

CHARACTERIZATION OF *PIC7* GENE FUNCTIONS IN ARABIDOPSIS
HORMONE RESPONSE

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ABSTRACT

CHARACTERIZATION OF *PIC7* GENE FUNCTIONS IN ARABIDOPSIS HORMONE RESPONSE

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As the major plant hormone, auxin regulates many aspects of plant growth and development. Recent studies indicate that the auxin response pathway interacts with other plant hormone signaling pathways such as cytokinin, ethylene, ABA and GA in multiple ways. Therefore, the final outcome of plant growth responses is due to the crosstalk among several plant hormones. To identify novel genes involved in the auxin response, our laboratory used a forward genetics approach to isolate ethyl methanesulfonate (EMS) mutagenized Arabidopsis mutants that were resistant to synthetic auxin picloram.

The objective of the research described here was to characterize one of the auxin (picloram) resistant mutants, *pic7-1*. The mutant gene *pic7-1* was identified as *adenine phosphoribosyltransferase1* (*APT1*). *APT1* involves in purine metabolism by converting adenine into adenosine monophosphate (AMP) through transferring a phosphoribosyl group from 5-phosphoribosyl 1-pyrophosphate (PRPP) to adenine. The mutation in *pic7-1* lies on the substrate-PRPP (5-Phosphoribosyl 1-pyrophosphate) binding domain of *APT1*, suggesting that mutation may alter the catalytic activity of the enzyme. This idea is further supported by the resistance of *pic7-1* to 2,6-diaminopurine (DAP) which is metabolized by *APT1* to its toxic nucleotide, 2,6-DAP riboside.

APT1 is also suggested to be involved in cytokinin metabolism by converting active cytokinin bases to cytokinin ribosides. Therefore, loss of *APT1* activity might lead to locally high level of active cytokinin. In *pic7-1*, transcript levels of cytokinin induced auxin biosynthetic genes, *CYP79B2*, *CYP79B3* and *NIT3* are elevated. In addition, morphological phenotypes between *pic7-1* and auxin over-producing mutants are similar, suggesting that auxin level is increased in *pic7-1* because of elevated active cytokinin. Resistance of *pic7-1* to exogeneous auxin as well as altered auxin signaling components indicates that auxin signaling may also be altered in *pic7-1*. Moreover, mis-regulated type-B Arabidopsis Response Regulators (ARRs) and cytokinin related responses in *pic7-1* indicate hampered cytokinin signaling in the mutant. Physiological characterization reveals that *APT1* is also involved in several other plant hormone signaling pathways such as abscisic acid, gibberellic acid and ethylene.

Arabidopsis expressing the reporter transgenes *APT1::APT1-GUS* and *APT1::APT1-GFP* reveals highly localized expression of *APT1* in vasculature, pericycle,

root tip and guard cells. APT1 is localized to cytoplasm, nucleus and the endoplasmic reticulum at the subcellular level. Expression of *APT1::APT1-GFP* complements the mutant phenotypes of *pic7-1*, further confirming that observed mutant phenotypes are caused by the mutation in *APT1*. The *APT1* gene generates at least two transcripts through alternative splicing although biological significance of these splice variants is to be characterized.

CHAPTER I

INTRODUCTION

Auxin and its regulations

Phytohormones play a major role in proper growth and development of plants and also involved in plant response to unpredictable and stressful environmental cues. During last few decades, due to the successful progress of plant science, several major plant hormones were identified. Auxin, cytokinin (CK), ethylene (ET), gibberellin (GA), and abscisic acid (ABA) are classified as five major plant hormones (Davies, 2004). Some other compounds such as jasmonates (Wasternack, 2007; Browse, 2009), brassinosteroids (BRs) (Vert et al., 2005), salicylic acid (SA) (Loake and Grant, 2007) and strigolactones (Gomez-Roldan et al., 2008; Umehara et al., 2008) with hormone properties have also been found recently. Among these, auxin is the best characterized hormone in plant growth and development.

Many decades ago Charles Darwin's experiments led to the discovery of auxin as a substance involved in phototropism (Darwin, 1880; Went, 1926). Since then many other functions are implicated to this vital plant hormone including embryogenesis (Cooke et al., 1993), apical dominance (Prasad et al., 1993), flower development (Cheng and Zhao, 2007), lateral root initiation (Casimiro et al., 2001) and gravitropism (Marchant et al., 1999). Auxin accomplishes these functions by regulating cell division, expansion

and differentiation (Perrot-Rechenmann C., 2010). As other plant hormones, auxin controls plant development by regulating gene transcription (Chapman et al., 2009).

Auxins are mostly synthesized in young leaves (Ljung et al., 2001), and also in mature leaves, cotyledons and roots (Ljung et al., 2005). Even though *de novo* auxin biosynthesis pathway in plants is not fully established yet, auxin synthesis is found to occur in tryptophan dependent as well as tryptophan independent pathways (Zhao, 2010). There are four proposed branches in tryptophan dependent pathways named after different intermediates: the tryptamine (TRM) pathway, the indole-3-acetamide (IAM) pathway, the indole-3-pyruvic acid (IPA) pathway, and the indole-3-acetaldoxime (IAOx) pathway. Even most of the enzymes catalyzing each of these pathways are still not identified to understand the physiological functions of redundancy in auxin biosynthetic pathways, recent findings have indicated that these pathways might function preferentially in certain growth processes, such as root development (IAOx and IPA pathways), shade avoidance (IPA pathway), and embryogenesis (IPA/TRM pathway) (Zhao, 2008; Normanly, 2010). Recently, increasing evidence has indicated that environmental factors can vary plant growth through affecting the auxin biosynthetic pathways. For example, TAA1, an aminotransferase which is involved in the conversion of L-tryptophan (L-Trp) to indole-3-pyruvic acid (IPA) (Tao et al., 2008), is essential for high temperature induced hypocotyl elongation in *Arabidopsis* (Yamada et al., 2009).

While almost all the plant tissues have the ability to synthesize auxin, it is known that auxin transport especially from the shoot apical meristem to the root is necessary for the normal growth and development of the plant such as lateral root development (Reed et al., 1998; Bhalerao et al., 2002). Auxin transport from cell to cell is regulated by auxin

influx and efflux carriers (Gao et al., 2008). These transporters can be categorized into three major classes. Auxin resistant1 /like aux1 (AUX1/LAX) influx carriers, pin-formed (PIN) efflux carriers and P-glycoprotein (PGP) efflux transporters (Shen et al., 2010). AUX1/LAX proteins require H^+ gradient for auxin transport across cell membrane (Swarup et al., 2004). After entering a cell, IAA efflux is dependent on several factors. PGPs are ATP driven efflux carriers (Noh et al., 2001). As the transmembrane auxin efflux carriers, PIN proteins facilitate the polar auxin transport in the plant, which is consistent with their asymmetric cellular distribution (Gälweiler et al., 1998). Also recently discovered PILS (PIN LIKES) proteins are involved in intracellular auxin transport (Barbez et al., 2012). Mutations in either of above mentioned transporters cause developmental defects highlighting their importance in development. *aux1* and *pin2* mutants cause agravitropism in developing roots (Muller et al., 1998; Parry et al., 2001). *pin1* mutants are unable to produce floral organs (Gälweiler et al., 1998). *lax3* mutant exhibits 40% reduction of lateral root formation and exclusively displays hookless defect during the apical hook formation among all the auxin influx carrier mutants (Swarup et al., 2008; Vandenbussche et al., 2010). A combination of AUX1 (Marchant et al., 2002), PIN1, PIN3 and PIN7 (Benkova et al., 2003; Laskowski et al., 2008; Lewis et al., 2011) controls the auxin movement from shoot to root, which has been shown to participate in the lateral root initiation and elongation. Therefore, auxin transport is a highly regulated process leading to proper development of the plant.

Auxin is known to regulate the transcription of auxin responsive genes by degrading a group of repressor proteins called Aux/IAAs via ubiquitin-proteasome pathway (Dharmasiri et al., 2004). Auxin responsive genes are generally under the

control of auxin responsive elements (ARE) that interact with transcription factors called auxin responsive factors (ARFs). In the absence of auxin, the ARFs bind to Aux/IAA repressors that inhibit the ARFs function through Aux/IAA-ARF heterodimerization via conserved C-terminal domain III and IV (Tiwari et al., 2003). Binding of auxin to its co-receptors TIR1/AFBs (Dharmasiri et al., 2005; Tan et al., 2007) and Aux/IAAs brings the repressor proteins into the close proximity of SCF protein complex which is composed of the auxin co-receptor F-Box protein, Skp1, Cullin1 and RBX1 proteins. A small protein molecule called ubiquitin is then covalently attached to the Aux/IAA proteins, targeting them for degradation by 26S proteasome pathway (Gray et al., 2001). Functionally, ARFs could activate or repress the auxin induced gene expression (Ulmasov et al., 1997a, 1999a). With ARFs as activators, derepression causes the transcription of auxin responsive genes (reviewed by Chapman and Estelle, 2009). Among them, three classes of genes called primary auxin responsive genes are induced immediately after the auxin treatment. They are Aux/IAA (Auxin/Indole Acetic Acid), SAUR (Small Auxin Up RNA) and GH3 (Gretchen Hagen 3) (Calderon-Villalobos et al., 2010).

In addition to the major natural auxin indole-3-acetic acid (IAA), phenylacetic acid (PAA), 4-chloro-indoleacetic acid, and indole-3-butyric acid (IBA) are also found in the plants (Ludwig-Müller et al., 2002). As auxins at high concentrations can be used as herbicides, many synthetic auxins that mimic naturally occurring auxins have been produced. 2, 4-dichlorophenoxyacetic acid (2,4-D), picloram, 1-naphthaleneacetic acid (NAA), 2-methoxy-3,6-dichlorobenzoic acid (dicamba) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) are examples for synthetic auxins (Woodward and Bartel, 2005). Even though auxin biology is an extensively studied field, there are more to be learned on

molecular mechanisms of auxin action. Clear understanding of the molecular mechanisms of auxin response will provide insight into auxin biology as well as into its applications mainly as an herbicide, which might also lead to engineering herbicide-resistant crop plants.

Cross talk between auxin and other hormones

Responding to internal and external stimuli, phytohormones modulate overlapping developmental processes throughout the life time of the plants. With the increasing knowledge of perception and signal transduction of each major plant hormone, a consensus has also been established: instead of solely dependent on one specific hormone, plant hormone responses are contributed to a sophisticated network of multiple hormone interactions, which have been functionally distinguished as additivity, synergism, or antagonism. In a tissue and developmental stage dependent manner, auxin crosstalk often plays a vital role in locally fine-tuning the developmental responses to environmental conditions, such as auxin biosynthesis induction for shade avoidance through ethylene pathway (Tao et al., 2008).

Auxin and Ethylene

One of the most characterized plant hormone crosstalks is the interaction between auxin and ethylene, including synergism in inhibition of root elongation (Rahman et al., 2001), induction of root hair (Rahman et al., 2002), antagonism in lateral root formation (Lewis et al., 2011) and hypocotyl development (Suttle, 1988).

As the mechanism of the most common crosstalk, auxin can regulate or be regulated by ethylene at biosynthetic level. On one hand, auxin boosts the transcriptional level of several ACC synthases (ACS), which catalyze the rate limiting step of ethylene biosynthesis (Abel et al., 1995; Stepanova et al., 2007), leading to the elevated ethylene synthesis (Hansen and Grossman, 2000). On the other hand, ethylene can also function as a positive regulator on auxin biosynthesis in the root tip. Discovery of several weak ethylene-insensitive (*wei*) mutants in Arabidopsis display the best evidence for tissue-specific induction of auxin biosynthesis through ethylene (Stepanova et al., 2005; Stepanova et al., 2008). Stepanova et al. (2005) showed that two genes *WEI12* (*ASA1*) and *WEI17* (*ASB1*), encoding alpha and beta subunits of anthranilate synthase which catalyzes the rate-limiting step of tryptophan synthesis, are transcriptionally upregulated by ethylene. Another WEI gene *WEI8* has also been shown to encode a tryptophan aminotransferase (TAA1), which produce the indole-3-pyruvic acid from tryptophan through the IPA auxin biosynthesis branch (Stepanova et al., 2008; Yamada et al., 2009). This induction of auxin biosynthesis is believed to be one of the mechanisms through which ethylene inhibits the root elongation.

In addition to positive regulation of auxin biosynthesis, ethylene also influence the expression of auxin influx and efflux facilitators in Arabidopsis root such as PIN1, PIN2, PIN4 and AUX1, enhancing the rootward polar auxin transport and thus the consequent high concentration of auxin in the elongation zone which results in reduced root cell expansion (Ruzicka et al., 2007; Negi et al., 2008). Another synergistic interaction between auxin and ethylene in initiation and elongation of root hairs was characterized through various genetic studies (Rahman et al., 2002). Their significance in

this process is obvious because root hair formation is largely impaired in auxin resistant mutants which also confer ethylene resistance, such as *aux1* (Pickett et al., 1990), *axr2* (Wilson et al., 1990), and *axr3* (Leyser et al., 1996). Similarly, exogenous ACC or IAA supplement can restore the root hair initiation in the *root hair defective 6* (*rhd6*) mutant (Masucci and Schiefelbein, 1994).

One well-studied plant growth response to ethylene is the triple response of the dark-grown plants which is characterized by the exaggerated apical hook, inhibited root and hypocotyl elongation, and thickened hypocotyls (Guzma'n and Ecker, 1990). Apical hook development in the hypocotyl has been highly utilized for the examination of auxin and ethylene crosstalk. The apical hook development consists of formation, maintenance and opening phases, while the first two phases are the products of unequal growth from altered cell division and elongation (Raz and Ecker, 1999). Various evidences suggest that the differential growth for apical hook formation can be achieved through the regulation of auxin transport, synthesis and maybe signaling by ethylene. For example, for the apical hook formation, HOOKLESS1 (HLS1) (Li et al., 2004) is necessary and found to be a convergent point between auxin and ethylene pathways. Ethylene regulates the expression of HSL1 and its repressor ARF2 (Li et al., 2004), and therefore controls the differential cell elongation in hypocotyls through modulating the auxin signaling pathway.

Auxin and GA

The overlapping roles of auxin and GA in plant growth are mainly in cell expansion, organ expansion and tissue differentiation. As an essential phytohormone, GA

regulates numerous developmental processes, such as promotion of seed germination and flowering, stem elongation and seed development (Sun and Gubler, 2004). It has been revealed that the level of endogenous GA as well as the cell response strategies to GA mainly decides the output of the GA regulations in plant (Richards et al., 2001). Similar to auxin, GA signaling cascade involves targeting transcriptional repressor proteins for 26S proteasome mediated degradation with the facility of SCF-E3 ubiquitin ligase complex, specifically using GID1 as a receptor (Griffiths et al., 2006; Willige et al., 2007) and SLY1 as the F-box protein in Arabidopsis (McGinnis et al., 2003; Dill et al., 2004) for repressors-DELLA (Olszewski et al., 2002) degradation. In Arabidopsis root development, auxin transport and signaling is crucial for GA stimulated root elongation. Fu and Harberd (2003) have shown that auxin promotes the DELLA destabilization in the root cells in response to GA, which is necessary for releasing the repressor effect and thus advancing the root growth.

The most known function of GA in plant development is to release dormancy and promote germination of seeds (Nambara et al., 1991). Radical protrusion for seed germination requires newly synthesized GAs (Jacobsen and Olszewski, 1993). There is very limited amount of knowledge on the role of auxin during seed germination. However, auxin positively regulates GA metabolism in many plant species through the induction of several GA oxidase genes such as GA20ox genes in Arabidopsis (O'Neill and Ross, 2002; Frigerio et al., 2006), which catalyzes the final step to produce bioactive GAs. Auxin induced GA biosynthesis is characterized to be the effect of degradation of AUX/IAA proteins and subsequent activation of ARF7 transcriptional factor (Teale et al., 2006). Conversely, the GA-deficient mutant characterization and microarray data

indicates that GA can up-regulate several putative auxin carriers genes and also regulate the abundance of the proteins, such as AUX1, PIN1, PIN2, PIN3 and PIN7 (Ogawa, 2003; Willige et al., 2011). Along with the up-regulation of several auxin biosynthesis genes such as CYP79B2, CYP79B3 by exogenous GA (Ogawa et al., 2003), it has been suggested that auxin level and transport is altered by GA during Arabidopsis seed germination.

Auxin and ABA

As a well-known stress hormone, ABA is synthesized by plants to cope with variable environmental conditions and biotic or abiotic stresses such as drought, salinity, extreme temperature and pathogen attacks (Dallaire et al., 1994). ABA response regulates the expression level of ABA responsive genes, whose promoter regions contain conserved *cis*-regulatory sequences (c/tACGTGGC) which are called ABA responsive elements (ABREs) (Hattori et al., 2002). In addition to stress responses, ABA is also essential for many fundamental development processes such as induction of seed dormancy, seed development, and vegetative growth (Finkelstein et al., 2002). Additionally, root growth is inhibited by ABA through inhibiting the cell division (Yin et al., 2009).

According to recent studies, auxin and ABA interactions are most prominent during seed germination and seedling development. Furthermore, auxin signaling components are closely involved in mediating ABA responses. Wang et al. (2011) have recently reported that Auxin Response Factor 2 (ARF2) down-regulates a homeodomain gene *HB33*, which positively regulates the ABA induced inhibition of seed germination

and primary root growth. In addition, down-regulation of *Auxin Response Factor 10* (*ARF10*) by *miR160* is shown to be required to decrease the ABA sensitivity, allowing seed germination and postgermination development (Nonogaki, 2007).

On the other hand, ABA modulates auxin response under different conditions. Under high salinity environment, auxin transport genes are altered through enhanced ABA level (Yu et al., 2010). ABA can also target auxin receptor family transcripts for degradation through inducing expression of *miR393* (Sunkar and Zhu, 2004). During the seed germination and following seedling development, embryonic axis elongation and cotyledon expansion are greatly inhibited by ABA. This developmental effect requires the transportation of auxin from root apex to the elongation zone with the facilitation of AUX1 and PIN2, as well as potentiated auxin signaling by ABA, which down-regulates Aux/IAA genes *AXR2/IAA7* in the same region in an auxin independent manner (Belin et al., 2009).

Auxin and Cytokinin

Auxin - cytokinin coordinated function in plant growth is among the earliest findings related to the plant hormone crosstalk: many plant development processes such as *de novo* organ formation from tissue culture rely on the balance of auxin to cytokinin ratio (Skoog and Miller, 1957).

As N⁶-substituted adenines, the roles of cytokinins in plant developments include seed germination, shoot and root development, delaying of leaf senescence (Riefler et al., 2006) and circadian clock (Hanano et al., 2006). In higher plants, isopentenyladenine (iP), zeatin and dihydrozeatin are the major natural cytokinins. The rate-limiting step of the

biosynthesis of these cytokinins is carried out by ATP/ADP-Isopentenyltransferase (IPT) (Kamada-Nobusada et al., 2009), which has seven members in *Arabidopsis thaliana* (Takei et al., 2001; Miyawaki et al., 2006). Similar to auxin, cytokinin is produced in shoot and root tissues of the plant, but young leaves have the highest capacity for biosynthesis (Miyawaki et al., 2004). Cytokinin homeostasis in *Arabidopsis* is regulated through irreversible degradation by seven Cytokinin Oxidase/Dehydrogenases (CKXs) (Sakakibara, 2006).

Cytokinin perception and signaling in plants is through a two-component pathway, which consists of two signaling factors; a histidine kinase as cytokinin receptor and an *Arabidopsis* response regulator ARR that are essential for the signal transduction and specific output (Hwang and Sheen, 2001). Three transmembrane proteins: *Arabidopsis* His Kinase 2 (AHK2), AHK3 and AHK4/WOL1 (Woodenleg 1)/CRE1 (Cytokinin Response 1) are identified as cytokinin receptors in *Arabidopsis* (Hwang and Sheen, 2001; Inoue, 2001; To and Kieber, 2008), which transfer the phosphorelay as the signal to the nucleus after binding with cytokinin. Subsequent phosphorylations of members of type-A and type-B *Arabidopsis* Response Regulators (ARRs) protein family lead to their activation (To et al., 2004; Mason, 2005). There are ten type-A ARRs (ARR3-ARR9, ARR15-ARR17) (To et al., 2004; To and Kieber, 2008) and eleven type-B ARRs (ARR1, ARR2, ARR10–ARR12, and ARR18) (Mason et al., 2005; Yokoyama et al., 2007) in the *Arabidopsis* genome. As positive regulators of cytokinin response, type-B ARRs function as transcription factors to promote the cytokinin response gene transcription, such as type-A ARRs and the Cytokinin Response Factors (CRFs) (Hwang and Sheen, 2001; Rashotte et al., 2006). Since type-A ARRs are negative regulators of cytokinin response,

a feedback loop is formed to modulate the magnitude of following responses (To et al., 2007). Type-B ARR along with CRFs mediate the cytokinin-dependent gene expression through targeting common genes (Rashotte et al., 2006).

Auxin-cytokinin crosstalk has been shown to regulate each other's function at metabolic level. Exogenous auxin treatments upregulate the transcription of *IPT5* and *IPT7* in Arabidopsis root (Miyawaki et al., 2004). In addition, auxin down-regulates the cytokinin biosynthesis in Arabidopsis by the isopentenyladenosine-5'-monophosphate (iPMP)-independent pathway (Nordstrom et al., 2004). Reciprocally, cytokinin is an essential positive regulator for auxin biosynthesis in developing root and shoot, possibly through induction of *CYP79B2/B3* and *NIT3* genes, which respectively encodes enzymes in IAOx pathway and the last step in auxin biosynthesis (Jones et al., 2010).

Either synergistically or antagonistically, the mutual correlations of auxin and cytokinin are indispensable to numerous fundamental plant processes, including vascular morphogenesis (Bishopp et al., 2011b), embryogenesis (Muller and Sheen, 2008) and inflorescence meristem establishment (Zhao et al., 2010). The molecular mechanism of the antagonistic interaction between auxin and cytokinin in root meristem maintenance is probably the best investigated. *SHY2/IAA3*, an Aux/IAA protein, is the convergent point for auxin and cytokinin to balance the rate of cell division and differentiation in the transition zone of root apex to control the meristem size (Dello Ioio et al., 2008). At the vasculature of the root meristem transition zone, cytokinin induces the expression of *SHY2* through AHK3/ARR1 and AHK3/ARR12 two-component signaling pathway (Dello Ioio et al., 2007). The induced *SHY2* down-regulates the expressions of *PIN* genes (*PIN1*, *PIN3* and *PIN7*) and leads to the subsequent redistribution of auxin in

surrounding tissues. Therefore, root meristem size is reduced because of cell division to cell differentiation transition of surrounding tissues. However, auxin destabilizes SHY2 to sustain the proper level of *PIN* genes and thus cell division for maintaining the meristem size (Dello Ioio et al., 2008). SHY2 functions through a feedback mechanism to attenuate its own level by negatively regulating cytokinin biosynthesis genes (Jones et al., 2010). The auxin and cytokinin antagonism is also prerequisite for the root stem cell niche specification from hypophysis during *Arabidopsis* embryogenesis (Muller and Sheen, 2008). Auxin suppresses the cytokinin response by inducing the expression of two type-A ARRs, ARR7 and ARR15 (Muller and Sheen, 2008), which are negative regulators of cytokinin signaling. The resulting high ratio of auxin to cytokinin is necessary for the root stem cell niche initiation.

Based on the previous studies on auxin and its correlations with other plant hormones, it is strongly suggested that auxin crosstalk is mediated through the metabolism, transport and signaling. However, most of the convergent points with which auxin fits into the plant hormone network are still yet to be defined. In our lab, to identify the components involved in auxin response, *Arabidopsis* mutants were screened against synthetic auxin, picloram. Characterization of these mutant genes will enable us to identify new components in auxin signaling pathways. The proposed research here will focus specifically on one such mutant isolated named *pic7-1*.

Preliminary studies have shown that the primary root growth of *pic7-1* (Figure 1,2) is resistant to 2,4-D and picloram, which indicates that *PIC7* gene is involved in auxin signaling pathway. *pic7-1* is a recessive mutation. The exact position of the mutant gene was located by map based cloning. Mapping was done using genetic polymorphism

between closely related *Arabidopsis* ecotypes *Columbia* (Col-0) and *Landsberg erecta* (Ler).

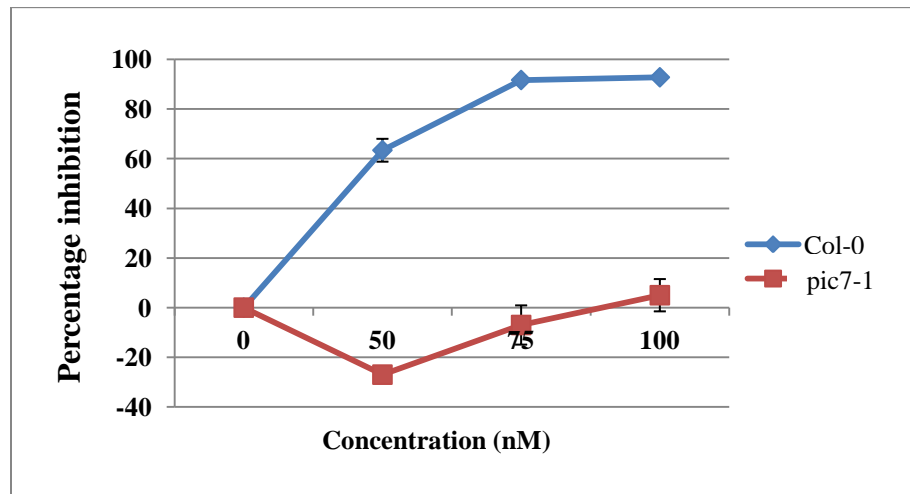


Figure 1. Primary root growth of *pic7-1* is resistant to 2,4-D. Four-day-old seedlings of *pic7-1* and wild type (Col-0) grown on *Arabidopsis thaliana* sucrose medium (ATS) were transferred onto ATS medium containing varying concentrations of 2,4-D. Length of the primary root was measured following four days of incubation in a growth chamber at 20°C (done by Dr. Sunethra Dharmasiri).

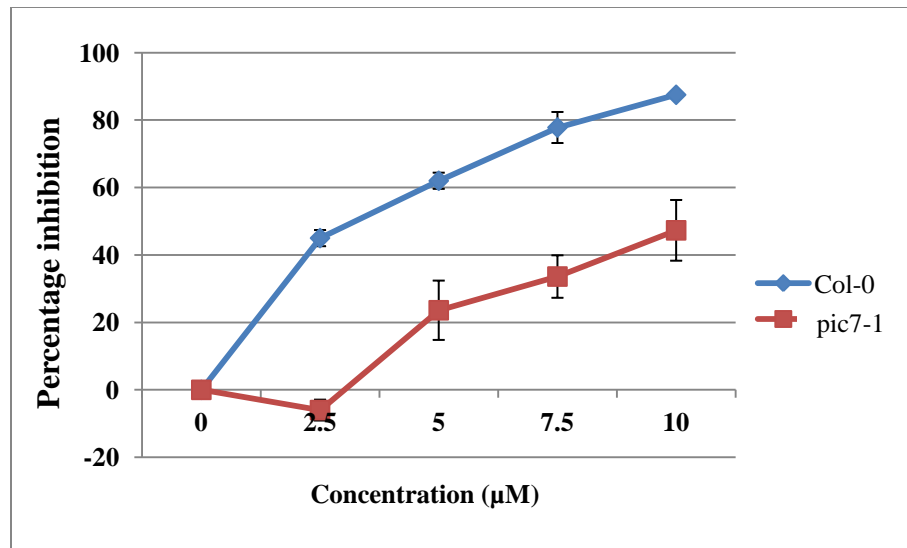


Figure 2. Primary root growth of *pic7-1* is resistant to picloram. Four-day-old seedlings of *pic7-1* and wild type (Col-0) grown on *Arabidopsis thaliana* sucrose medium (ATS) were transferred onto ATS medium containing varying concentrations of picloram. Length of the primary root was measured following four days of incubation in a growth chamber at 20°C. (done by Dr. Sunethra Dharmasiri)

The locus for the *pic7-1* mutation was narrowed down to a region between BACs F17L21 and T22C5 representing *Arabidopsis* chromosome I. This genetic window contained F17L21, T17H3 and T22C5 with an approximate total size of 270kb. Candidate genes were cloned and sequenced. Comparison of sequence data with the wild type genome sequences revealed a single base pair change in the fifth exon of Adenine phosphoribosyltransferase1 (*APT1*) (EC 2.4.2.7) gene. With a C1650T nucleotide substitution in the *APT1* gene, a missense mutation in the amino acid sequence was produced, which replaced the threonine (T) at position 197 with an isoleucine (I) residue in *APT1* (Figure 3).

1624	TGATGACCTC ATTGCCACGG GTGGGA	C	TCT CGCTGCTGCA ATCCGACTAC	1673	Col -0	
1624	TGATGACCTC ATTGCCACGG GTGGGA	I	TCT CGCTGCTGCA ATCCGACTAC	1673	<i>pic7-1</i>	
157	EYGTDTIEHVGAVEP	GE RAIIDDLIATGG	T	LAAAIRLLERVGVKIVECACVIELP	212	Col -0
157	EYGTDTIEHVGAVEP	GE RAIIDDLIATGG	I	LAAAIRLLERVGVKIVECACVIELP	212	<i>pic7-1</i>

Figure 3. Alignments of nucleotide and amino acid sequences of *APT1* from wild type and *pic7-1*. In *pic7-1* background, the 1650th nucleotide (underlined as red) of the *APT1* gene (Col-0) had been changed from cytosine (C) to thymine (T). Consequently in *pic7-1*, the threonine (T) at position 197 was converted to isoleucine (I) (underlined) due to a missense mutation.

APT1 is known to be primarily involved in purine metabolism pathway (Allen et al., 2002). Here *APT1* converts adenine into adenosine monophosphate (AMP) by transferring a phosphoribosyl group from 5-phosphoribosyl 1-pyrophosphate (PRPP) to adenine in the presence of Mg^{2+} (Nygaard, 1983). Mutation in *APT1* abolishes 99% of total adenine phosphoribosyltransferase activity in plants, thus *APT1* is the major contributor for the APT activity (Moffatt and Somerville, 1988). Since AMP is the precursor for ATP and also nucleic acid substrate dATP, mutation in the *APT1* may hamper many biochemical pathways including nucleotide synthesis. In plant, it is also found that *APT1* can utilize cytokinin bases as the substrate (Mok and Mok, 2001). It has been shown that *APT1* isolated from wheat germ can act on cytokinin substrate such as benzyladenine (BA) and kinetin *in vitro* (Chen et al., 1982) and Arabidopsis plant with *APT1* activity deficiency showed strongly reduced conversion rate of BA into

benzyladenosine monophosphate-BAMP (Regan and Moffatt, 1990). Since the base form of cytokinin is thought to be the active form among the others (Laloue and Pethe, 1982), APT1 would have a potential role in down-regulating the level of active forms of cytokinin in plant cells through conversion of cytokinin bases into cytokinin ribosides. Given cytokinin ribosides are utilized as transport forms of cytokinin (Kudo et al., 2010), plant with deficient APT1 activity might display higher local activity of cytokinin and hampered long distance distribution of the hormone.

One of the major phenotypes of previously described *apt1-3* mutant is male sterility. This is mainly due to the defects in pollen development (Moffatt and Somerville, 1988). Interestingly, APT1 is proposed to produce three splice variants named AT1G27450.1, AT1G27450.2 and AT1G27450.3 according to The Arabidopsis Information Resource (TAIR) (<http://www.arabidopsis.org>). However, alternative splicing of this gene has not yet been proven experimentally.

In the present work, *pic7-1* was studied extensively with respect to auxin as well as other plant hormone signaling cascades using genetic, physiological and biochemical techniques. I hypothesize that defective auxin response in *pic7-1* is attributed to the mutation in *APT1*. Secondly, since APT1 is involved in purine metabolism, it might also affect plant growth and development through other plant hormone signaling pathways such as cytokinin, ethylene, ABA and GA. Thirdly, there might be multiple splice variants produced by *APT1* in Arabidopsis. To address these hypotheses, specific aims of this work were to study the auxin signaling cascade in *pic7-1* including the degradation of Aux/IAA protein and the transcription levels of auxin inducible genes, to unravel the expression levels of cytokinin signaling components and cytokinin inducible genes, to

characterize other major plant hormone related phenotypes in *pic7-1*, and also to understand the expression and sub-cellular localization patterns of APT1 in Arabidopsis.

CHAPTER II

MATERIALS AND METHODS

Plant materials and growth conditions

All the experiments described here were done using *Arabidopsis thaliana* (L.) Heynh. Var. *Columbia* (Col-0) obtained from the Arabidopsis Biological Resource Center, Ohio State University. Seeds were surface sterilized with 40% bleach with 0.1% TritonX-100 and rinsed with sterile distilled water thoroughly. Seeds were plated on either *Arabidopsis thaliana* medium with 1% sucrose (ATS), pH 5.6 (Lincoln et al., 1990), or where specified on 0.5X Murashige and Skoog medium (MS; Murashige and Skoog, 1962; Sigma) with 1% sucrose, pH 5.6. The plates were incubated at 4°C for 24 hours and then transferred to a growth chamber at 21°C under continuous illumination with Sylvania cool white fluorescent bulbs (Dharmasiri et al., 2003). All experiments on sterile medium were performed in the same growth chamber. Further cultivation for mature plants was carried out in a plant room at 25°C under continuous illumination.

Morphometric Characterization

The mutant and wild type plants were grown under similar growth conditions as indicated and phenotypical defects related to the mutation were analyzed by morphological comparison between mutant and the wild type Col-0.

Physiological Characterization

Primary root elongation in response to plant hormones

For root growth assays, surface sterilized seeds from wild type or mutant were stratified at 4° C for 24 hours before transferred into the growth chamber at 21°C. Four-day-old seedlings grown on vertically oriented ATS plates were then transferred onto solid ATS medium with or without auxin (50 nM-100 nM IAA, 75 nM 2,4-D, 10 µM picloram, 5 µM-20 µM IBA, 50 nM NAA) and incubated for additional four days before measuring the primary root length. Primary root growth responses to other major hormones-ABA (5 µM-20 µM), cytokinin (10 µM kinetin, 5 µM-10 µM zeatin) or ethylene precursor 1-aminocyclopropane carboxylic acid-ACC (1 µM-10 µM) were tested at range of concentrations as indicated in the results section. Root length was measured for the calculation of percentage inhibition as described.

Secondary root initiation

Seeds were grown on ATS medium for four days and transferred onto ATS medium with or without different auxins (20 nM-85 nM IAA, 25 nM-100 nM NAA) as described in each experiment and allowed to grow for another four days in vertically oriented plates under continuous white light. The total number of lateral roots and adventitious roots per seedling was counted using a stereomicroscope (Nikon SMZ1500). For lateral root number, ten to fifteen seedlings were analyzed for each genotype. Adventitious root formation was calculated as the percentage of seedlings with more than one adventitious root.

Induction of root hairs

To examine the induction of root hairs by 20 nM IAA or 10 μ M ACC, four-day-old vertically grown seedlings were transferred onto solid media with or without IAA or ACC. After four days, number of root hairs (from root tip to 5 mm above it) was counted on a minimum of 10 seedlings per treatment.

Phenotype of dark-grown seedlings

To monitor the involvement of *PIC7* gene in the triple response induced by ACC, sterilized seeds of mutant and wild type were plated directly onto the solid media containing 10 μ M ACC or 50 μ M AgNO₃ (inhibitor of ethylene signaling) and kept at 4°C for 48 hours before transferred to 21°C. The petri dishes were covered with aluminum foil for completely dark growth condition. Apical hook formation, hypocotyl inhibition and radial swelling were examined after 72 hours incubation.

De-etiolation response to cytokinin was performed similarly to triple response assays. Seeds were grown in the dark for four days vertically on ATS media with 10 μ M kinetin and the length of hypocotyls was measured for the calculation of percentage inhibition as described.

Hypocotyl Elongation under high temperature or GA

To test the effect of *pic7-1* mutation on the high-temperature induced hypocotyl elongation, wild type and mutant seeds were planted and grown vertically on ATS at 21°C or 29°C under continuous illumination. Ten or more nine-day-old seedlings were used for hypocotyl length measurement for each treatment. For GA induced hypocotyl

elongation, mutant and wild type seeds were plated directly onto solid ATS medium with or without 1 μM GA, and kept vertically under continuous illumination at 21°C for seven days before imaging and measurement.

Germination assays

Surface sterilized seeds were plated on ATS media containing 0.5 μM ABA, 360 mM mannitol, 160 mM NaCl, 50 μM GA or 0.1 mM 2,6-diaminopurine (DAP) respectively. Plates were stratified at 4°C for 48 hours and transferred into the growth chamber at 21°C with continuous illumination. During six-seven days period, number of seeds with protruded radical or green cotyledons and the number of total seedlings were counted, and percentage germination and percentage green cotyledons were calculated.

