The Arabidopsis *AtIPT8*/*PGA22* Gene Encodes an Isopentenyl Transferase That Is Involved in De Novo Cytokinin Biosynthesis[1]

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Cytokinin plays a critical role in plant growth and development by stimulating cell division and cell differentiation. Despite many years' research efforts, our current understanding of this hormone is still limited regarding both its biosynthesis and signaling. To genetically dissect the cytokinin pathway, we have used a functional screen to identify Arabidopsis gain-of-function mutations that enable shoot formation in the absence of exogenous cytokinins. By using a chemical-inducible activation tagging system, we have identified over 40 putative mutants, designated as *pga* (*plant growth activators*), which presumably were affected in key components of cytokinin biosynthesis and signaling pathway. Here, we report a detailed characterization of *pga22*, a representative mutant from this collection. A gain-of-function mutation in the *PGA22* locus resulted in typical cytokinin responses. Molecular and genetic analyses indicated that *PGA22* encodes an isopentenyl transferase (IPT) previously identified as AtIPT8. Plants of the *pga22* mutant accumulated at remarkably higher levels of isopentenyladenosine-5′-monophosphate and isopentenyladenosine when analyzed by mass spectrometry, suggesting that AtIPT8/PGA22 is a functional IPT that may direct the biosynthesis of cytokinins in planta via an isopentenyladenosine-5′-monophosphate-dependent pathway.

Cytokinin plays an important role in many aspects of plant growth and development, such as regulating shoot and root growth, and controlling apical dominance and leaf senescence as well as flowering time. At the cellular level, it is generally believed that cytokinin executes its function by stimulating cell division and cell differentiation (Davies, 1995). Despite its critical role in plant growth and development, cytokinin is the least understood hormone among the so-called classical plant phytohormones with respect to its biosynthesis and signaling (Abel et al., 2000; Ross and O'Neill, 2001). Significant efforts have been made to elucidate the molecular and cellular mechanisms of cytokinin actions. For instance, extensive genetic screens, which have been carried out under conditions of high concentrations of exogenous cytokinins, have not yielded any mutations that are affected mainly in the cytokinin pathway. These results were presumably related to cross talk among different pathways, particularly between the ethylene and cytokinin pathways, or to the fact that cytokinin also partially evokes ethylene responses.

During the last several years, considerable progress has been made in efforts to elucidate the molecular mechanism of cytokinin signaling (for review, see D'Agostino and Kieber, 1999; Mok and Mok, 2001; Haberer and Kieber, 2002; Sheen, 2002). A major breakthrough was the use of an in vitro shoot formation bioassay to identify key components in cytokinin signal transduction (Kakimoto, 1996). A high cytokinin to auxin ratio has been shown to promote shoot formation from explants of certain species (Skoog and Miller, 1957; Sugiyama, 1999). In principle, an overactive key regulator may evoke cytokinin responses, thus leading to shoot regeneration in the absence of externally supplied cytokinins. This working hypothesis led to the identification of the Arabidopsis *CKI1* (*Cytokinin Independent 1*) gene, which encodes a putative receptor-like His kinase (Kakimoto, 1996). Conversely, loss-of-function mutations in similar loci may render mutant plants or explants insensitive to exogenous cytokinins. By screening such cytokinin-insensitive mutants using the shoot

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formation assay, Kakimoto and colleagues identified the Arabidopsis *cre1* (*cytokinin receptor 1*) mutant, which showed an attenuated response to the hormone (Inoue et al., 2001). Moreover, CRE1 is capable of binding to the phytohormone in yeast (*Saccharomyces cerevisiae*; Inoue et al., 2001) and bacterial (Suzuki et al., 2001; Yamada et al., 2001) cell-based bioassays. Thus, these results provided direct evidence that CRE1 is a bona fide cytokinin receptor.

Recent studies revealed that the cytokinin signal transduction pathway probably includes two branches: the hormone-dependent CRE1 branch and the hormone-independent CKI1 branch (Hwang and Sheen, 2001). In either case, the pathway or branch is presumably activated through a series of His-to-Asp phosphorelays, which are known to include several His-containing phosphotransfer factors and a large number of the so-called "response regulators" (Brandstatter and Kieber, 1998; Imamura et al., 1998). The Arabidopsis genome contains five His-containing phosphotransfer factors (AHP) and 22 response regulators (ARR; for review, see D'Agostino and Kieber, 1999; Schaller, 2000). Similar to Arabidopsis His kinases (AHKs), all AHPs and ARRs contain a highly conserved His residue in the kinase domain and an Asp residue in the receiver domain. It appears that an upstream signal is transmitted to and amplified by sequential transferring of phosphoryl groups between these conserved His and Asp residues located in AHKs, AHPs, and ARRs, leading to altered gene expression and eventually global physiological responses (Sakai et al., 2000, 2001; Hwang and Sheen, 2001; Suzuki et al., 2002; for review, see Haberer and Kieber, 2002; Sheen, 2002). However, little detail is known about this phosphorelay during cytokinin signaling, and other critical components of the pathway still remain to be identified.

Our current knowledge on cytokinin biosynthesis in plants is largely deduced from studies on a possibly analogous system in *Agrobacterium tumefaciens*. Cells of *A. tumefaciens* are able to infect certain plant species by inducing tumor formation in host plant tissues (Van Montagu and Schell, 1982; Hansen and Chilton, 1999). To do so, these *A. tumefaciens* cells synthesize and secrete cytokinins, which mediate the transformation of normal host plant tissues into tumors or calli. This process is facilitated by the *A. tumefaciens* tumor-inducing plasmid, which contains genes encoding the necessary enzymes and regulators for cytokinin biosynthesis (for review, see Van Montagu and Schell, 1982; Saito et al., 1992; Hansen and Chilton, 1999). Biochemical and genetic studies revealed that Gene 4 of the tumor-inducing plasmid encodes an isopentenyl transferase (IPT), which converts AMP and dimethylallyl-diphosphate (DMAPP) into isopentenyladenosine-5′-monophosphate (iPMP), the active form of cytokinins (Akiyoshi et al., 1984; Barry et al., 1984). Overexpression of the *ipt* gene in a variety of transgenic plants has been shown to cause an increased level of cytokinins and elicit typical cytokinin responses in the host plants (Hansen and Chilton, 1999). Therefore, it has been postulated that plant cells use machinery similar to that of *A. tumefaciens* cells for cytokinin biosynthesis. Although ipt-like enzymatic activity has been detected in crude extracts of various plant tissues (e.g. Horgan, 1975; Chen and Melitz, 1979), homologous genes from Arabidopsis were only identified recently by a bioinformatic approach (Kakimoto, 2001; Takei et al., 2001). These Arabidopsis homologs were designated as *AtIPT1* through *AtIPT9* (Kakimoto, 2001). With the exception of AtIPT2 and AtIPT9, recombinant proteins of the other seven *AtIPT* genes were able to catalyze the production of active cytokinins in *Escherichia coli* cells (Takei et al., 2001). Moreover, overexpression of *AtIPT4* in transgenic Arabidopsis plants elevated cytokinin levels and elicited typical cytokinin responses in planta and under tissue culture conditions (Kakimoto, 2001). These phenotypes are similar to those observed with overexpression of the *A. tumefaciens ipt* gene.

