1\)-3 or \(arr3,4,5,6\ axr1\)-3 is statistically significant for all measurements (\(P < 0.01\); ANOVA with Bonferroni multiple comparison post-test).

5.3 Discussion

The fact that ARR5 accumulated both in \(axr1\) and in MLN4924 treated wild-type seedlings (Figure 4.5 and 4.7) suggested that the decreased cytokinin sensitivity of \(axr1\) mutants might be caused by increased endogenous ARR5 levels. Indeed, introduction of the loss-of-function \(arr5\)-1 mutation reversed the cytokinin resistance of \(axr1\)-3 mutant, but this reversion remained only partial (Figure 5.1, 5.2 and 5.3). Introduction of additional loss-of-function mutations, \(arr3\), \(arr4\) and \(arr6\), did enhance the reversion effect of \(arr5\)-1, but the difference between \(arr3,4,5,6\ axr1\)-3 and \(arr5\)-1 \(axr1\)-3 is not large, suggesting that other type-A ARRs might also contribute to the cytokinin insensitive phenotypes of the \(axr1\) mutant. To further address this question, higher order loss-of-function type-A ARR mutants need to be constructed in the \(axr1\) background. However, this is a difficult task because some type-A ARR genes are linked with \(AXR1\) (\(AXR1\),...
ARR3, ARR4, ARR7 and ARR15 are linked on chromosome 1) and others are linked to each other (ARR5, ARR9 and ARR17 are linked on chromosome 3 and ARR8 and ARR16 are linked on chromosome 2). In addition, even the weak axr1-3 mutant has reduced seed yield.

Another approach is to examine the protein levels and degradation rates of other type-A ARR proteins in the axr1 mutant background or MLN4924 treated wild-type seedlings. For these analyses, ectopically overexpressed tagged versions of all type-A ARRs are needed because type-A ARRs have highly similar sequences and low endogenous protein levels, which make it difficult to generate specific antibodies. Finally, it also remains possible that part of the cytokinin resistance of the axr1 mutant might be caused by increased accumulation of cytokinin response repressors other than the type-A ARR family.

The cytokinin-induced ARR6 expression was observed to decrease in the arr5-1 mutant (Figure 5.1). This seems to be contradicting with the notions that ARR act as a negative feedback regulator of cytokinin signaling and that ARR6 is a cytokinin inducible gene. While the cause for this unexpected result is currently unknown, it may be related to the potentially antagonistic functions of the ARR5/6 gene product pair (To et al., 2004). ARR5 and ARR6 share the highest homology with each other compared to other members of the type-A ARR gene family (To et al., 2004). The loss-of-function arr5-1 mutant has an altered rosette morphology with reduced rosette size in short day conditions, and this phenotype was not enhanced but was instead absent in the arr5-1 arr6 double mutant (To et al., 2004), suggesting an antagonistic function of these two gene products. A recent report indicated the expression of ARR5 and ARR6 is affected by auxin in a highly tissue specific and opposing manner in the presence of cytokinin (Kakani and Peng, 2011). These reports suggested that although ARR5 and ARR6 are the most closely related gene pair in the type-A ARR gene family, their products might play antagonistic functions and that their expression are not only regulated by cytokinin signaling pathway. Besides auxin, it remains possible that other stimuli might also contribute to the regulation of ARR6.
expression. Thus, the decrease of cytokinin-induced ARR6 expression in the arr5-1 mutant might not only be a result of the disturbed cytokinin signaling, but a combined result of disturbing multiple regulatory functions.

5.4 Materials and methods

The arr5-1 and arr3,4,5,6 mutants (To et al., 2004) were in the Col-0 background and were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University). To construct arr5-1 axr1-3 and arr3,4,5,6 axr1-3 plants, the stigma of emasculated flowers on axr1-3 plants was pollinated by pollen from arr5-1 or arr3,4,5,6 plants, and F1 individuals with wild-type phenotypes were selected for self-pollination. To isolate homozygous arr5-1 axr1-3 and arr3,4,5,6 axr1-3 mutants, F2 individuals with axr1-3 mutant phenotypes were screened by PCR genotyping for the respective T-DNA insertions in ARR genes. Primers used for genotyping are listed in Appendix C. RNA isolation, quantitative RT-PCR analyses and cytokinin response assays for root and anthocyanin accumulation were done as described in Chapter 3.
Chapter 6: ARR1, a type-B ARR, accumulates in the *axr1* background