Anthocyanin extraction and determination

Cytokinin induced anthocyanin formation was performed by transferring four-day-old light grown seedlings onto ATS medium with or without 10 μM kinetin for another eight days under continuous illumination at 21°C. Around 50 mg of shoot tissue were collected from mutant and wild type seedlings for different treatment. The tissue ground in liquid nitrogen was extracted in 1% HCl in methanol (v/v) at 4°C overnight with gentle shaking. After extraction, half volume of distilled water and one volume of chloroform were added into the extraction solution, vortexed and centrifuged at 3,000 rpm for 2 minutes. The upper aqueous phase was used for spectrophotometric quantification at 530 nm. The relative anthocyanin contents were expressed as $A_{530} \text{ g}^{-1}$ FW (Bieza and Lois, 2001).

Pollen analysis

To study the pollen viability, several experiments were carried out as follows. For pollen number, 5 anthers were collected separately from Col-0 and *pic7-1* plants. They were stained with neutral red for visualization. Pollen grains were taken out by crushing the anthers and counted under the light microscope (Olympus - BH2).

For pollen staining assays, pollen grains were collected from minimum of 10 open flowers placed in 8% w/v sucrose solution and briefly vortexed to release pollen. After removing flowers, pollens were pelleted by brief centrifugation and the supernatant was discarded. Pollen grains were then resuspend in either 0.02% neutral red or Alexander staining (composition) for 5 minutes and observed under the microscope (Olympus – BH2).

For *in vitro* pollen germination assay, flowers were directly dipped on the pollen germination medium for 16 hours, which contains 18% Sucrose, 0.01% boric acid, 1 mM MgSO_4 , 1 mM CaCl_2 , 1 mM $\text{Ca}(\text{NO}_3)_2$, and 0.5% agar (pH 7) for 16 hours. The germinated pollens were counted under the microscope. Pollen grains were counted at least 4 different fields to obtain the mean value.

To prepare the pollen for SEM imaging, pollen grains collected from open flowers were fixed in a 2.5% (w/v) glutaraldehyde in 0.1 M PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , pH7.4) for 24 hours at room temperature. Then the pollen grains were washed with 4% glucose in 0.1 M PBS for 15 minutes, dehydrated through an ascending series of ethanol (30–100% ethanol), and an ascending series of acetone: ethanol (30–100% acetone). Then pollen grains were mounted on a stub

with double side tape, sputter coated with gold, and observed and photographed with the FEI Helios 400 Dual Beam scanning electron microscope.

Molecular and Genetic Characterization

Analysis of the effect of mutation on auxin regulated Aux/IAA degradation

Previous studies have used *HS::AXR3NT-GUS* reporter system to study the stability of Aux/IAA17 protein in mutant backgrounds (Gray et al., 2001). To test whether the degradation of Aux/IAA proteins in the *pic7-1* mutant background was affected, mutant *pic7-1* was crossed to *HS::AXR3NT-GUS* transgenic line. The F1 plants were self-pollinated to obtain the F2 and subsequently homozygous F3 generation (done by Dr. Sunethra Dharmasiri). Four-day-old *pic7-1* seedlings containing the *HS::AXR3NT-GUS* construct in the mutant background were given a heat shock of 37°C in liquid ATS for 2 hours and transferred to ATS at room temperature with gentle agitation. Seedlings were then stained for GUS activity after incubating for 0 min, 60 minutes and 90 minutes at room temperature. The histochemical or quantitative results obtained from *pic7-1* were compared with Col-0 to determine the effect of *pic7-1* mutation on the stability of Aux/IAA proteins.

Histochemical staining

To visualize the recombinant protein with GUS reporter, a histochemical staining procedure was performed based on Jefferson et al. (1987). Collected seedlings with GUS reporter genes were first washed with distilled water and fixed using GUS fixer (0.3 M mannitol, 10 mM 4-morpholineethanesulfonic acid (MES), 0.3% formaldehyde) for 40

minutes with gentle shaking, followed by three washes of five minutes each with 1 ml of 100 mM phosphate buffer (pH 7.0). Fixed seedlings were then incubated in GUS staining buffer (0.1 M 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide cyclohexylammonium, 100 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide) at 37 °C until color developed after vacuum infiltration.

Quantitative β -glucuronidase assay

For quantitative β -glucuronidase assays, roots of seedlings were frozen in liquid nitrogen immediately after collection. After quick grind, tissues were homogenized in GUS extraction buffer (100 mM phosphate buffer, pH 7.0, EDTA, 0.1% sodium lauryl sarcosine, 10 μ M β -mercaptoethanol). After centrifugation at 10,000 x g for 10 minutes, supernatant was collected. The total protein in the extract was estimated by Bradford method (Bradford, 1976). Equal amounts of total protein from each treatment were incubated for 1 hour in the assay buffer containing 4-methylumbelliferyl- β -D-glucuronide hydrate (4-MUG) dissolved in the extraction buffer. The reactions were stopped by adding 0.2 M sodium carbonate and quantified using a luminometer at 460 nm for fluorescence signals (Turner, Sunnyvale, CA, Model number-9200-002). All the experiments were carried out in triplicate.

Analysis of auxin induced gene expression using DR5::GFP reporter system

The expression of GFP (green fluorescence protein) under *DR5* which is a synthetic auxin responsive promoter was used to demonstrate auxin dependent gene

expression. The mutant plants were crossed into Col-0 plants that contain the *DR5::GFP* reporter gene construct. The resulting F1 generation was self-pollinated to obtain the F2 and F3 generation (done by Dr. Sunethra Dharmasiri). Plants from the F4 generation that is homozygous for both the mutation and the reporter gene construct were treated with 85 nM 2,4-D or 10 μ M picloram and observed under an epifluorescence microscope. The expression of GFP in the wild type was compared to that of the mutants to determine if the mutation causes a difference in GFP gene expression.

Analysis of cell division activity using *CyclinB::GUS* reporter system

The expression of GUS reporter gene under the promoter of a cell division marker *CyclinB* was used to demonstrate cell division activity in *pic7-1*. The mutant plants were crossed into Col-0 plants that contain the *CyclinB::GUS* reporter gene construct. The resulting F1 generation was self-pollinated to obtain the F2 and F3 generation. Plants from the F4 generation that is homozygous for both the mutation and the reporter gene construct were grown at 21°C under continuous illumination for seven days and stained as described. The expression pattern of GUS in the wild type was compared to that of the mutants to determine if the mutation causes a difference in cell division.

Expression of *APT1::APT1-GUS* and *APT1::APT1-GFP* in plants

Function of a protein is closely related to its expression and sub-cellular localization pattern. To study the expression pattern of APT1, coding region of the *APT1* gene along with 5' leading sequence (2000bp upstream of ATG) was amplified from wild type Col-0 using Phusion DNA polymerase (NEB) using primers:

PIC7-prom-SalI –F (5'-CACCGTCGACTTAATAGGTAGATCTTGATGTATATG-3')

PIC7.3-XbaI-R (5'-TCTAGATTCTTGTTTAGCAGCCGAC-3'). The PCR amplified DNA fragment was cloned into the EcoRV site of pBluescript II SK (Stratagene) and sequenced to verify the fidelity of sequence. The *APT1* DNA fragment was released with SalI and XbaI and cloned into the same sites in pBI101-GUS or pBI101-GFP to generate *APT1::APT1-GUS* and *APT1::APT1-GFP* recombinant plasmids. These constructs were shuttled into *Agrobacterium tumefaciens* strain GV3101 and then were transformed into wild type Col-0 and *pic7-1*. Further screening was carried out for homozygous lines from T3 generation.

Characterization of spatial and temporal expression pattern of *APT1* gene

Wild type seedlings carrying *APT1::APT1-GUS* were used to examine the expression pattern of *PIC7 (APT1)* gene. Homozygous *APT1::APT1-GUS* seedlings that were grown for five, seven, twelve days were collected and stained with GUS staining buffer as described. To study the sub-cellular localization of *APT1*, wild type seedlings carrying *APT1::APT1-GFP* construct were examined at sub-cellular level using Olympus FV1000 confocal microscopy.

To test the possible effect of auxin and cytokinin on expression of *APT1* gene, four-day-old seedlings carrying the *APT1::APT1-GUS* were transferred to ATS liquid media containing 20 μ M 2,4-D, 20 μ M IAA, 20 μ M kinetin or 20 μ M zeatin. Seedlings were incubated for 2 or 6 hours following treatment under continuous illumination with gentle agitation. All the treatments were carried out in 6 well microtiter plates.

Protein expression, extraction and purification from *E.coli*

According to Arabidopsis thaliana resource center (<http://www.arabidopsis.org/>) annotations, *APT1* may produce 3 splice variants possibly encoding 3 different proteins. To construct splice variants expression plasmid, two of them-*APT1.1* and *APT1.2*, were amplified using cDNA prepared from wild type Col-0. . Primers used are PIC7.1-pGEX-BamHI-F (5'-AAGATCTCTCGGATCCAAGACAATGC-3'), PIC7.1-SalI-R (5'-TTCTCGTCGACTTTCTTGTTAAGCAGC-3') and PIC7.2-pGEX-BamHI-F (5'-AGTGGATCCAGTGAAATGGCGACTG-3'). Both cDNAs were cloned into the EcoRV site of pBluescript II SK (Stratagene) vector and sequenced to verify the fidelity. Each cDNA was then cloned into the *BamHI-SalI* site of pGEX-4T-3 vector and transformed into *E. coli* (BL21) with CaCl₂ method (Bergmans et al., 1981).

To generate constructs expressing mutant splice variants, plasmids from pGEX-4T-3-APT1.1/pGEX-4T-3-APT1.2 were used for the site-directed mutagenesis (Stratagene) with sequence specific primers, PIC7-SDM-F (5'-GTGGGATTCTCGCTGCTGCAATCCGAC-3') and PIC7-SDM-R (5'-GTCGGATTGCAGCAGCGAGAATCC CAC-3') and transformed into BL21.

To purify GST-APT1.1, GST-APT1.2, GST-APT1.1, GST-APT1.2 recombinant proteins, *E. coli* (BL21) carrying the respective recombinant plasmids were grown overnight and 0.5 ml of overnight culture was inoculated into 30 ml liquid LB containing carbenicillin (100 µg/ml). After 4 hours incubation, 1 mM isopropyl thiogalactoside (IPTG) (Sigma Aldrich Inc. MO) was added to induce recombinant protein expression at 30°C for another 4 hours. Bacterial cells were pelleted at 9000 x g

for 10 minutes at 4°C (Eppendorf Centrifuge 5810R), resuspended in 7 ml of phosphate buffered saline (PBS, pH = 8.0) and sonicated (Branson Sonifier 250). The extract was incubated for 10 minutes with 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma Aldrich Inc. MO) and 0.1% Tween-20 (Sigma Aldrich Inc. MO). After centrifugation at 10,000 x g for 10 minutes (Eppendorf Centrifuge 5810R), cell debris was removed and glutathione-agarose beads (GST beads) (Sigma Aldrich Inc. MO) that were previously hydrated in PBS were added to the supernatant. The mixture was incubated at 4°C overnight with gentle agitation. Beads were recovered by centrifugation and washed three times each for 10 minutes with 10 ml of PBS containing 0.5% Tween-20. Washed beads were re-suspended in 150µl of PBS and 10µl of the suspension was used for confirmation of the expression using SDS-PAGE protein electrophoresis.

Expression of 35S::APT1.1-Myc and 35S::APT1.2-Myc in plants

To study the physiological role of *APT1.1* and *APT1.2*, both were amplified using cDNA prepared from wild type Col-0. Primers used are

PIC7.1-myc-BamHI-F (5'-TAGGATCCCTCAGTCCAAAGACAATGC-3'),

PIC7.1-myc-SalI-R (5'-TCCAGTGTCGACTAAAGCAGCCGAC -3'), and

PIC7.2-BamHI-F (5'-GTCGGATCCGTGAAATGGCGACTG-3')

Both cDNAs were cloned into the BamHI-SalI sites of modified pBluescript vector containing Myc tag and sequenced to verify the fidelity. The *APT1.1* or *APT1.2* DNA fragment was released with BamHI and KpnI and cloned into the same sites in pROKII vector to produce *35S::APT1.1-Myc*, *35S::APT1.2-Myc* constructs. These constructs were shuttled into *Agrobacterium tumefaciens* strain GV3101 and then were transformed

into wild type Col-0 and *pic7-1*. Further screening was carried out for homozygous lines from T3 generation. Defect in male sterility observed with *pic7-1* was assessed with plants over-expressing *35S::APT1.1-Myc* and *35S::APT1.2-Myc*.

Western blotting for Myc or GFP tagged proteins

Total protein of leaves from independent lines of T1 plants carrying *35S::APT1.1-Myc* and *35S::APT1.2-Myc* transgenes in wild type was extracted using EZ buffers (Appendix III). Protein samples were separated using 12.5% polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) (Pall Corporation, FL) membrane. Western blotting was carried out using anti-Myc (Covance, 1:10,000) as the primary antibody and anti-mouse IgG (Invitrogen, 1:10,000) as the secondary antibody. For GFP western blotting, anti-GFP primary antibody (Invitrogen, 1:2000) and anti-rabbit IgG (Sigma, 1:2000) secondary antibody were used.

After the transfer, the membrane was blocked with 5% non-fat milk in Tris buffered saline containing 0.1% Tween 20 (TBST) for one hour on a slowly moving shaker, and then washed three times with TBST. After one hour incubation with primary antibody solution, the membrane was washed three times with TBST (each 5 minutes). The blot was then incubated with secondary antibody solution for one hour and washed 4 times with TBST (1st wash for 15 minutes, three washes each 5 minutes). The Myc blot was developed using PIERCE ECL western blotting substrate and exposed to X-ray film according to manufacturer's instructions. The GFP blot was developed using PIERCE ECL Plus western blotting substrate and exposed to X-ray film according to manufacturer's instructions.

RNA isolation and Quantitative Real Time PCR (qRT-PCR)

Seven-day-old *pic7-1* and Col-0 seedlings were treated with liquid ATS supplemented with 20 μ M 2,4-D for 6 hour at room temperature on a shaker with gentle agitation. Then the shoot and root tissues were separately collected for each treatment and frozen in liquid nitrogen. Total RNA was extracted from shoot or root by using TriReagent (Sigma, St.Louis, MO) according to manufacturer's instructions. The isolated total RNA was treated with RNase-free DNaseI to remove any DNA contamination. Complementary DNA was synthesized from total RNA with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) in a 20 μ l total volume following the manufacturer's instructions. Each qRT-PCR reaction contains 25 μ l reaction mixture including 5 μ l of 1:100 dilution of cDNA (8ng of RNA equivalent), 12.5 μ l of SYBR GreenER_ qPCR SuperMix Universal (Thermo Scientific), 2 μ l of 10 μ M forward/reverse primers and 5.5 μ l water.

To study the effect of *pic7-1* mutation on auxin inducible gene expression, induction of *GH3.3*, *GH3.5*, *SAUR19* genes were studied in response to 2,4-D treatment using qRT-PCR. As the key components in cytokinin signaling pathways, the expression of *ARR1*, *ARR10*, and *ARR12* gene were compared in *pic7-1* to the wild type. Transcript levels of *CYP79B2*, *CYP79B3* and *NIT3* in mutant were also examined as cytokinin-regulated auxin synthetic genes. As a control, a housekeeping gene, ubiquitin associate protein UBA gene, was also amplified from all the samples. Primers used for each gene are given below:

Table 1. Oligonucleotide primers for qRT-PCRs

(* denotes the primers made by Thilanka Jayaweera)

	Primer Name	Primer Sequence (5' to 3')	Primer Length (base pairs)	Melting Temperature (°C)
1	qGH3-3-F	TGGGACATCAGCTGGTGAAAGG	22	62.9
2	qGH3-3-R	TGTCTAATCCGGGCACGTAGAG	22	61.9
3	qGH3-5-F	CCATCTCTGAGTTCCTCACAAGC	23	61.3
4	qGH3-5-R	TCCTCTTCGATTGTTGGCATTAGC	24	61.8
5	qSAUR19-F	GAAGAGATATTTGGTGCCGCTCTC	24	61.9
6	qSAUR19-R	ACTGAGCAGAGCTTGAAATGACG	23	61.8
7	qARR1-F	AGACCGTCGTCGAGATGTTTCC	22	62.7
8	qARR1-R	GCTCTGTTGCATTTCTGTTACTTCG	24	61.5
9	qARR10-F	TGATGGCTTCTGATGCTGGTTCC	23	63.4
10	qARR10-R	TCAGATTGGCTCTGTTCTGTGTC	24	63
11	qARR12-F	CAACTCCACGATGAAGCAGGAAC	23	62.3
12	qARR12-R	ACCCTCCATATCCCAAATCTCCTC	24	61.6
13	qCYP79B2-F	TCAAACCCACCATTAAGGAGCTTG	24	61.7
14	qCYP79B2-R	TTCCACGGCGTTTGATGGATTG	22	62.4
15	qCYP79B3-F	ACCGGAAAGAGAGGATGTGCTG	22	62.7
16	qCYP79B3-R	CGCTAGCATCATGGTCGTTATCGC	24	64.5
17	qNIT3-F	CAGGTTATTGGCGTTGACCCATC	23	62.4
18	qNIT3-R	AACTTCTCCGCCTTGTCTAAAGTG	24	61.4
19*	UBA-F	AGTGGAGAGGCTGCAGAAGA	20	58.1
20*	UBA-R	CTCGGGTAGCACGAGCTTTA	20	56.7

The PCR program consisted 55°C for 10 minutes, 95°C for 2 minutes, 40cycles of 95°C for 15 seconds and 60°C for 1 minute. Before the trials, serial cDNA dilutions were used to examine the amplification efficiency of each primer pair for correcting the cycle threshold value. Relative expression levels were calculated using the comparative C_T method (User Bulletin 2, ABI Prism 7700 Sequence Detection System). 2^{-ΔΔCT} values of control samples were normalized to 1.

Length measurements and calibration

Root and hypocotyl growth were measured using a standard 1 mm scaled ruler. For small seedlings or seeds, digital pictures were taken and analyzed with NIH ImageJ software for the relative length. Length of root cells was analyzed using Olympus Fluoview software.

For calculating the percentage inhibition in root growth assay or hypocotyl inhibition assay, means of root or hypocotyl length for different treatments were calculated after the measurement. Percentage inhibition was calculated using following equation:

$$\% \text{ inhibition} = [(\bar{Y} - \bar{X}) / \bar{Y}] * 100\%$$

Standard error of percentage inhibition was calculated using following equation:

$$SE = \frac{\bar{X}}{\bar{Y}} \sqrt{\frac{SD_x^2}{n_x \bar{X}^2} + \frac{SD_y^2}{n_y \bar{Y}^2}}$$

(\bar{X} : average of inhibited values. \bar{Y} : average of uninhibited values. n_x : number of inhibited values. n_y : number of uninhibited values. SD_x : standard deviation of inhibited values. SD_y : standard deviation of uninhibited values.)

Microscopy

For imaging roots carrying *DR5::GFP* and *APT1::APT1-GFP*, Olympus FV1000 confocal microscope was used and images were analyzed using Olympus Fluoview software (Olympus, Melville, NY). The objectives used were PlanApo 60x oil (NA= 1.4), UPlanApo 40x oil (NA= 1), and XLUMPlanF1 20x water (NA = 0.95). Gain and dynamic range settings were calibrated on control GFP expressing roots and then kept unchanged for recording of images of the mutant roots with various treatments. For imaging GUS stained seedlings, Nikon SMZ1500 stereo microscope (Nikon, Melville, NY) and Olympus BH2-RFCA compound microscope were used.

For imaging the roots from independent lines of *pic7-1* carrying *APT1::APT1-GFP* in response to root growth inhibition by picloram and zeatin, Olympus BH2-RFCA compound microscope was used.

Statistical analysis

To perform the two factor analysis of variance (ANOVA), the data were checked for the assumptions of normality and homoscedasticity. Tukey's HSD test was used to identify the statistically significant difference between means. All analyses were performed using software R (<http://www.r-project.org/>). In addition, Student's T-test was also used when necessary using JMP9.

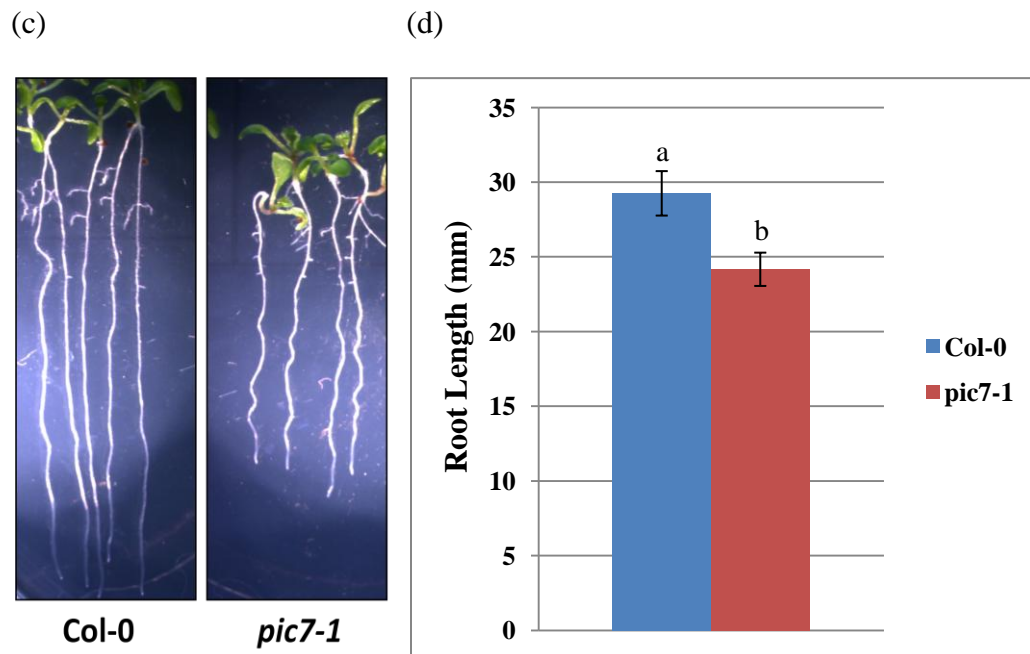
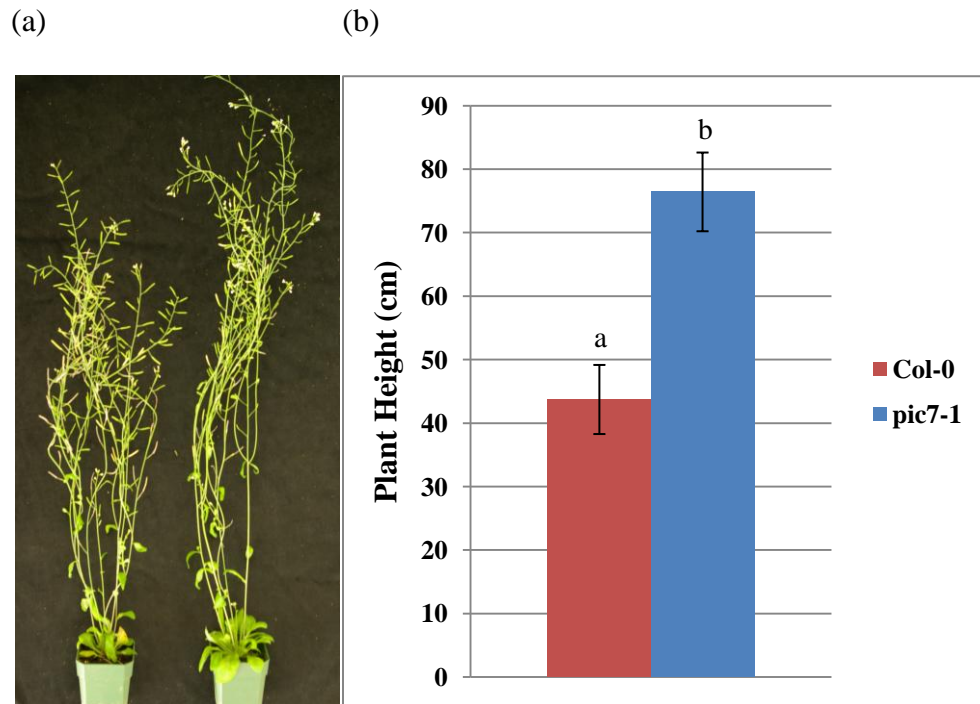
CHAPTER III

RESULTS

Morphometric characterization

According to the morphological comparisons, *pic7-1* plants showed defects in growth and development. Mature mutant plants were significantly taller compared to wild type plants (Figure 4a,b). However, roots of the *pic7-1* seedlings were significantly shorter than the wild type (Figure 4c,d), and correspondingly the measurements of mature cortical cell length showed that *pic7-1* had significantly shorter cells compared to the wild type (Figure 4e) that might contribute to the shorter root phenotype. In addition, *pic7-1* plants had small siliques and produced a few numbers of seeds per plant (Figure 5a,b). By close observations of the floral organs, it was obvious that *pic7-1* produced less number of pollen grains compared to wild type Col-0 (Figure 6a). After the neutral red staining, wild type pollen had numerous uniform shaped small vacuoles stained as red, while *pic7-1* contained many dead pollen grains which were not stained. Although a smaller number of *pic7-1* pollen grains were stained, they still displayed defective vacuole phenotypes such as fewer large vacuoles with irregular shape and size (Figure 6b,c). The scanning electron microscopy also revealed the deformed pollen grains in *pic7-1* (Figure 6b,c). According to Alexander staining, *pic7-1* had less number of viable

pollen grains which were stained as purple (Figure 6d,e). Pollen germination assays confirmed the sterility of *pic7-1* pollen (Figure 7a,b).



(Figure 4-continued)

(e)

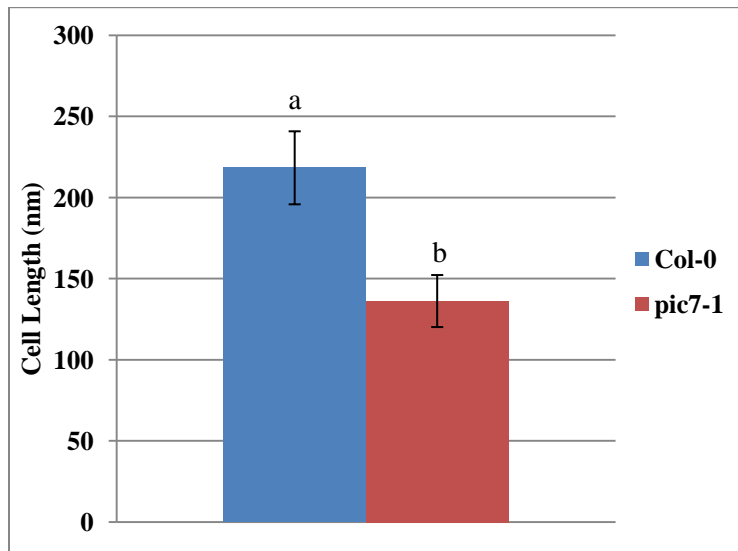


Figure 4. Shoot and root growth of *pic7-1*. (a,b) *pic7-1* plants grow taller than the wild type. When plants were grown under similar growth conditions *pic7-1* plants had a taller stature compared to the wild type. Plants were grown under continuous light for sixty days at 25°C. Means with different letters “a, b” indicate statistically significant difference ($P < 0.05$), student t-test. (c,d) Roots of *pic7-1* seedlings are shorter compared to the wild type when grown on ATS media. Seedlings were grown under continuous illumination at 21°C for eight days prior to the root measurements. Means with different letters “a, b” indicate statistically significant difference ($P < 0.05$), student t-test. (e) Length of the mature cortical cells of the primary root are shorter compared to the wild type. Seedlings were grown under continuous illumination at 21°C for eight days, roots were imaged using FV1000 confocal microscope under differential interference contrast (DIC) objective lens and mature cortical cell length was measured using Olympus

fluoview software. Means with different letters “a, b” indicate statistically significant difference ($P < 0.05$), student t-test. Error bars represent standard deviations of the means.

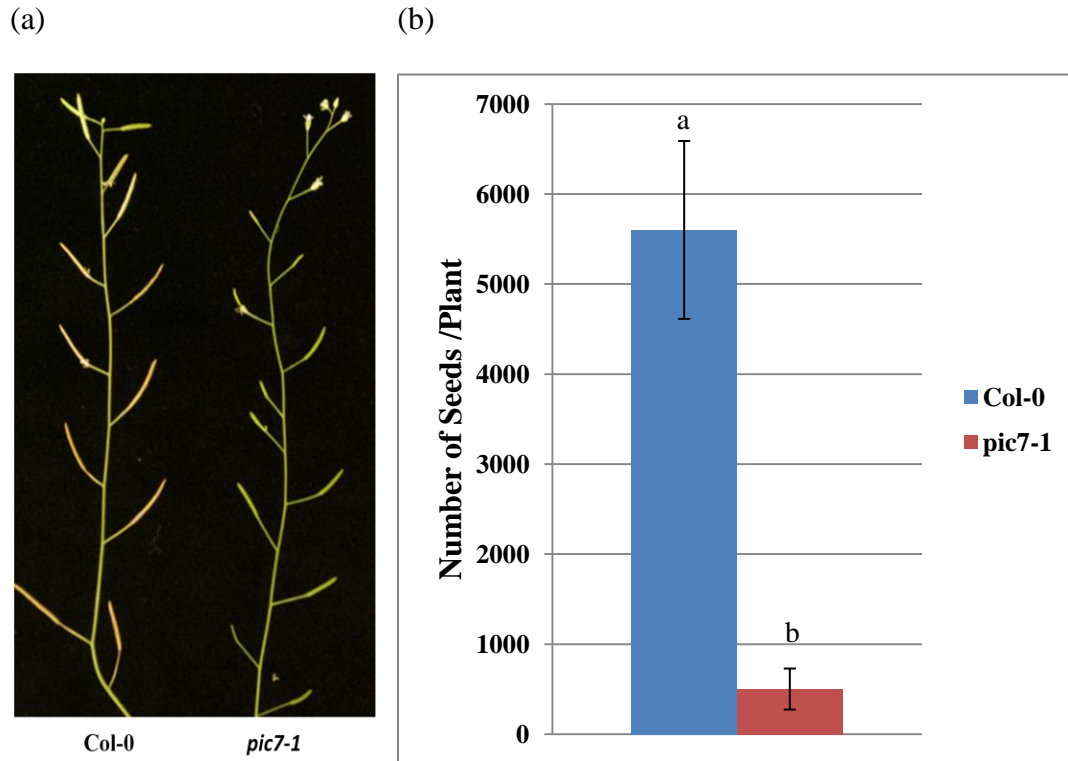
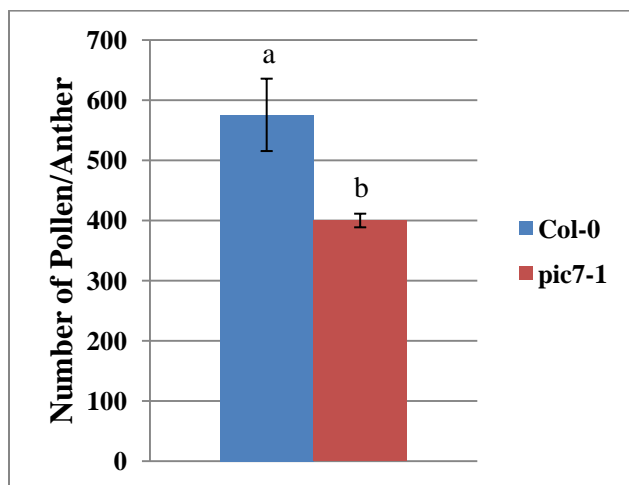
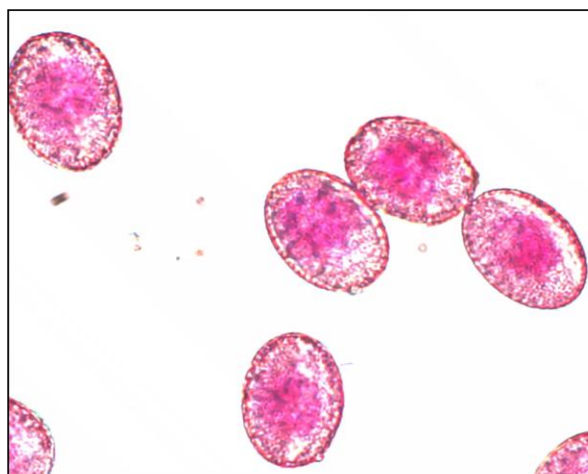
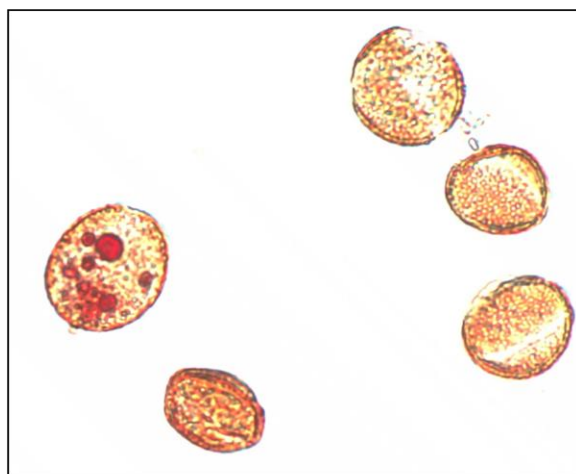


Figure 5. Sterility of *pic7-1*. (a) *pic7-1* produces shorter siliques. When plants were grown under similar growth conditions, *pic7* mutant plants produced siliques that were shorter in length compared to that of Col-0. Plants were grown under 24 hours light conditions for sixty days at 25°C. (b) *pic7-1* produces significantly less number of seeds. Seeds were collected from 10 mature senesced *pic7-1* and wild type Col-0 plants. Seeds were weighed and compared the weight of 10 seeds to calculate the total seeds number. (done by Dr. Sunethra Dharmasiri). Means with different letters “a, b” indicate statistically significant difference ($P < 0.05$), student t-test. Error bars represent standard deviations of the means.