By using genetic approaches, two mutations that affect cytokinin biosynthesis were recently identified from petunia (*Petunia hybrida*; Zubko et al., 2002) and Arabidopsis (Catterou et al., 2002). The petunia mutant *sho* (*shooting*), identified by a 35S enhancer tag, displayed the shooty phenotype characteristic of cytokinin responses. The *sho* mutant phenotype was found to be caused by increased levels of isopentenyladenosine (iPA) and derivatives in mutant plants (Zubko et al., 2002). Subsequent molecular analysis indicated that *SHO* encodes an ipt-like enzyme (Zubko et al., 2002). The Arabidopsis mutant *hoc* (*high organogenic capacity*) showed a phenotype similar to that of *sho* (Catterou et al., 2002). However, *hoc* appears to be a recessive mutation, whose wild-type (WT) allele, yet to be identified, may negatively regulate the cytokinin biosynthetic pathway.

To dissect the cytokinin signal transduction pathway, we have carried out a functional screening to identify key components of the hormone action. The screen, in principle, was similar to that developed by Kakimoto (1996), who identified the *cki1* gain-of-function mutation. An improvement introduced by us was the use of a chemical-inducible promoter/enhancer (Zuo et al., 2000a; Zuo et al., 2002) rather than a constitutive enhancer; therefore, mutants that displayed severely abnormal plant growth and development or lethality could be recovered. In addition to the shooty phenotype, i.e. cytokinin-independent shoot formation, we also extend our screening criteria to include mutations that were capable of promoting embryonic callus formation. As reported previously, we collectively designated these two classes of mutants as *pga* (*plant growth activators*; Zuo et al., 2002). Among the *pga* mutants with a shooty phenotype, we expect that a mutation would affect either cytokinin biosynthesis or transduction of

the hormonal signal. Here, we present genetic and molecular evidence showing that PGA22 may represent a functional IPT. Overexpression of *PGA22* caused a massive increase in cytokinin levels in mutant plants, thus evoking typical cytokinin responses.

RESULTS

Identification of the *plant growth activator* 22 (*pga*22) Mutant

To identify gain-of-function mutations related to cytokinin signaling and somatic embryogenesis, we carried out a functional screen by using a chemical-inducible activation-tagging vector (the LexA-VP16-estrogen receptor vector pER16; see Zuo et al., 2000a, 2002). In this screen, *A. tumefaciens ABI* cells carrying pER16 were used to transform Arabidopsis (the Wassilewskija [Ws] ecotype) root explants according to Koncz et al. (1989). Subsequently, the *A. tumefaciens*-infected root explants were cultured on the screening medium (SCM; containing auxin indole-3-acetic acid, kanamycin, and 17-β-estradiol without cytokinins). Under our screening conditions, WT explants were not able to generate shoots or embryogenic calli. However, a gain-of-function mutation may cause the activation of the cytokinin signal transduction pathway, thus producing green calli or shoots; alternatively, a gain-of-function mutation may promote a vegetative-to-embryogenic transition, thus leading to the formation of somatic embryos. In a large-scale screen, we have identified over 40 putative mutants, which displayed two distinctive phenotypes characterized as "shooty" or "embryogenic." We collectively named these two classes of mutants as *plant growth activators* (see also Zuo et al., 2002). Results on the embryogenic mutants, represented by two mutant alleles of the *PGA6/WUSCHEL* (*WUS*) gene, have been published previously (Zuo et al., 2002). Here we report the characterization of *pga22*, a typical shooty mutant.

When cultured on the screen medium, WT root explants did not appear to undergo apparent cell divisions. Similar to most of the *pga* mutants, *pga22* was identified by its capability to form green calli and subsequently develop into shoots on the SCM. Following standard tissue culture manipulations (for details, see Zuo et al., 2002), *pga22* seeds were harvested from the mutant plants after being transferred to soil. To confirm the phenotype observed in the primary screening, putative *pga22* seeds were germinated on Murashige and Skoog medium (Murashige and Skoog, 1962), and roots or leaves excised from the mutant seedlings were cultured on a 2,4-dichlorophenoxyacetic acid-containing medium to induce callus formation. The callus was subsequently transferred onto the SCM as described before. In the absence of the inducer (SCM without 17-β-estradiol), neither WT nor *pga22* root explants showed any apparent cell division (Fig. 1A). When cultured on the SCM in the presence of the inducer, however, *pga22* root explants were able to develop rapidly dividing green calli (Fig. 1A), which subsequently differentiated into shoots (Fig. 1B). These shoots were indistinguishable from those generated from WT root explants cultured on a standard shoot regeneration medium containing both auxin and cytokinin. Upon removal of the inducer, these shoots were able to grow and develop into morphologically normal plants that were fertile and set seeds (Fig. 1C). These observations indicated that an inducer-dependent gain-of-function mutation in the *PGA22* locus was sufficient to activate the organogenesis pathway.