### 6.1 Introduction

Although overexpression of type-B ARRs increases cytokinin sensitivity, it does not significantly influence plant development (Hwang and Sheen, 2001; Sakai et al., 2001), suggesting that type-B ARRs are produced as inactive forms in plants and require activation by cytokinin-induced phosphorylation. Consistent with this, when a mutated ARR2, in which the conserved Asp residue in the receiver domain is mutated to an unphosphorylatable Asn residue, is introduced into protoplasts, the cytokinin-induced mobility shift of ARR2 on SDS-PAGE gels (which signifies phosphorylation) is not detected and the cytokinin-dependent transactivation of *ARR6* is reduced (Kim et al., 2006a). Studies in bacteria and yeast have shown that mutating the conserved Asp (D) residue of a response regulator to a glutamate (E) residue can mimic the aspartyl-phosphate and activates the protein (Klose et al., 1993; Brown et al., 1994). In Arabidopsis, transgenic plants overexpressing a phospho-mimic ARR2 (*ARR*D80E) show up-regulated expression of type-A *ARR* genes, suggesting the substitution of the conserved Asp80 to Glu creates a dominant-active form of ARR2 which causes plants to become partially independent of exogenously applied cytokinin (Hass et al., 2004).

An Arabidopsis line ectopically expressing a phospho-mimic ARR1 (*ARR*1*D94E*) has been constructed in our lab. The *ARR1*D94E in this line mimics the phosphorylated form of the ARR1 protein and indeed the *35S::ARR1*D94E plants showed constitutively up-regulated cytokinin responses in several assays, including root elongation, type-A *ARR* gene expression and cytokinin independent callus induction (Figure 6.1a, 6.2 and 6.3a).

In this chapter, the *35S::ARR1*D94E plants were crossed with the strong *axr1*-12 mutant and phenotypes of the double homozygous *axr1*-12 *35S::ARR1*D94E plants were examined. The objective of this work was to determine if type-A
ARRs are the only components of the cytokinin signaling pathway that are stabilized by loss-of-function of the AXR1 protein. Increased ARR1 accumulation in the axr1 mutant background was detected, suggesting that at least this type-B ARR is also degraded by a proteasome-dependent mechanism that involves CRL E3 ligases.

6.2 Results

The steady state ARR5 expression levels were first examined in Col-0, 35S::ARR1\textsuperscript{D94E}, axr1-12 and axr1-12 35S::ARR1\textsuperscript{D94E} plants. The 35S::ARR1\textsuperscript{D94E} plants showed constitutively up-regulated cytokinin responses as determined by elevated ARR5 expression level (Figure 6.1a). This elevated ARR5 expression was enhanced in the axr1-12 35S::ARR1\textsuperscript{D94E} line (Figure 6.1a), suggesting that the axr1-12 mutation increases the influence of ARR1\textsuperscript{D94E} expression. The most straightforward explanation for this is that the ARR1\textsuperscript{D94E} protein was more abundant in the axr1-12 background.

To test this, I next examined the ARR1 protein level in these lines and found that it accumulates to a higher level in axr1-12 35S::ARR1\textsuperscript{D94E} plants compared to the 35S::ARR1\textsuperscript{D94E} line (Figure 6.2b). These results suggested that ARR1\textsuperscript{D94E} is stabilized in the axr1-12 background which provided an explanation for the enhanced ARR5 expression. An increased endogenous ARR1 protein level was also observed in the axr1-12 mutant compared to the wild type Col-0 (Figure 6.2b). The ARR5 transcript level in the axr1-12 mutant seems to be decreased slightly compared to Col-0 (Figure 6.1a), suggesting that, although ARR1 is stabilized, the axr1-12 mutant still shows decreased cytokinin sensitivity to endogenous cytokinins (also refer to Figure 3.11).
Figure 6.1 ARR1 is stabilized in the axr1-12 mutant.

(a) Steady-state ARR5 transcript levels in eight-day-old Col-0, 35S::ARR1<sup>D94E</sup>, axr1-12, and axr1-12 35S::ARR1<sup>D94E</sup> plants.

(b) ARR1 protein levels in five-day-old Col-0, 35S::ARR1<sup>D94E</sup>, axr1-12, and axr1-12 35S::ARR1<sup>D94E</sup> plants.

The cytokinin responses of these lines were then tested. Theoretically, a constitutive cytokinin response mutant, similar to a cytokinin overproducer line, will be able to form callus on auxin-containing medium without exogenous cytokinin. Indeed, after 39 days of incubation on medium containing 2 or 5 μM NAA, hypocotyl explants excised from the 35S::ARR1<sup>D94E</sup> plants formed calli, while the wild-type explants did not (Figure 6.2). For the hypocotyl explants excised from the axr1-12 35S::ARR1<sup>D94E</sup> line, I observed increased formation of green calli on 2, 5 and 10 μM NAA medium compared to the 35S::ARR1<sup>D94E</sup> line (Figure 6.2), suggesting that the stabilized ARR1<sup>D94E</sup> caused stronger callus formation. Interestingly, the hypocotyl explants of the axr1-12 plants formed green calli on medium containing 10 μM NAA (Figure 6.2). This may be caused by the combined effect of the decreased auxin sensitivity and decreased cytokinin sensitivity of the axr1-12 mutant. The altered hormonal sensitivity levels in the mutant apparently require 10 μM NAA for callus induction.
Figure 6.2 Cytokinin-independent callus induction.