(a)

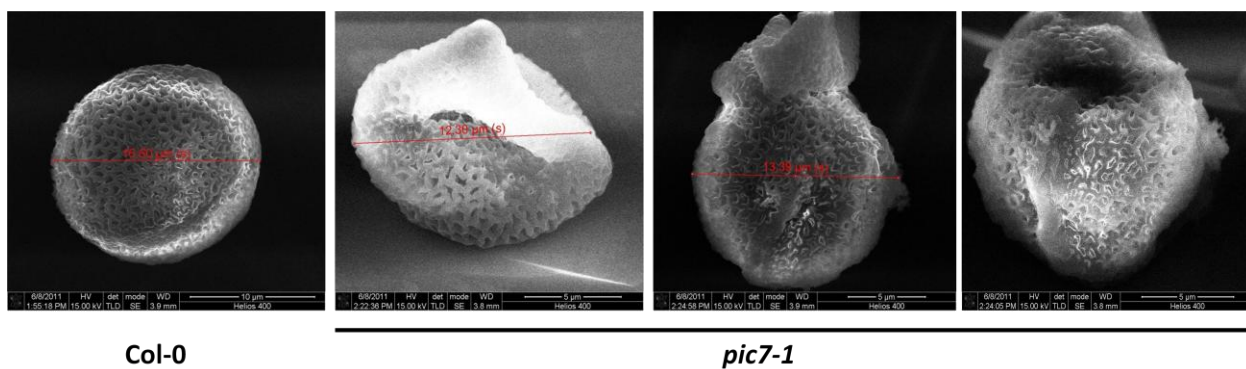


(b)

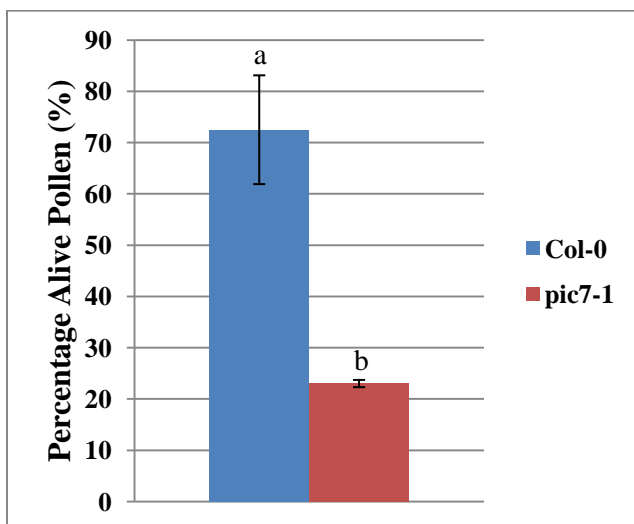
**Col-0***pic7-1*

(Figure 6-continued)

(c)



(d)



(Figure 6-continued)

(e)

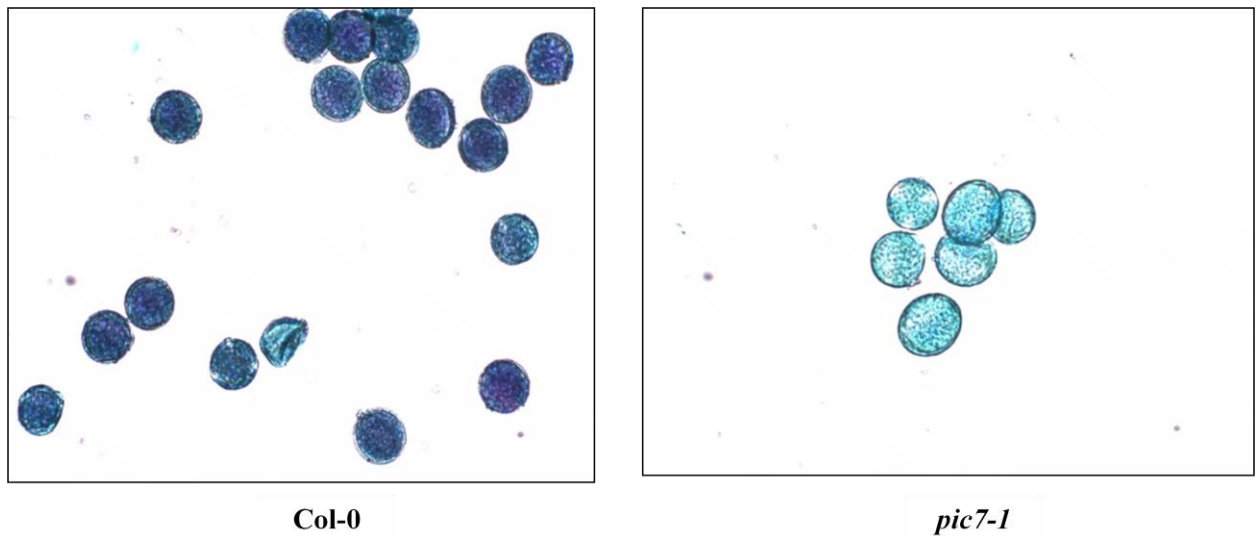
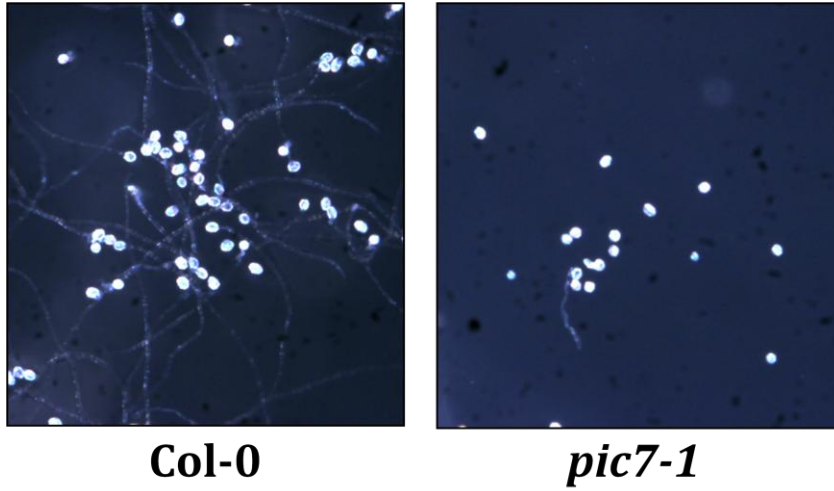


Figure 6. Male sterility of *pic7-1*. (a) *pic7-1* produces less number of pollen grains compared to wild type. 5 Anthers were collected separately from Col-0 and *pic7-1* plants. After stained with neutral red, pollen grains were taken out by crushing the anthers and then counted under the light microscope. Means with different letters “a, b” indicate statistically significant difference ($P < 0.05$), student t-test. (b,c) Pollen grains of *pic7-1* are deformed. Pollen grains were photographed (b) at 40X magnification using Olympus BH2 light microscope after neutral red staining or (c) using SEM (FEI Helios 400 Dual Beam). (d,e) *pic7-1* has less viable pollen grains. Means with different letters “a, b” indicate statistically significant difference ($P < 0.05$), student t-test. Pollen grains were stained with Alexander stain and photographed at 10X magnification using Olympus BH2 light microscope. Viable pollen grains were stained purple in color while sterile ones were stained green in color. Error bars represent standard deviations of the means.

(a)



(b)

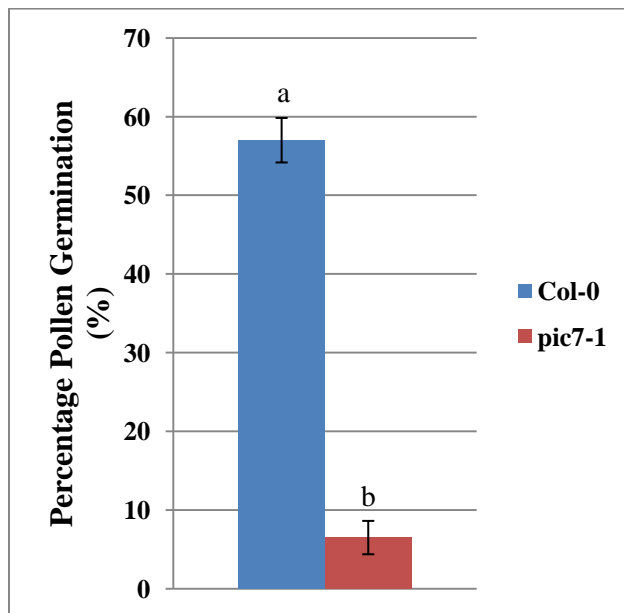


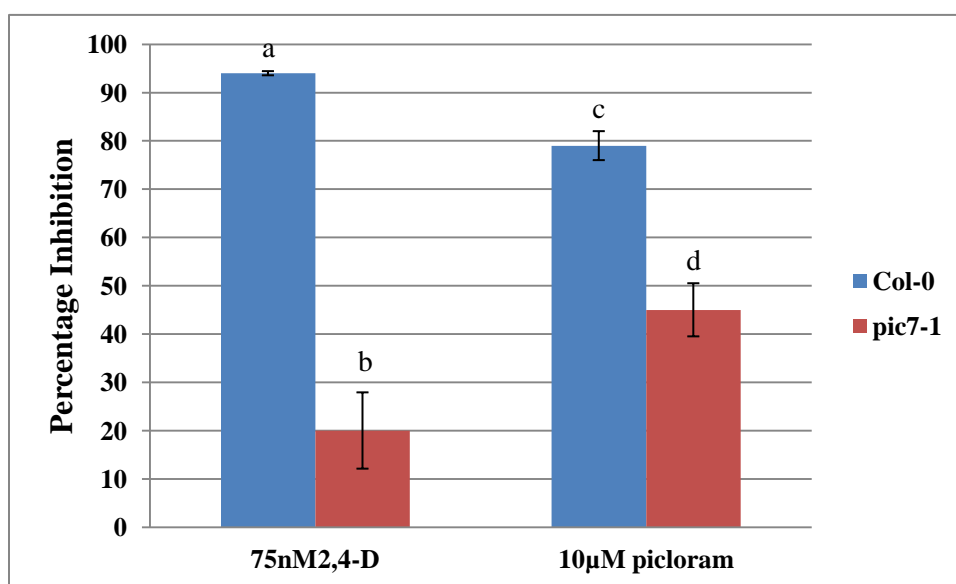
Figure 7. *pic7-1* has less fertile pollen grains. (a) Pollen grains were germinated on pollen germination media for 16 hours and imaged using Olympus BH2 microscope (40X). (b) Germinated pollen grains were counted under the microscope. *pic7-1* has significantly

less alive pollen grains than wild type Col-0. Means with different letters “a, b” indicate statistically significant difference ($P < 0.05$), student t-test. Pollen grains were counted at least 4 different fields to obtain the mean. Error bars represent standard deviations of the means.

Auxin responses were defective in *pic7-1*

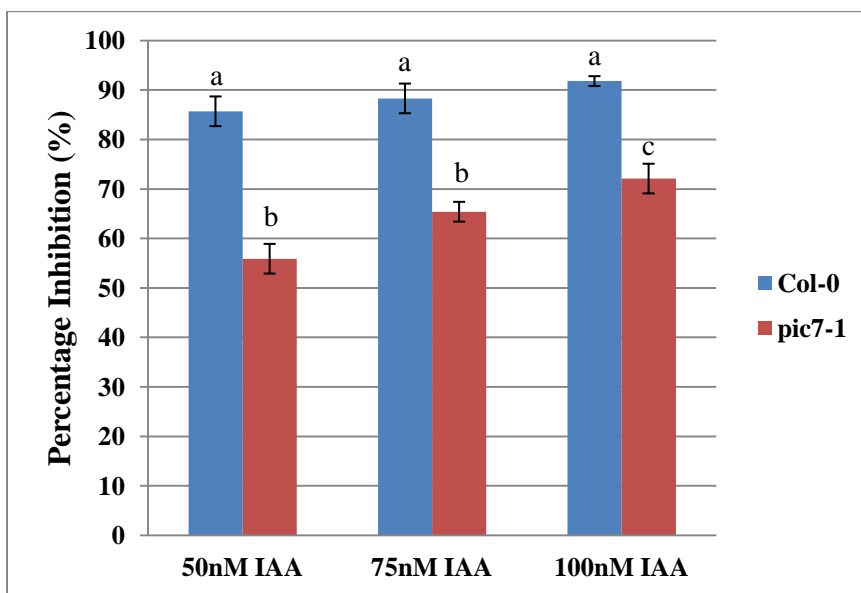
Primary root growth is inhibited by high concentrations of auxin (Leyser et al., 1996). Root growth assays done on *pic7-1* revealed that the mutant was highly resistant to natural as well as synthetic auxins. While *pic7-1* was highly resistant to synthetic auxin 2,4-D and picloram (Figure 8a), its resistance to natural auxin IAA and IBA was moderate (Figure 8b,c). It also showed resistance to another synthetic auxin NAA, which does not need a carrier protein to get into the cell (Figure 8d) suggesting that *pic7-1* mutation is involved mainly in auxin signaling rather than auxin transport.

(a)

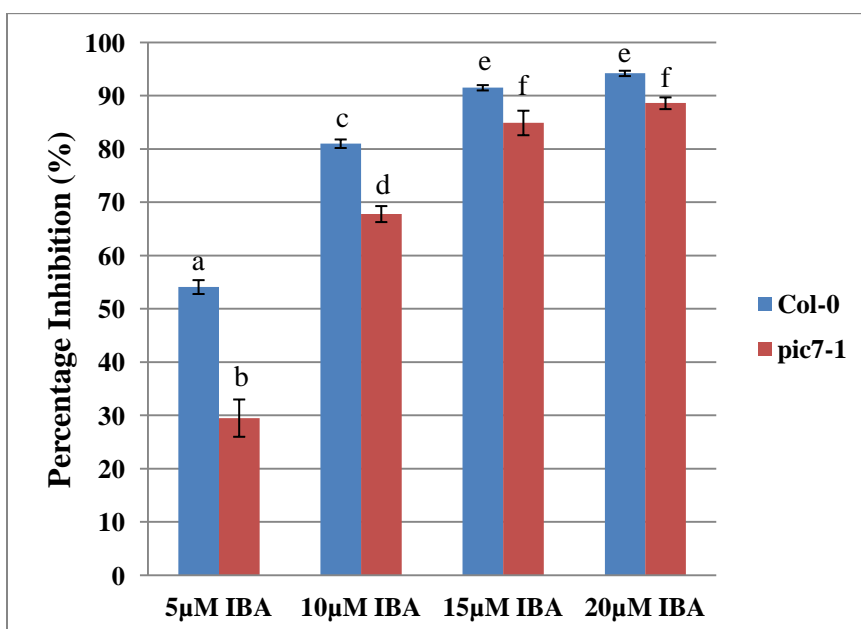


(Figure 8-continued)

(b)



(c)



(Figure 8-continued)

(d)

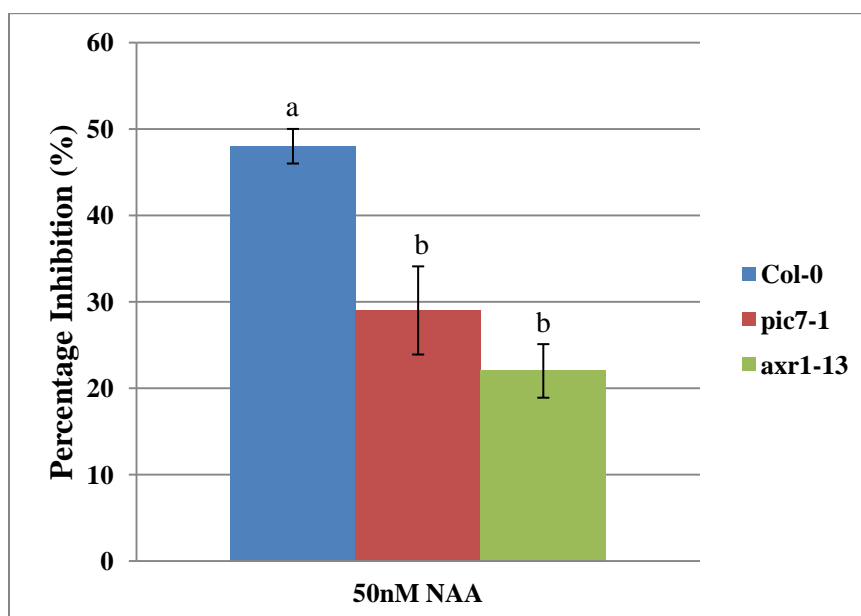


Figure 8. Primary root growth of *pic7-1* is resistant to natural and synthetic auxins. Four-day-old seedlings of *pic7-1* and wild type (Col-0) grown on ATS medium were transferred onto ATS media containing varying concentrations of (a) 2,4-D, picloram, (b) IAA, (c) IBA and (d) NAA. Length of the primary root was measured following four days of incubation vertically in a growth chamber under continuous illumination at 21°C. (a) With 2,4-D treatment, a two-way ANOVA indicated interaction between genotype and treatment ($F=295.05$; $df=1, 32$; $P<0.001$). With picloram treatment, A two-way ANOVA indicated interaction between genotype and treatment ($F=93.68$; $df=1, 32$; $P<0.001$). Means with different letters “a, b, c, d” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. (b) A two-way ANOVA indicated interaction between genotype and treatment ($F=86.60$; $df=1, 48$; $P<0.001$). Means with different letters “a, b, c” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. (c) A

two-way ANOVA indicated interaction between genotype and treatment ($F=26.679$; $df=1, 90$; $P<0.001$). Means with different letters “a, b, c, d, e, f” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. (d) A two-way ANOVA indicated interaction between genotype and treatment ($F=6.195$; $df=2, 54$; $P<0.005$). Means with different letters “a, b” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. Error bars represent standard errors of the means.

Auxin-Dependent Gene Transcription in *pic7-1* background

Auxin-dependent gene transcription was examined using the *DR5::GFP* reporter gene system, which contains a synthetic promoter *DR5* that is induced in the presence of auxin. In Arabidopsis wild type seedlings, *DR5::GFP* is highly induced in the quiescent center and columella where auxin is naturally highly present. F3 seedlings homozygous for *pic7-1* and *DR5::GFP* were treated with auxins such as picloram or 2,4-D. While *pic7-1* exhibited no significant difference on GFP level under control condition compared to the wild type, GFP expression induction by synthetic auxin treatment were lower in *pic7-1* background compared to that in the wild type Col-0 background. These results indicate that auxin-dependent gene transcription is impaired in *pic7-1* (Figure 9).

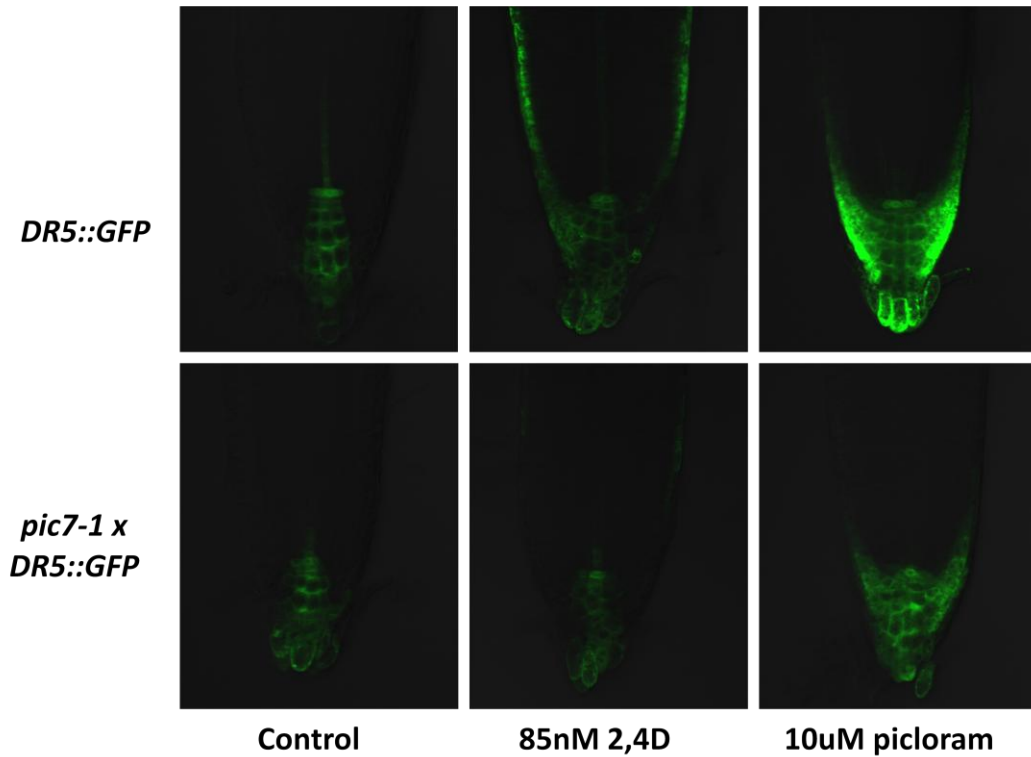


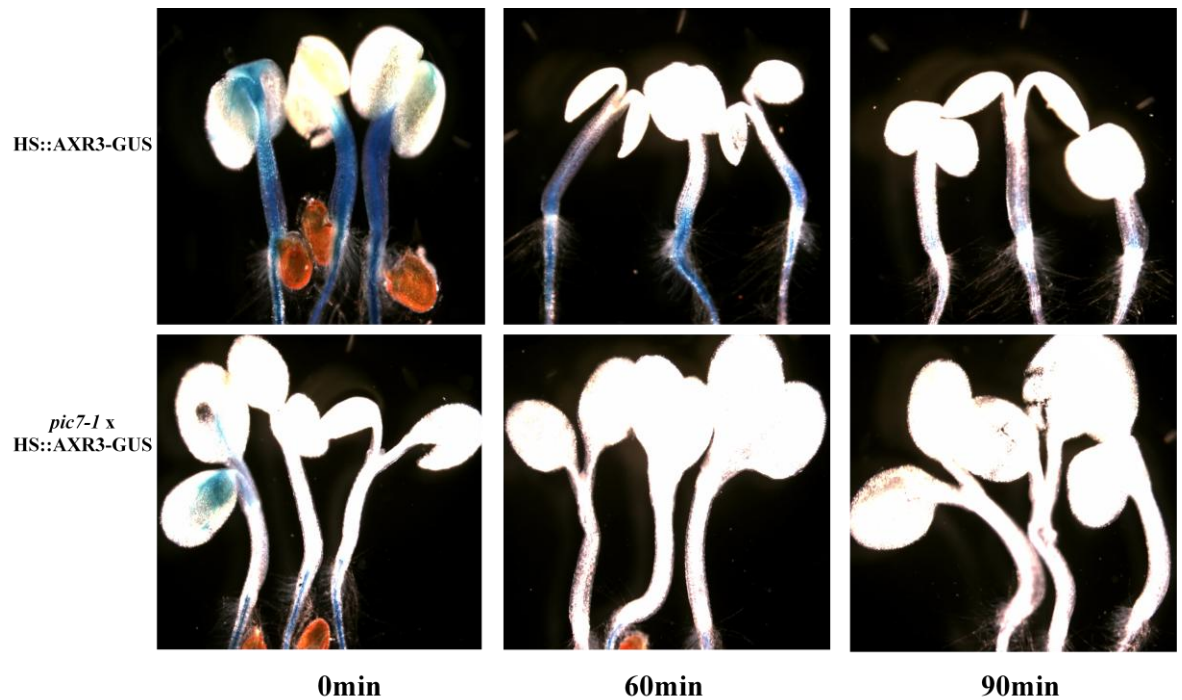
Figure 9. Induced *DR5::GFP* expression in *pic7-1*. Four-day-old light grown wild type Col-0 or homozygous *pic7-1* seedlings carrying auxin inducible reporter gene *DR5::GFP* were used. Seedlings were treated with mock (ethanol/DMSO), 85 nM 2,4-D or 10 μ M picloram for 12 hours on solid agar plates and imaged using Olympus FV1000 confocal microscope.

Stability of the AXR3-GUS Recombinant Protein in *pic7-1* Background

Aux/IAA proteins have a very short life span and are rapidly degraded in the presence of auxin (Worley et al., 2000; Zenser et al., 2003). In most of the currently known auxin response mutants, the stability of the Aux/IAA repressor proteins are enhanced. The stability of Aux/IAA proteins in *pic7-1* background was studied using the *HS::AXR3NT-GUS* line (Gray et al., 2001). Seedlings that are homozygous for both *pic7-*

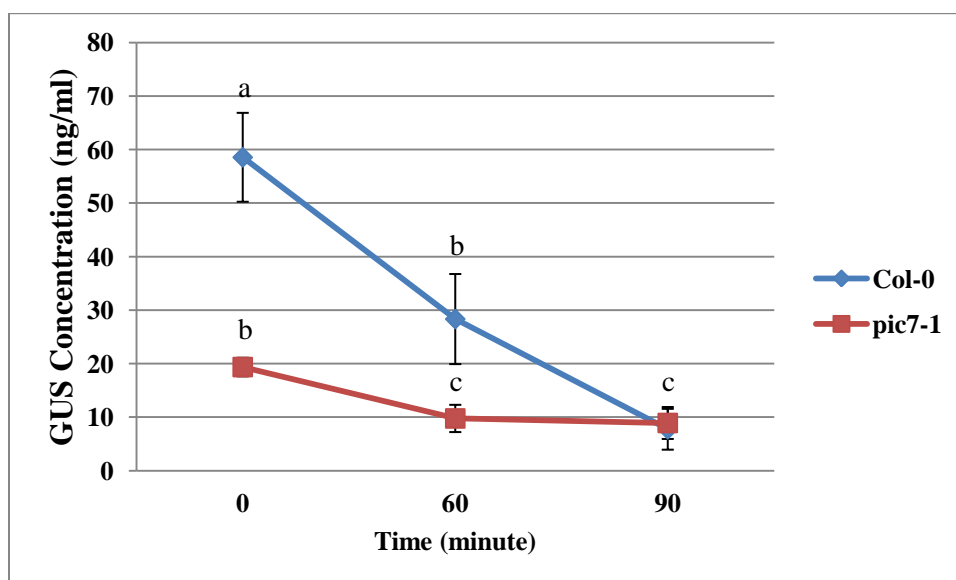
1 and *HS:AXR3NT-GUS* were selected and used for the experiment. Compared to wild type, the recombinant protein was present less abundant in the shoot (Figure 10a,b) but more in the root of *pic7-1* background (Figure 10c,d) at 0 minute after 2 hours heat shock induction of *HS::AXR3NT-GUS* gene. This indicates that AXR3-GUS protein was rapidly degraded in the shoots but stabilized in the roots of the mutant background. However, the rate of degradation of AXR3-GUS in the root of *pic7-1* was probably faster than that of the wild type after 60 and 90 minutes incubation at room temperature, especially in the mature region of the root.

(a)



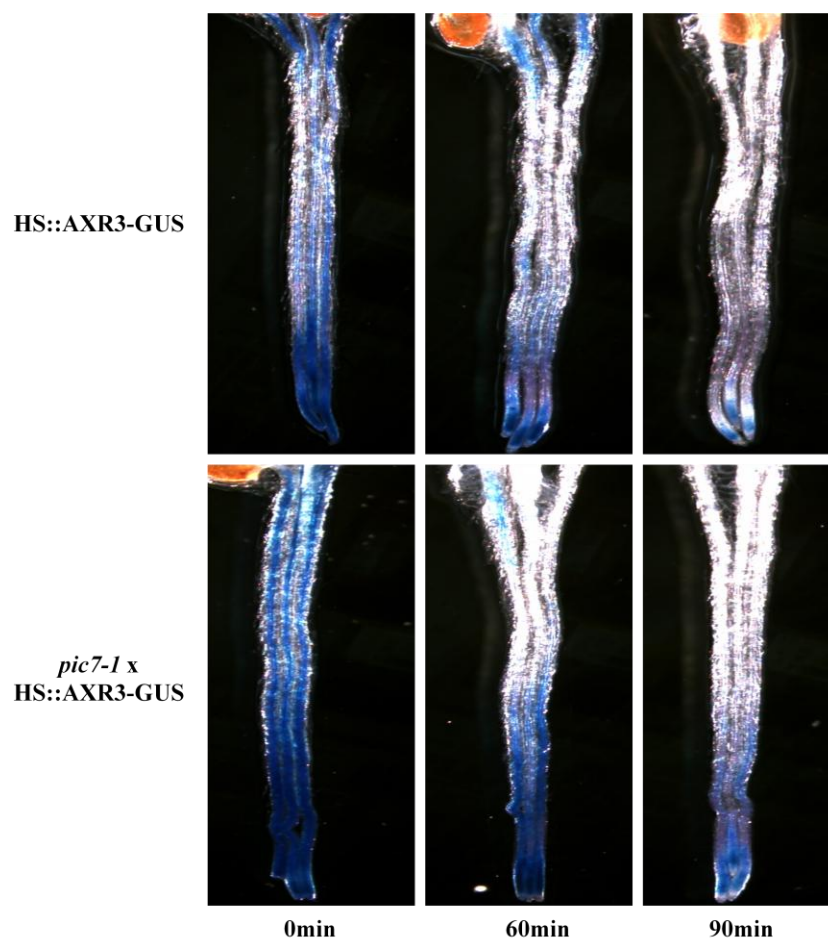
(Figure 10-continued)

(b)



(Figure 10-continued)

(c)



(Figure 10-continued)

(d)

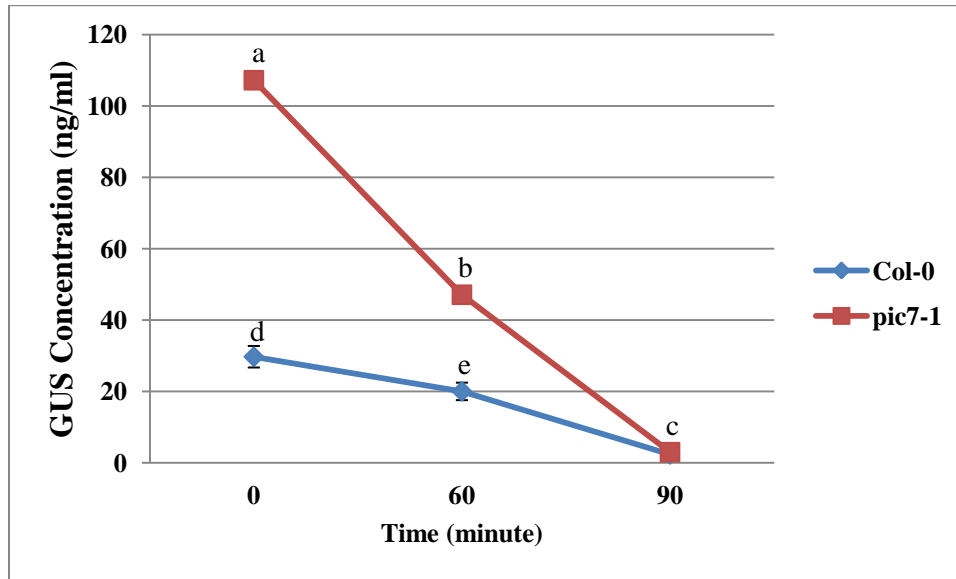


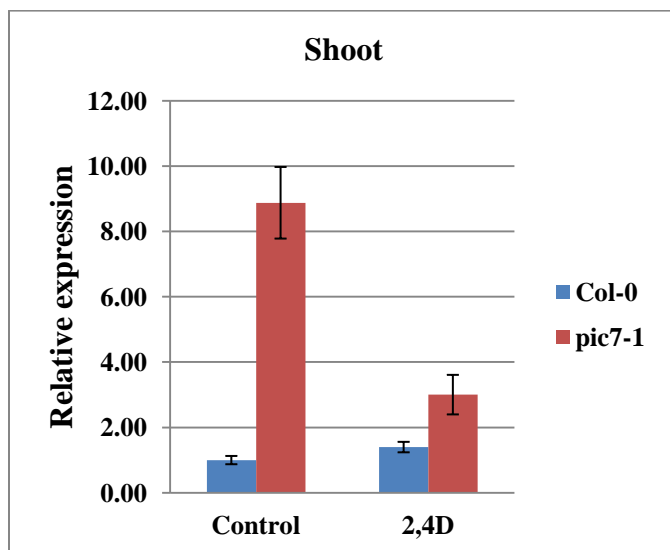
Figure 10. Degradation of AXR3NT-GUS in *pic7-1*. Four-day-old light grown wild type Col-0 and *pic7-1* seedlings carrying *HS::AXR3NT-GUS* were heat shocked for 2 hours and transferred to ATS at room temperature for further incubation. Seedlings were fixed after indicated time intervals and stained for GUS activity to indicate the degradation of AXR3NT-GUS in (a) shoot and (c) root. (b,d) For quantitative GUS assay, total protein was extracted from shoot and root separately at indicated time points and used in MUG assay as described in methods. (b) A two-way ANOVA indicated interaction between genotype and treatment ($F=26.99$; $df=1, 12$; $P<0.001$). Means with different letters “a, b, c” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. (d) A two-way ANOVA indicated interaction between genotype and treatment ($F=771.5$; $df=1, 12$; $P<0.001$). Means with different letters “a, b, c, d, e” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. Error bars indicate standard deviations from the mean.

Auxin induced gene expression in *pic7-1*

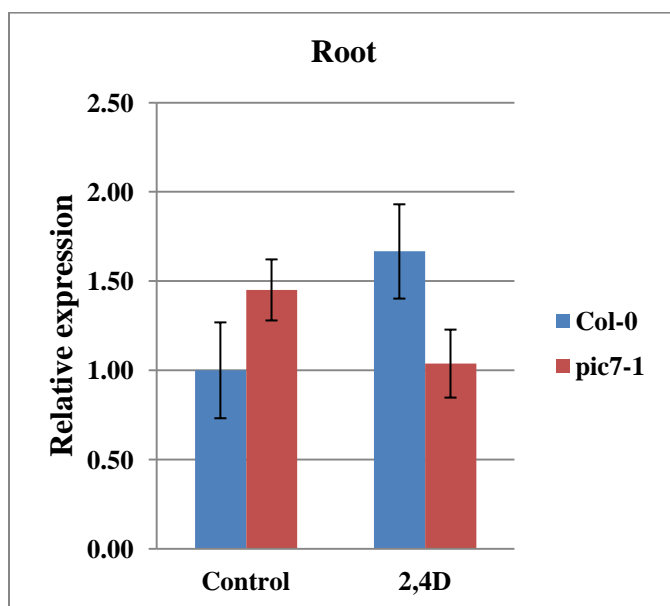
Aux/IAA, *SAUR* and *GH3* gene families are primary auxin responsive genes and the expression of these genes is induced within short period of time following auxin treatment (Calderon-Villalobos et al., 2010). To examine the effect of *pic7-1* mutation on the transcription of primary auxin responsive genes, transcript levels of *SAUR19*, *GH3-3* and *GH3-5* genes were analyzed using qRT-PCR using total RNA isolated from either shoots or roots of seven-day-old seedlings after 6 hours of 20 μ M 2,4-D treatment.

SAUR19 expression was differentially regulated in wild type and *pic7-1* (Figure 11a,b). Prior to the auxin treatment, transcript levels of *SAUR19* were higher in both shoot and root of *pic7-1* than wild type Col-0. But when *SAUR19* was induced by 2,4-D in wild type, it was down-regulated by auxin in *pic7-1* background. Similar to *SAUR19*, *GH3-3* and *GH3-5* gene expression levels were higher in *pic7-1* background originally (Figure 11c-f). Even though 2,4-D treatment greatly up-regulated *GH3-3* and *GH3-5* expression levels both in wild type and *pic7-1* background, the fold of induction was less in *pic7-1* compared to that of the wild type. Despite quantitative differences, induction patterns of *GH3-3* and *GH3-5* in wild type Col-0 were similar to that in *pic7-1* background in both shoot and root.

(a)

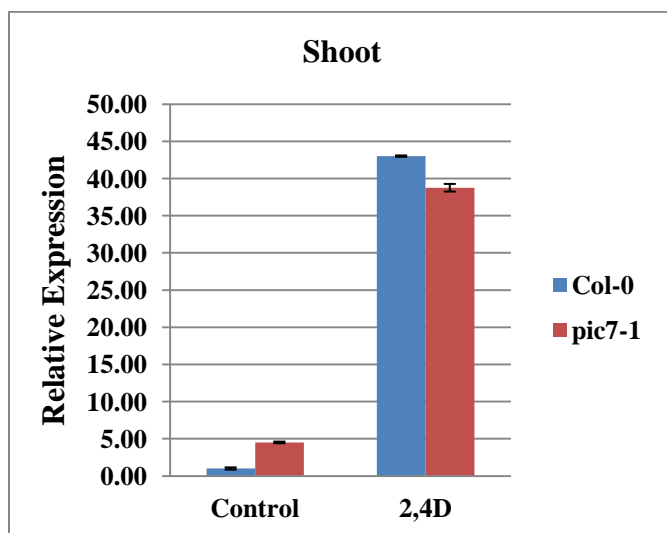


(b)

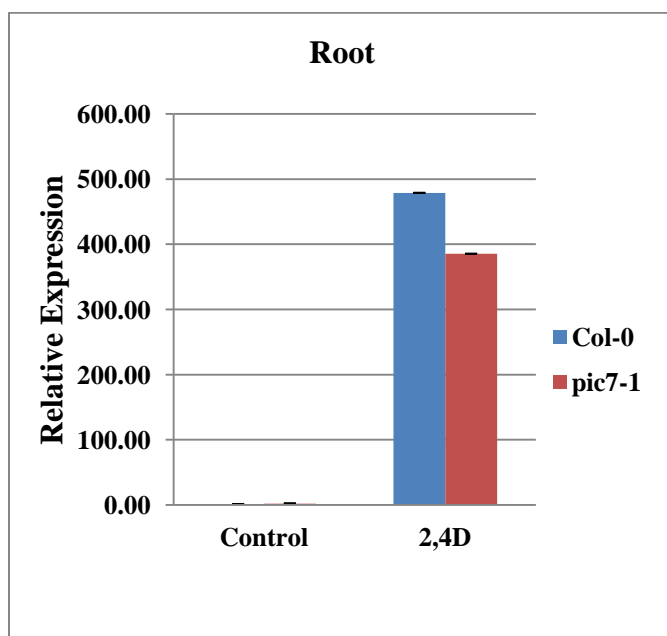


(Figure 11-continued)

(c)

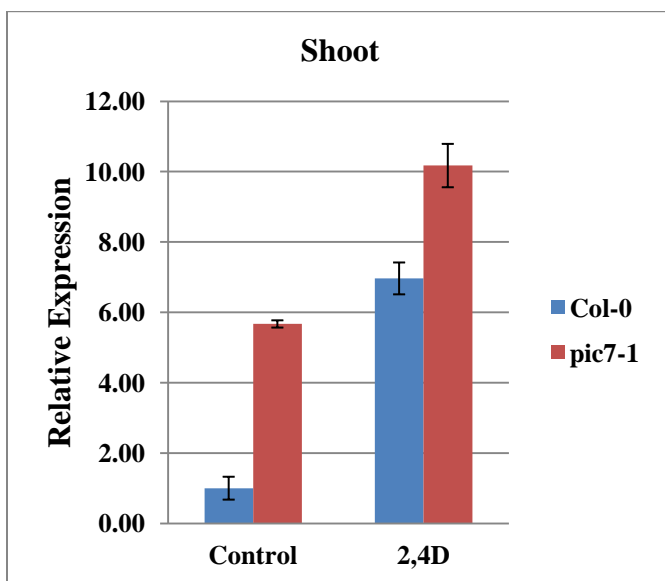


(d)



(Figure 11-continued)

(e)



(f)

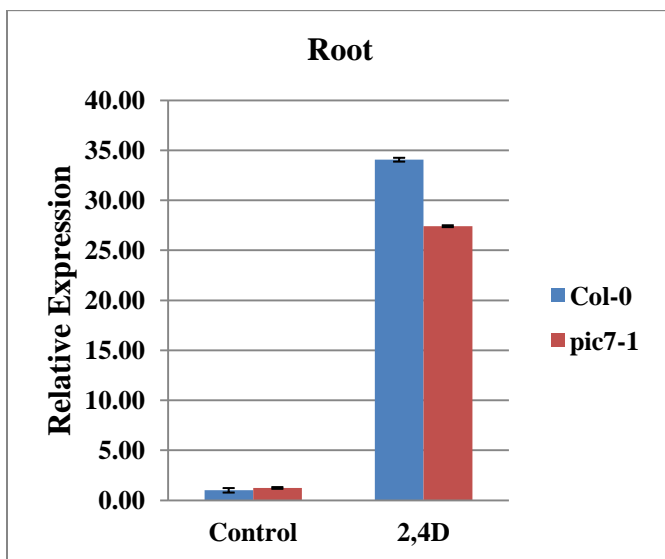


Figure 11. Quantitative RT-PCR analysis of primary auxin responsive genes. Total RNA was extracted from shoots and roots separately using seven-day-old wild type (Col-0) and

pic7-1 seedlings treated with 2,4-D for 6 hours in liquid ATS medium. Expression levels of *SAUR19* (a:shoot,b:root), *GH3-3* (c:shoot,d:root) and *GH3-5* (e:shoot,f:root) were examined. Relative mRNA levels of individual genes were normalized to *AtUBA* (*ATIG04850*) gene expression. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean.