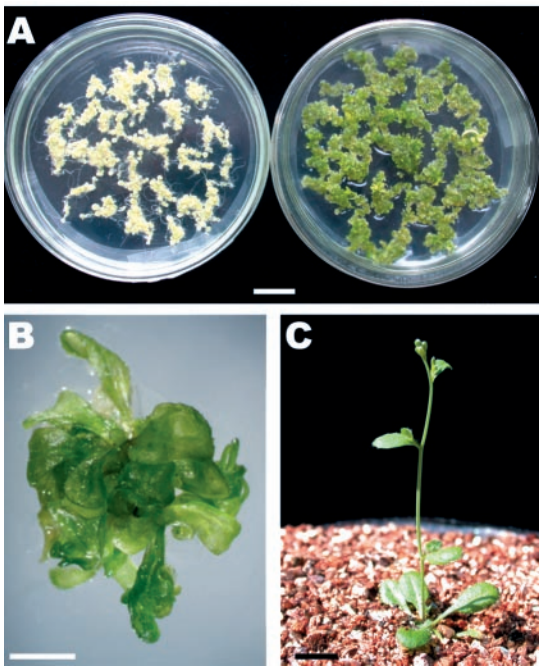

Figure 1. Cytokinin-independent shoot formation of *pga22* explants. A, Root explants derived from *pga22* seedlings were cultured on the noninductive SCM (without cytokinins and 17-β-estradiol; left) or on the inductive SCM (containing 17-β-estradiol but without cytokinins; right) for 20 d. B, Shoots regenerated from *pga22* root explants cultured on the inductive SCM were transferred onto a Murashige and Skoog medium (35 d). C, A 45-d-old plantlet derived from shoots shown in B was transferred into soil. Bar = 1 cm.

The *pga22* Gain-of-Function Mutant Phenotype

To assay *PGA22* functions during plant growth and development, *pga22* seeds were germinated on Murashige and Skoog medium with or without the inducer. When germinated on the noninductive medium, no apparent growth and development abnormality was observed (Fig. 2A), suggesting that mutations in the *pga22* genome did not appear to affect the normal function of the gene. On the inductive medium, however, *pga22* mutant plants showed severe morphological abnormality. The mutant roots were extremely short, the cotyledons remained pale yellow after germination, and true leaf initiation was

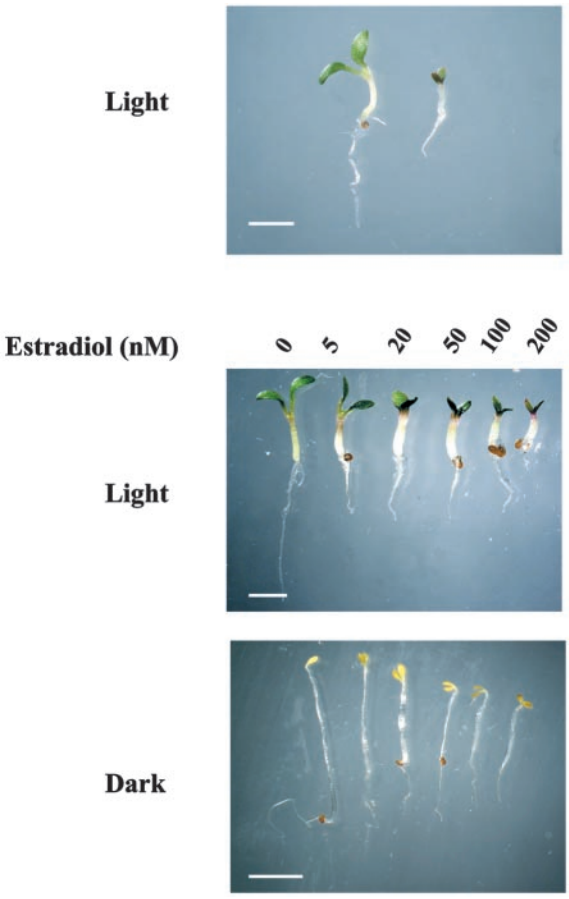

Figure 2. The *pga22* gain-of-function mutant phenotype. Homozygous T2 *pga22* seeds were germinated in the presence or absence of 17-β-estradiol in the light or dark as indicated. Photographs were taken 6 d after germination. A, Germinated in the absence (left) or presence of 10 μM 17-β-estradiol (right). B and C, Germinated in the absence (far left) or presence of different concentrations of 17-β-estradiol in the light (B) or dark (C), respectively, as indicated. Bar = 5 mm.

rarely observed (Fig. 2A). Shortly after germination, plant growth and development were completely arrested and mutant plants eventually died. Note that the above-described phenotype was routinely observed when *pga22* mutant seeds were germinated in the presence of 17-β-estradiol in concentrations higher than 0.2 μM.

In studies on *pga6* and other *pga* mutants, we have observed that the penetration of a mutant phenotype was strictly dependent on the inducer dosage (Zuo et al., 2002). As highlighted above, this dosage dependency, however, was not apparent in *pga22*, within the range of inducer concentrations routinely used (0.2–10 μM). To further explore the effects of the *pga22* gain-of-function mutation on plant growth and development, we carried out a titration experiment with inducer concentrations lower than routinely used. Under such test conditions, the *pga22* mutant showed typical cytokinin responses, including shorter roots, enlarged hypocotyls, and dark-green cotyledons (Fig. 2B). When germinated in the dark, *pga22* seedlings displayed a characteristic de-etiolated phenotype, including shorter hypocotyls and shorter roots, opened cotyledons, and the absence of apical hooks (Fig. 2C). The strength of phenotype was strictly dependent on inducer concentrations in both light and dark conditions. These observations suggested that a gain-of-function mutation in the *PGA22* locus was able to evoke cytokinin responses, and that PGA22 is probably a key component in the cytokinin biosynthesis or signaling pathway. In addition, these data also suggested that the *PGA22* gene was tagged by the O^{LexA}*-46* promoter of the pER16 vector.

PGA22 Encodes an IPT

Genetic analysis showed that the 17-β-estradiol-dependent mutant phenotype and WT phenotype segregated in a ratio of 3:1, indicating that the mutation was dominant in a single genetic locus. Segregation analysis also suggested that the mutant genome contained a single T-DNA insertion. Because the *pga22* gain-of-function phenotype was strictly dependent on the inducer, the *PGA22* gene should be tagged by the O^{LexA}*-46* promoter of pER16 inserted in the mutant genome. Taking advantage of this, we cloned the O^{LexA}*-46* promoter-tagged mutant genomic sequences by thermal asymmetric interlaced-PCR (Liu et al., 1995). Sequence analysis indicated that the T-DNA inserted 362 bp upstream from the putative translation start codon of a putative open reading frame (ORF; Fig. 3A).