Hypocotyls of six-day-old seedlings were excised and transferred to MS full strength medium containing 2% sucrose and the indicated concentrations of NAA. Photos were taken after 39 days of treatment. Scale bar = 5mm.

The constitutively up-regulated cytokinin responses in the 35S::ARR1<sup>D94E</sup> plants led to the development of short roots and increased root hair formation. These phenotypes seem to be reversed in the axr1-12 35S::ARR1<sup>D94E</sup> plants (Figure 6.3a and 6.3b). The homozygous axr1-12 35S::ARR1<sup>D94E</sup> plants had a similar shoot size as the 35S::ARR1<sup>D94E</sup> plants (Figure 6.3c). Although the anthocyanin levels in both the 35S::ARR1<sup>D94E</sup> and axr1-12 35S::ARR1<sup>D94E</sup> plants were higher than in the wild type, the anthocyanin accumulated less in the axr1-12 35S::ARR1<sup>D94E</sup> plants compared to the 35S::ARR1<sup>D94E</sup> plants (Figure 6.3d).
Figure 6.3 Root, shoot and anthocyanin phenotypes.

(A) Root phenotypes of seven-day-old Col-0, 35S::ARR\textsuperscript{D94E}, axr1-12 and axr1-12 35S::ARR\textsuperscript{D94E} plants. Seeds were germinated on MS/2 medium and grown vertically for seven days before photography. Scale bar = 5mm.

(B) Representative root hair phenotypes of seven-day-old Col-0, 35S::ARR\textsuperscript{D94E}, axr1-12 and axr1-12 35S::ARR\textsuperscript{D94E} plants. Scale bar = 1mm.

(C) Representative shoot phenotypes of seven-day-old Col-0, 35S::ARR\textsuperscript{D94E}, axr1-12 and axr1-12 35S::ARR\textsuperscript{D94E} plants. Scale bar = 1mm.

(D) Anthocyanin accumulation in seven-day-old Col-0, 35S::ARR\textsuperscript{D94E}, axr1-12 and axr1-12 35S::ARR\textsuperscript{D94E} plants. Anthocyanin levels were calculated as mean A\textsubscript{520} nm/FW ± SD of three independent samples (11~17 seedlings per sample) and data was normalized to that of Col-0.

Cytokinin has been reported to delay leaf senescence (Richmond, 1957; Nooden et al., 1979; Gan and Amasino, 1995; McCabe et al., 2001). During leaf
senescence, the earliest and most significant change in cell structure is the breakdown of chloroplasts and the loss of chlorophyll, thus the chlorophyll content is often used as a marker for leaf senescence (Lim et al., 2007). Leaf senescence is a highly regulated process and is influenced by age, internal- and environmental-signals (Lim et al., 2007). Since the 35S::ARR1D94E plants have delayed leaf development compared to the wild type and axr1-12 mutant, I performed a detached-leaf senescence test on cotyledons which have less developmental differences than the mature leaves within the four tested lines. Significantly delayed senescence was observed in the 35S::ARR1D94E line and this delay was maintained in the axr1-12 35S::ARR1D94E line (Figure 6.4).
Figure 6.4 Detached-leaf senescence test on cotyledons.

(A) Delayed senescence in 35S::ARR1<sub>D94E</sub> and axr1-12 35S::ARR1<sub>D94E</sub> plants. Cotyledons of six-day-old seedlings grown on MS/2 medium plates were detached, placed in sterile water in a plate and incubated for the indicated times in darkness at room temperature. Scale bar = 5mm.

(B) Chlorophyll content in cotyledons incubated in dark for the indicated times. Data were presented as mean ± SD of three independent samples (each containing 12 cotyledons). For each line, the chlorophyll content was normalized to that of D0.
6.3 Discussion

The observation that both ARR1<sup>D94E</sup> and endogenous ARR1 are more abundant in the axr1-12 background (Figure 6.1b) suggested that ARR1, a type-B ARR, is also degraded by a RUB-regulated CRL E3 ligase. A recently described auf1 mutant, which contains a mutation in an F-box protein called AUXIN UP-REGULATED F-BOX PROTEIN 1 (AUF1), has increased ARR1 protein levels and is hypersensitive to cytokinin in root growth and type-A ARR gene expression assays (Zheng et al., 2011). ARR1 might be targeted for 26S proteasome degradation by a CRL E3 ligase assembled with AUF1. As indicated by the name, the expression of AUF1 is strongly up-regulated by auxin (Zheng et al., 2011). Thus, the potential degradation of ARR1 by this CRL E3 ligase provides one more layer of interaction between cytokinin and auxin signaling. ARR2, another type-B ARR, has also been reported to be degraded by the proteasome pathway (Kim et al., 2012). Whether ARR2 is also potentially targeted for proteasome-dependent degradation through an AXR1 regulated CRL E3 ligase remains to be elucidated.