***pic7-1* shows defects in lateral root, adventitious root and root hair development**

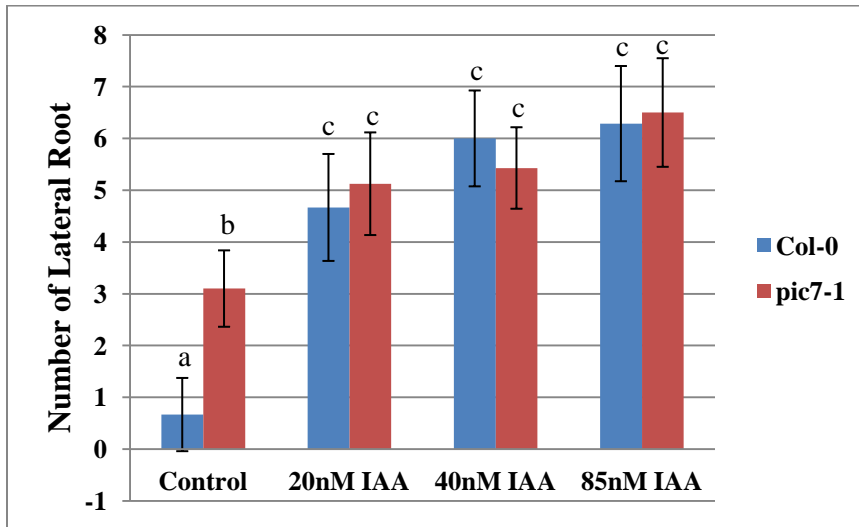
Lateral root development is found to be controlled by auxin, for example, a well-organized auxin transport is the prerequisite for normal lateral root formation (Reed et al., 1998; Bhalerao et al., 2002). To test the effect of auxin on lateral root development in *pic7-1*, four-day-old light grown seedlings were transferred onto ATS medium containing elevated concentration of natural auxin IAA. In un-supplemented media *pic7-1* had significantly more lateral roots compared to the wild type. IAA induced the number of lateral roots in both *pic7-1* and Col-0, but the degree of induction was obviously less in *pic7-1* compared to wild type (Figure 12a,b). Since the possibility that the less sensitivity of *pic7-1* to IAA was due to impaired uptake of IAA, a synthetic auxin NAA which doesn't need influx transporter (Yamamoto and Yamamoto, 1998) was also used to induce lateral roots (Figure 12c). Similar to IAA, level of lateral roots induction by NAA was still less in *pic7-1* compared to the wild type Col-0.

The secondary roots derived from the shoot are called adventitious roots. Adventitious roots are rare and emerge occasionally in the shoot system of dicotyledonous such as *Arabidopsis* (Osmont et al., 2007). Similar to lateral roots, *pic7-1*

had more adventitious roots compared to wild type when grown on un-supplemented media (Figure 12d,e).

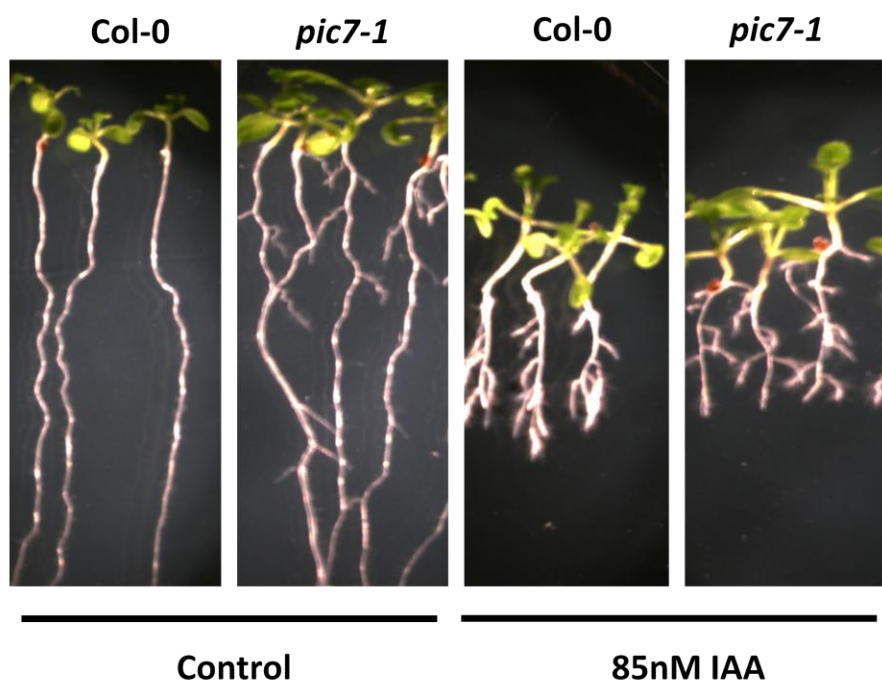
As for optimizing the absorption surface area and anchorage of root, epidermal originated root hair formation and development is yet another phenotype controlled by auxin (Masucci et al., 1994). Therefore, the root hair development was examined in *pic7-1*. The mutant produced strikingly less number of root hairs compared to Col-0. However, as low as 20 nM IAA treatment rescued the root hair deficiency of *pic7-1* similar to the wild type Col-0 under control condition. Additionally, 20 nM IAA treatment induced more root hair formation in *pic7-1* than in the wild type Col-0 (Figure 12f,g).

(a)

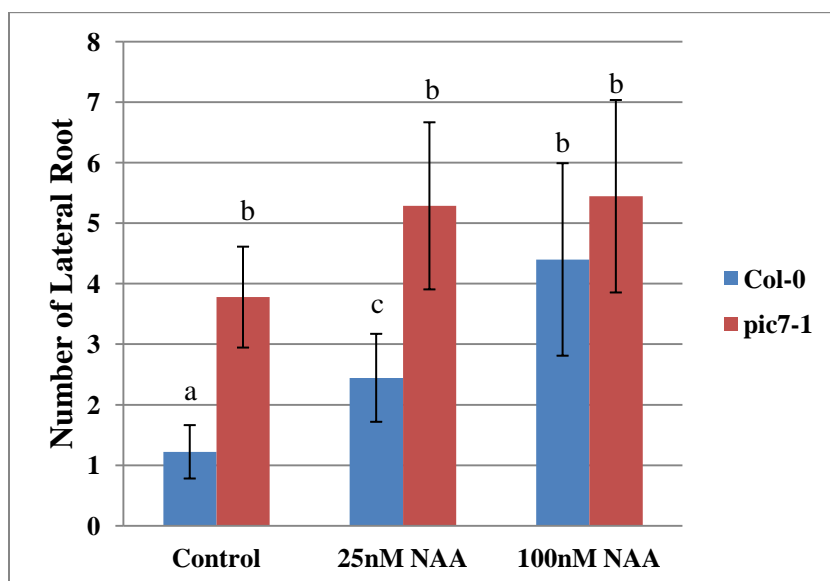


(Figure 12-continued)

(b)

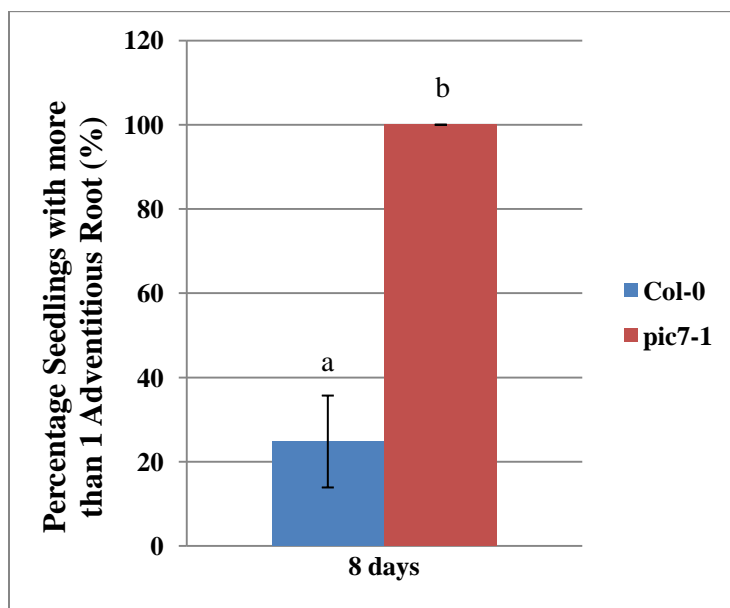


(c)



(Figure 12-continued)

(d)

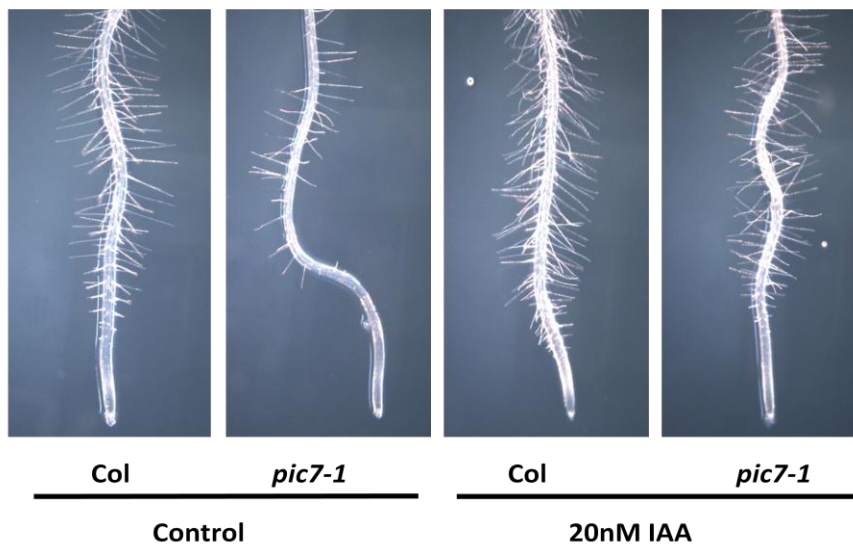


(e)



(Figure 12-continued)

(f)



(g)

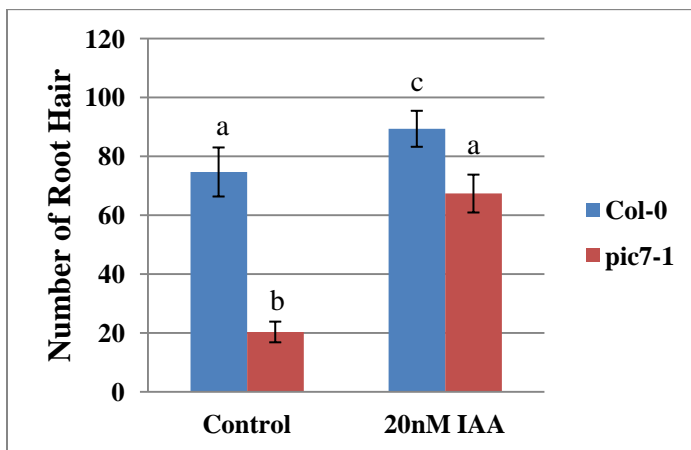


Figure 12. *pic7-1* shows defects in lateral root, adventitious root and root hair development. Four-day-old Col-0 and *pic7-1* seedlings were transferred onto indicated concentrations of IAA or NAA media and grown for another four days prior to counting the number of lateral roots or root hairs. (a,b) IAA induces lateral root formation. A two-

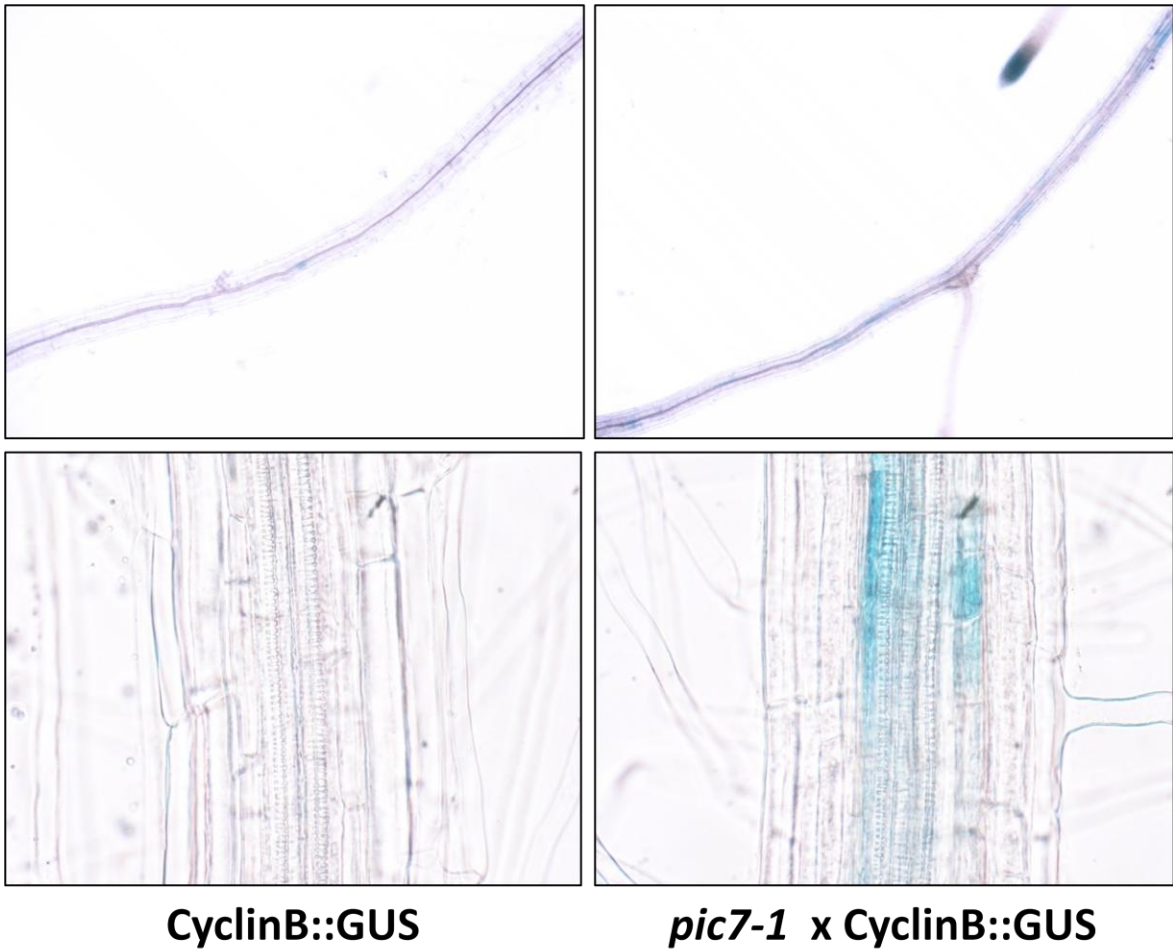
way ANOVA indicated interaction between genotype and treatment ($F=3.763$; $df=1, 40$; $P<0.05$). Means with different letters “a, b, c” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. (c) NAA induces lateral root formation. A two-way ANOVA comparing genotype and treatment revealed a significant main effect of genotype ($F=43.599$; $df=1, 36$; $P<0.001$) but indicated no interaction between genotype and treatment ($F=1.974$, n.s.). Means with different letters “a, b, c” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. (d,e) Increased number of adventitious root in *pic7-1*. Seedlings were grown for eight days on ATS media prior to the counting. Means with different letters “a, b” indicate statistically significant difference ($P < 0.05$), student t-test. (f,g) *pic7-1* produces less number of root hairs but IAA treatment recovers the defect. A two-way ANOVA indicated interaction between genotype and treatment ($F=19.56$; $df=1, 8$; $P<0.005$). Means with different letters “a, b, c” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. Error bars represent standard deviations from the means.

Cell division marker *CyclinB::GUS* activity was high in *pic7-1*

Since the *pic7-1* has more lateral roots compared to the wild type, mitotic marker *CyclinB::GUS* which has the peak GUS accumulation during the G2 phase in the cell cycle (Colon-Carmona et al., 1999) was crossed into *pic7-1* to study the cell division. After seedlings homozygous for both *pic7-1* and *CyclinB::GUS* were stained for GUS activity, it was apparent that numerous lateral roots with high GUS staining at the tip were emerged and developed to some extent in *pic7-1* while there were only a couple lateral root primordia in wild type Col-0. In addition, in wild type, GUS expression was

restricted to lateral root initiation sites, but *pic7-1* background had areas with high GUS expression along the primary root including lateral root primordia (Figure 13a). In Arabidopsis root, daughter cells generated by stem cell undergo additional divisions in the apical meristem (Scheres, 2007). There were also more *CyclinB::GUS* positive cells with higher GUS expression in the apical meristem of the *pic7-1* primary root compared to the wild type (Figure 13b). These results suggest enhanced cell division activity in *pic7-1*.

(a)



(Figure 13-continued)

(b)

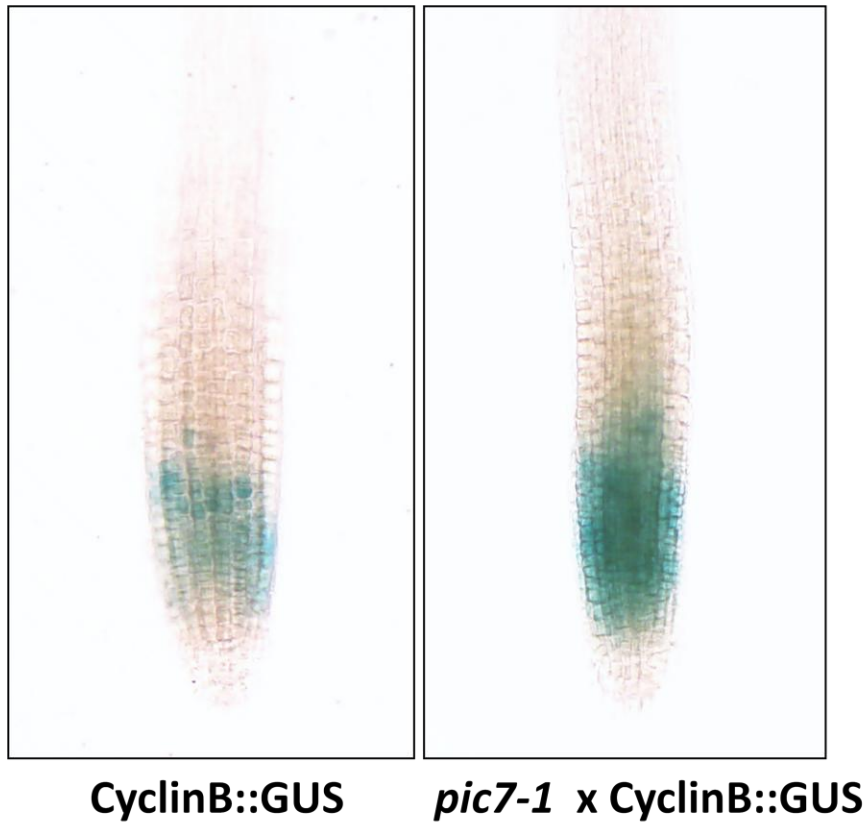
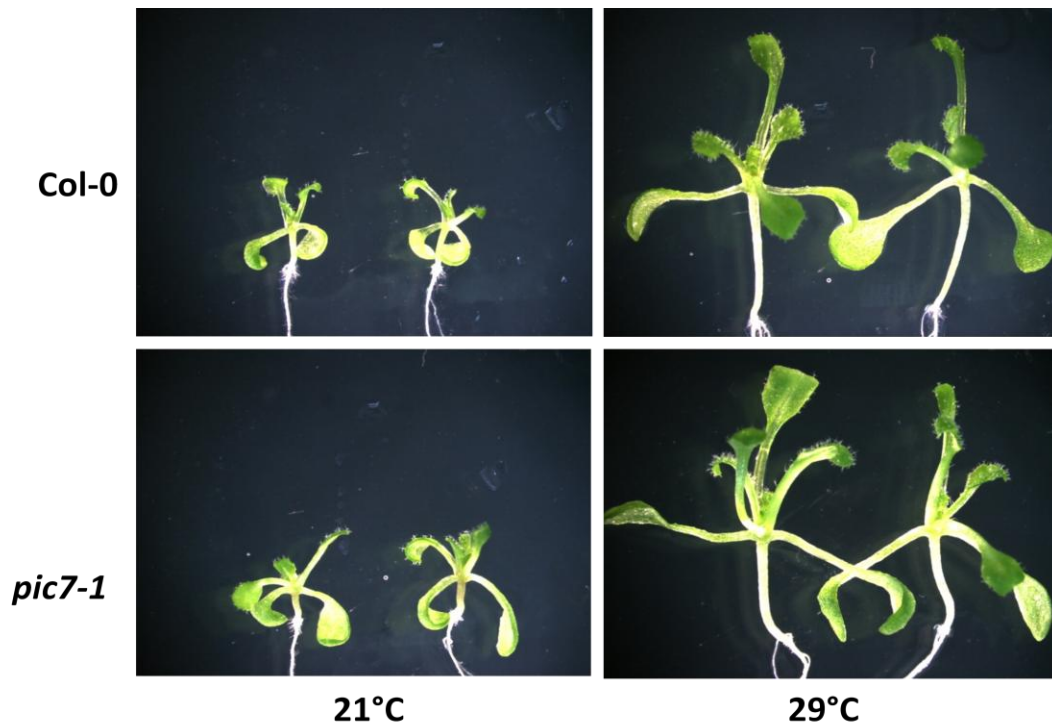


Figure 13. Increased *CyclinB::GUS* activity in *pic7-1*. (a) *pic7-1* has GUS expression along the primary root but in wild type GUS expression is restricted only to lateral root primordia. (b) *pic7-1* has very high GUS expression at the apical meristem area of the primary root. Seedlings homozygous for both *pic7-1* and *CyclinB::GUS* were grown on ATS media for eight days and stained for GUS activity.

High Temperature induced hypocotyl elongation was impaired in *pic7-1*

Previous studies have shown that *Arabidopsis* seedlings show dramatic hypocotyl elongation when grown at high temperatures due to increased auxin biosynthesis (Gray et al., 1998). To test whether *pic7-1* is defective in high temperature induced auxin biosynthesis, growth responses were tested at high temperatures (29°C) under continuous illumination. According to the results, high temperature failed to induce *pic7-1* hypocotyl elongation to the same extent as the wild type Col-0 (Figure 14a,b), suggesting defective high temperature induced auxin biosynthesis in mutant background.

(a)



(Figure 14-continued)

(b)

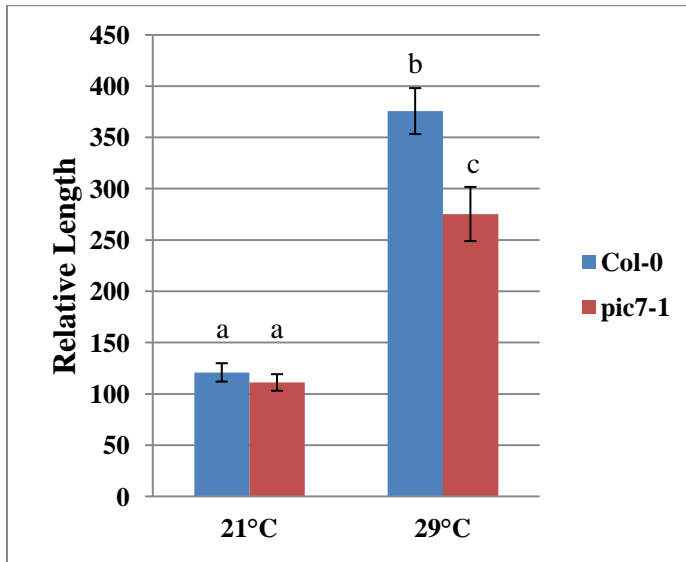


Figure 14. High temperature induced hypocotyl elongation is altered in *pic7-1*. (a,b)

Hypocotyl length of *pic7-1* is significantly shorter compared to the wild type at high temperature. Col-0 and *pic7-1* seedlings were grown on ATS media for nine days at 29°C and compared with the seedlings grown at 21°C. A two-way ANOVA indicated interaction between genotype and treatment ($F=41.41$; $df=1, 24$; $P<0.001$). Means with different letters “a, b” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. Error bars represent standard errors of the means.

Cytokinin responses were defective in *pic7-1*

Auxin and cytokinin are known to tightly crosstalk with each other in controlling many growth and developmental processes such as root development (Dello Ioio et al., 2008). Also due to the potential involvement of *APT1* in cytokinin metabolism in plant, *pic7-1* was tested for cytokinin response using both synthetic cytokinin, kinetin, and natural cytokinin, zeatin. Here primary root growth inhibition by high concentration of kinetin and zeatin were tested. *pic7-1* showed resistance to kinetin (Figure 15a) and zeatin (Figure 15b), where primary root length of wild type Col-0 was at least 70 % inhibited but *pic7-1* less inhibited. This suggests cytokinin signaling in *pic7-1* mutant is impaired.

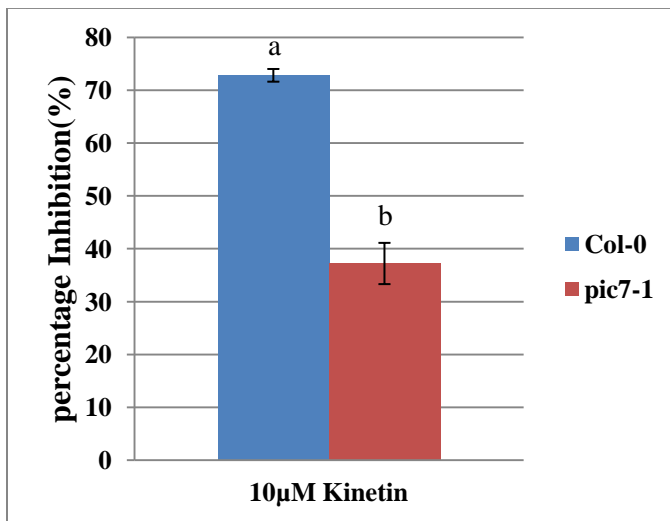
According to Deikman and Ulrich (1995), anthocyanin accumulation is induced by cytokinin. When grown in continuous light, *pic7-1* accumulated more anthocyanin than wild type Col-0. Treatment with 10 μ M kinetin induced the accumulation of anthocyanin in both wild type and *pic7-1* (Figure 16a,b), but the level of induction was higher in *pic7-1* than that in the wild type.

Chory et al., (1994) have reported that cytokinin induces de-etiolation in dark grown seedlings, which is evident by inhibition of hypocotyl elongation, petiole elongation, faster opening of cotyledons and development of true leaves. When *pic7-1* was treated with kinetin, it was significantly more resistant to the inhibition of hypocotyl elongation by kinetin than wild type (Figure 17). However, other de-etiolation phenotypes of *pic7-1* were similar to wild type.

Seed size is affected cytokinin. The cytokinin receptor triple mutant *ahk2-5/ahk3-7/cre1-2* has significantly larger seeds than wild type (Riefler et al., 2006). It is also

known that the early endosperm is the location with high cytokinin biosynthesis activity and expression of cytokinin responsive genes (Day et al., 2008). Therefore, the seed size of the *pic7-1* mutant was measured. In both length and width scales, seeds of *pic7-1* were larger compared to wild type Col-0 seeds (Figure 18a,b). Among many factors regulating cotyledon development, cytokinin controls the cotyledon size by regulating cell division and expansion (Stoynova-Bakolova et al., 2004). *pic7-1* had larger cotyledons compared to wild type Col-0 when grown on un-supplemented media (Figure 18c,d), suggesting the possibility of altered cytokinin signaling in *pic7-1*.

(a)



(Figure 15-continued)

(b)

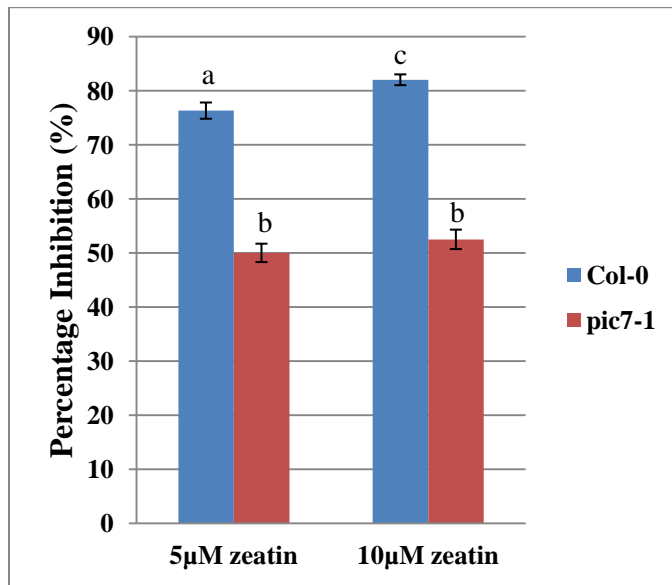


Figure 15. Primary root growth of *pic7-1* is resistant to natural and synthetic cytokinins. Four-day-old seedlings of *pic7-1* and wild type Col-0 grown on ATS media were transferred onto ATS medium containing (a) kinetin or (b) zeatin at indicated concentrations. Length of the primary root was measured following four days of incubation under continuous illumination at 21°C. (a) A two-way ANOVA indicated interaction between genotype and treatment ($F=111.72$; $df=1, 28$; $P<0.001$). Means with different letters “a, b” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. (b) A two-way ANOVA indicated interaction between genotype and treatment ($F=102.858$; $df=1, 42$; $P<0.001$). Means with different letters “a, b, c” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. Error bars represent standard errors of the means.

(a)



(b)

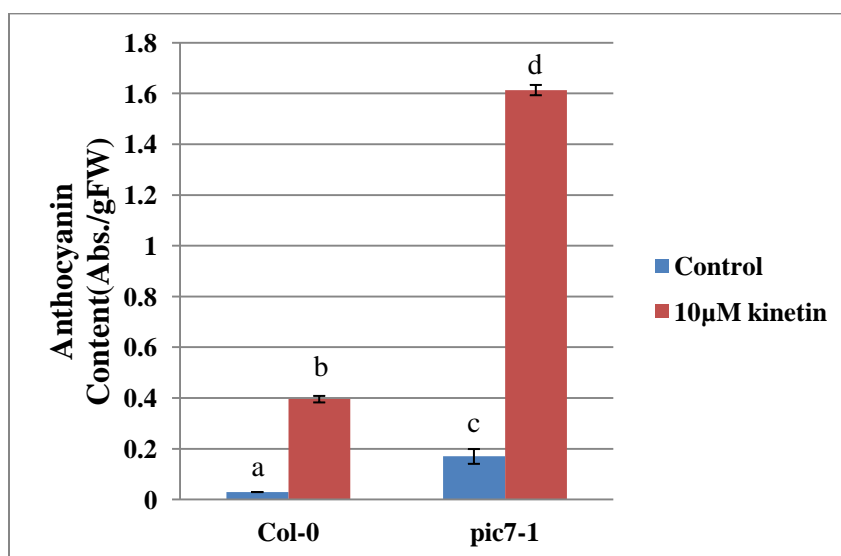
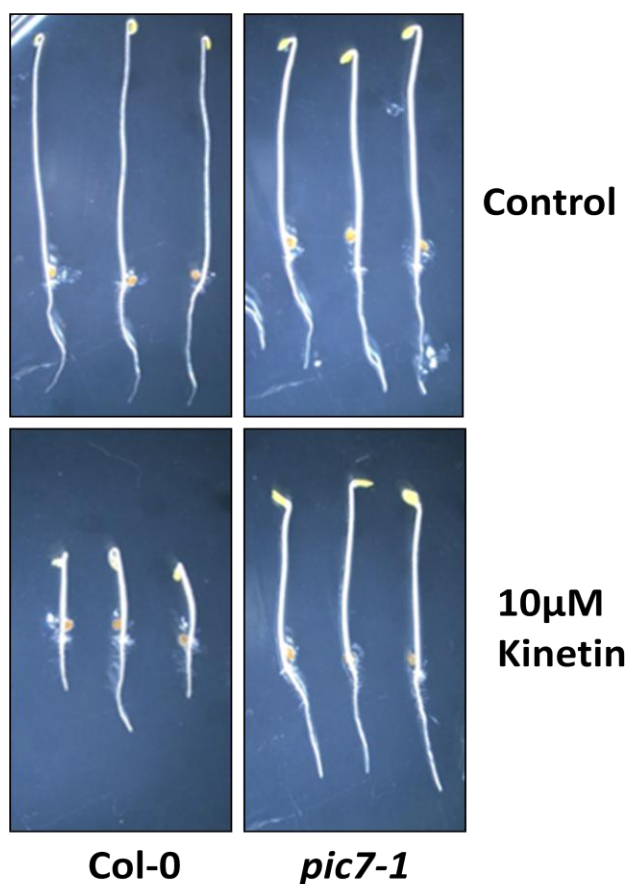


Figure 16. Enhanced cytokinin induced anthocyanin accumulation in *pic7-1*. (a) The mutant accumulates more anthocyanin than the wild type. (b) When treated with kinetin,

pic7-1 produces eight fold more anthocyanin than the wild type. Seedlings were grown on ATS media for four days and transferred onto ATS comprised of 10 μ M kinetin. Shoot was separated from root and anthocyanin was extracted and measured as described in methods. A two-way ANOVA indicated interaction between genotype and treatment ($F=2444$; $df=1, 8$; $P<0.001$). Means with different letters “a, b, c, d” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. Error bars represent standard deviations of the means.

(a)



(Figure 17-continued)

(b)

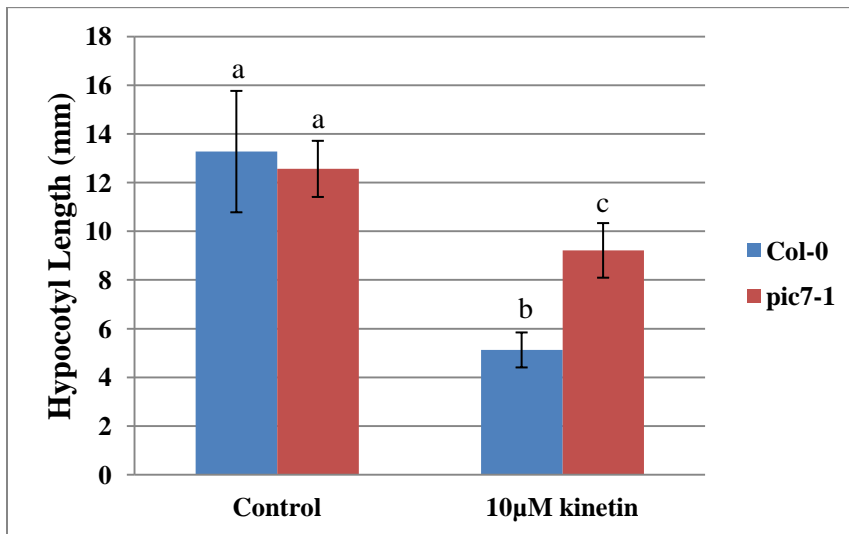
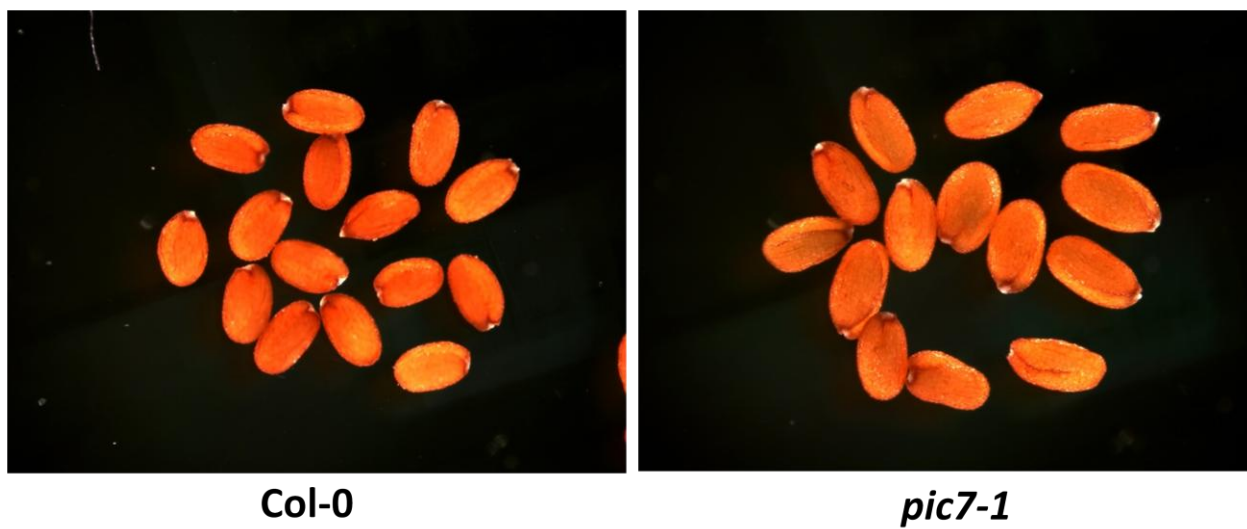


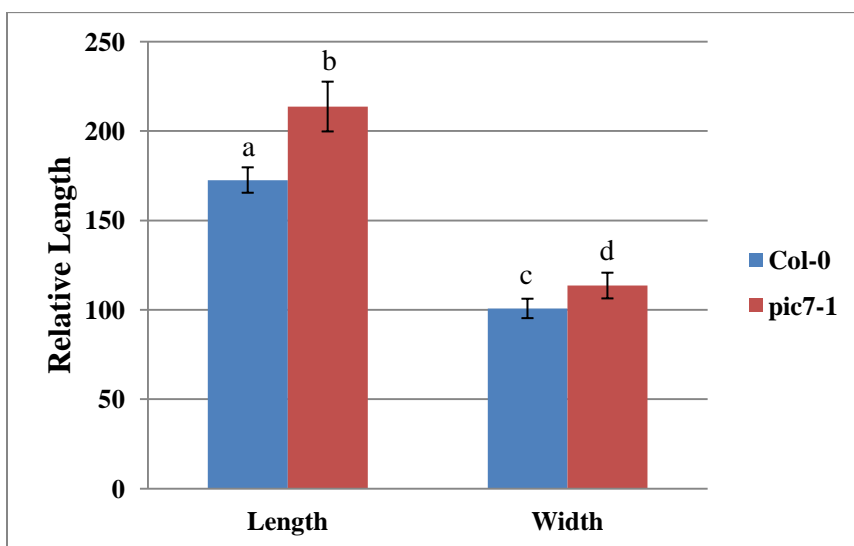
Figure 17. Cytokinin fails to completely de-etiolate dark grown *pic7-1*. (a,b) *pic7-1*

showed longer hypocotyls when grown in dark for four days on media containing 10 µM kinetin. A two-way ANOVA indicated interaction between genotype and treatment ($F=21.31$; $df=1, 36$; $P<0.001$). Means with different letters “a, b, c” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. Error bars represent standard deviations of the means.