To verify if this ORF represents the *PGA22* gene, a genomic DNA fragment spanning the entire ORF and part of flanking 5′-untranslated region and 3′-untranslated region was cloned into pER10, a 17-β-estradiol-inducible expression vector (Zuo et al., 2000a, 2002). The resulting construct was used to transform root explants derived from WT plants. The transformed root explants were placed on the SCM or a control medium without cytokinins and 17-β-estradiol as described before. The explants formed neither green calli nor shoots on the control medium (Fig. 3B). In contrast, on the SCM, green callus formation could be observed after 10 to 15 d (Fig. 3C), and shoots were generated after 3 to 4 weeks (Fig. 3D). Similar results were obtained using a genomic fragment containing additional upstream sequences of the gene up to the T-DNA insertion site (see Fig. 3A), with a slightly lower regeneration efficiency (data not shown). All of the above-mentioned *pga22* gain-of-function phenotypes were observed in the pER10-*ORF1* transgenic plants in an inducer-dependent fashion (Fig. 3E). Taken together, these data demonstrated that the O^{LexA}*-46* promoter-tagged ORF represented the *PGA22* gene.

The *PGA22* gene, interrupted by an intron of 252 bp, encodes a polypeptide of 329 amino acids, with

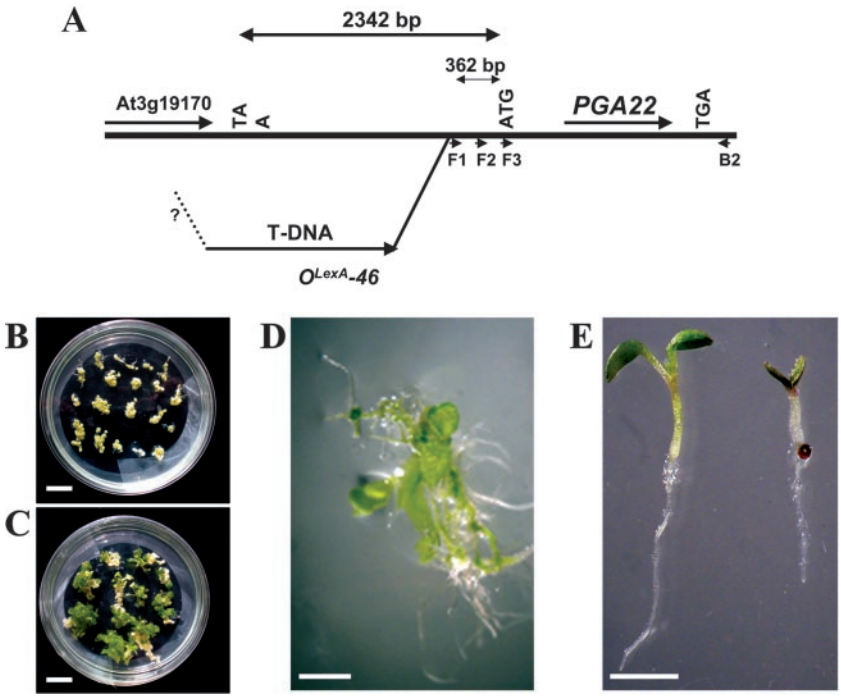

Figure 3. The *pga22* mutant genome and genetic confirmation of the *PGA22* gene. A, Schematic diagram showing the insertion site of the T-DNA upstream from the *AtIPT8/PGA22* gene (not in scale). Arrows indicate the directions of transcription. The insertion site of the right border (RB) was unclear (question mark). F1 through F3 and B2, Positions and orientations of primers used for PCR-amplification of the *AtIPT8/PGA22* gene (see text for more details). B, WT root explants were transformed with pER10-*AtIPT8* (the genomic DNA fragment spanned from the F2 to B2 region as shown in A) and cultured on the SCM without 17-β-estradiol for 15 d. C, WT root explants were transformed with pER10-*AtIPT8* (the genomic DNA fragment spanned from the F2 to B2 region as shown in A) and cultured on the SCM containing 10 μM 17-β-estradiol for 25 d. D, A 40-d-old pER10-*AtIPT8* shoot grown on Murashige and Skoog medium. E, T_1 seeds of pER10-*AtIPT8* transgenic plants were germinated in the absence (left) and presence of 10 μM 17-β-estradiol. Figure shows 7-d-old seedlings. Bar = 2 cm (B and C); bar = 1 cm (D and E).

an estimated molecular mass of 37.2 kD and a pI of 8.03. A database search revealed that PGA22 was identical to the previously identified AtIPT8 (Arabidopsis IPT; GenBank accession nos. BAB02956 [Takei et al., 2001] and BAB59034 [Kakimoto, 2001]). *AtIPT8* and several other *IPT*-like genes were identified by an in silica approach based on partial homology of these Arabidopsis genes with the *A. tumefaciens ipt*. Detailed annotation and phylogenetic studies on these *AtIPT* genes have been reported by Takei et al. (2001) and Kakimoto (2001). Hereafter, we will refer to the PGA22 gene/protein as AtIPT8 and the mutant/mutation as *pga22*.

AtIPT8 Is Mainly Expressed in Roots

To better understand its function, we analyzed the *AtIPT8* expression pattern in WT plants. Although the *AtIPT8* expression was easily detected in *pga22* treated with various concentrations of the 17-β-estradiol inducer, we were unable to detect its expression in WT plants by northern-blot analysis (see below). Therefore, we used reverse transcription-PCR (RT-PCR) followed by Southern-blot analysis to assess *AtIPT8* expression in WT plants. Figure 4 shows that *AtIPT8* could only be detected in roots under our assay conditions, suggesting that AtIPT8 is likely involved in cytokinin biosynthesis in roots.

Consistent with the inducer dosage-dependent mutant phenotype (see Fig. 2), *AtIPT8* expression was strictly dependent on the inducer concentration (Fig. 5A) as well as the time of induction (Fig. 5B). Similar to those observed in the LexA-VP16-estrogen receptor-green fluorescent protein transgenic lines (Zuo et al., 2000a), the *ATIPT8* induction was saturated at 5 μM 17-β-estradiol and an incubation time of 12 to 24 h (see Fig. 5, A and B). We have reported previously that in the two alleles of *pga6*, the inserted O^{LexA}-*46* DNA segment could act as a functional promoter as well as an enhancer under our induction conditions (Zuo et al., 2002). Similarly, the two distinctive transcripts detected by the *AtIPT8* probe (indicated by arrows in Fig. 5A) presumably represented those initiated from the O^{LexA}-*46* promoter (the longer transcript) and the native *AtIPT8* promoter (the shorter transcript).