All the tested phenotypes, except for the root length and root hair phenotypes, supported the view that the cytokinin insensitivity of axr1 mutant is caused by stabilized ARR5 which acts upstream of ARR1. The root phenotypes are reversed in the axr1-12 35S::ARR1<sup>D94E</sup> plants compared to that in the 35S::ARR1<sup>D94E</sup> plants (Figure 6.3a and 6.3b). However, root phenotypes can be explained by decreased ethylene sensitivity of the axr1 mutant. Ethylene production is increased by exogenous cytokinin treatment and adds to the cytokinin phenotypes (Cary et al., 1995). Overexpression of ARR1<sup>D94E</sup> induces constitutive cytokinin responses and the ethylene production, as a downstream event of this overexpression, should also be increased and could contribute to the short root and increased root hair formation phenotypes in the 35S::ARR1<sup>D94E</sup> plants. The introduction of axr1-12 mutation influenced not only cytokinin signaling but also ethylene signaling. The decreased ethylene sensitivity caused
by the *axr1* mutation could mask most, if not all, of the upstream changes and lead to the reversion of cytokinin-related root phenotypes.

The anthocyanin level in the *axr1-12 35S::ARR1<sup>D94E</sup>* plants was lower than that in the *35S::ARR1<sup>D94E</sup>* plants but higher than that in the wild type and *axr1-12* mutant (Figure 6.3d). This might be explained by the pleiotropic character of the *axr1* mutation. Multiple phytohormones have been reported to be involved in anthocyanin biosynthesis: cytokinin positively regulates anthocyanin biosynthesis by modulating the expression of anthocyanin biosynthesis genes *PAL1*, *CHI*, *CHS* and *DFR* (Deikman and Hammer, 1995); ethylene and gibberellin suppress anthocyanin accumulation through their respective signaling pathways; the other two hormones, jasmonate (JA) and abscisic acid (ABA) synergistically enhance sugar-induced anthocyanin biosynthesis (Loreti et al., 2008; Das et al., 2012). The *axr1-12* mutant has decreased sensitivity to all these hormones (Tiryaki and Staswick, 2002), and the observed anthocyanin level in the *axr1-12 35S::ARR1<sup>D94E</sup>* plants could be a balanced result of all these effects.

Although the delayed senescence of the *35S::ARR1<sup>D94E</sup>* plants was not blocked by the *axr1-12* mutation (Figure 6.4), it was not enhanced as in the callus induction assay (Figure 6.2). Leaf senescence is a complex event which is regulated by multiple phytohormone pathways. Besides cytokinin, auxin has also been reported to negatively regulate leaf senescence: auxin levels increase during senescence and the auxin signaling factor, ARF2 (AUXIN RESPONSE FACTOR 2) is involved in this process (Lim et al., 2007). Three other hormones, ethylene, abscisic acid (ABA) and methyl jasmonate (MeJA) are reported to positively regulate senescence (Lim et al., 2007). Since the *axr1* mutant is a pleiotropic mutant which shows decreased sensitivity to all these hormones (Tiryaki and Staswick, 2002), the observed senescence phenotype of the *axr1-12 35S::ARR1<sup>D94E</sup>* plant might be a combined effect of all these influences.

In summary, the data presented in this chapter suggest that, in addition to the type-A response regulator ARR5, the type-B response regulator ARR1 is also
targeted for 26S proteasome degradation through a RUB-regulated CRL E3 ligase.

6.4 Materials and methods

Plant materials

To generate 35S::ARR1$^{D94E}$ plants, the full-length ARR1 cDNA was PCR-amplified from an Arabidopsis cDNA library using attB primers and cloned into pDONR221 using BP clonase enzyme mix (Invitrogen). The resulting pENTR-ARR1 clone was used as a template for site-directed mutagenesis with forward and reverse primer 5’-GAT GTT CAT ATG CCT GAG ATG GAC GGT TTC AAG-3’, which introduce the C-to-G mutation, and thus a D to E substitution at the position 94. The $ARR1^{D94E}$ fragment was then recombined into pEarlyGate 100 binary vector (Earley et al., 2006) using LR clonase enzyme mix (Invitrogen). The pEarlyGate100-$ARR1^{D94E}$ construct was introduced into C58C1Rif Agrobacterium strain, which was used to transform Col-0 plants through the floral dip transformation (Clough and Bent, 1998). To generate double homozygous 35S::ARR1$^{D94E}$ axr1-12 plant, the axr1-12 plant was crossed with the 35S::ARR1$^{D94E}$ plant.