(a)



(b)



(Figure 18-continued)

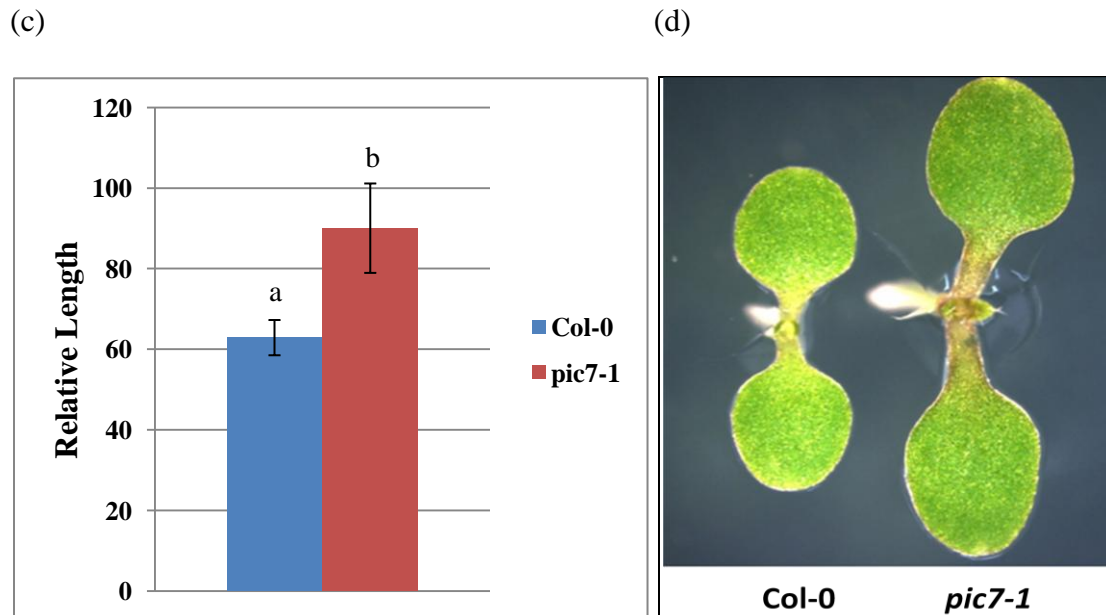


Figure 18. *pic7-1* has larger seeds and cotyledons than those of wild type. (a,b) Dried seeds were imaged using light microscope and length/width of the seeds was measured using imageJ software. Means with different letters “a, b, c, d” indicate statistically significant difference ($P < 0.05$), student t-test. (c,d) Seedlings were grown on ATS media for four days and imaged using light microscope. The length of the cotyledons was measured using imageJ software. Means with different letters “a, b” indicate statistically significant difference ($P < 0.05$), student t-test. Error bars represent standard deviations of the means.

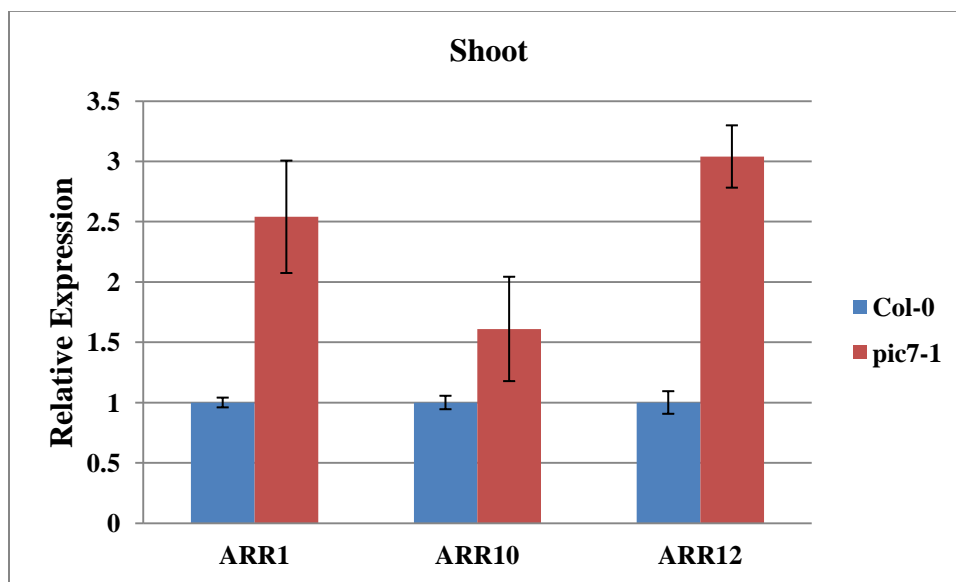
Cytokinin induced gene expression in *pic7-1*

Arabidopsis response regulators (ARRs) are transcription factors that respond to cytokinin and mediate its signaling (To et al., 2004; Mason, 2005). Among them three type-B ARRs, ARR1, ARR10 and ARR12 have been shown to display indispensable

functions in cytokinin signaling throughout Arabidopsis life cycle (Ishida et al., 2008). Since *pic7-1* revealed a series of severe defects in cytokinin responses, the transcript levels of *ARR1*, *ARR10* and *ARR12* were analyzed in *pic7-1* background using qRT-PCR. In roots of *pic7-1*, *ARR1* expression was higher than wild type Col-0 but there was little difference in *ARR10* and *ARR12* expression (Figure 19a). However in shoots, expression of all three *ARRs* was higher when compared to the wild type Col-0 (Figure 19b).

It is also known that cytokinin induces auxin biosynthesis by up-regulating the transcription of auxin biosynthesis genes (Jones et al., 2010). Therefore, transcript levels of cytokinin induced auxin biosynthesis genes, *CYP79B2*, *CYP79B3* and *NIT3* were analyzed in *pic7-1* background. Interestingly, transcript levels of all three genes were more abundant in mutant than in wild type, and interestingly the expression level difference between the mutant and wild type was greater in shoot than that in the root (Figure 20a,b).

(a)



(Figure 19-continued)

(b)

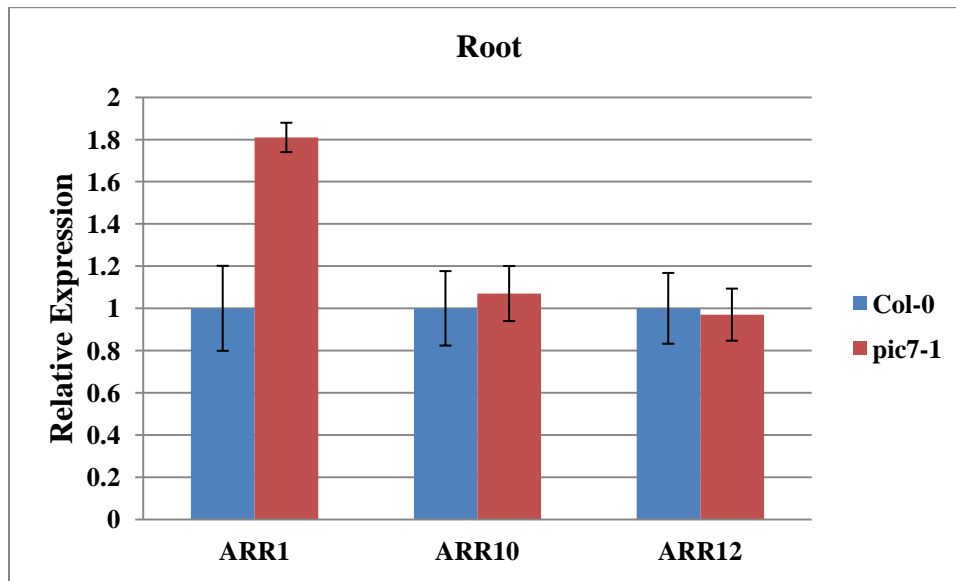
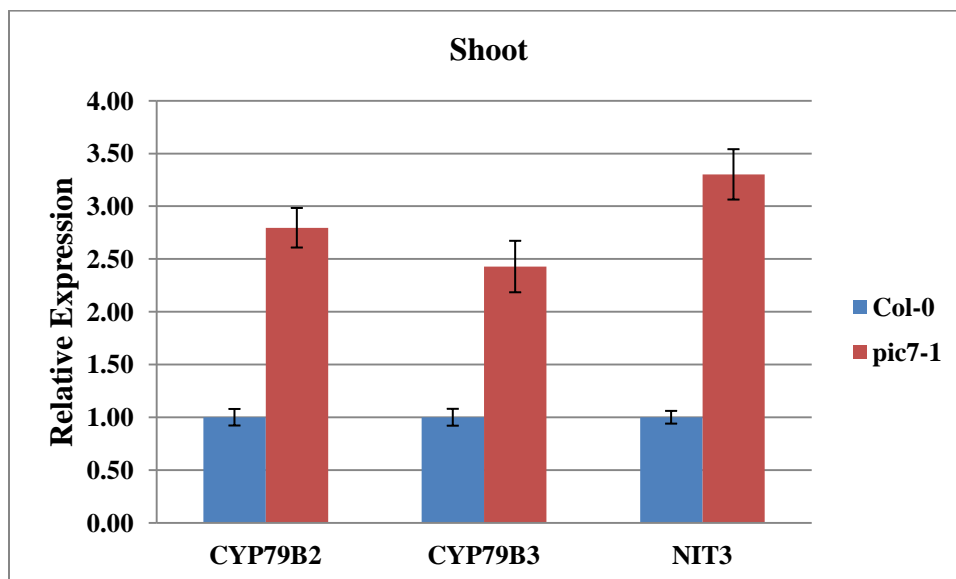


Figure 19. Real-time quantitative RT-PCR analysis of *ARRs* involved in cytokinin signaling. Total RNA was extracted from shoots and roots separately using seven-day-old wild type Col-0 and *pic7-1* seedlings. (a) Expression levels of *ARR1*, *ARR10* and *ARR12* in shoot were examined. (b) Expression levels of *ARR1*, *ARR10* and *ARR12* in root were examined. Relative mRNA levels of individual genes normalized to *AtUBA* (*AT1G04850*) gene are shown. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean.

(a)



(b)

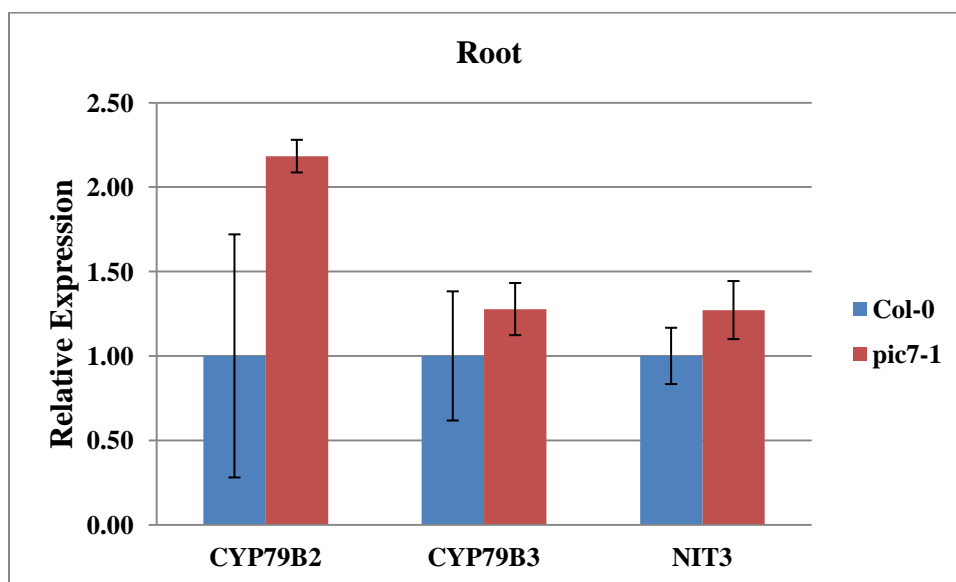


Figure 20. Real-time quantitative RT-PCR analysis of cytokinin induced auxin biosynthesis genes. Total RNA was extracted from shoots and roots separately using seven-day-old wild type Col-0 and *pic7-1* seedlings. (a) Expression level of *CYP79B2*,

CYP79B3 and *NIT3* in shoot were examined. (b) Expression level of *CYP79B2*, *CYP79B3* and *NIT3* in root were examined. Relative mRNA levels of individual genes normalized to *AtUBA* (*AT1G04850*) gene are shown. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean.

Ethylene signaling in *pic7-1*

Previous studies have shown that some auxin-responsive mutants exhibit cross-resistance to the plant hormone ethylene (Pickett et al., 1990). To test whether *pic7-1* also shows the similar trait, seedlings were exposed to the precursor of ethylene, 1-aminocyclopropane-carboxylic acid (ACC) and primary root growth inhibition by ethylene was tested. At 1 μ M ACC, primary root of wild type Col-0 was inhibited by 65% while *pic7* was only inhibited by 35% (Figure 21). The classical ethylene response that has been studied is the triple response shown by dark grown seedlings in the presence of ethylene. Exaggeration of apical hook, inhibition of the hypocotyl and radial swelling of the hypocotyls are considered as triple response (Guzma'n and Ecker, 1990). When *pic7-1* seedlings were grown in dark in the presence of 10 μ M ACC, non-exaggerated apical hook and less shortened hypocotyls compared to wild type were observed (Figure 22a,b). Also, the apical hook opens up faster when dark grown wild type Col-0 seedlings are treated with ethylene signaling inhibitor AgNO₃ (De Grauwe et al., 2005). In *pic7-1*, the apical hook was partially open in response to AgNO₃, suggesting that it is less sensitive to the inhibitor (Figure 22a). In addition, similar to auxin, ethylene is known to induce root hair formation in Arabidopsis roots (Rahman et al., 2002). ACC treatment rescued the root hair deficiency in *pic7-1* similar to the level of wild type Col-0 under control

condition and also induced more root hair formation in *pic7-1* than in wild type Col-0 (Figure 23a,b). Altogether these results suggest that the ethylene signaling is dampened in *pic7-1* mutant background.

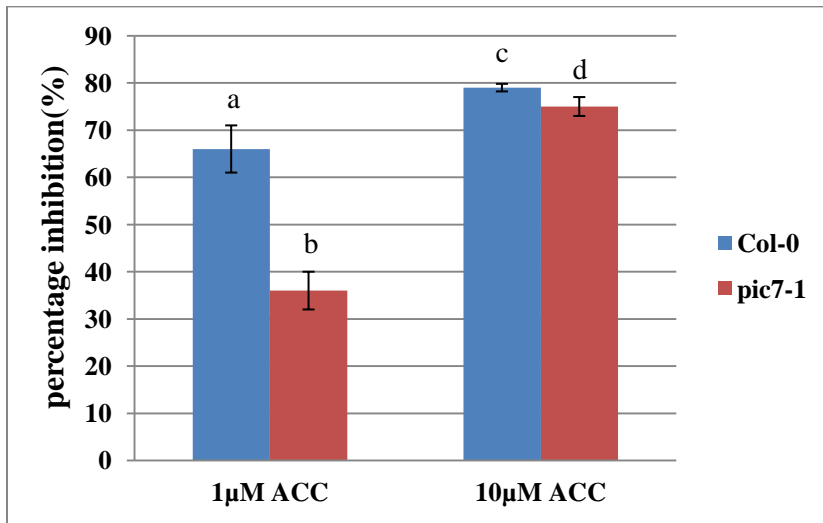
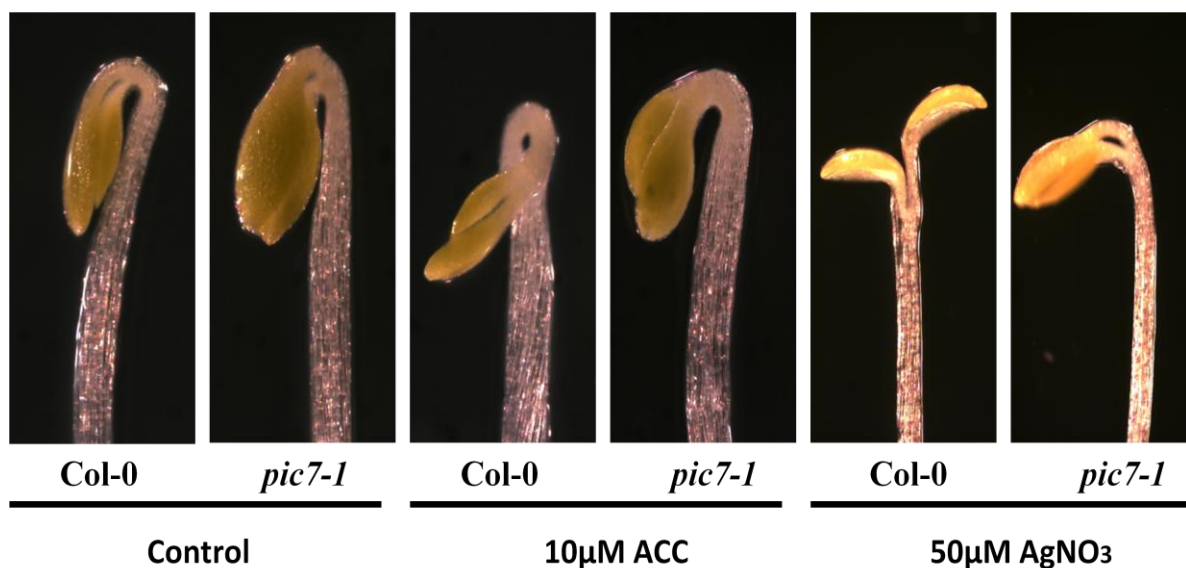


Figure 21. Primary root growth of *pic7-1* is resistant to low concentrations of ethylene precursor ACC. Four-day-old seedlings of *pic7-1* and wild type Col-0 grown on ATS media were transferred onto ATS media containing different concentrations of ACC. Length of the primary root was measured following four days of incubation under continuous illumination at 21°C. A two-way ANOVA indicated interaction between genotype and treatment ($F=47.38$; $df=1, 48$; $P<0.001$). Means with different letters “a, b, c, d” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. Error bars represent standard errors of the means.

(a)



(b)

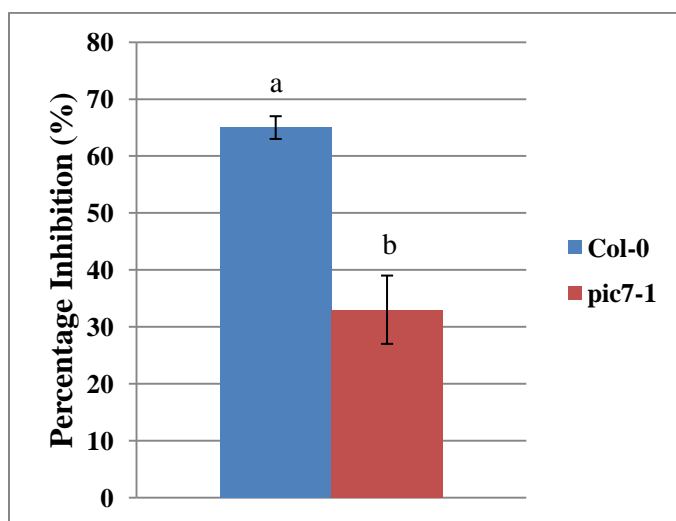
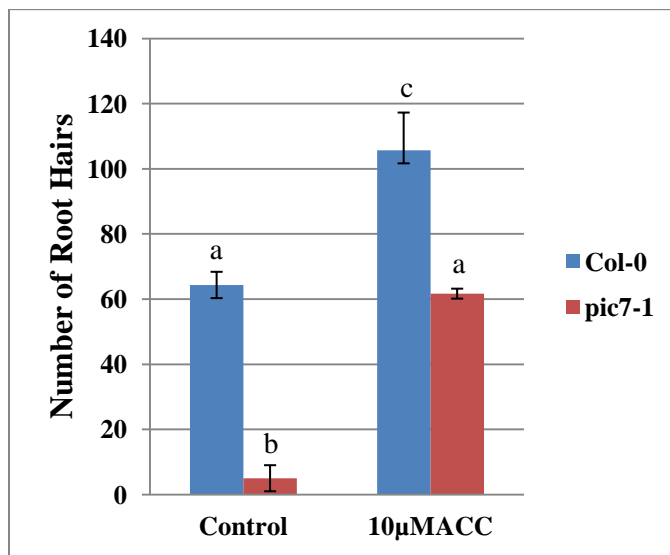


Figure 22. Ethylene signaling is defective in *pic7-1*. (a) Apical hook formation in *pic7-1*. Ethylene fails to exaggerate apical hook in *pic7-1* compared to wild type Col-0. The ethylene signaling inhibitor AgNO₃ opens up the apical hook in wild type completely but only partially in *pic7-1*. (b) Hypocotyl of *pic7-1* was less sensitive to ACC inhibition of

hypocotyl elongation than wild type Col-0. Seedlings were grown on ATS, or ATS containing 10 μ M ACC or 50 μ M AgNO₃ for four days in dark. A two-way ANOVA indicated interaction between genotype and treatment ($F=14.892$; $df=1, 16$; $P<0.005$). Means with different letters “a, b” indicate statistically significant difference ($P < 0.05$), Tukey’s HSD. Error bars represent standard errors of the means.

(a)



(Figure 23-continued)

(b)

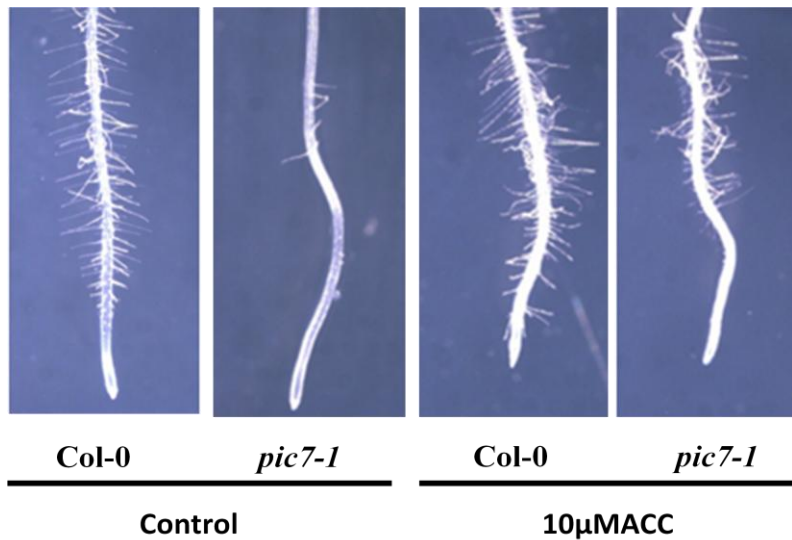


Figure 23. Ethylene induced root hair formation is defective in *pic7-1*. (a,b) *pic7-1* seedlings had less number of root hairs when grown on control media. ACC treatment induced the root hair formation in both wild type and mutant. And the level of induction was more in *pic7-1* compared to the wild type. Seedlings were grown on ATS media for four days and transferred onto ATS media containing 10 μM ACC for additional four days growth. The number of root hairs (from root tip to 5 mm above it) was counted using image J software. A two-way ANOVA comparing genotype and treatment revealed a significant main effect of genotype ($F=189.546$; $df=1, 8$; $P<0.001$) but indicated no interaction between genotype and treatment ($F=4.174$, n.s.). Means with different letters “a, b, c” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. Error bars represent standard deviations of the means.

Absciscic acid responses are impaired in *pic7-1*

Absciscic acid is a plant hormone that acclimates under certain stresses and mediates stress signaling as well as plant tolerance to stresses (Xiong et al., 2002). It has been reported many auxin resistant mutants also show defects in ABA responses suggesting the crosstalk between auxin and ABA signaling (Strader et al., 2008; Berlin et al., 2009). To test whether *pic7-1* is also involved in this interaction, primary root growth inhibition by ABA was examined as described in methods. *pic7-1* showed resistance to all the ABA concentrations tested in contrast to wild type (Figure 24).

Delaying or blocking germination and early seedling development is a well known characteristic of ABA (Lopez-Molina et al., 2001). Hence the germination inhibition and post-germination arrest of *pic7-1* was tested on a medium containing high concentration of ABA. While *pic7* did not differ from wild type Col-0 in ABA inhibition of germination, it was more resistant to post-germination arrest as shown by percentage of green cotyledons (Figure 25a).

Salinity and osmotic stresses are two major stresses that rapidly increase the ABA level in the cell and regulate stress responsive genes through ABA in the plants (Xiong et al., 1999b; Zhang et al., 2006). Hence post-germination arrest of *pic7-1* was tested against salinity stress induced by NaCl and osmotic stress promoted by mannitol. Intriguingly, *pic7-1* was resistant to both salinity and osmotic stresses (Figure 25b,c).

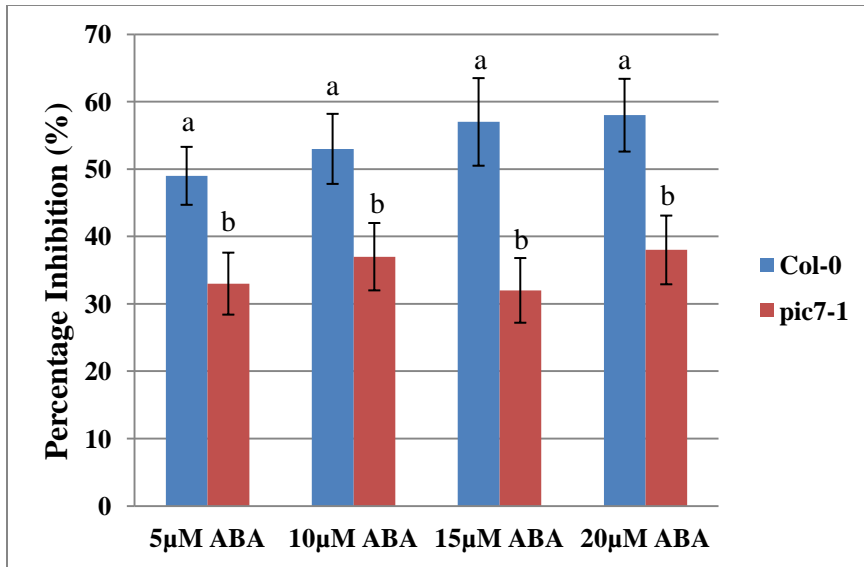
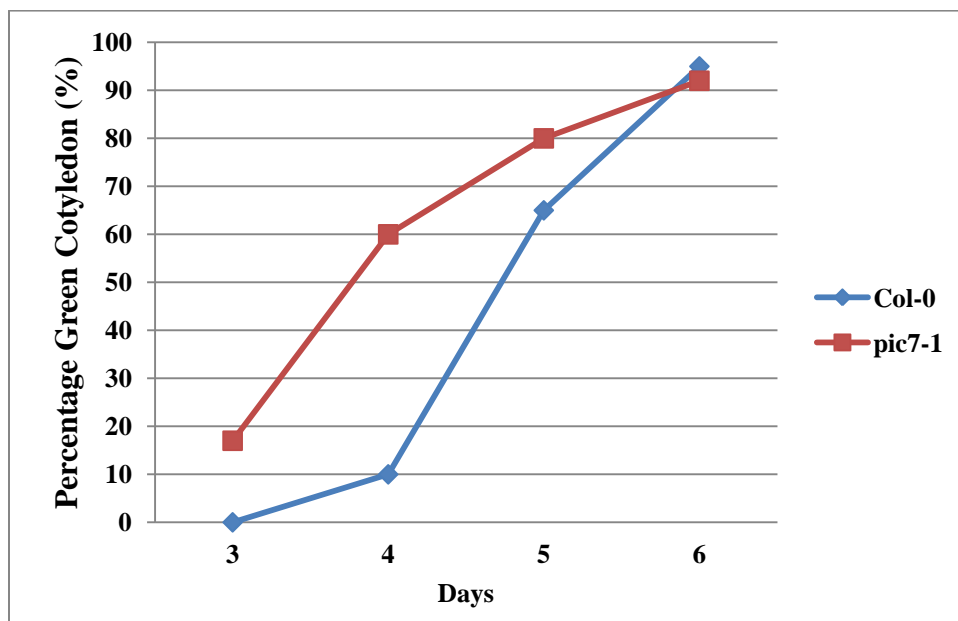
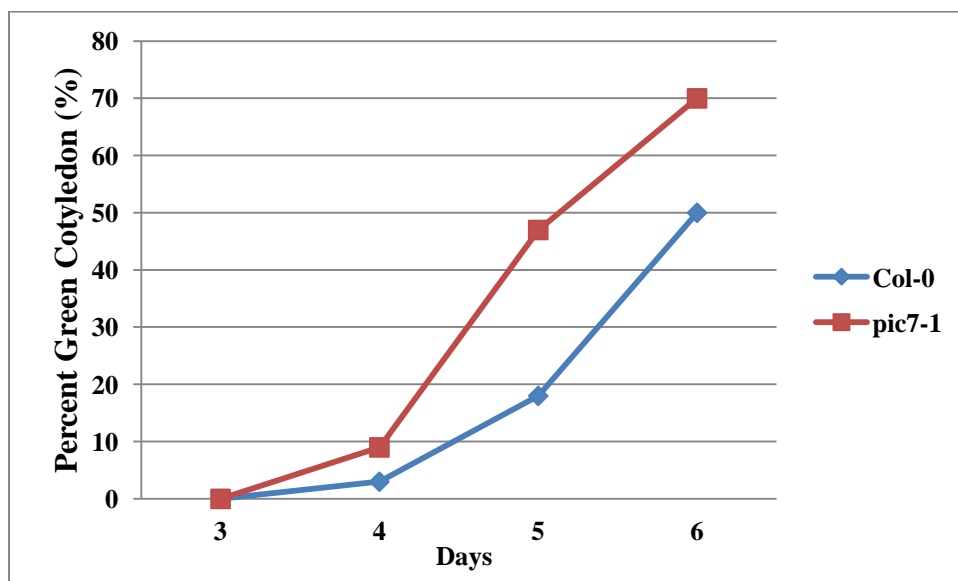


Figure 24. Primary root growth of *pic7-1* is resistant to ABA. Four-day-old seedlings of *pic7-1* and wild type Col-0 grown on ATS media were transferred onto ATS medium containing different concentrations of ABA. Length of the primary root was measured following four days of incubation in a growth chamber under continuous illumination at 21°C for calculating the percentage inhibition as described in methods. A two-way ANOVA indicated interaction between genotype and treatment ($F=6.073$; $df=1, 70$; $P<0.001$). Means with different letters “a, b” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. Error bars represent standard errors of the means.

(a)

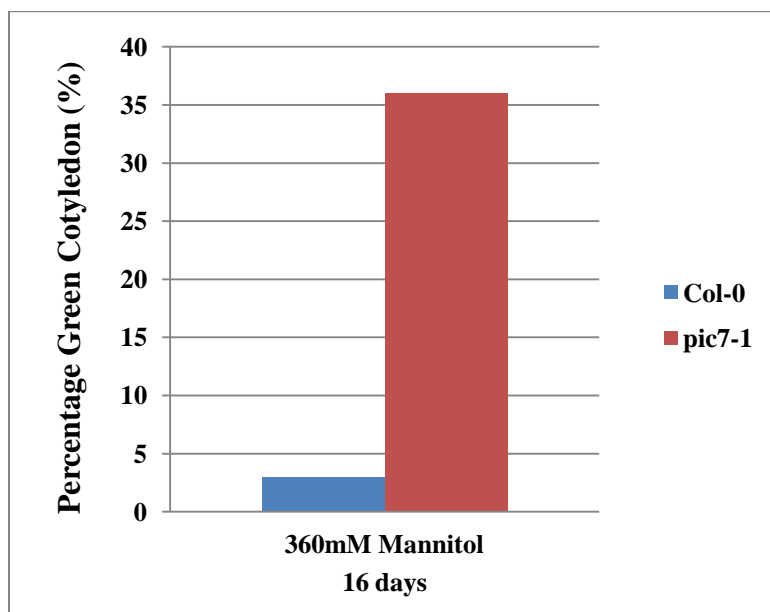


(b)



(Figure 25-continued)

(c)



(d)

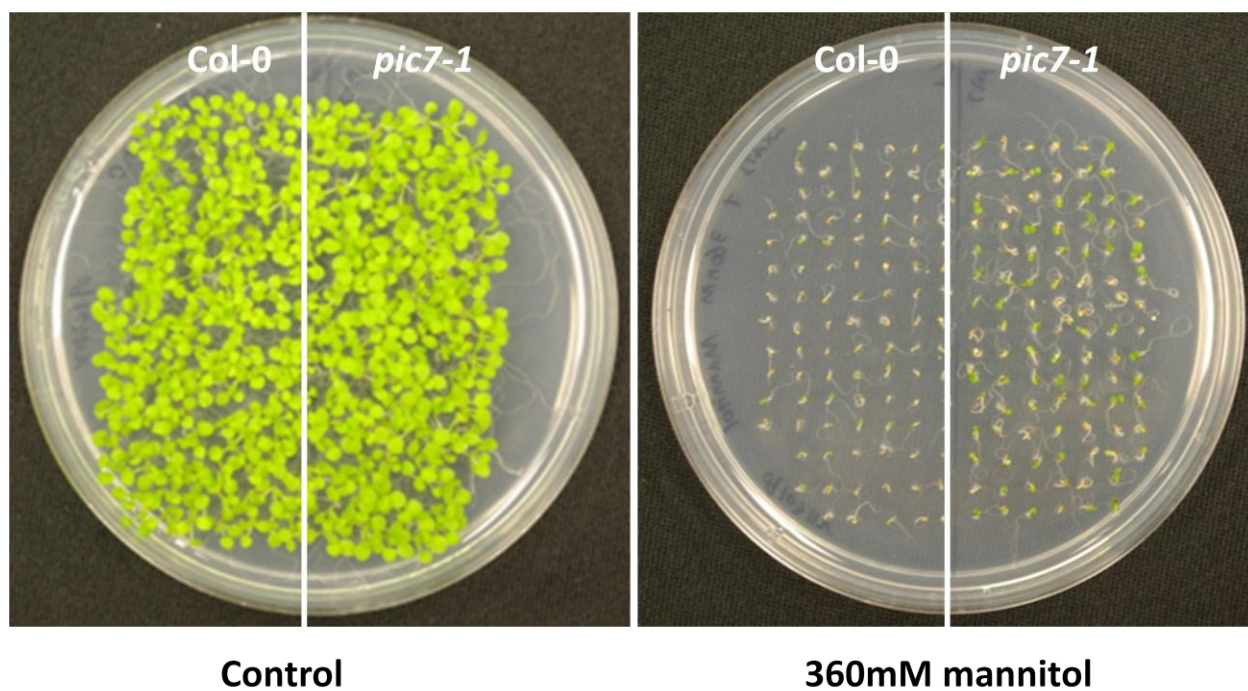


Figure 25. *pic7-1* is resistant to ABA, NaCl and mannitol induced post-germination inhibition. Seeds were sterilized and plated on ATS media containing (a) 0.5 μ M ABA, (b) 160 mM NaCl or (c,d) 360 mM Mannitol. Seeds stratified for two days at 4°C were grown horizontally at 21°C for sixteen days under continuous illumination. Seedlings producing green cotyledons were counted after indicated time periods of growth and calculated as a percentage of germinated seedlings.