The *pga22* Gain-of-Function Mutation Induces Expression of Type A *ARR* Genes

The cytokinin signal is believed to be transduced via a His-Asp phosphorelay pathway involving the cytokinin receptors CRE1 (Inoue et al., 2001) and, possibly, CKI1 (Kakimoto, 1996), and a series of conserved His kinases including AHPs and ARRs (for review, see Schaller, 2000; Haberer and Kieber, 2002; Sheen, 2002). One of the physiological consequences of the cytokinin action is to stimulate cell division (Davies, 1995), presumably mediated by CycD3, a D-type cyclin (Riou-Khamlichi et al., 1999). Although the precise functions of these proteins are not well understood, expression of type A *ARRs* has been shown to be induced by cytokinin applications (Brandstatter and Kieber, 1998; Imamura et al., 1998). To further characterize the *pga22* mutant phenotype at the molecular level, we analyzed expression of several above-mentioned cytokinin marker genes. As shown in Figure 6, expression of *ARR5*, a type A *ARR*

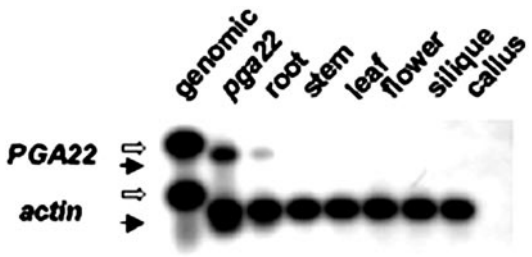

Figure 4. Expression pattern of the *AtIPT8/PGA22* gene. Five microliters of RT-PCR mixtures (see "Materials and Methods" for details) was separated by a 1.2% (w/v) agarose gel and then blotted onto a nylon membrane. The membrane was hybridized with an *AtIPT8* (a genomic DNA fragment spanned the F3 and B2 region; see Fig. 3) probe and an actin 3 DNA probe. Fifty nanograms of Arabidopsis genomic DNA was used in the PCR to serve as a control (the "genomic" lane). RNA prepared from *pga22* seedlings (T_3 homozygous, 3 weeks old) treated with 5 μM 17-β-estradiol for 12 h was used for RT, and the PCR products of the primer pair F3/B2 were diluted 50 times before loading (the "*pga22* " lane). Other lanes: RNA prepared from various tissues/organs of WT plants was used for RT. Solid and white arrows indicate cDNA and genomic fragments, respectively.

gene, was strongly induced in *pga22* mutant plants upon induction of *AtIPT8*. Identical results were obtained with the expression of *ARR6*, another type A *ARR* gene. In contrast, expression of *ARR1*, a type B *ARR* gene, was not affected (data not shown). Moreover, expression of *CycD3* was also increased in *pga22* upon induction of *AtIPT8*. Along with morphological analyses of the mutant highlighted before, these data indicate that the *pga22* gain-of-function mutation is sufficient to activate the cytokinin signal transduction pathway.

AtIPT8 Directs iPMP Biosynthesis in Vivo

Previous studies suggested that many of the *AtIPT* genes (except *AtIPT2* and *AtIPT9*) were able to catalyze cytokinin synthesis in *E. coli* cells (Takei et al., 2001) and in vitro (Kakimoto, 2001). Moreover, overexpression of *AtIPT4* (Kakimoto, 2001) or *AtIPT8* (i.e. the *pga22* mutant, this study) was capable of inducing shoot formation independent of exogenous cytokinins. These results strongly suggested that AtIPTs play an important role in cytokinin biosynthesis. It has been demonstrated that ipt catalyzes the biosynthesis of isopentenyladenosine-5′-monophosphate (iPMP) from AMP and DMAPP and the corresponding nucleotide iPA (Taya et al., 1978), but there is as yet no experimental evidence to support the operation of a similar biosynthetic pathway in higher plants.

To test if AtIPT8 is a functional IPT in planta, we analyzed the cytokinin levels in the *pga22* mutant as compared with those of WT plants. Upon inducer treatment, the iPMP and iPA levels increased more than 19- and 38-fold, respectively, in the mutant plants upon a 24-h induction. By contrast, we only observed a minor increase in levels of the zeatin type of ribosides and ribotides after induction (Fig. 7). Moreover, little alterations in the content of the free bases were observed during the course of the experiment.

The above results suggest that AtIPT8 may act as a functional IPT, which directly catalyzes iPMP synthesis in planta. As a consequence, overexpression of the *AtIPT8* gene in *pga22* caused elevated iPMP and iPA levels, leading to cytokinin responses.

DISCUSSION

In a functional screen aimed at the dissection of cytokinin and auxin signaling pathways, we have identified two classes of novel mutants. Whereas the first class of mutants, represented by two alleles of *pga6*, appears to be involved in embryogenesis (Zuo et al., 2002), the second class of mutants is shown to be affected in key components in cytokinin signaling. Here, we have presented several lines of evidence showing that the Arabidopsis *AtIPT8* locus is directly