Protein isolation, antibody generation and immunoblotting analysis

Protein extraction and immunoblotting analyses were done as described in Chapter 4. The ARR1-specific antibody was generated against a 55 amino acid-long peptide (position 348-402). This peptide has 47% amino acid sequence identity with ARR1’s closest homologue ARR2. The antisera were generated in rabbits and affinity-purified before use (Strategic Diagnostics). The secondary antibody used was HRP-conjugated anti-rabbit IgG goat antibodies (Santa Cruz Biotechnology).
Detached-leaf senescence test on cotyledons

Cotyledons of six-day-old light-grown seedlings were excised and transferred to Petri dishes with distilled water. Samples were incubated in the dark at room temperature for the indicated times. At each time interval, the cotyledons were photographed on moistened filter paper and 12 cotyledons per line were frozen in liquid nitrogen as one sample for chlorophyll extraction. The frozen cotyledons were incubated overnight with 80% (v/v) acetone at 4°C in the dark. Absorbance at 647 and 664 nm was measured using Ultrospec 2000 (Pharmacia), and the chlorophyll content was calculated according to Graan and Ort (Graan and Ort, 1984).

Other assays

Cytokinin independent callus induction assay was done as described in Chapter 3, except that the induction medium contained 2% sucrose and different concentrations of NAA (Sigma, prepared as stock solutions in DMSO) as indicated in the legend of Figure 6.2. Plates were kept in growth chamber at 22°C for 39 days. RNA isolation and gel blot analyses were done as described in Chapter 2. Anthocyanin quantification was done as described in Chapter 3.
Chapter 7: Summary and hypotheses for proteolytic control of the cytokinin signaling pathway

Although it has been reported that axr1 mutations affect the cytokinin response (Timpte et al., 1995; Tiryaki and Staswick, 2002), the molecular basis of this decreased sensitivity has remained unknown. In this study, loss of AXR1 function was shown to cause cytokinin insensitivity by directly impacting the primary cytokinin response pathway. More specifically, loss of AXR1 leads to the stabilization of the negative-feedback regulator ARR5. The degradation rate of ARR5, a representative of the type-A ARR family, was attenuated by chemical suppression of the RUB conjugation pathway (Figure 4.5). Furthermore, ARR5 accumulated both in axr1 and in wild-type seedlings treated with the RUB pathway inhibitor, thus providing an explanation for the decreased cytokinin sensitivity caused by loss of AXR1 function (Figure 4.5, 4.6 and 4.7) and this was confirmed by the partial suppression of axr1 cytokinin resistance by the arr5-1 mutation (Figure 5.1, 5.2 and 5.3). However, the higher order mutant arr3,4,5,6 did not revert the axr1 phenotype significantly more than arr5-1 alone, even though ARR4 and ARR6 were also shown to be unstable proteins that are targeted for proteasome-dependent proteolysis (Figure 5.2, 5.3 and To et al., 2007; Ren et al., 2009; Ryu et al., 2009). Thus, it appears that one or more other type-A ARRs, not considered here, also contribute to the axr1 cytokinin phenotypes. Alternatively, it remains possible that part of the axr1 cytokinin resistance is caused by the increased accumulation of cytokinin response repressors other than the type-A ARR family.

It has been shown that cytokinins promote both the transcription of type-A ARRs and the stability (of at least a subset) of type-A ARR proteins thus engaging a robust feedback inhibition mechanism that limits the strength of the response (D'Agostino et al., 2000; To et al., 2007; Ren et al., 2009; Ryu et al., 2009). Here in the axr1 mutants this feedback-inhibition mechanism is enhanced due to increased type-A ARR accumulation which overrides the response
activation mechanism thus leading to cytokinin insensitivity. Essential to this cytokinin resistance phenotype is that it becomes stronger in response to cytokinin treatment.

Results from this study also indicated that a positive regulator of cytokinin signaling, ARR1, is stabilized when the RUB modification pathway is interrupted. However, the expected activation of cytokinin responses was not observed in the axr1 mutants that have decreased cytokinin sensitivity, suggesting that the effect of stabilized type-A ARRs can override the influence of stabilized ARR1 in the axr1 mutants.