Gibberellin responses are defective in *pic7-1*

According to previous findings, gibberellic acid (GA) antagonizes ABA (Razem et al., 2006) and shows crosstalk with auxin at metabolic and signaling level (Fu and Harberd, 2003; Nemhauser et al., 2006). Hence the germination of *pic7-1* was examined on media containing high concentrations of GA₃, which is a natural form of active GA. When wild type Col-0 was greatly inhibited, *pic7-1* showed high resistance to GA in germination inhibition (Figure 26). GA is also known to enhance the hypocotyl elongation of light grown seedlings by influencing the cell elongation (Cowling and Harberd, 1999). At 1 μ M GA₃, *pic7-1* hypocotyl was longer than wild type, suggesting its hypersensitivity to GA in hypocotyl elongation (Figure 27).

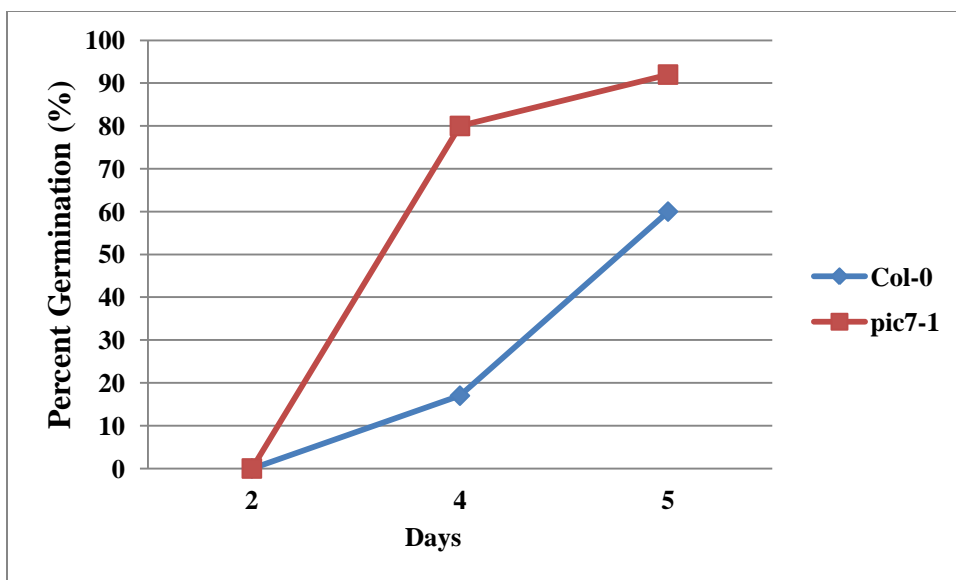


Figure 26. *pic7-1* is resistant to high concentration of GA induced germination inhibition.

Seeds were sterilized and plated on ATS media containing 50 μM GA₃. Seeds that were stratified for two days at 4°C were grown at 21°C horizontally for five days under continuous illumination. Radical protrusion was counted as germination every day up to five days of growth and calculated as a percentage of total seeds number.

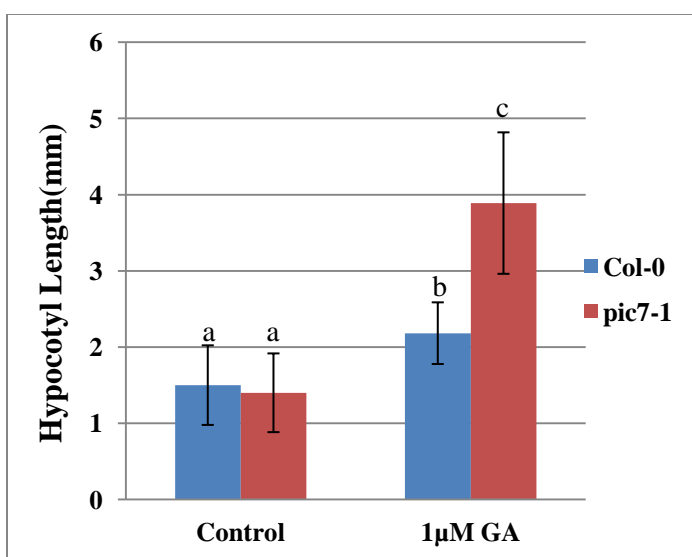


Figure 27. Induction of hypocotyl elongation in *pic7-1* by GA. Low concentrations of GA₃ induces the *pic7-1* hypocotyl elongation more than that of the wild type. Seedlings were grown on ATS or ATS containing 1 μ M GA for seven days and hypocotyl length was measured using imageJ software. A two-way ANOVA indicated interaction between genotype and treatment (F=20.28; df=1, 32; P<0.001). Means with different letters “a, b, c” indicate statistically significant difference (P<0.05), Tukey’s HSD. Error bars represent standard deviations of the means.

The substrate binding domain of APT1 is highly conserved

The mutation in *pic7-1* lies on the substrate-PRPP (5-Phosphoribosyl 1-pyrophosphate) binding domain of APT1 suggesting a possible alteration in catalytic activity of the enzyme (Thiemann et al., 1998). In *Arabidopsis thaliana*, *APT* family consists of five members and all share the highly conserved PRPP binding domain (Figure 28).

Tissue specific expression pattern and localization of APT1

In order to study the expression pattern and localization of APT1 in Arabidopsis *APT1::APT1-GUS* and *APT1::APT1-GFP* transgenic constructs in wild type Col-0 background were used. According to the GUS expression data, APT1 localized mainly in the vascular tissue throughout the plant (Figure 29a-c,g) and were absent in the apical meristem (Figure 29e,f). In addition to the vascular localization, high level of APT1-GFP was also observed in the pericycle of the root (Figure 31b) as well as in the elongation zone and apical meristem of the root tip (Figure 31a). Less APT1-GFP expression was

evident in the epidermal cells (Figure 31e). In both GUS and GFP translational gene constructs, high level of expression was seen in the dividing cells of the lateral root primordia (Figure 30a-g,33a-e). After the lateral root emergence, APT1-GFP protein localized mainly to the vascular tissue, pericycle and the tip of the secondary root (Figure 33f,g). APT1-GUS localized similar to APT1-GFP, except the absence in the apical meristem of lateral roots (not shown). In the shoot, protein was highly present just below the shoot apex, at the shoot root juncture and in guard cells (Figure 29a,d,31c,d). According to sub-cellular localization of APT1-GFP, it was localized to the nucleus and cytoplasm of the cell (Figure 32). In dividing cells, APT1 expressed throughout the cytoplasm and nucleus (Figure 32a-e). However in differentiated cells it mainly localized in the nucleus and also aggregated along the cell membrane visualized as bright spots (Figure 32f,g).

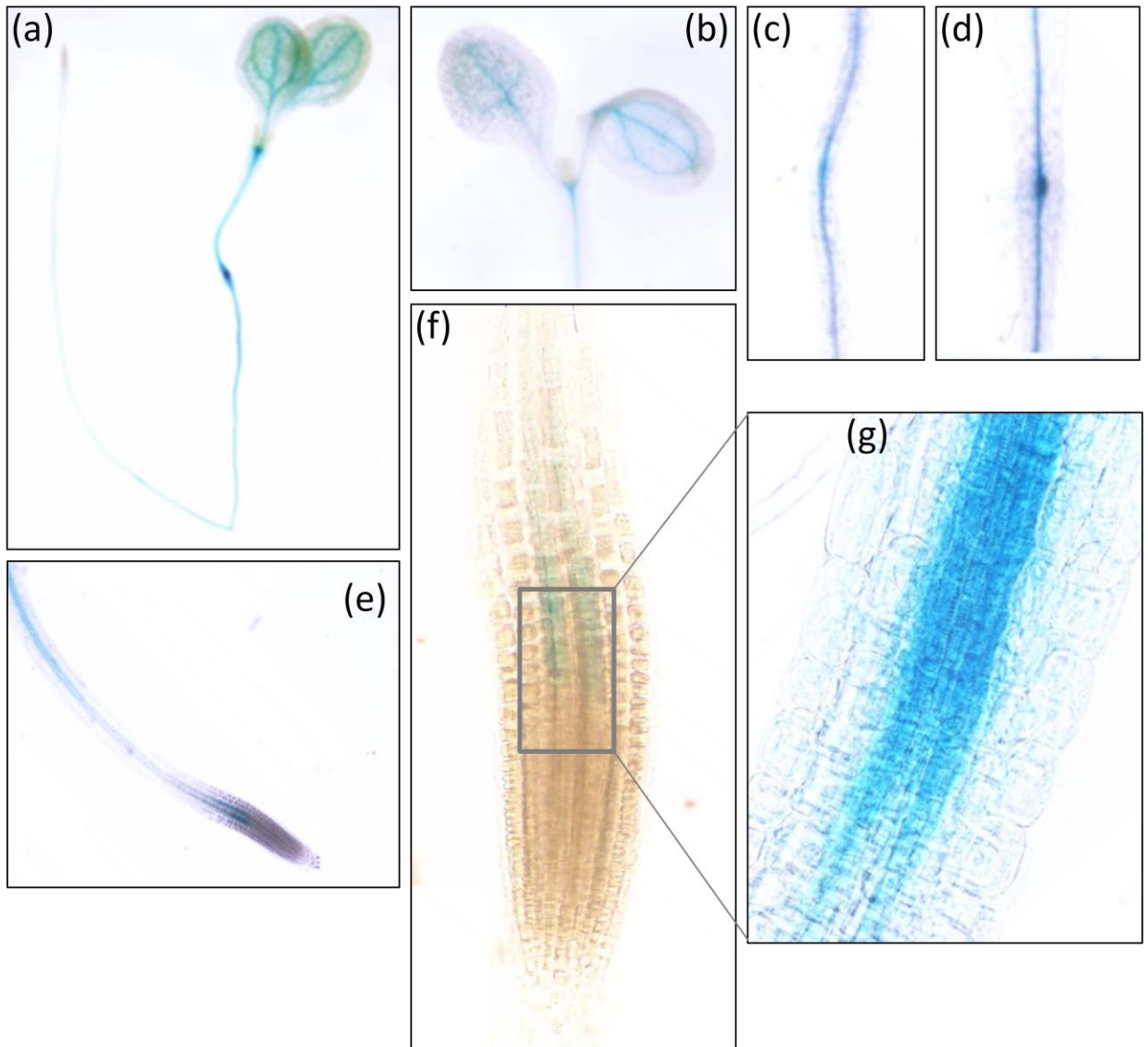


Figure 29. Expression pattern of APT1-GUS in five-day-old Arabidopsis seedlings. (a) APT1-GUS expresses mainly in the vascular tissue of the seedlings. Enlarged images show the APT1-GUS expression in vasculature of (b) cotyledon, (d) shoot and (c,e) root. (d) It is also highly present in shoot-root junction. (f,g) APT1-GUS expresses in the basal meristem of the root tip area where cells start expanding.

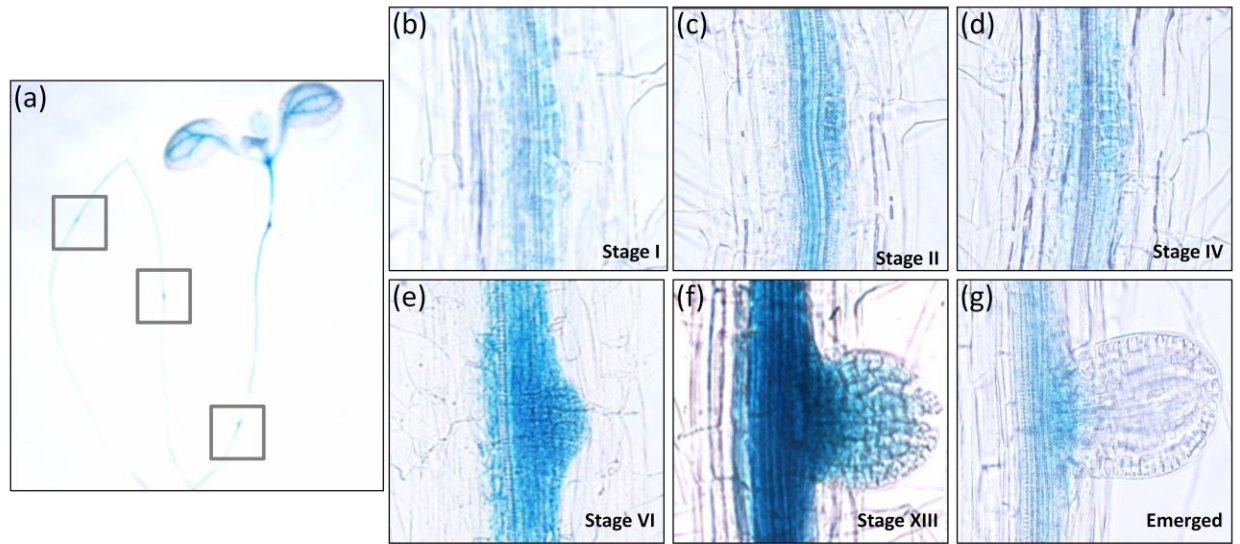


Figure 30. Expression of APT1-GUS in lateral root primordia. (a) APT1-GUS is highly expressed in the lateral root initiation sites visualized as dark blue spots along the root. (b-d) Magnified images show the expression of APT1-GUS in dividing cells at different stages of lateral root primordial development.

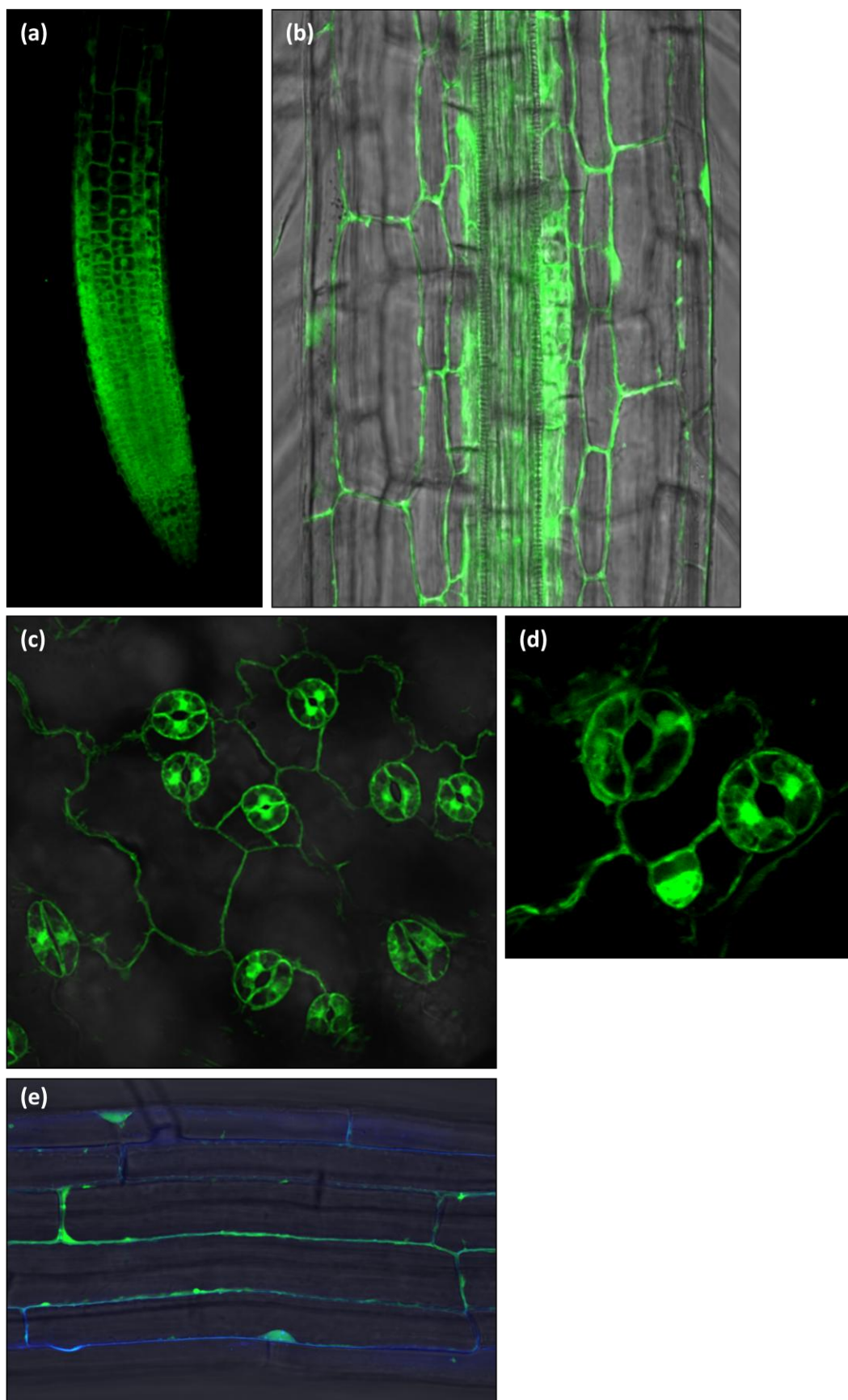


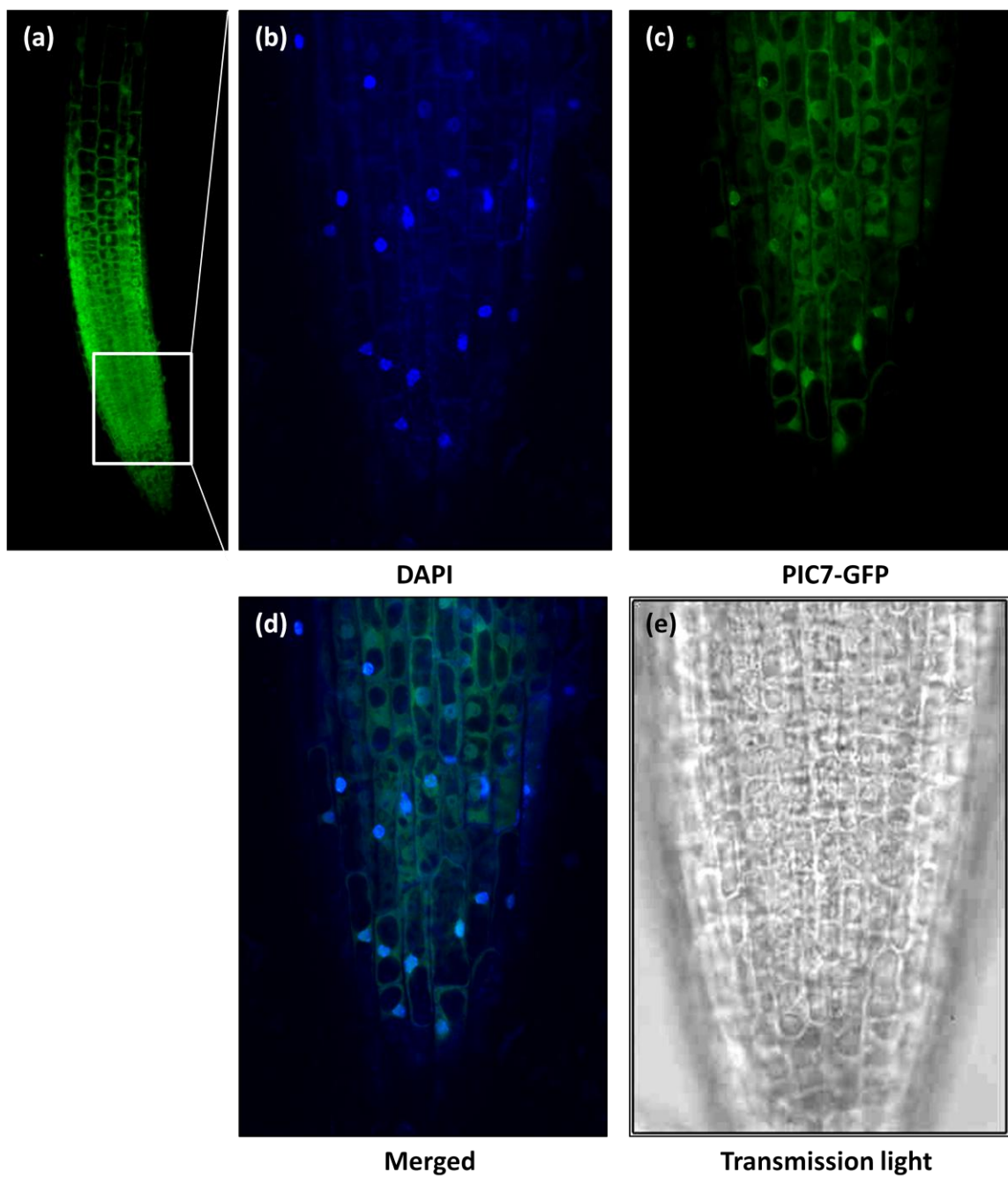
Figure 31. Expression pattern of APT1-GFP in five-day-old Arabidopsis seedlings.

APT1-GFP expressed throughout the seedling. High level of APT1-GFP was observed in

(a) the root tip, (b) vasculature, (b) pericycle, (c) guard cells and (d) meristemoid cells.

Low level of APT1-GFP was observed in (e) the epidermal cells. Image was taken using

Olympus FV1000 confocal microscope.



(Figure 32-continued)

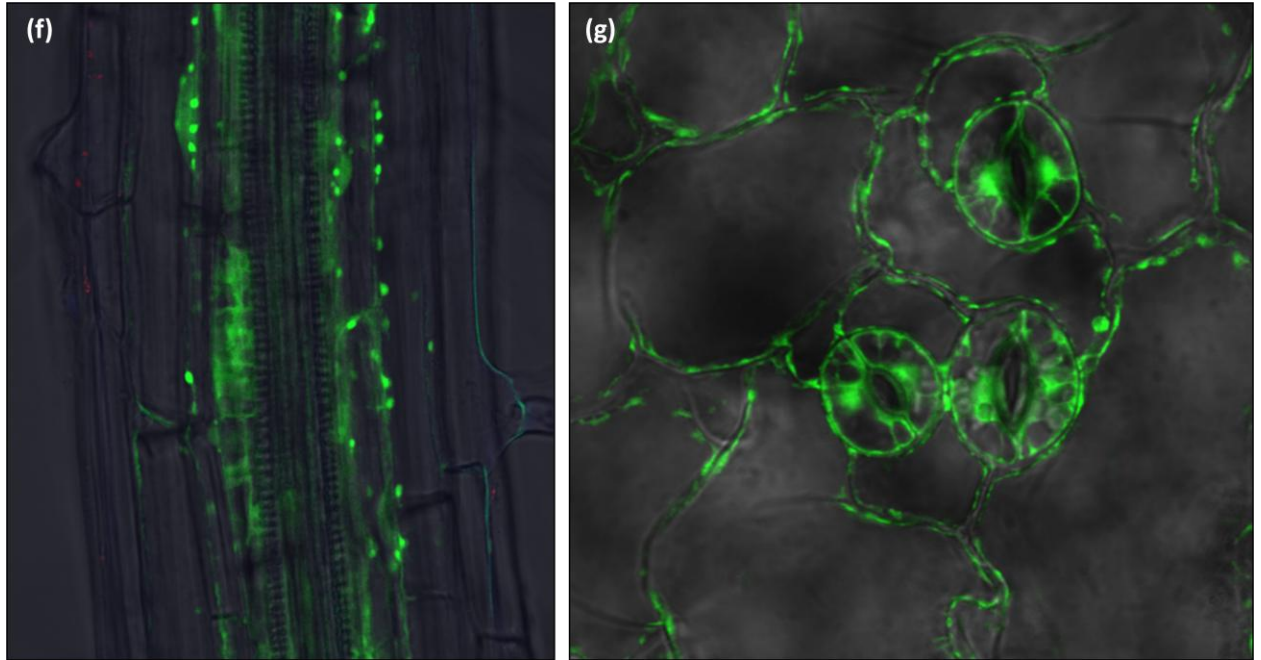


Figure 32. Cellular localization of APT1-GFP. (a-e) APT1-GFP is localized to the nucleus and cytoplasm. In dividing cells GFP expression is observed throughout the cytoplasm and nucleus. (f,g) APT1-GFP is mainly localized in the nucleus of the mature cells as well as unknown aggregate structure along the plasma membrane. Image was taken using Olympus FV1000 confocal microscope.

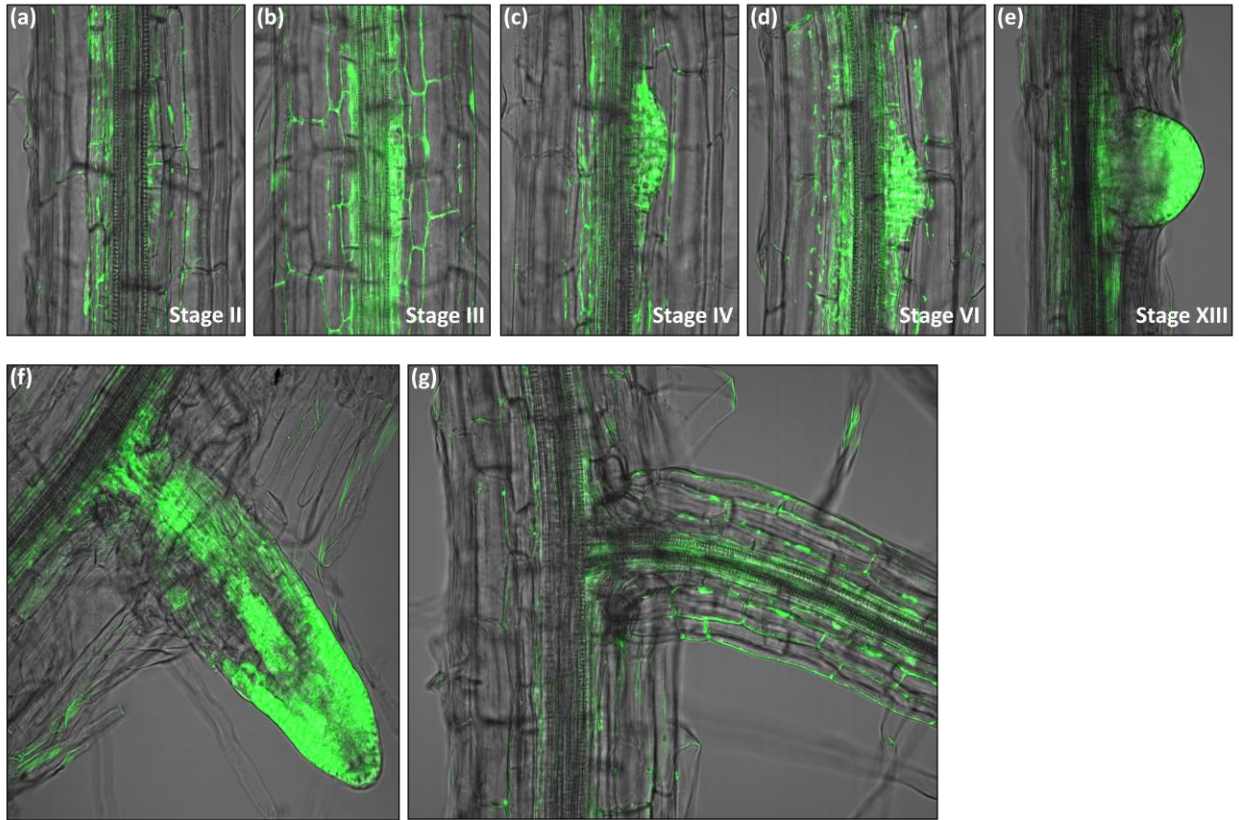


Figure 33. Expression of APT1-GFP in lateral root primordia. APT1-GFP is highly expressed in the lateral root initiation sites. (a-e) Expression of APT1-GFP in dividing cells at different stages of lateral root primordia. (f) APT1-GFP expression in emerged lateral root. GFP expression is mainly localized to the root tip and vasculature of the newly formed lateral root. (g) APT1-GFP expression in mature lateral root. Expression of APT1-GFP is similar to the primary root. Image was taken using Olympus FV1000 confocal microscope.

Induction of APT1 expression by auxin and cytokinin

To examine the regulation of APT1 expression by auxin, *APT1::APT1-GUS* seedlings were treated with synthetic auxin 2,4-D or natural auxin IAA. Both auxins were able to induce the APT1-GUS expression (Figure 34). Induction was evident throughout the root and the highest expression was seen in basal meristem and elongation zone of the root. According to the tested time points, it takes between 2 to 6 hours for the induction of APT1-GUS by auxin. In order to test the effect of cytokinin on APT1 expression, *APT1::APT1-GUS* seedlings were treated with synthetic cytokinin kinetin or natural cytokinin zeatin. Both cytokinins induced the APT1-GUS expression (Figure 35). Cytokinin did not significantly induce APT1-GUS expression in the basal meristem and elongation zone of the root, but rather enhanced the expression in the mature area of the root. Additionally, the induction was prominent in shoot. The time period taken for the induction was 2 hours suggesting APT1's faster response to cytokinin than auxin.

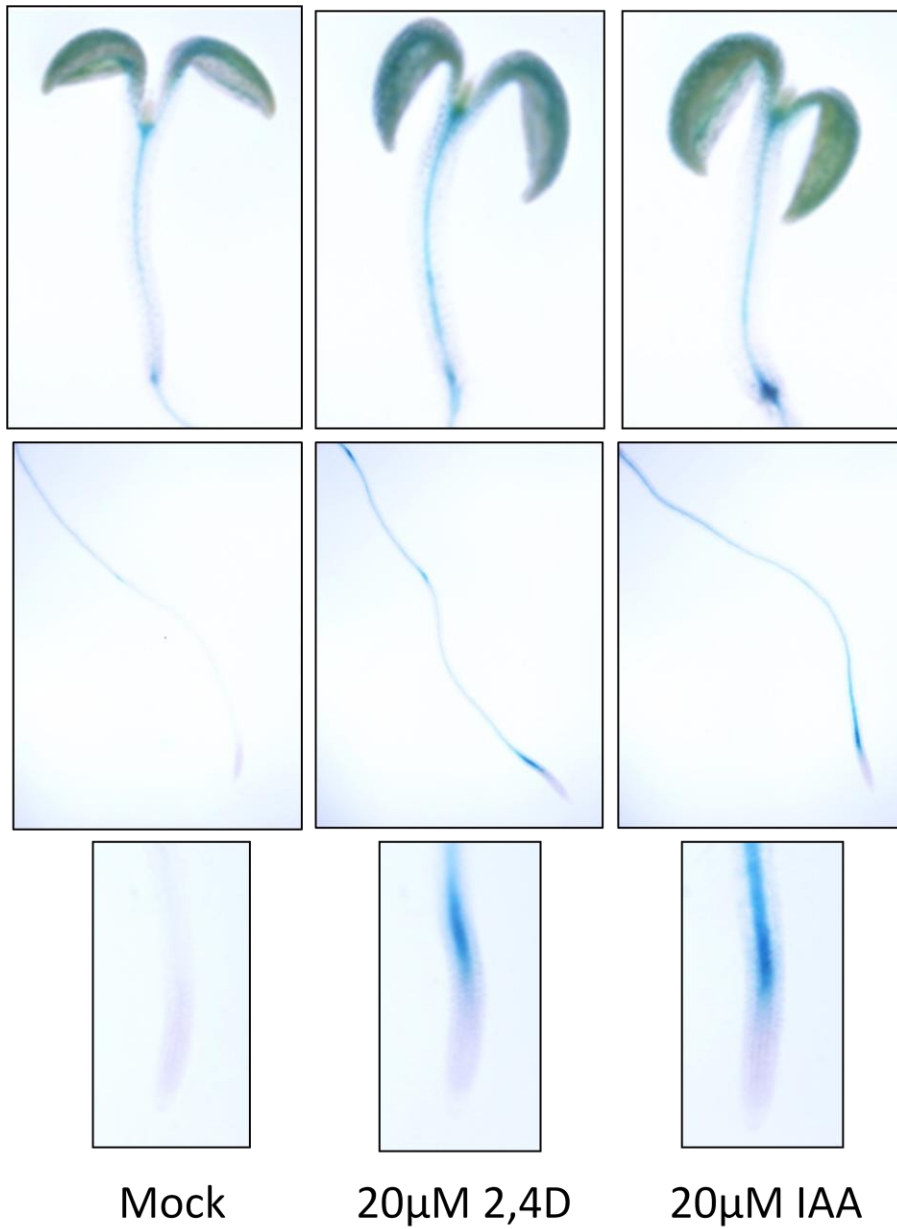


Figure 34. Auxin induces APT1 expression. To check the auxin effect on APT1 expression, four-day-old seedlings carrying *APT1::APT1-GUS* construct were treated with 20 μM 2,4-D or 20 μM IAA for 6 hours before fixing and stained for GUS activity.

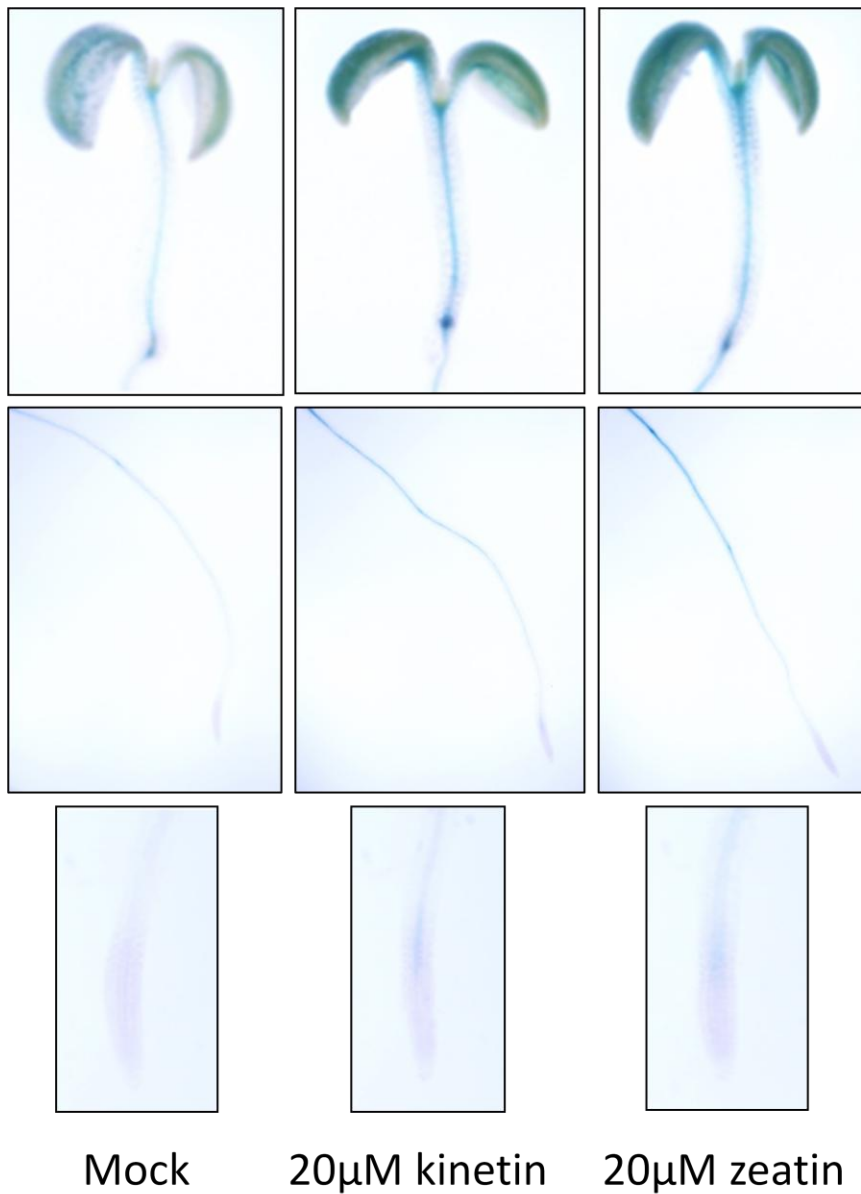
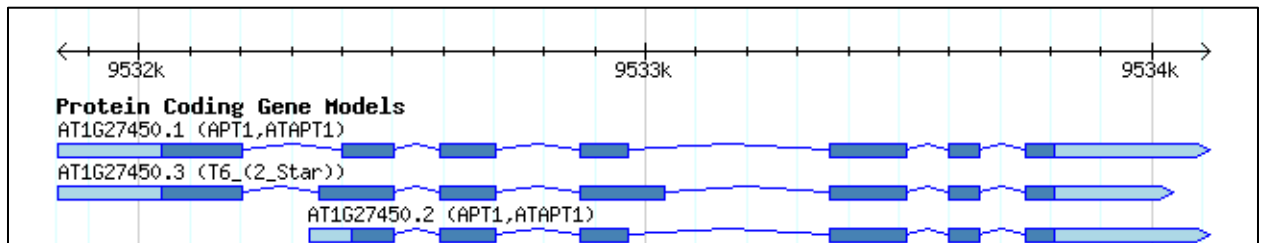


Figure 35. Cytokinin induces APT1 expression. To check the cytokinin effect on APT1 expression, four-day-old seedlings carrying *APT1::APT1-GUS* construct were treated with 20 μM kinetin or 20 μM zeatin for 2 hours before fixed and stained for GUS.