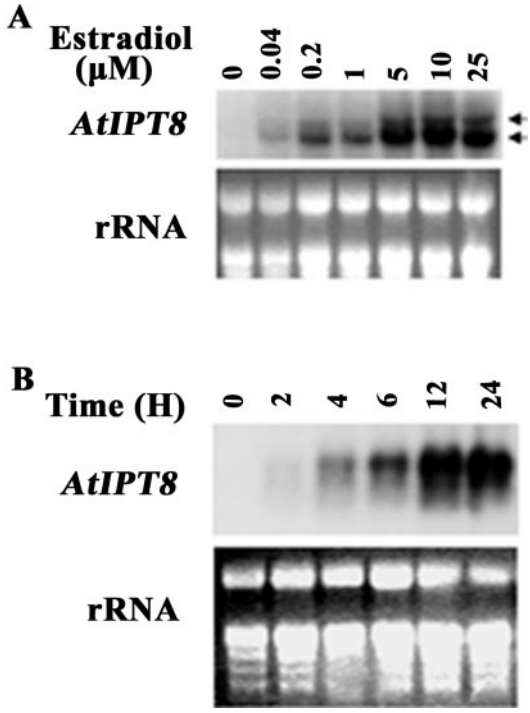

Figure 5. Inducible expression of *AtIPT8* in *pga22* mutant plants. A, Dosage dependency of the *AtIPT8* induction. Three-week-old *pga22* seedlings were treated for 16 h with varying concentrations of 17-β-estradiol (indicated on the top). Ten micrograms of RNA prepared from the treated seedlings was used for northern-blot analysis by using *AtIPT8* as a probe. Two transcripts with distinctive sizes were observed (indicated by arrows at the right side), presumably representing transcription initiation from two different sites (see text for detail). B, Time course of the *AtIPT8* induction. Three-week-old *pga22* seedlings were treated with 10 μM 17-β-estradiol for different time as indicated on the top of the photograph. See A for other technical details. Note that the two transcripts of slightly different sizes could be observed with a shorter exposure.

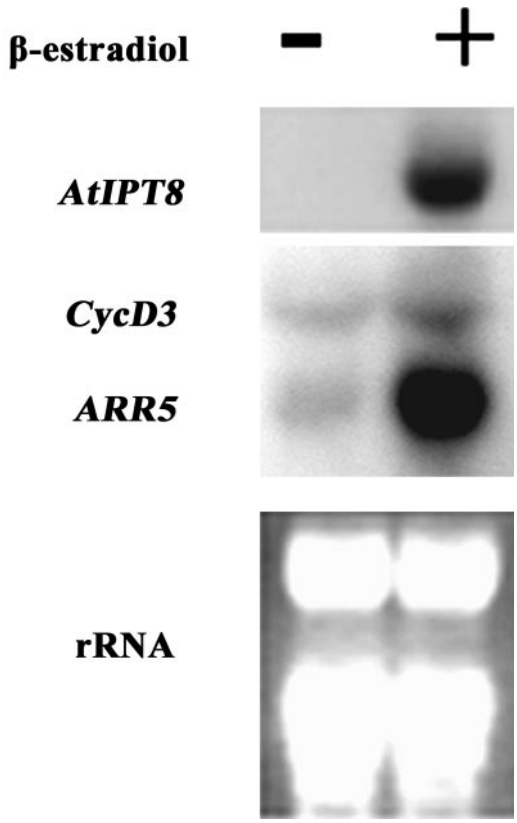

Figure 6. Activation of cytokinin marker genes in *pga22* conditional gain-of-function mutant. Three-week-old *pga22* seedlings were cultured in the absence (−) or the presence (+) of 10 μM 17-β-estradiol for 12 h. Ten micrograms of RNA was used for northern-blot analysis by using *AtIPT8*, *CycD3*, and *ARR5* as probes. See Figure 5 for other technical details.

involved in cytokinin biosynthesis. First, a gain-of-function mutation in the *PGA22* locus resulted in cytokinin-independent shoot formation, suggesting that the WT gene is a key component in biosynthesis or signaling. Second, overexpression of the *AtIPT8* gene caused typical cytokinin responses, thus demonstrating its physiological function. Last, the *AtIPT8* gene encodes a putative protein sharing amino acid sequence homology with the *A. tumefaciens* ipt enzyme, implying a possible role in cytokinin biosynthesis. Measurement of hormone levels in the mutant plants showed a remarkable increase of iPMP and iPA levels after induction, suggesting that AtIPT8 is likely a functional IPT.

Initially identified in *Dictyostelium discoideum* and several bacteria including *A. tumefaciens*, ipt has been demonstrated to be an enzyme that converts AMP and DMAPP into iPMP, a critical intermediate of cytokinin biosynthesis in these organisms (Taya et al., 1978). Although enzymatic activities similar to those of ipt have been detected in crude extracts prepared from various plant cells and tissues (Haberer and Kieber, 2002), it was only recently that *ipt*-like genes have been identified by an in silica approach upon the completion of the Arabidopsis genome (Arabidopsis Genome Initiative, 2000). These Arabidopsis homologs, designated as AtIPT1 through AtIPT9, appeared to have certain structural features of the bacterial ipt, based on phylogenetic analyses (Kakimoto, 2001; Takei et al., 2001). Except AtIPT2 and AtIPT9, recombinant proteins derived from the other seven *AtIPT* genes were capable of catalyzing cytokinin synthesis in vitro. In addition, overexpression of *AtIPT4* was able to promote shoot regeneration in the absence of external cytokinins, strongly arguing that the gene product was involved in cytokinin biosynthesis. Interestingly, AtIPT4 appeared to catalyze the formation of isopentenyladenosine-5′-triphosphate (iPTP) and isopentenyladenosine-5′-diphosphate (iPDP) from ADP and ATP in an in vitro assay (Kakimoto, 2001). This finding is apparently inconsistent with the conventional view that ipt catalyzes the formation of iPMP by utilizing AMP and DMAPP as substrates. In addition, the lack of detectable alterations in iPMP levels in *A. tumefaciens*-infected plant tissues and in *ipt* overexpression transgenic plants (Åstot et al., 2000, and refs. therein) further argues that an iPMP-