Based on these observations, I propose an AXR1-dependent multilevel proteolytic control of cytokinin signaling: both the negative regulator ARR5 (and probably other type-A ARRs) and the positive regulator ARR1 (and probably other type-B ARRs) are targeted for 26S proteasome-dependent degradation through AXR1-regulated CRL E3 ligases (Figure 7.1). According to the current cytokinin signaling model, phosphorylation of ARR1 activates its function and this activation depends on cytokinin signaling. So, under normal conditions without exogenous cytokinin signal, there is a balance between the unphosphorylated ARR1 and phosphorylated ARR1 and the amount of phosphorylated ARR1 is controlled by a small reaction rate constant k1. Upon cytokinin treatment, the reaction rate constant is greatly increased to k2, thus a large amount of phosphorylated ARR1 is formed to initiate downstream cytokinin responses. In our model, under normal conditions, both ARR5 and ARR1 levels are precisely controlled by the AXR1-regulated CRL E3 ligases to maintain the cell’s normal capability to respond to cytokinin. Once AXR1 function is interrupted, as in the axr1 mutants, both ARR5 and ARR1 are stabilized. The stabilized ARR1 will potentially increase the overall amount of phosphorylated ARR1, but this increase will be limited by the small reaction rate constant k1. Accordingly, the expected increased cytokinin response caused by ARR1 stabilization will be limited. At the same time, the stabilized ARR5 will repress the phosphor-relay of cytokinin signaling which is expected to decrease the cytokinin-stimulated
reaction rate constant $k_2$, thus decreasing the cell’s capability to respond to cytokinin. Altogether, even if the total ARR1 level is increased in $axr1$ plants, the actually formed phosphorylated ARR1 amount upon cytokinin treatment is expected to be lower than that in the wild type resulting in decreased cytokinin sensitivity as observed in the $axr1$ mutants. On the other hand, in the $axr1$-12 $35S::ARR1^{D94E}$ plants, the overexpression of the phosphor-mimic $ARR1^{D94E}$ can greatly increase the active “phosphorylated” ARR1 amount, because the unphosphorylatable $ARR1^{D94E}$ form is not affected by any phosphorylation/dephosphorylation equilibrium, which blocks the negative influence of stabilized ARR5 in $axr1$-12. This then is predicted to exaggerate the influence of stabilized ARR1 in the $axr1$-12 background. Indeed, some of the constitutive cytokinin response phenotypes associated with $35S::ARR1^{D94E}$ expression was observed to be increased in the $axr1$-12 $35S::ARR1^{D94E}$ plants.

Accordingly, our model predicts that any genetic change that globally influences CRL E3 ligase activity, like the $axr1$ mutant and the $cul1$-6 mutant (Moon et al., 2007) will result in a cytokinin insensitive phenotype; only genetic change influencing ARR1-specific CRL E3 ligase activity, like in the $auf1$ mutant (Zheng et al., 2011), would show the specific effect of stabilized ARR1 and give a cytokinin hypersensitive phenotype.
Figure 7.1 Model for AXR1 function in cytokinin signaling pathway.

The question marks indicate that either the identity of the factor or the exact position where it functions is unknown. Dashed blue arrows indicate the possible dephosphorylation of ARRs.
Recent advances in plant hormone signaling revealed that the ubiquitin-proteasome pathway plays a central role in the signaling pathways for auxin, jasmonate, gibberellin, ethylene and abscisic acid (Santner and Estelle, 2009). Except for abscisic acid signaling, which is regulated by a RING E3 ligase, the other four hormone signaling pathways are all regulated by CRL-E3 ligases, more specifically by the founding member SCF-type E3 ligases (assembled by S phase kinase-associated protein 1 (SKP1), cullin 1 (CUL1), F-box protein, and RING-box 1 (RBX1)). Interestingly, the ethylene signaling pathway is regulated at multiple points by SCF E3 ligases assembled from different F-box proteins. Our results suggest that CRL E3 ligase-mediated protein degradation also plays an important role in the Arabidopsis cytokinin signaling pathway. AUF1 assembled SCF E3 ligase probably participates in the regulation of ARR1 stability (Zheng et al., 2011). Whether ARR5 is regulated by an SCF E3 ligase or other types of CRL E3 ligases remains to be shown. Similar to the ethylene signaling pathway, cytokinin signaling is also regulated by proteolytic control at multiple points. A common character of these two hormone response pathways is the involvement of two-component elements. Perhaps this type of multilevel proteolytic control is also a common character for the regulation of two-component signaling in plants.

Analysis of the proteolytic control of the cytokinin response pathway will allow a better understanding of how cytokinin signaling is integrated in the regulation of plant growth and development, and ultimately will help to develop predictable models of plant growth that will be instrumental for modern agriculture.