Characterization of splice variants of *APT1*

APT1 is proposed to produce three splice variants named *AT1G27450.1*, *AT1G27450.2* and *AT1G27450.3* according to The Arabidopsis Information Resource (TAIR) (<http://www.arabidopsis.org>). However, only two splice variants [*AT1G27450.1* (*APT1.1*), *AT1G27450.2* (*APT1.2*)] were able to be amplified using wild type Col-0 cDNA. To understand the function of splice variants in Arabidopsis, they were over-expressed in both mutant and wild type backgrounds as Myc tagged proteins. According to the western blot analysis of T1 plants, both *APT1.1* and *APT1.2* expresses in wild type Col-0 background (Figure 36c,d). Over-expression of *APT1.1* and *APT1.2* in mutant background recovers the sterile phenotype of *pic7-1* at the T2 generation shown by their silique length (Figure 36e).

(a)



(Figure 36-continued)

(b)

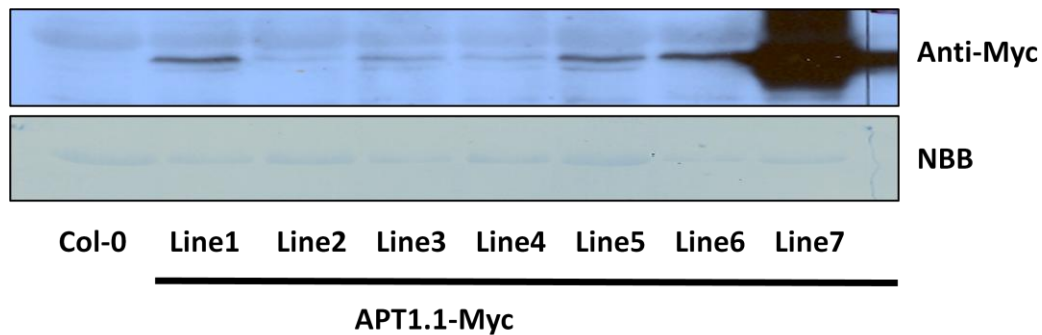
AT1G27450.1	MQTIIISPLVSHRLCLARAVPCNRLLNNHHRAPPSIRLSNHRSTTSRLRFS-----
AT1G27450.3	MQTIIISPLVSHRLCLARAVPCNRLLNNHHRAPPSIRLSNHRSTTSRLRFSSAGSLCVCK
AT1G27450.2	-----
AT1G27450.1	-----SAAASRDSEMAT EDVQDPRIAKIASSIRVIPDFPKPGIMFQDIT TLLLDTEAF
AT1G27450.3	SDAEYFAKAAASRDSEMAT EDVQDPRIAKIASSIRVIPDFPKPGIMFQDIT TLLLDTEAF
AT1G27450.2	-----MATEDVQDPRIAKIASSIRVIPDFPKPGIMFQDIT
	TLLLDTEAF

AT1G27450.1	KDTIALFVD RYKDKGISVVAGVEARGFIFGPPIALAIGAKFVPMRKP KKLPG-----
AT1G27450.3	KDTIALFVD RYKDKGISVVAGVEARGFIFGPPIALAIGAKFVPMRKP KKLPGTFLFLPLV
AT1G27450.2	KDTIALFVD RYKDKGISVVAGVEARGFIFGPPIALAIGAKFVPMRKP KKLPG-----

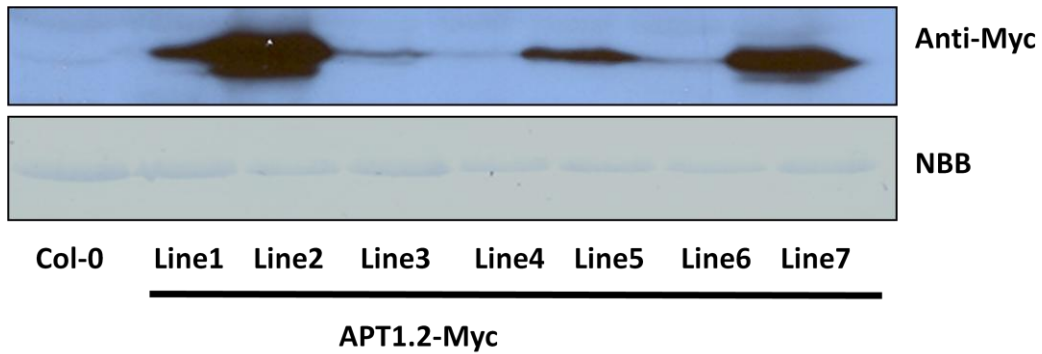
AT1G27450.1	-----KVISEEYSLEYGTD TIEMHVGAVEPGERAI IIDD LIATGGTLA
AT1G27450.3	ACDFDIKLHERSQLGSWKVISEEYSLEYGTD TIEMHVGAVEPGERAI IIDD LIATGGTLA
AT1G27450.2	-----KVISEEYSLEYGTD TIEMHVGAVEPGERAI IIDD LIATGGTLA

AT1G27450.1	AAIRLLERVGVKIVECACVIELPELKGKEKLG ETSLFVLVKSAA
AT1G27450.3	AAIRLLERVGVKIVECACVIELPELKGKEKLG ETSLFVLVKSAA
AT1G27450.2	AAIRLLERVGVKIVECACVIELPELKGKEKLG ETSLFVLVKSAA

(c)



(d)



(e)

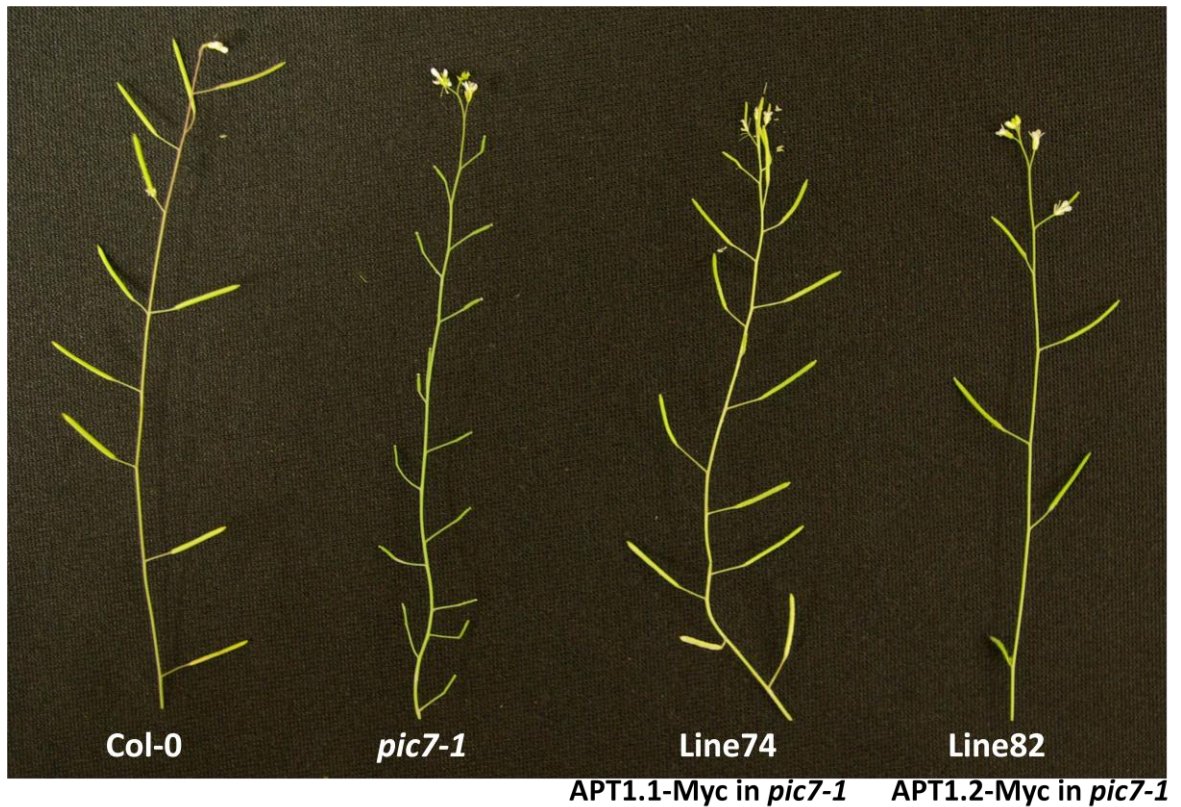


Figure 36. (a) Analysis of splice variants of *APT1*. According to Arabidopsis thaliana resource center (<http://www.arabidopsis.org/>) annotations, *APT1* produces 3 splice variants. (b) Nucleotide alignment of splice variants of *APT1*. (c,d) Western blot analysis of APT1.1-Myc and APT1.2-Myc expression in wild type background. Total protein was

extracted from leaves of independent T1 plants, separated by SDS PAGE and western blot analysis was done using anti-myc antibody. (e) Complementation of sterility of *pic7-1* by *35S::APT1.1-Myc* and *35S::APT1.2-Myc*. Siliques of *pic7-1* plants (T2 generation) carrying *35S::APT1.1-Myc* and *35S::APT1.2-Myc* were compared with siliques of *pic7-1* and Col-0.

Effect of mutation on enzyme activity

The mutation in *pic7-1* is present in both splice variants. In order to check the enzyme activity of *APT1.1* and *APT1.2* and the effect of mutation on their activity, they were cloned into pGEX4T-3 vector and expressed as Glutathion S-transferase (GST) tagged proteins in BL21 bacteria cells. Mutant versions both referred as *apt1.1* and *apt1.2* were also expressed in bacteria as GST tagged proteins with the same method (figure 37).

It has been reported that 2,6-diaminopurine (DAP) is converted to its corresponding nucleotide by APT1 and will only be toxic once converted (Moffatt and Somerville, 1988). On media containing DAP, wild type seeds with the APT1 activity got arrested at post germination stage because of toxicity (Figure 38c). As expected, *pic7-1* did not get arrested after the germination, possibly because DAP was not converted due to the loss of enzyme activity in the mutant protein (Figure 38a,b). This strongly suggests that *pic7-1* lacks the adenine phosphoribosyltransferase activity.

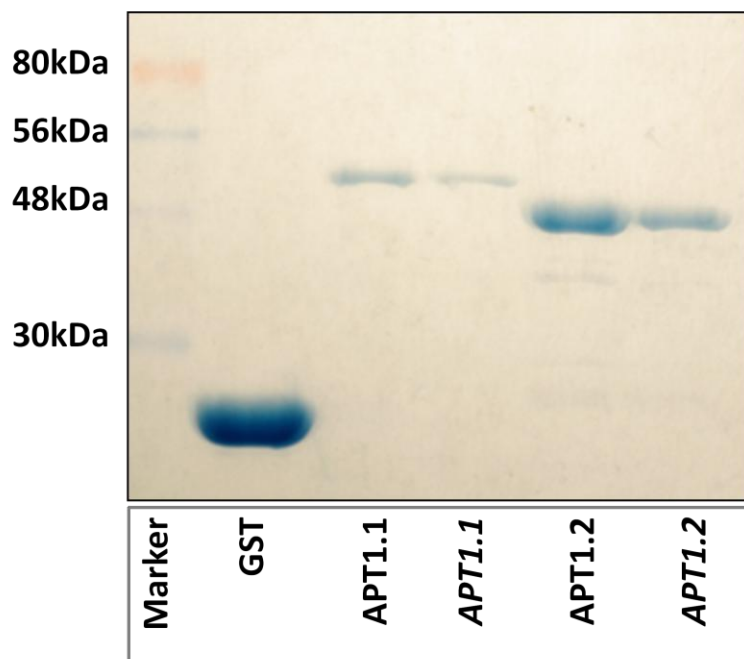
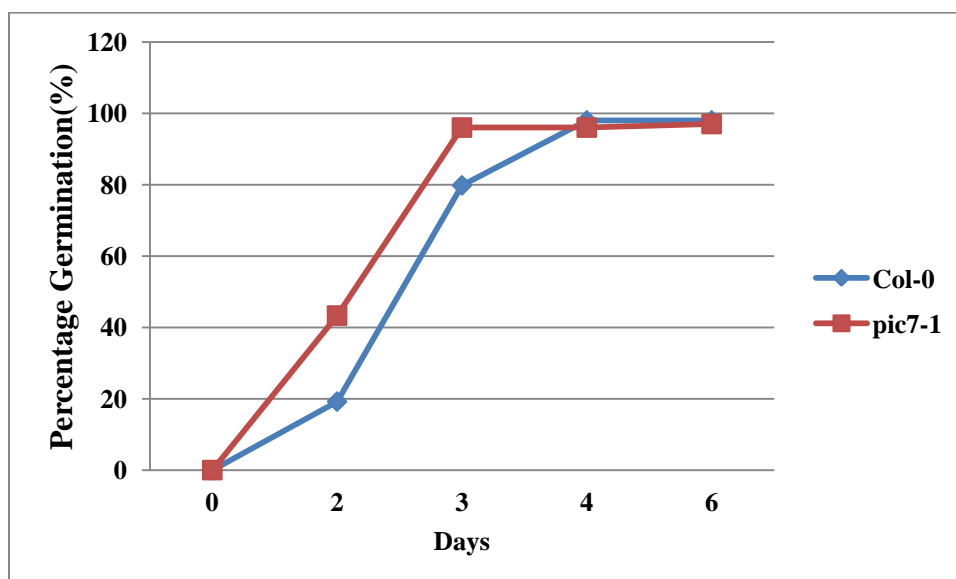
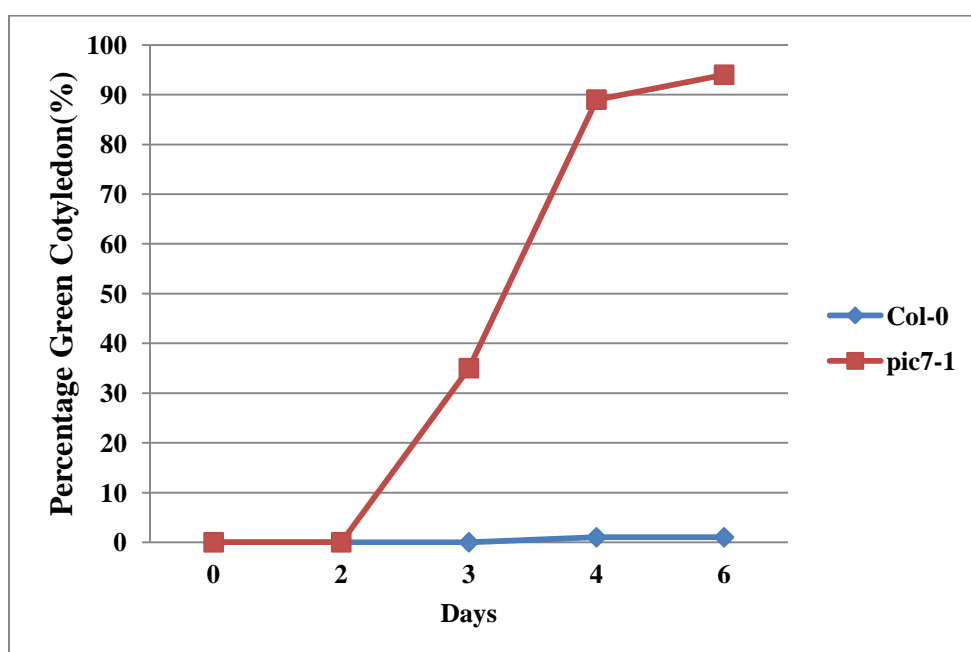


Figure 37. Expression of splice variants of *APT1* in bacteria. Proteins were expressed in *E.coli* BL21 cells as GST tagged proteins and purified using Glutathion agarose beads. (Lane1). Molecular weight marker, (Lane2). GST, (Lane3). GST-APT1.1, (Lane4). GST-APT1.1, (Lane5). GST-APT1.2, (Lane6). GST-APT1.2.

(a)



(b)



(Figure 38-continued)

(c)

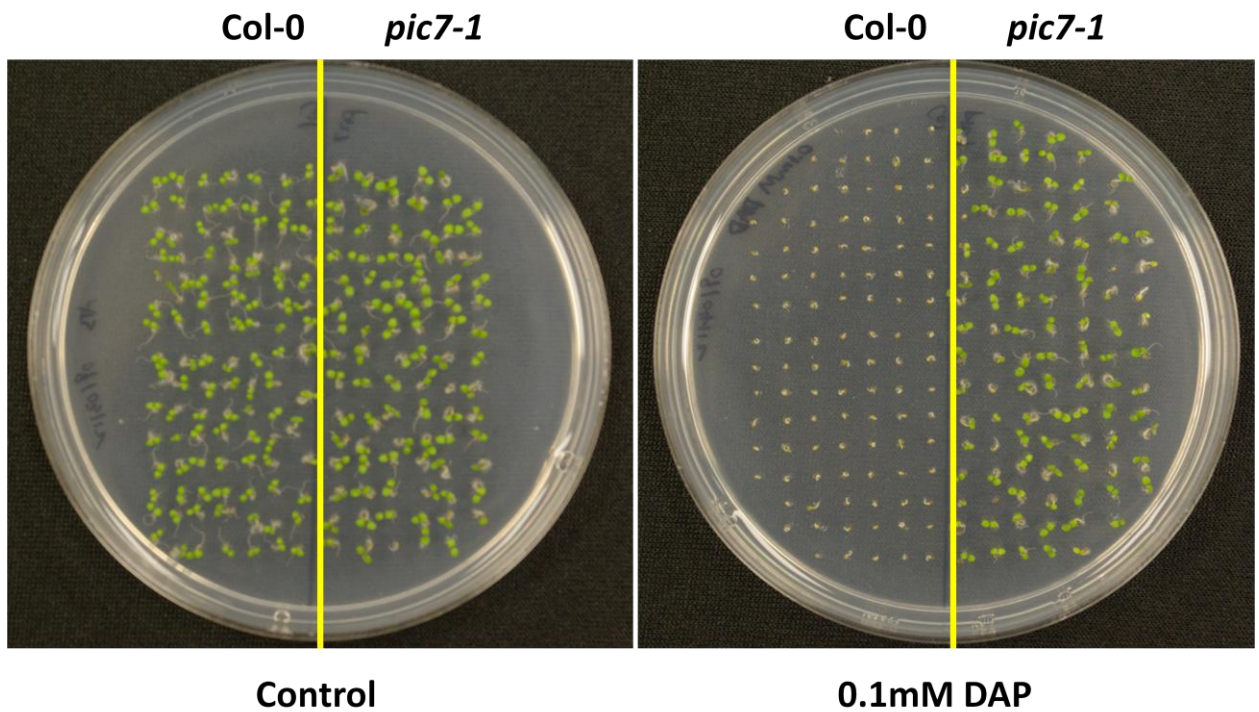


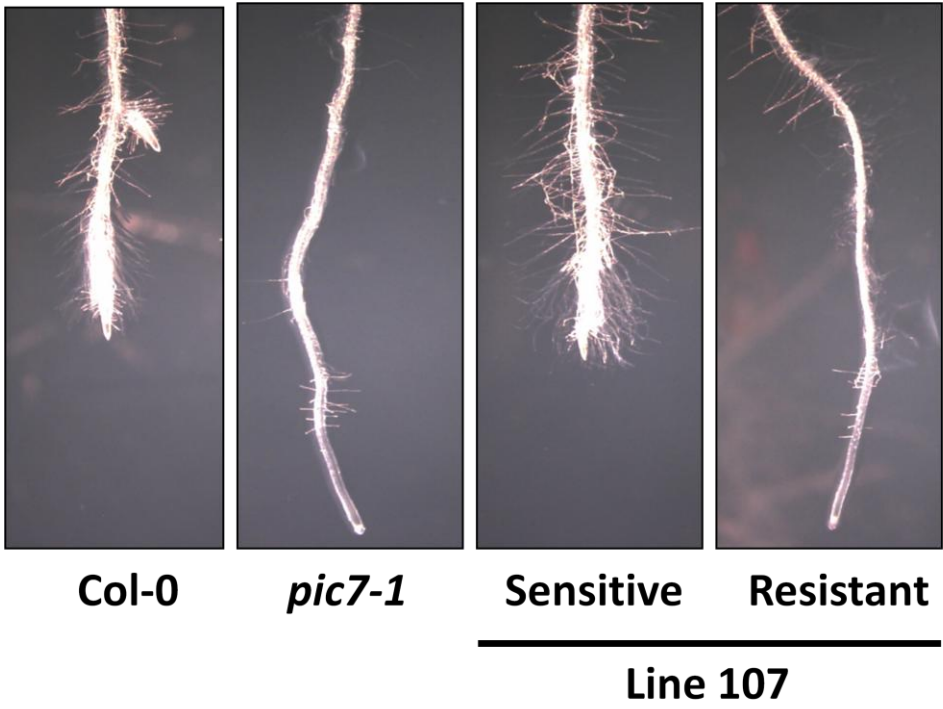
Figure 38. Post-germination inhibition by 2,6-diaminopurine (DAP). (a) Both *Col-0* and *pic7-1* germinate almost at the same rate on 0.1 mM DAP media. (b) *pic7-1* is greatly resistant to DAP induced post germination arrest when compared to the wild type. (c) Seeds were sterilized and plated on ATS media containing 0.1 mM DAP. Seeds that were stratified for two days at 4°C were grown at 21°C for up to six days under continuous illumination. Seedlings producing green cotyledons were counted up to six days of growth and calculated as a percentage of total number of seeds.

APT1::APT1-GFP* and *APT1::APT1-GUS* recovered mutant phenotypes of *pic7-1

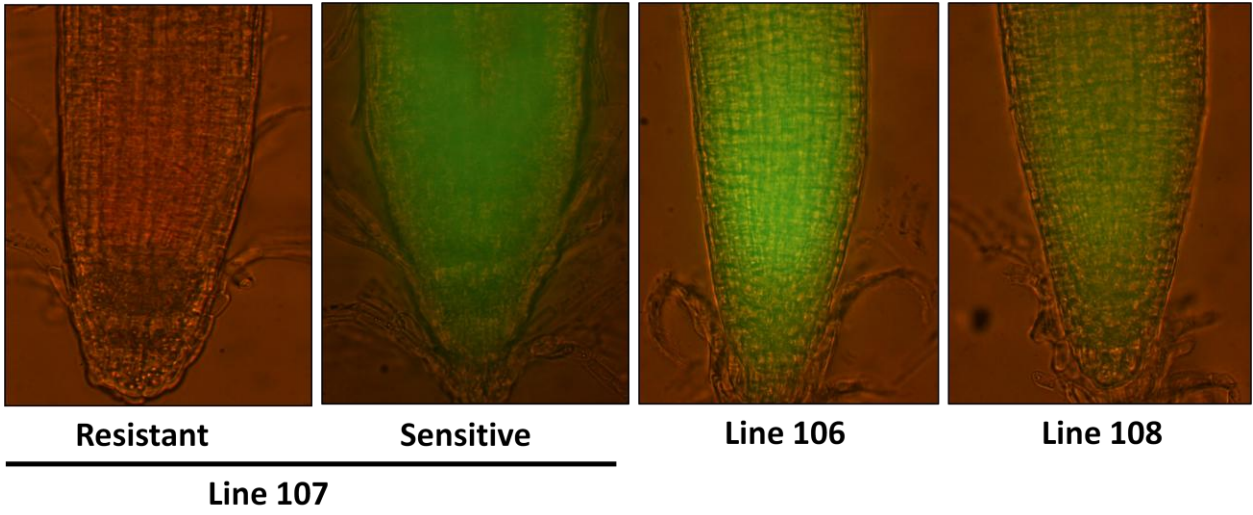
Previously described *APT1::APT1-GFP* expressed in *pic7-1* background was tested for the complementation of mutant phenotypes. When several independent lines of T3 generation were tested on media containing 10 μ M picloram or 10 μ M zeatin, the plants carrying transgene (Figure 39b,c) became sensitive to picloram and zeatin (Figure 39a,d,e). 75% of the seedlings of line 107 which is heterozygous (Figure 39b) for *APT1::APT1-GFP* were sensitive to picloram (Figure 39a) and the sensitive seedlings were positive for GFP expression while the resistant ones were negative. Expression level of APT1-GFP complements the recovery level where highly expressing line 106 was recovered completely to wild type level but less expressing line 108 was less recovered compared to line 106 (Figure 39c,d). However, both lines recovered the cytokinin sensitivity to wild type level (Figure 39e).

Previously described *APT1::APT1-GUS* expressed in *pic7-1* background was also tested for the complementation of mutant phenotypes. When several homozygous independent lines of T3 generation were tested on media containing 10 μ M picloram or 10 μ M zeatin, the plants became sensitive to zeatin (Figure 39g) but showed partial recovery on picloram (Figure 39f).

(a)

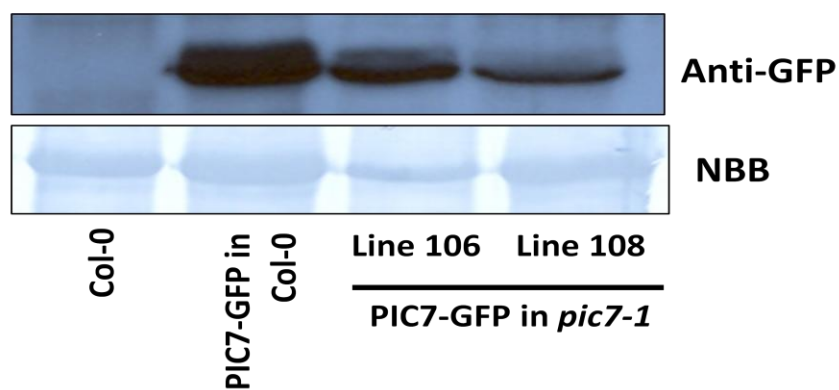


(b)

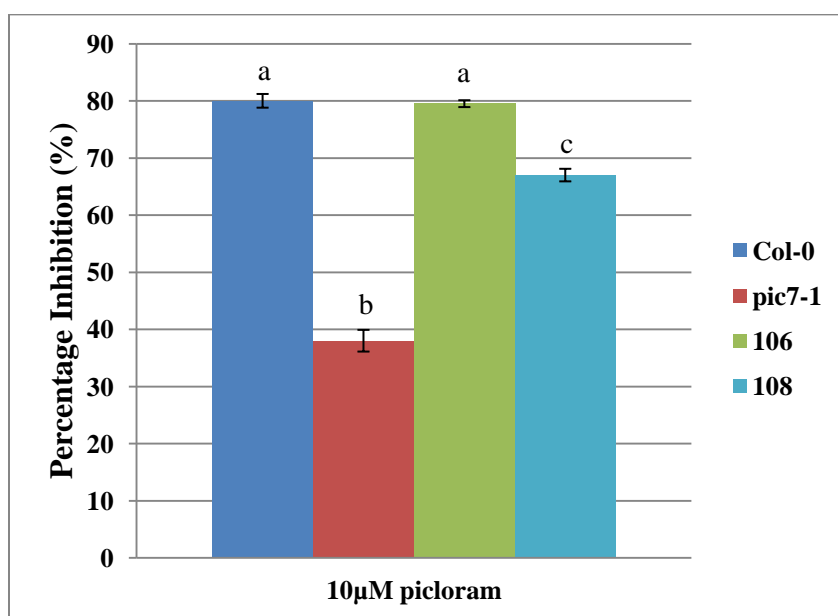


(Figure 39-continued)

(c)

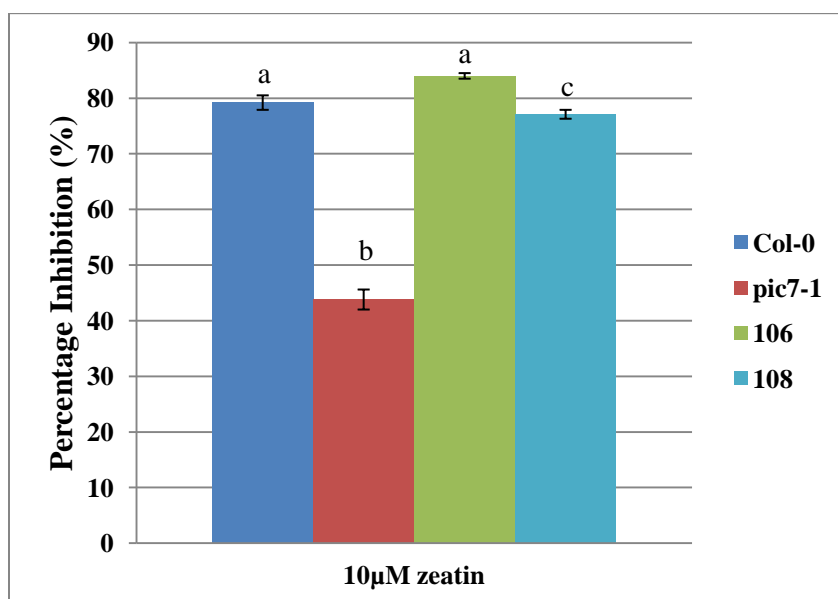


(d)

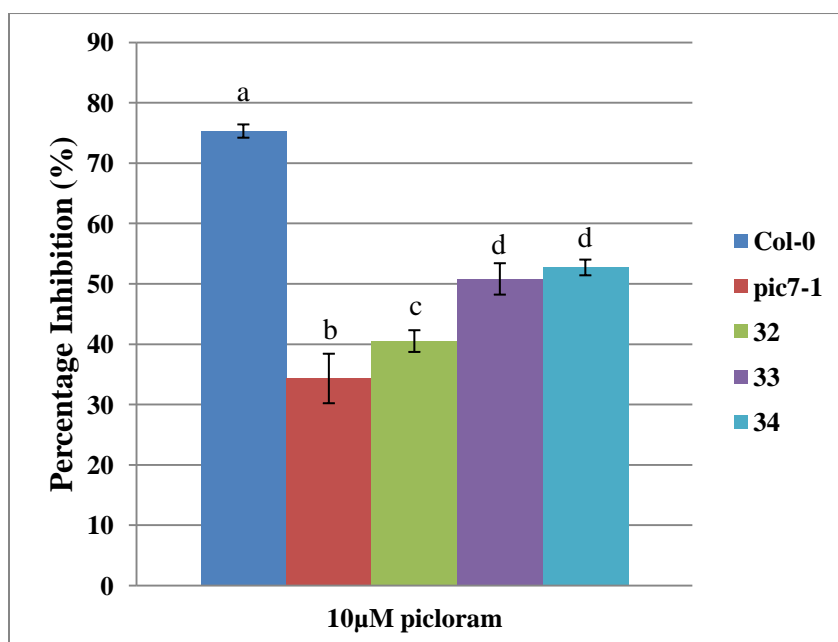


(Figure 39-continued)

(e)



(f)



(Figure 39-continued)

(g)

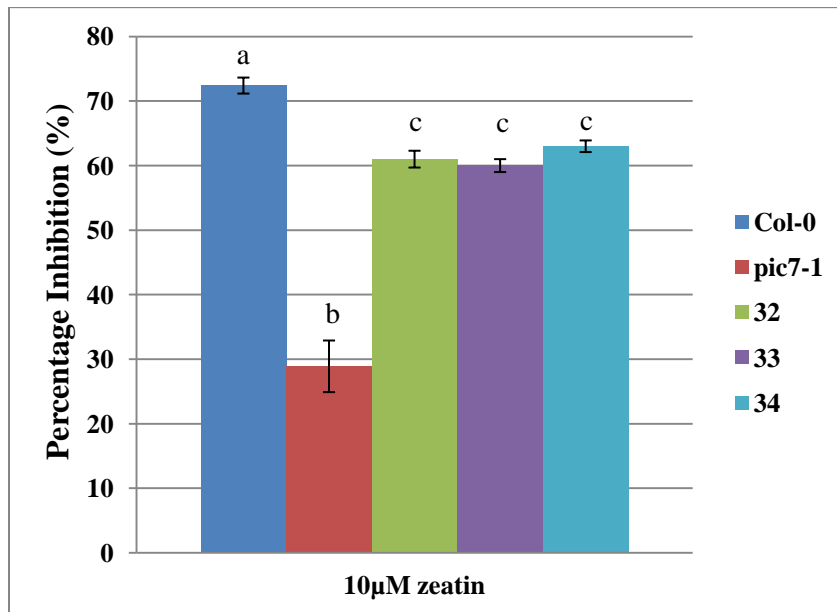


Figure 39. Expression of *APT1::APT1-GFP* or *APT1::APT1-GUS* in *pic7-1* complements *pic7-1* phenotypes. (a,d) Four-day-old seedlings grown on ATS media were transferred onto media containing 10 μ M picloram. Length of the primary root was measured following four days of incubation vertically in a growth chamber under continuous illumination at 21°C. A two-way ANOVA indicated interaction between genotype and treatment ($F=187.94$; $df=3, 48$; $P<0.001$). Means with different letters “a, b, c” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. (b) Expression of *APT1::APT1-GFP* in three independent lines. Four-day-old seedlings were imaged using Olympus BH2-RFCA epifluorescence microscope. Line 107 is heterozygous for *APT1::APT1-GFP* and line 106 and 108 are homozygous for the transgene. (c) Western blot analysis of APT1-GFP in *pic7-1* background. Total protein was extracted from seedlings of independent T3 plants, separated by SDS PAGE and western blot analysis

was done using anti-GFP antibody. (e) Four-day-old seedlings grown on ATS media were transferred onto media containing 10 μ M zeatin. Length of the primary root was measured following four days of incubation vertically in a growth chamber under continuous illumination at 21°C. A two-way ANOVA indicated interaction between genotype and treatment ($F=185.56$; $df=3, 48$; $P<0.001$). Means with different letters “a, b, c” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. (f,g) Expression of *APT1::APT1-GUS* in *pic7-1* background complements mutant phenotypes. Line 32, line 33 and line 34 are homozygous for the transgene. Four-day-old *pic7-1* seedlings carrying *APT1::APT1-GUS* grown on ATS media were transferred onto media containing (f) 10 μ M picloram or (g) 10 μ M zeatin. Length of the primary root was measured following four days of incubation vertically in a growth chamber under continuous illumination at 21°C. (f) A two-way ANOVA indicated interaction between genotype and treatment ($F=31.34$; $df=4, 50$; $P<0.001$). (g) A two-way ANOVA indicated interaction between genotype and treatment ($F=43.10$; $df=4, 50$; $P<0.001$). Means with different letters “a, b, c, d” indicate statistically significant difference ($P<0.05$), Tukey’s HSD. Error bars represent standard errors of the means.

CHAPTER IV

DISCUSSION

As a pivotal plant hormone, auxin regulates a wide range of fundamental developmental processes in plant. Despite many years of studies, the molecular mechanisms of plant auxin response have not being completely understood. One of the reasons is that, in addition to auxin biosynthesis, transport and signaling, tremendous synergistic and antagonistic functional crosstalk with other plant hormones and environmental cues also regulate auxin responses, which determine the final outcome in plant development. For example, through regulating the auxin biosynthesis and its transport, ethylene inhibits cell expansion and thus the primary root growth in *Arabidopsis* (Ruzica et al., 2007). Auxin regulates the lateral root formation in response to ABA by inducing ABI3 which is a transcriptional factor mediating ABA signaling in the development of lateral root primordia (Brady et al., 2003). Also auxin and cytokinin function antagonistically in the root to decide the apical meristem size, in which cytokinin represses the auxin signaling and alters its transport (Zhang et al., 2011). These studies suggest that control of plant growth development is a complex process involving many hormone response pathways working coordinately. To identify novel genes in plant auxin response, a forward genetic approach using the synthetic auxin picloram was employed. Here characterization of one of the mutant, *pic7-1*, is described.

***pic7-1* encodes an adenine phosphoribosyltransferase1 (APT1)**

Before the identification of *pic7-1*, the segregation pattern of F2 seeds from the cross between *pic7-1* and wild type Col-0 suggested that *pic7-1* was a recessive mutation. *pic7-1* was located to the north arm of chromosome 1 in the annotation unit T17H3 and identified as an allele of *apt1*. *APT1* is among five *APT* members in Arabidopsis genome (Allen et al., 2001) and it is the first isolated *APT* gene from Arabidopsis (Moffatt et al., 1994). APT catalyzes a one-step purine salvage to convert adenine into AMP with the presence of PRPP (Nygaard, 1983). In *pic7-1*, a C1650T point mutation leads to a substitution of threonine (T) at position 197 with an isoleucine (I) residue (Figure 3), which is the last amino acid in the highly conserved PRPP binding domain GERAIIDDLIATGGT (Figure 26) (Schnorr et al., 1996). Therefore, the mutation in *pic7-1* might result in abolition or partial loss of the enzyme activity in the plant. Alleles of *apt1* were first isolated for deficient APT activity using forward genetic method by selecting for resistance to 2,6-diaminopurine (DAP), which is only toxic once converted to the nucleotide form by APT (Moffatt and Somerville, 1988). These mutants had only 1% to 15% APT activity compared to wild type (Moffatt and Somerville, 1988), which might indicate that APT1 has greater adenine metabolic activity than the other APTs (Allen et al., 2002). To examine whether the APT activity is largely impaired in *pic7-1*, seed germination assay on DAP was carried out. Similar to other *apt1* mutants (Moffatt and Somerville, 1988), more than 90% of *pic7-1* seeds were able to germinate and further develop green cotyledons, indicating that *pic7-1* is resistant to DAP (Figure 38b,c). This result strongly suggests that APT1 activity is severely hampered in *pic7-1*. Therefore,

there is a high possibility that adenine and also active cytokinin levels are elevated in *pic7-1*.