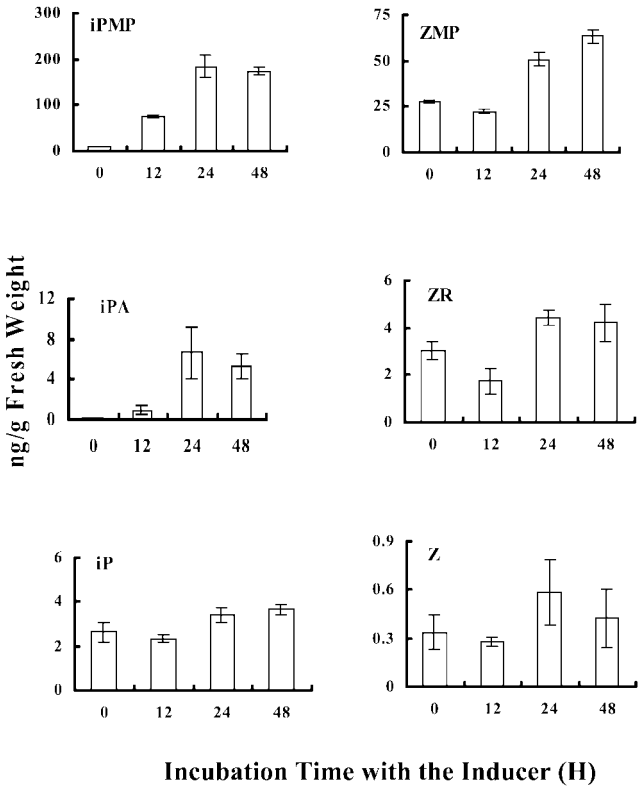

Figure 7. AtIPT8 catalyzes the production of iPMP and iPA. Cytokinin levels in *pga22* mutant plants. Three-week-old *pga22* seedlings were treated with 5 μM 17-β-estradiol for varying time periods. H, Hours, as indicated below the graph. Cytokinins extracted and purified from the frozen materials were measured by liquid chromatography/mass spectrometry analyses. The experiment was repeated three times, and the data shown here are mean values of the three experiments.

independent mechanism operates in plant cells, or that bacterial ipts function differently as compared with their plant counter-partners. A recent study on the petunia *SHO* gene revealed that the *ipt*-like gene was capable of promoting biosynthesis of iPA and derivatives (Zubko et al., 2002), which may potentially originate from not only iPMP but also from iPTP and/or iPDP (Kakimoto, 2001; Haberer and Kieber, 2002).

The fact that overexpression of *AtIPT8* leads to the accumulation of high levels of iPMP and iPA in planta strongly suggests that plant IPTs are, at least in part, functionally analogous to bacterial ipt, which directs the biosynthesis of iPMP from AMP and DMAPP. Consistent with this notion, K_m values of AMP and DMAPP (185 and 50 μM, respectively) for recombinant AtIPT1 are comparable with those of *A. tumefaciens* ipt; moreover, the plant enzyme catalyzed synthesis of iPMP (Takei et al., 2001). On the other hand, phylogenetic studies showed that AtIPT8, AtIPT4, and AtIPT1 are the most closely related members in the same subfamily (Kakimoto, 2001; Takei et al., 2001), making it difficult to understand why these three proteins have different enzymatic activities. The conflicting results obtained from these three studies are presumably because of different assay conditions used in the studies and the different genes investigated (*AtIPT1*, *AtIPT4*, and *AtIPT8*). Nevertheless, AtIPT8 is clearly involved in the synthesis of iPMP in planta, although it remains to be clarified whether or not the substrates of the ipt-like enzyme are AMP and DMAPP in vivo. In addition, it is also important to elucidate the potential involvement of iPDP and/or iPTP in this pathway.

Recently, Catterou et al. (2002) identified an Arabidopsis mutant designated as *hoc*. The *hoc* recessive mutation rendered shoot formation from explants in the absence of exogenous cytokinins (Catterou et al., 2002), a phenotype similar to that of the dominant-positive *pga22* mutant. Analysis of hormone levels revealed that an elevated cytokinin level in *hoc* plants caused the mutant phenotype. In contrast to *pga22*, in which the iPMP and iPA levels significantly increased but other cytokinins remained largely unchanged, *hoc* contained an elevated amount of most of the major cytokinins, particularly in roots. Because the iPMP concentration was not determined in the *hoc* mutant, it is unclear if *HOC* acts in the iPMP-dependent pathway (Catterou et al., 2002). Similar to that of *sho* (Zubko et al., 2002), the elevated amount of iPA and other derivatives may be derived from precursors other than iPMP. Nevertheless, the phenotypic differences between *pga22* and *hoc* suggest that these two loci function differently in the cytokinin biosynthesis pathway. Considering the nature of the mutant (Catterou et al., 2002), it appears that *HOC* acts as a negative regulator other than a biosynthetic enzyme in the cytokinin biosynthesis pathway. Molecular characterization of the *HOC* gene will help to clarify its own function and aid our understanding of the cytokinin biosynthetic pathway.

In a previous study, we have shown that overexpression of *A. tumefaciens ipt* caused the accumulation of cytokinins via an iPMP-independent pathway, which leads to the formation of ZMP rather than iPMP (Åstot et al., 2000). In *pga22* mutant plants, although the iPMP level showed a remarkable increase, the ZMP level increased only marginally, suggesting that AtIPT8 functions in an iPMP-dependent pathway. The different enzymatic activities of the *A. tumefaciens* ipt and AtIPT8 can be likely attributed to structural differences because the two proteins share only 12% homology. On the basis of the above-discussed results, we propose that Arabidopsis, perhaps other higher plants as well, may have several pathways for cytokinin biosynthesis, including iPMP-independent and -dependent pathways. On the other hand, it is reasonable to assume that the di- and tri-phosphates of both the iP and Z type of cytokinin are potential intermediates upstream of the ribotides (see also Kakimoto, 2001). Clearly, the role of iPDP and iPTP in cytokinin biosynthesis must be further elucidated to better understand the entire pathway.

AtIPT8 is expressed at a very low level, detectable only in roots by RT-PCR combined with Southern-blot analysis. As a consequence, no expressed sequence tag clone was found in all public databases. Although its precise expression pattern remains to be confirmed by in situ hybridization, *AtIPT8* appears to be predominately expressed in roots where cytokinins are generally believed to be synthesized (Davies, 1995; Mok and Mok, 2001). As expected, overproduction of the hormone in *pga22* resulted in the activation of the cytokinin signal transduction pathway, thereby leading to typical cytokinin responses of the mutant. Interestingly, we were unable to identify any T-DNA insertion mutants in the *AtIPT8* gene by searching over 50,000 independent transgenic Arabidopsis lines from public databases and our own collections, implying that a knockout mutation in this gene may be embryo lethal, rendering it impossible to recover a loss-of-function *pga22* mutation by conventional methods. A screen for the *pga22* suppressor mutants is expected to identify weaker mutation alleles of the *AtIPT8* gene and, possibly, important components in cytokinin biosynthesis, transport, and signal transduction, thus providing invaluable genetic tools to study AtIPT8 functions and the entire cytokinin biosynthetic and signaling pathway.