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### Appendices

**Appendix A: Primers used for fine mapping.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Chr</th>
<th>Position (bp)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Size (bp)</th>
<th>Restriction enzyme</th>
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<tr>
<td>F20D22*</td>
<td>1</td>
<td>1,070,575</td>
<td>CCCAAGTGACGTCTGGTTTC</td>
<td>AACAAAATGAGTTTCTCTGCATG</td>
<td>201 190</td>
<td>None</td>
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<tr>
<td>1,241,207**</td>
<td>1</td>
<td>1,241,207</td>
<td>GCCCAACCAATTGGTACAC</td>
<td>ATACCAACTCCCATCCATATT</td>
<td>190 190</td>
<td><em>Hin</em> (Col-0)</td>
</tr>
<tr>
<td>1,297,710**</td>
<td>1</td>
<td>1,297,710</td>
<td>AGTTACTAGCGAAGATGAAA</td>
<td>AATGTTATGCAGTTCTTTTT</td>
<td>200 200</td>
<td><em>EcoR</em> (Col-0)</td>
</tr>
<tr>
<td>1,362,041**</td>
<td>1</td>
<td>1,362,041</td>
<td>AAGAGAGAGAAGAATCGAAG</td>
<td>CATATCCATATTTATTTTG</td>
<td>200 200</td>
<td><em>Sal</em> (Col-0)</td>
</tr>
<tr>
<td>1,381,924**</td>
<td>1</td>
<td>1,381,924</td>
<td>TATATAAGAAGACATTCAATT</td>
<td>ATGAGAAGGGCGCAATTGAA</td>
<td>200 200</td>
<td><em>Dpn</em> (Col-0)</td>
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<tr>
<td>ATEAT1*</td>
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<td>1,431,412</td>
<td>GCCACTGCGTGAATGATATG</td>
<td>CGAACAGCCAACATTAATTCCC</td>
<td>172 157</td>
<td>None</td>
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<td>1,458,698**</td>
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<td>1,458,698</td>
<td>TGAGTCTCCGTAAAGATTAG</td>
<td>CGCTTACTATTTAATGTCG</td>
<td>188 188</td>
<td><em>Hin</em> (Col-0)</td>
</tr>
<tr>
<td>1,488,401**</td>
<td>1</td>
<td>1,488,401</td>
<td>AAAAGCAGTGAGTGAAATG</td>
<td>GTAGGACTGAAGGTTAAAAAGG</td>
<td>204 204</td>
<td><em>EcoR</em> (Col-0)</td>
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<td>1,603,470**</td>
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<td>1,603,470</td>
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<td>TTTGGTTTCTGCTTTACTTT</td>
<td>200 200</td>
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<td>1,717,961**</td>
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<td>1,717,961</td>
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<td>TTATAACCCCTTTGCTCA</td>
<td>200 200</td>
<td><em>Pst</em> (Col-0)</td>
</tr>
<tr>
<td>2,376,118**</td>
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<td>2,376,118</td>
<td>TCAATCATCTTTCTAGACC</td>
<td>CACACCCCGTTCTCCTAT</td>
<td>246 246</td>
<td><em>Dpn</em> (Col-0)</td>
</tr>
<tr>
<td>HYL1 ID***</td>
<td>1</td>
<td>3,137,769</td>
<td>CATCCTCCTCAACCTACCTGATCA</td>
<td>TATGCGTGTGCTTCTGCTTCTCC</td>
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<td>None</td>
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<td>ICE10*</td>
<td>1</td>
<td>4,015,381</td>
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<td>GACTCTATGGAAGCTCCTTG</td>
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<td>None</td>
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<td>MSAT1.3*</td>
<td>1</td>
<td>8,322,321</td>
<td>GGAACTGTTGCTGGAAGTGAAG</td>
<td>CGATTGCACTAAAAGCCTCTC</td>
<td>266 230</td>
<td>None</td>
</tr>
</tbody>
</table>

*MSAT database ([http://www.inra.fr/internet/Produits/vast/msat.php](http://www.inra.fr/internet/Produits/vast/msat.php)). ***Marker was identified in this work.

**Markers were designed based on single nucleotide polymorphism information ([http://polymorph.weigelworld.org/cgi-bin/webapp.cgi](http://polymorph.weigelworld.org/cgi-bin/webapp.cgi)).
### Appendix B: Primers used for AXR1 sequencing and genotyping.

<table>
<thead>
<tr>
<th>Name</th>
<th>Intended use (position within the gene)</th>
<th>Primer sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>AXR1 5' FP</td>
<td>soi1 sequencing; axr1-3 genotyping (1-28)</td>
<td>TTTATCAGTTCACCGGAGGCAAAAAATCG</td>
</tr>
<tr>
<td>AXR1 M RP1</td>
<td>soi1 sequencing; axr1-3 genotyping (1400-1376)</td>
<td>AGGTATGTGCTTATGTGCAGCGGCC</td>
</tr>
<tr>
<td>AXR1 M FP1</td>
<td>soi1 sequencing (1201-1228)</td>
<td>ATAATCCATGGCCTGAACTCAAGAGGTC</td>
</tr>
<tr>
<td>AXR1 M RP2</td>
<td>soi1 sequencing (2599-2573)</td>
<td>CACCTGTAATCTCGTCCGCTAAATAC</td>
</tr>
<tr>
<td>AXR1 M FP2</td>
<td>soi1 sequencing; axr1-12 genotyping (2402-2432)</td>
<td>TTGTTTTCTAATGTCAACGTTTACCTATTG</td>
</tr>
<tr>
<td>AXR1 3' RP</td>
<td>soi1 sequencing; axr1-12 genotyping (3711-3684)</td>
<td>GTGAATGGGCTTACAGTGGCCCATTTT</td>
</tr>
<tr>
<td>AXR1 seq 1</td>
<td>soi1 sequencing (505-526); axr1-3 genotyping</td>
<td>GAGGTGTGGCCAATCAAAAGCC</td>
</tr>
<tr>
<td>AXR1 seq 2</td>
<td>soi1 sequencing (1003-1022)</td>
<td>TGTTCGCATCTCTGTAAAGG</td>
</tr>
<tr>
<td>AXR1 seq 3</td>
<td>soi1 sequencing (1702-1721)</td>
<td>ACTTGACTTAGAATGTTAC</td>
</tr>
<tr>
<td>AXR1 seq 4</td>
<td>soi1 sequencing (2158-2177)</td>
<td>CAGTCTATTTTCACATTGG</td>
</tr>
<tr>
<td>AXR1 seq 5</td>
<td>soi1 sequencing (2898-2917); axr1-12 genotyping</td>
<td>TGTCGCAGTGTTGCAATGGG</td>
</tr>
<tr>
<td>AXR1 seq 6</td>
<td>soi1 sequencing (3303-3322)</td>
<td>TTTATGGGAAGTGCTAGC</td>
</tr>
<tr>
<td>AXR1 seq7</td>
<td>soi1 sequencing (301-320)</td>
<td>TCCGAGGCTTTGAAGAATCT</td>
</tr>
<tr>
<td>AXR1 seq7R</td>
<td>soi1 sequencing (320-301)</td>
<td>AGATTCCTCAAGCGCTCGGA</td>
</tr>
<tr>
<td>AXR1 seq8</td>
<td>soi1 sequencing (612-631)</td>
<td>AGAGAAGAAGATGGTTAG</td>
</tr>
</tbody>
</table>
### Appendix C: Primers used for genotyping and real-time RT-PCR.