One major defect observed in *pic7-1* was less seed production (Figure 5) than wild type Col-0 which might be due to pollen abortion. From several pollen assays, it was evident that *pic7-1* had less viable pollens (Figure 6d,e), abnormal vacuolation (Figure 6b) and deformed cell wall (Figure 6c). Since pollen numbers, vacuole fragmentation and cell wall formation are decided before second mitotic phase (Yamamoto et al., 2003; Sundberg and Østergaard, 2009), it is possible that pollen development in *pic7-1* is aborted before the second mitotic phase. The reproductive defect in *pic7-1* is reminiscent of the male sterility of the first isolated *apt1* mutant *apt1-3*, which also has infertile pollens with abnormal vacuoles and cell wall (Regan and Moffatt, 1999; Zhang et al., 2002). This phenotype is common to other *apt1* alleles too (Moffatt and Somerville, 1988). Results of this study along with previous work suggest that *APT1* enzyme activity is essential for proper pollen development. Since *pic7-1* might have higher level of adenine and also cytokinin base form caused by the impaired enzymatic activity, altered cytokinin and adenine metabolism might both contribute to the pollen development deficiency, which is also proposed by Zhang et al. in 2002.

Auxin responses are defective in *pic7-1*

Mutants that are defective in auxin responses are resistant to the inhibition of primary root elongation in response to exogenous auxin (Leyser et al., 1996; Ruegger et al., 1998; Strader et al., 2008). Similarly, *pic7-1* was also resistant to synthetic as well as natural auxins (Figure 8), in which the most resistance is against synthetic auxin 2,4-D

compared to synthetic auxin picloram and natural auxins IAA and IBA. Resistance of *pic7-1* to synthetic auxin NAA (Figure 8d) which does not need carrier proteins to get in to cells (Dellbarre et al., 1996) suggests that PIC7 may regulate auxin response by modulating auxin signaling rather than auxin transport as shown in Figure 40.

To test the effect of *pic7-1* mutation on the auxin induced gene transcription, auxin inducible reporter line *DR5::GFP* (Ulmasov et al., 1997a; Ulmasov et al., 1997b) was crossed into *pic7-1*. In *pic7-1* background the *DR5::GFP* expression was slightly low compared to the wild type under control condition (Figure 9). In the presence of 2,4-D and picloram, GFP expression in the wild type Col-0 was greatly induced but in *pic7-1* it was less induced, suggesting impaired auxin response in *pic7-1*. Similar observations have been reported previously with respect to auxin signaling mutants (Dharmasiri et al., 2005) as well as auxin biosynthesis mutants (Stepanova et al., 2008).

HS::AXR3 NT-GUS is another reporter gene that has been used to study auxin induced Aux/IAA degradation (Gray et al., 2001). Domain one and two of repressor protein Aux/IAA17 has been fused to *GUS* reporter gene under the heat inducible promoter HS (Gray et al., 2001). To test whether *pic7-1* mutation interferes with auxin induced Aux/IAA degradation, *HS::AXR3 NT-GUS* transgenic line was crossed into *pic7-1*. According to the results, AXR3NT-GUS degradation in *pic7-1* was affected (Figure 10) and differentially regulated in shoot and root. In *pic7-1*, shoot had less AXR3NT-GUS expression immediately after heat shock while root had more AXR3NT-GUS at the same time point than the wild type Col-0. During the subsequent incubation at room temperature after the heat shock, high rate of degradation was observed in root while shoot had less rate of degradation compared to wild type Col-0. It can be argued that the

less amount of AXR3NT-GUS observed in shoot of *pic7-1* following heat shock is due to its high rate of degradation during heat shock. Similarly, low rate of degradation in root during heat shock results in high level of AXR3NT-GUS immediately after heat shock. However, if that is tenable, the same rates of degradation should continue during the cold incubation after heat shock which is contrary to the observations. Therefore, it is more likely that induction of AXR3NT-GUS by heat shock is affected in *pic7-1*. However, further experiments are needed to confirm the above claim. Most importantly, if the degradation rate of AXR3NT-GUS in root is higher in *pic7-1* than the wild type, *DR5::GFP* expression should be higher in *pic7-1* background compared to wild type, but the observations suggest the opposite. However, similar observation in auxin resistant mutant *ibr5-1* has suggested TIR1 independent pathway to regulate auxin responsive gene expression (Strader et al., 2008), which might attribute to the differential regulations of AXR3NT-GUS degradation and *DR5::GFP* expression in *pic7-1*. Also since only root tip has been observed for *DR5::GFP* expression, examination of *DR5::GFP* in other areas of the seedlings might give a better picture about the auxin response in *pic7-1*.

To clarify the reporter gene expression data, early auxin responsive genes, *SAUR19*, *GH3-3* and *GH3-5* which are induced by auxin (Hagen and Guilfoyle, 2002; Spartz et al., 2012) were analyzed by qRT-PCR. Expression of all the genes was higher in *pic7-1* compared to wild type Col-0, suggesting increased auxin activity in *pic7-1* (Figure 11). Differential regulation of auxin signaling in shoot and root predicted using *HS::AXR3NT-GUS* reporter gene was not evident with endogenous gene expression data. The contradictory results obtained for reporter gene expression and endogenous gene expression might be due to several facts. *DR5* which is composed of auxin regulatory

elements might not include the other regulatory elements such as enhancers and suppressors influencing the expression of the endogenous gene. Also as discussed above examination of areas other than root tip is important to draw conclusions about *DR5::GFP* expression. Since there are 29 different AUX/IAA proteins in Arabidopsis (Liscum and Reed, 2002) regulating the expression of auxin responsive genes, it is possible that other AUX/IAAs with altered levels in *pic7-1* might also contribute to higher level of *SAUR19*, *GH3-3* and *GH3-5* found in shoot of *pic7-1*.

With 2,4-D treatment, expression of *SAUR19* was induced in wild type Col-0 but decreased in *pic7-1* background. In contrast to *SAUR19*, *GH3-3* and *GH3-5* expression was induced in both wild type Col-0 and *pic7-1* backgrounds by 2,4-D treatment. However, the fold of induction of both genes in *pic7-1* was less compared to wild type (Figure 11e). This pattern is similar to *DR5::GFP* induction by 2,4-D and picloram. Altogether, these data strongly suggest the impaired auxin signaling in *pic7-1*. *GH3-3* and *GH3-5* are known to be involved in auxin homeostasis by producing auxin conjugates to buffer the free IAA levels (Kim et al., 2011). Since *pic7-1* had more *GH3-3* and *GH3-5* transcripts overall than wild type Col-0 under the control condition, it can be speculated that *pic7-1* has more IAA to induce the responsive gene expression, which might be due to the enhanced auxin biosynthesis in *pic7-1*. Experiments are underway to find out the IAA content in shoot and root of *pic7-1*.

APT1 is a negative regulator of secondary root formation

Lateral root development is a well characterized phenomenon extensively controlled by auxin. Auxin induces the lateral root formation by regulating all stages of

lateral root development, from priming to initiation until emergence and further development (Casimiro et al., 2003; Peret et al., 2009). Lateral roots initiate from the pericycle right next to the vascular tissue. Auxin is first transported to lateral root founder cells to trigger cell division followed by creating an auxin gradient in the lateral root primordia to help develop lateral root. Both shoot and root derived auxin is important for this process (Overvoorde et al., 2010) and many auxin signaling mutants show less number of lateral roots (Goh et al., 2012). On the contrary, auxin over-producing mutants such as *sur1* have more lateral roots (Boerjan et al., 1995). Similar to them, *pic7-1* also has more lateral roots compared to the wild type Col-0 (Figure 12a,b). Exogenous auxin supplement induced the lateral root formation in wild type Col-0 whereas the degree of induction in *pic7-1* was less compared to wild type, suggesting its defective auxin signaling. In addition to more lateral root formation, more adventitious root formation is also a typical characteristic of the auxin over-producing mutants. For example, *sur1*, *sur2* and *yucca* mutants all have more adventitious roots when grown on un-supplemented media (Boerjan et al., 1995; Barlier et al., 2000; Zhao et al., 2001). *pic7-1* also has more adventitious roots compared to wild type, suggesting the possibility of higher level of auxin in the mutant background (Figure 12d,e). According to the previous studies, cytokinin can induce IAA biosynthesis (Jones et al., 2010). Therefore, as discussed in the introduction, accumulation of active cytokinin in *pic7-1* might be the cause of high IAA level as shown in Figure 40.

APT1 negatively regulates cell division

The *CyclinB::GUS* has been used as a marker for cell division and it has been widely useful to identify dividing cells in the lateral root primordia (Ferreira et al., 1994; Smith and Fedoroff, 1995). According to the increased *CyclinB::GUS* expression along pericycle of the root in *pic7-1* (Figure 13a), it contains higher number of actively dividing cells when compared to the wild type. Therefore, the higher number of lateral roots and adventitious roots found in *pic7-1* may be due to the induced cell division of the pericycle cells.

Auxin over-producing mutant *yucca6* is a taller plant compared to wild type Col-0 (Kim et al., 2007) and some of the other auxin over-producing mutants such as previously mentioned *sur1* have shorter primary roots (Boerjan et al., 1995). *pic7-1* plants are also taller than wild type Col-0 and produce a shorter primary root (Figure 4). Since the *CyclinB::GUS* activity was increased in the root tip of *pic7-1* seedlings (Figure 13b), it is more likely that *pic7-1* may have increased cell division in the root. It is known that auxin promotes cell division and cytokinin enhances cytokinesis when auxin is present. Therefore, potential higher levels of active cytokinin and auxin in *pic7-1* caused by enzyme deficiency may result in higher rate of cell division. Moreover, according to cell length measurements, *pic7-1* had shorter cells compared to wild type (Figure 4e) suggesting lack of cell elongation as the reason for shorter root.

***APT1* is a novel component in cytokinin (CK) homeostasis**

Previous studies with APT1 activity reveal the capacity of APT1 to utilize cytokinin as its substrate, and *APT1* mutants harbor significantly reduced conversion of cytokinin to the corresponding nucleotide (Regan and Moffatt, 1990; Allen et al., 2002).

This raises a possibility that APT1 is required to regulate the level of active form of cytokinin in the plant cells. And APT1 deficiency might lead to altered cytokinin sensitivity of the plant. Therefore, responses of *pic7-1* to two cytokinins, kinetin and zeatin, were examined to clarify this scenario. Firstly, *pic7-1* showed less sensitivity to both cytokinins in inhibition of primary root elongation (Figure 15a,b), which is the hallmark action of cytokinin on roots (Cary et al., 1995). To further confirm the APT1's involvement in cytokinin response, *APT1::APT1-GFP* transgene was introduced into the *pic7-1* mutant plants. As expected, root growth of multiple independent transgene lines in response to cytokinin was reversed to the similar level as wild type Col-0 (Figure 39e). Similar to *pic7-1*, plants with disturbed function of cytokinin receptor AHK4/CRE1 (Inoue et al., 2001; Ueguchi et al., 2001b) or type B ARRs, ARR10 and ARR12 (Yokoyama et al., 2007) which belong to the family of cytokinin two-component signaling system (Hwang and Sheen, 2001), have impaired root growth response to cytokinin. This suggests that mutation in *pic7-1* may have altered cytokinin homeostasis which results in the resistant root growth response to cytokinin. Moreover, kinetin failed to de-etiolate dark grown *pic7-1* seedlings to the extent as wild type Col-0, particularly in the inhibition of hypocotyl elongation (Figure 17), suggesting the *pic7-1* resistance to cytokinin regulated shoot development. Since it has been shown that combination of cytokinin receptor AHK3 with either AHK2 or AHK4/CRE1 mediate de-etiolation in *Arabidopsis* triggered by exogenous cytokinin (Riefler et al., 2006) and increased cytokinin level in *amp* mutant induces deetiolated features in the dark grown seedlings (Chin-Atkins et al., 1996), it further confirms that APT1 is involved in the cytokinin homeostasis as shown in Figure 40.

In addition to root growth and de-etiolation defects, *pic7-1* showed altered response to cytokinin mediated anthocyanin induction which is used as cytokinin responsive marker (Deikman and Hammer, 1995). As shown in Figure 16a,b, anthocyanin level was higher in *pic7-1* shoot than in wild type Col-0 on the control media. Exogenous cytokinin elevated anthocyanin accumulation 4 fold in *pic7-1* but 2 fold in wild type Col-0, indicating the hypersensitivity of *pic7-1* to cytokinin in anthocyanin induction. Elevated levels of cytokinin response regulator expression might explain the higher cytokinin level seen in *pic7-1* as discussed below.

***APT1* may be mediating cytokinin response through major type-B ARRs**

Since three type-B ARRs, ARR1, ARR10 and ARR12 function redundantly as essential positive regulators in cytokinin signal transduction (Ishida et al., 2008), qRT-PCR was carried out using *pic7-1* shoot and root tissues to examine whether the cytokinin response defects in *pic7-1* is related to altered transcript level of these three major ARRs. Excitingly, *pic7-1* contained significantly higher levels of all three transcripts in the shoot (Figure 19a) and high level of ARR1 in the root (Figure 19b). It is important to notice that type-B ARRs are not known to be induced by cytokinin. Therefore it cannot be concluded that predicted higher level of cytokinin in *pic7-1* induces the ARRs. However it is still possible that enhanced cytokinin signaling may induce ARRs expression.

Recently, it's revealed that these three ARRs positively mediate cytokinin induced anthocyanin biosynthesis redundantly and the induction fold is dramatically dropped in single, all combinations of double and triple mutants compared with the wild type (Das et al., 2012). Therefore, it's highly possible that boosted *ARR1*, *ARR10* and

ARR12 levels caused by the *pic7-1* mutation results in the *pic7-1* shoot hypersensitivity to exogenous cytokinin in anthocyanin accumulation. At the same time, anthocyanin synthesis is also regulated by other factors such as sugar (Jeong et al., 2010) and light (Guo et al., 2008). It is still early to conclude the causes for high anthocyanin content in *pic7-1* under control condition without cytokinin treatment.

By comparing the phenotypes of *pic7-1* with *arr1-3/arr10-5/arr12-1* triple null mutants, it's striking that both of them display defects in largely overlapping growth responses. The only opposite phenotypes between them was in shoot elongation where *pic7-1* had larger stature (Figure 1a,b) while *arr1-3/arr10-5/arr12-1* exhibited reduced height (Argyros et al., 2008) contrast to the wild type Col-0, which is as anticipated for opposite regulated levels of *ARR1*, *ARR10* and *ARR12* in *pic7-1* and *arr1-3/arr10-5/arr12-1*. However, it's interesting that both mutants had similar defects in infertility (Figure 5), bigger seeds (Figure 18a,b), more anthocyanin accumulation (Figure 16), more adventitious roots (Figure 12d), shorter root (Figure 4c,d), resistance to cytokinin in de-etiolation (Figure 17) and inhibition of primary root elongation (Figure 15) (Ishida et al., 2008; Argyros et al., 2008). Even though major profiles of cytokinin responsive genes were altered in *arr* triple mutants, knowledge is scarce on the intermediate components and the molecular mechanisms for each of these *ARRs* regulations on specific developmental outputs and the effects of their interactions. Also it is evident that there are other novel mechanisms through which *ARRs* act on the cytokinin output independent of their well-known roles as transcriptional activators in two-components signaling pathway (Marhavy et al., 2011), such as action of *ARR2* and *ARR12* on PIN1 localization even in the presence of transcription inhibitor. Therefore, it is arbitrary to

exclude the possibility that these developmental defects are due to the altered major type-B *ARRs* as a result of *pic7-1* mutation.

It's also notable that among *ARR1*, *ARR10* and *ARR12*, transcript level of *ARR1* was up-regulated in *pic7-1* throughout the seedling (Figure 16). According to Sakai et al., (2001), plant over-expressing *ARR1* has shorter roots as *pic7-1* while *arr1* null mutant shows longer root than the wild type Col-0 under normal growth condition. This suggests that *APT1* may control root growth through *ARR1*.

Tissue specific expression of APT1

Function of a protein is highly correlated with its expression pattern and localization. In order to study that of *APT1*, 2kb region of the *APT1* 5' leading sequence and its genomic DNA was used to generate *APT1::APT1-GUS* and *APT1::APT1-GFP* transgenic constructs. Both transgenic constructs showed the same expression pattern in transgenic seedlings with few exceptions (Figure 29,31). The localization of APT1 in vascular tissue and pericycle was consistent with both reporter constructs. In addition to that, PIC7-GFP expressed in the root apical meristem and epidermis cells throughout the root as well as in the guard cells. This may be due to the fact that large size of the marker protein GUS obstacles the proper localization in these tissues while GFP is relatively small. Since there are proteins that translocate to the sites they function from the original synthetic site, it is possible that APT1 belongs to this type of proteins and its translocation to other tissues is blocked because GUS is a significantly bigger tag than the GFP tag.

High expression of APT1 in the cell division and elongation zones of the root tip (Figure 29g,31a) might explain the short root phenotype of *pic7-1* as the mutant *apt1* might result in dysfunctional cell elongation and thus shorter cells compared to the wild type. Expression of APT1 in the pericycle region (Figure 31b) correlates with high *CyclinB::GUS* expression in the pericycle of *pic7-1* and its production of high number of lateral roots. Similar correlation can be observed in the shoot root junction (Figure 29a,d) where *pic7-1* produces more adventitious roots. APT1 expression was enhanced in the dividing cells of the root tip as well as lateral root primordia (Figure 30,33), indicating that expression of APT1 increases where the cell division takes place. After the stage VIII when lateral root emerges, the localization pattern of APT1-GFP changed to the tip, vascular region and pericycle of the newly emerged lateral root (Figure 30f,g,33e-g), suggesting the importance of the enzyme APT1 in cell division. In addition, the presence of APT1 in the epidermal cells (Figure 31e) seems to play a critical role in root hair formation as *pic7-1* only produces very few numbers of root hairs (Figure 12f,g). Also, high expression of APT1-GFP in stomata (Figure 31c,d) signifies the importance of APT1 in guard cell functions, which can be further explained as *pic7-1* influences auxin, ABA, cytokinin and ethylene signaling which are involved in stomatal movements and related functions (Tanaka et al., 2006). However stomatal movement related phenotypes need to be studied in *pic7-1* to further support the expression data.

APT1 localizes to both nucleus and cytoplasm

According to previous studies APT1 has been suggested to be localized to the cytosol in both plant and parasite cells (Allen et al., 2002; Zarella-Boitz et al., 2004).

APT1-GFP expression data clearly suggests that APT1 might be localized to both cytoplasm and nucleus (Figure 32a-e). Cytosolic localization of APT1 was mainly observed in the dividing cells such as root tip and lateral root primordia (Figure 32,33). When the cells are mature, APT1 tends to localize predominantly to the nucleus of the cells. This differential localization was very clear in the basal meristem of the root tip and in dividing stomata (Figure 31a,d). Epidermal cells also have nuclear localized APT1 (Figure 31e). However it is important to notice that the cytosolic fraction of the mature plant cells is very limited due to the presence of a large vacuole, hence the detection of APT1 expression in the cytosol is limited. Another interesting observation was bright APT1-GFP aggregate spots along the cell membrane (Figure 32f,g). Similar expression patterns have been reported for mitochondrial markers (Werner et al., 2003) as well as for vesicles trafficking (Lewis and Lazarowitz, 2009). Web-like expression pattern was also observed in *pic7-1* (data not shown) which is similar to reported ER associated protein expression pattern (Werner et al., 2003). Therefore, APT1-GFP expression and localization has to be further examined with these organelle specific markers and other available techniques to identify the exact localization pattern of APT1.

Auxin and Cytokinin induces *APT1* expression

Besides primary auxin responsive genes, expression of other genes are also regulated by auxin (Lee et al., 2009). According to *APT1::APT1-GUS* expression data, auxin induced the APT1 expression to a great extent (Figure 34). Expression was mainly induced in the vascular tissue throughout the plant but the highest induction was seen in the basal meristem and elongation zone of the primary root tip. This induction pattern

further states the important role of APT1 in the auxin response. Cytokinin also induces wide variety of genes following its application (Kiba et al., 2005). Similar to auxin, cytokinin also induced APT1 expression (Figure 35). However, the induction was very less in the basal meristem and elongation zone of the root tip but it was more evident in the vasculature of mature region in the root and also in the shoot.

Mutation in APT1 triggers altered auxin and cytokinin response in *pic7-1*

To check the possible existence of other mutations in *pic7-1* background which leads to the altered hormone responses, *APT1::APT1-GFP* transgene was introduced into the *pic7-1* mutant plants. As expected, root growth of multiple independent transgenic lines in response to auxin and cytokinin were reversed to the similar level as wild type Col-0 (Figure 39d,e), indicating the altered auxin and cytokinin response in primary root growth in *pic7-1* is attributed to mutation in APT1 as shown in Figure 40. Experimental evidence shows that *APT1::APT1-GUS* transgene could only recover the defective primary root growth responding to auxin and cytokinin in *pic7-1* to certain extent, in which cytokinin response is recovered more than the auxin response (Figure 39f,g). Along with the localization difference between *APT1::APT1-GFP* and *APT1::APT1-GUS* transgene lines, it can be suggested that the apical meristem localization of APT1 is of more importance to auxin response than to cytokinin response in primary root growth. With the evidence that cytokinin induced APT1 highly in the differentiation zone of the root while auxin induced APT1 more in the elongation zone and basal meristem in *APT1::APT1-GUS* transgene lines, it is highly possible that APT1 mediates growth responses to auxin and cytokinin in a spatial differential pattern.

***APT1* as a convergent point between auxin and cytokinin signaling crosstalk**

Experimental evidence from this study indicates the profound contributions of *APT1* to both auxin and cytokinin signaling pathways. And many developmental processes that *pic7-1* is defective in are regulated by both hormone signaling pathways. For example, auxin biosynthesis and transport (Cheng et al., 2006) as well as cytokinin synthesis and perception (Werner et al., 2003; Kinoshita-Tsujimura and Kakimoto, 2011) are indispensable for proper pollen formation and maturation (Figure 6). Similar to cytokinin signaling receptor triple mutants *ahk2-5 ahk3-7cre1-2* (Riefler et al., 2006), auxin response factor 2 mutant *mnt* has drastically increased seed size (Schruff et al., 2006) (Figure 18a,b). Cotyledon expansion (Figure 18c,d) could be increased by improper auxin distribution (Strader and Bartel, 2009) or reduced by cytokinin in light (Stoynova-Bakalova et al., 2004). Noticeably, *ARR1*, which was up-regulated in *pic7-1* (Figure 19), is a key component for mediating cytokinin-auxin antagonistic interaction in the basal meristem of Arabidopsis root for determining the size of root meristem and the subsequent root growth (Dello Ioio et al., 2007). Also it has been reported that *ARR12* which was also up-regulated in *pic7-1* is involved in cytokinin induced degradation of auxin efflux carrier, PIN1 (Marhavy et al., 2011). All these lead to a postulation that *APT1* might mediate the crosstalk between auxin and cytokinin in various developmental processes.

To further investigation this postulation at molecular level, qRT-PCR was carried out to monitor the transcript levels of several cytokinin induced auxin biosynthetic genes, *CYP79B2*, *CYP79B3* and *NIT3* (Jones et al., 2010). According to Figure 20, transcript

levels of all three genes were much higher in the shoot of *pic7-1* than in wild type Col-0, and only *CYP79B2* was up-regulated in the root of *pic7-1* while *CYP79B3* and *NIT3* in *pic7-1* did not differ from the wild type Col-0. Thus, it is indicated that *pic7-1* mutation might result in increased auxin biosynthesis which is induced by the elevated cytokinin level in the mutant as described in the introduction. Besides suggesting APT1's action on regulating cytokinin level, this result further supports that APT1 is involved in auxin cytokinin interaction at metabolic level as shown in Figure 40.

Expression pattern of *APT1* in response to auxin and cytokinin further elucidates the roles of *APT1* in auxin and cytokinin interactions. In *APT1::APT1-GUS* seedlings, APT1-GUS was highly induced by cytokinins within 2 hours treatment (Figure 35) while it was not induced by auxins (not shown). It is also surprising to notice that the *APT1* expression pattern extensively overlaps with expression of ARR1, ARR10 and ARR12, which were defective at transcriptional level in *pic7-1* (Mason et al., 2005). Therefore, *APT1* might mediate quick response to cytokinin through a tight regulation of ARR1, ARR10 and ARR12 in plants. Auxins notably induced APT1-GUS level slower than cytokinins probably within 2 hours to 6 hours treatment, and also exhibited similar and differential tissue specific induction pattern compared to the wild type. As shown in Figure 34,35, both auxin and cytokinin upregulated APT1-GUS level in vasculature throughout the plant. However, the APT1-GUS induction by auxins was much higher in the basal meristem and elongation zone in the root than by cytokinins. Therefore, APT1 might be a novel gene functioning as the co-regulatory gene/protein for auxin and cytokinin crosstalk and might mediate growth response toward auxin and cytokinin in differential spatial and temporal manners.

***APT1* is involved in auxin crosstalk with other hormones through differential mechanisms**

As auxin extensively interacts with other major hormones at metabolic, transport and signaling levels, it is highly possible that *pic7-1* also harbors altered responses to other hormones. To confirm this possibility and further elucidate the underlying mechanisms, responses of *pic7-1* to other major plant hormones ethylene, GA and ABA were carried out.

The best characterized auxin ethylene interaction is the synergistic effect on the inhibition of primary root growth (Rahman et al., 2001), in which ethylene inhibits the root growth through regulating auxin biosynthesis (Swarup et al., 2007), transport (Ruzicka et al., 2007) and signaling (Stepanova et al., 2007) in the root elongation zone. In addition, it is uncertain on the requirement of fully functioning ethylene signaling for its action on root growth through auxin pathway (Stepanova et al., 2007). Therefore, the altered auxin response in primary root growth is suggested to be the reason for the resistance of *pic7-1* to the ethylene induced inhibition of root elongation (Figure 18). Another synergistic interaction between auxin and ethylene is in the initiation of root hairs. Previous studies on this process indicate that auxin distribution and signaling are the direct factors determining the root hair initiation, while ethylene affects indirectly through the auxin level in the cell (Rahman et al., 2002). In *pic7-1*, the less root hair defect was recovered by exogenous auxin (Figure 12f,g) or ACC (Figure 23) treatment to the similar extent as wild type under control condition, suggesting that ACC rescues the root hair defect in *pic7-1* through recovering the intracellular auxin level that is

impaired by the *APT1* mutation. Moreover, triple response to ethylene in *pic7-1* was also disturbed as resistance to the inhibition of hypocotyl elongation (Figure 22b) and insensitivity to the exaggeration of apical hook (Figure 21a). Auxin and ethylene antagonistically control the hypocotyl length in dark in which ethylene inhibits the hypocotyl elongation but auxin promotes the growth (Suttle, 1988). Additionally, for exaggerating the apical hook, ethylene acts through modulating auxin transport (Lehman et al., 1996), biosynthesis (Vandenbussche et al., 2010) and probably signaling (Zadnikova et al., 2010) as well. From the experimental results, the hypocotyl elongation (not shown) and apical hook formation in *pic7-1* did not differ from the wild type Col-0 under the control condition but was highly resistant to ACC treatment (Figure 21), which is highly similar to the triple response of many ethylene signaling mutants such as *ein5-1*, *ein6* and *ein 7* (Roman et al., 1995). Therefore, it is more likely that ethylene signaling pathway rather than auxin pathway for hypocotyl and apical hook development is affected in *pic7-1*.

As a stress hormone, ABA extensively crosstalks with auxin synergistically or antagonistically at multiple levels, such as targeting the same gene/protein (Li et al., 2011) and regulating transport or signaling levels (Belin et al., 2009). Hence *pic7-1* might display altered responses to ABA. According to the seed germination and seedling development response to ABA, *pic7-1* was more resistant to ABA induced green cotyledon and primary root arrest than the wild type Col-0 (Figure 24,25a). Further, response of *pic7-1* to two environmental stresses, salinity and osmotic stresses, were carried out because ABA is highly induced to mediate plant growth responses to both stresses (Xiong et al., 1999b; Zhang et al., 2006). Consistent with ABA growth assay

result, *pic7-1* is less sensitive to both salinity and osmotic stresses compared to the wild type Col-0 (Figure 25b,c). All these results further confirm the involvement of *APT1* in auxin and ABA crosstalk as well as in mediating stress responses in Arabidopsis.

Distinct from stem elongation which is attributed to both cell division and elongation, hypocotyl elongation is solely due to cell elongation (Gendreau et al., 1997). Auxin (Lincoln et al., 1990; Leyser et al., 1996) and GA (Chiang et al., 1995; Peng et al., 1997) have all been shown to be core regulators of this process. Although hypocotyl of *pic7-1* seedling was not different from that of the wild type Col-0 in length on control medium, exogenous GA treatment induced the elongation of hypocotyl to more extent in *pic7-1* than wild type Col-0 (Figure 27), suggesting a hypersensitivity of *pic7-1* in GA-induced hypocotyl elongation. With a recent finding that GA and auxin act on hypocotyl elongation independently (Clare et al., 2000), the sensitivity of *pic7-1* to GA in hypocotyl elongation might be a direct outcome of deficient GA signaling pathways. In addition, a well-known GA function in the plant is to promote the embryogenesis to seed germination transition (Razem et al., 2006). However, based on the experimental evidence, this effect is dose dependent and the seed germination will be inhibited if the GA concentration is relatively high. With 50 μ M GA treatment, germination of wild type Col-0 was delayed, but *pic7-1* was more resistant to seed germination inhibition by high concentration of GA (Figure 26) suggesting altered GA response in *pic7-1*.

Multiple splice variants of *APT1* in Arabidopsis

From experimental evidence, *APT1.1* and *APT1.2* are produced by *APT1* in Arabidopsis. The capability of recovering sterile phenotype of *pic7-1* back to the wild

type indicates the redundant function of *APT1.1* and *APT1.2* in regulating male fertility in *Arabidopsis*. The annotated *APT1.3* was not amplified which might be due to the low expression level of *APT1.3*. It is also possible that *APT1.3* might be expressed under specific environmental condition or at specific developmental stages later than the seedling stage from which the cDNA of wild type was isolated. Moreover, it could also be an error in the splice variants predictions.

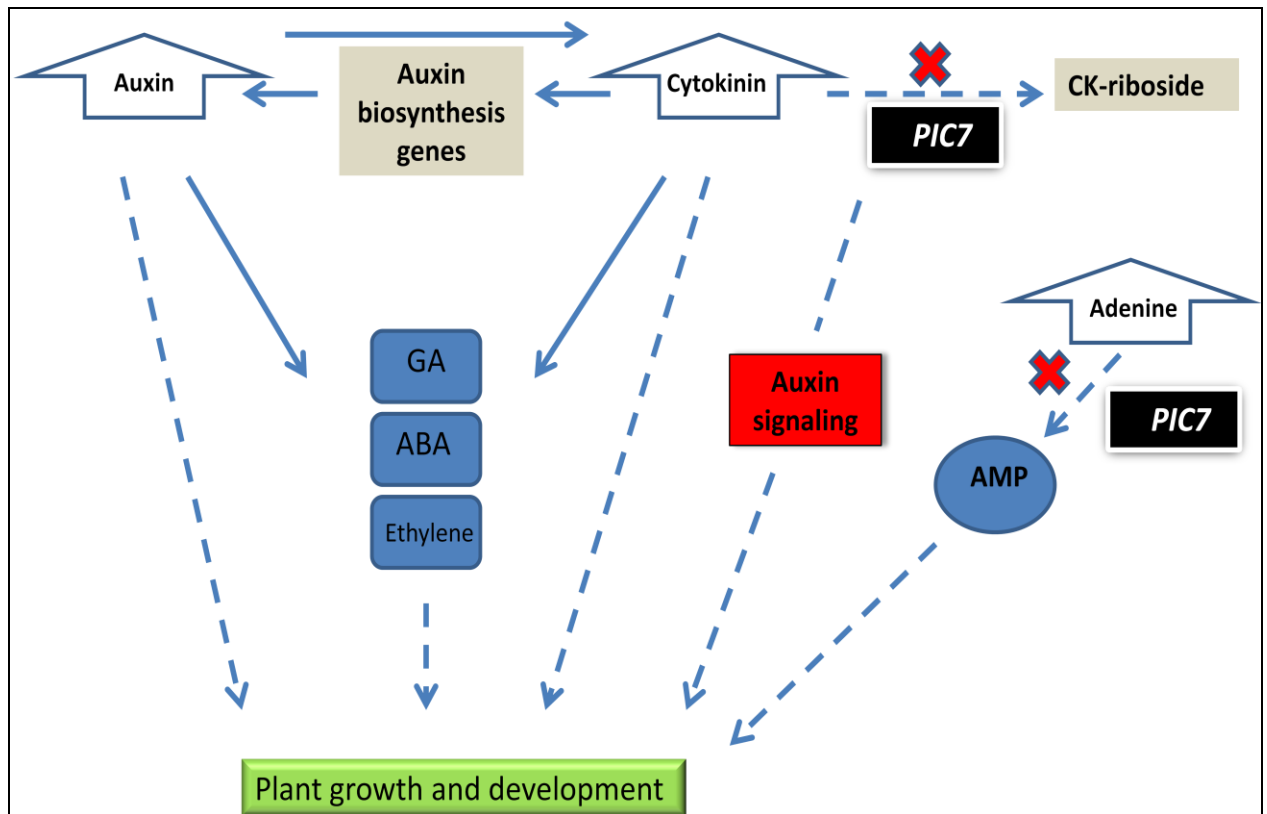


Figure 40. A schematic diagram to illustrate possible functions of *APT1* gene in Arabidopsis hormone response. In *pic7-1*, loss of APT1 activity may lead to reduced conversion from active cytokinin to the transportable form, CK-riboside. Therefore, the elevated local activity of cytokinin and hampered long distance distribution of the hormone may attribute to the altered cytokinin response in *pic7-1*. Moreover, auxin level may be up-regulated by high level of cytokinin through the induction of auxin biosynthesis genes. High level of auxin and altered auxin signaling cascade may lead to the altered auxin response in *pic7-1*. In addition, the distorted responses to other major hormones such as GA, ABA and ethylene might be due to the crosstalk of these major hormones with auxin and cytokinin. Since APT1 converts adenine to AMP, impaired

purine metabolism in *pic7-1* may be also responsible for the altered plant growth and development in *pic7-1*.

Conclusions and future directions

In this study, the auxin resistant mutant *pic7-1* was characterized. My hypotheses were that defective auxin response in *pic7-1* is attributed to the mutation in *APT1*. Secondly, since *APT1* is involved in purine metabolism, it might also affect plant growth and development through other plant hormone signaling pathways such as cytokinin, ethylene, ABA and GA. Thirdly, there might be multiple splice variants produced by *APT1* in Arabidopsis. According to the experimental evidence, my hypotheses were supported. *pic7-1* confers altered auxin signaling caused by the *APT1* mutation and it might also have higher auxin level. *APT1* is mainly localized to the vasculature, the cytoplasm and nucleus of the dividing cells and nucleus of the mature cells, which shows potential linkage with growth defects in *pic7-1*. *APT1* is a novel component in cytokinin homeostasis and might mediate cytokinin response through major type-B ARRs. *APT1* might function as a convergent point between auxin and cytokinin signaling crosstalk. *APT1* is involved in auxin crosstalk with other hormones through differential mechanisms. Finally, two splice variants are produced by *APT1* in Arabidopsis and regulating male fertility in a redundant manner.

There are many promising directions toward which this project proceeds. Measurement of the endogenous free IAA and its conjugates in different tissues will be of great importance to build up a solid model for auxin homeostasis regulation by *APT1*. It is also interesting to characterize the phenotypic differences where *APT1* localizes

between wild type Col-0 and *pic7-1* in finer temporal and spatial scales to further uncover the function of *APT1* in plant development. In addition, enzymatic activity comparison between wild type and mutant APT1 is necessary to understand the effect of mutation on the enzyme activity, which is helpful to further confirm the contribution of enzyme activity to hormone responses. In addition, further characterization of each splice variant of *APT1* will give insight into the mechanisms through which *APT1* regulates wide range of developmental processes. Finally, the endogenous cytokinin measurements as well as further characterization of cytokinin response regulators will help understand the involvement of *APT1* in cytokinin homeostasis and auxin cytokinin crosstalk. Therefore, the overall deeper and detailed understanding of the underlying mechanisms through which *APT1* mediates auxin as well as other hormone signaling will further fill up the gap in auxin signaling and crosstalk.

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