MATERIALS AND METHODS

Screening of *pga* Mutants

Screening of *pga* mutants has been described in detail in a previous report (Zuo et al., 2002). The *pga22* mutant, in the Ws background, was out-crossed twice with Ws WT plants. Homozygous or heterozygous F_2 or F_3 progenies were used in all experiments.

Plant Materials, Growth Conditions, and Plant Transformation

The Ws and Columbia ecotypes of Arabidopsis were used. Plants were grown under a 16-h-light/8-h-dark cycle at 22°C on solid A medium (1× Murashige and Skoog salts, 3% [w/v] Suc, and 0.8% [w/v] agar) supplemented with the appropriate antibiotics and/or the inducer 17-β-estradiol. Transformation of root explants (derived from Ws or Columbia WT plants) was carried out according to Koncz et al. (1989). Treatment of plants with 17-β-estradiol was carried out as described previously (Zuo et al., 2000a).

Analysis of Cytokinin Concentrations

Three-week-old seedlings homozygous for the *pga22* locus were sprayed with 5 μM 17-β-estradiol in 0.1% (v/v) Tween 20 and incubated for varying periods of time before the seedlings were immediately frozen in liquid nitrogen and stored at −80°C. The extraction and purification of cytokinins were essentially the same as described by Åstot et al. (1998). In brief, frozen tissues were grounded with mortar and pestle in liquid nitrogen. Fresh mass (500 mg) was extracted overnight in Bieleski solvent (Bieleski, 1964) in the presence of heavy labeled internal standards: $^2H_5$-Z, $^2H_5$-ZR, $^2H_5$-Z9G, $^2H_6$-iP, ^{15}N, $^2H_5$-ZMP, and ^{15}N, $^2H_6$-iPMP (Apex International, Haniton, UK) as internal tracers for quantification. After two rounds of ion-exchange chromatography steps (strong cation-exchange cartridge and DEAE-Sephadex combined with C18 cartridges), the samples were split into two fractions. The first fraction was directly loaded onto an immuno-affinity chromatography column (OlChemIm Ltd., Olomouc, Czech Republic) to purify cytokinin free bases, ribosides, glucosides, and the second ribotides. The second fraction was treated with alkaline phosphatase and subsequently immunopurified to obtain cytokinin ribotides. Derivatization (propionylation) was performed according to Åstot et al. (1998). All samples were evaporated in vacuo and stored at −20°C until further analysis.

Cytokinin levels were estimated by liquid chromatography/mass spectrometry analysis in selective reaction monitoring mode. Chromatographic separation was performed using a Symmetry Shield RP_{18} column (3.5 μm), 2.1× 150 mm. At a flow rate of 0.2 mL min^{-1}, the following binary gradient was used: 0 to 3 min, isocratic elution of 10% (v/v) B; 3 to 20 min, a linear gradient to 90% (v/v) B; followed by a 2-min isocratic elution of 90% (v/v) B. Solvent A consisted of 1% (v/v) formic acid in water, and solvent B consisted of 1% (v/v) formic acid in acetonitrile. Effluents from the chromatographic column were introduced to a Micromass Quattro Ultima mass spectrometer (Jeol, Tokyo) via an electrospray ion source (capillary voltage + 3.2 kV, cone voltage + 60V, source temperature 110°C, desolvation temperature 250°C, cone gas flow 220 L h^{-1}, desolvation gas flow 740 L h^{-1}, collision energy 20 units, and dwell time 0.35 s).

Molecular Manipulations

All molecular manipulations were performed according to standard methods (Sambrook et al., 1989). The O^{LexA}-*46* promoter-tagged genomic sequence in the *pga22* genome was identified by thermal asymmetric interlaced-PCR as previously described (Liu et al., 1995; Zuo et al., 2002). The *AtIPT8* genomic clones were obtained by PCR using the primer pairs pga22F1/pga22B2 (clone long [L]) and pga22F2/pga22B2 (clone short [S]). At the 5′ end, the clone L (1,638 bp) started exactly at the T-DNA insertion site, presumably in the *AtIPT8* promoter region, whereas the clone S (1,482 bp) started 127 bp upstream from the putative ATG. The PCR products, amplified by PWO DNA polymerase (Roche Diagnostics Hong Kong, Hong Kong), were cloned into the *Sma*I site of a pBlueScript SK vector (Strategene, La Jolla, CA). Both clones, characterized by extensive restriction digests and DNA sequencing, were released by *Xho*I/*Spe*I digestion and inserted into the same sites of pER10 (Zuo et al., 2000a; 2002). The resulting constructs were transformed into *Agrobacterium tumefaciens* strain GV3101, which was used for subsequent root transformation experiments. Although both clones were functional for cytokinin-independent shoot formation, clone S appeared to be slightly more efficient.

Primers used in the PCR (non-plant sequences are in lower case and restriction sties used in cloning are underlined): pga22F1, 5′ ggactagtgtcgactcgagGACATTGTTAAGAGCATGAATGGTAAC; pga22F2, 5′ ggactagtgtcgactcgagCCGTATGAAATGTCCTTTGACATATCA; pga22F3, 5′ ATGCAAAATCTTACGTCCACATTC; pga22B2, 5′ tctagagtcgacgactagtAAATCGAGGTGCAAAAATCTTAAACATC; Act3F1, 5′ GTATGTGGCTATTCAGGCTGT; and Act3B1, 5′ CTGGCGGTGCTTCTTCTCTG. The pga22F3 primer starts at the putative translation start codon.

DNA Southern and RNA northern-blot analyses were carried out as previously described (Zuo et al., 2000a, 2001).

RT-PCR

RT-PCR was carried out as previously described with modifications (Zuo et al., 2000b). One microgram of total RNA was used for RT primed by oligo(dT). SuperScript II (Life Technologies/Gibco-BRL, Rockville, MD) was used for the RT reaction according to the manufacturer's instructions. After incubation with RNase H for 30 min at 37°C, 1 μL of the reaction mixture was used for subsequent PCR using appropriate primer pairs (pga22F3/pga22B2, see above; Act3F1/Act3B1). The reaction was cycled at 94°C for 30 s, 56°C for 1 min, and 72°C for 1.5 min 25 to 35 times. Five microliters of the PCR products was subjected to Southern-blot analysis using radioactive *AtIPT8* and *actin3* DNA fragments as probes. The *AtIPT8* expression was undetectable if the PCR was cycled less than 30 times.

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