<table>
<thead>
<tr>
<th>Name</th>
<th>Intended use</th>
<th>Primer sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kan FP*</td>
<td>ipt-161 genotyping</td>
<td>TTGTCAAGACCACCTGTCC</td>
</tr>
<tr>
<td>Kan RP*</td>
<td>ipt-161 genotyping</td>
<td>ACCGTAAAGCAGAGGAGC</td>
</tr>
<tr>
<td>ARR3 FP</td>
<td>arr3 genotyping</td>
<td>GGAACTAGTAGCAATTATCTCTTATCTTTTC</td>
</tr>
<tr>
<td>ARR3 RP</td>
<td>arr3 genotyping</td>
<td>CACAGAGGTTAAGCTGCACATTTT</td>
</tr>
<tr>
<td>ARR4 FP</td>
<td>arr4 genotyping</td>
<td>CTCTCTCTACAGTTACTATAAGGCTGC</td>
</tr>
<tr>
<td>ARR4 RP</td>
<td>arr4 genotyping</td>
<td>GGAGCGCGGAGAGATTAAGGAGGAGC</td>
</tr>
<tr>
<td>ARR5 FP</td>
<td>arr5-1 genotyping (with EcoRI site)</td>
<td>ATCCTACTCTTTGATATGGCTGAGGT</td>
</tr>
<tr>
<td>ARR5 RP</td>
<td>arr5-1 genotyping (with SalI site)</td>
<td>AACGCGCAAGATCTGAGTTATACAGT</td>
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<tr>
<td>ARR6 FP</td>
<td>arr6 genotyping</td>
<td>CAACATTCTGGATCAATGGCTGAGT</td>
</tr>
<tr>
<td>ARR6 RP</td>
<td>arr6 genotyping</td>
<td>CGGAGAGCTCAGATCTTTTCGCGGAG</td>
</tr>
<tr>
<td>attB1 SP/ARR5</td>
<td>pDONR221 cloning</td>
<td>GGGGACAAGTTTGTACAAAAAGAGCGAGTTTATGCTGAGGTTTTCGAGGTTTTCGAGGTT</td>
</tr>
<tr>
<td>attB2 ASP/ARR5</td>
<td>pDONR221 cloning</td>
<td>GGGGACCACTTTGTACAAAAAGAGCGAGTTTATGCTGAGGTTTTCGAGGTTTTCGAGGTT</td>
</tr>
<tr>
<td>ARR4</td>
<td>real-time RT-PCR</td>
<td>ATCGGAGGAATCTG</td>
</tr>
<tr>
<td>ARR4</td>
<td>real-time RT-PCR</td>
<td>CTCTCGATGACAATCGATC</td>
</tr>
<tr>
<td>ARR5**</td>
<td>real-time RT-PCR</td>
<td>ATGTCTTCAGAGAACATCTTGCTG</td>
</tr>
<tr>
<td>ARR5**</td>
<td>real-time RT-PCR</td>
<td>TCACAGGCTTTCAATAAGAATATCTGC</td>
</tr>
<tr>
<td>ARR6</td>
<td>real-time RT-PCR</td>
<td>TCACCGGATCTCTTCTCTATG</td>
</tr>
<tr>
<td>ARR6</td>
<td>real-time RT-PCR</td>
<td>GCAAGAAGATACTCTGAGCA</td>
</tr>
</tbody>
</table>

* Primers have been described (Van der Graaff et al., 2001).

** Primers have been described (Rashotte et al., 2006).
References


senescence, seed size, germination, root development, and cytokinin metabolism. The Plant Cell **18**, 40-54.


quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3, RESEARCH0034.


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