The Plant Cell, Vol. 13, 2115–2125, September 2001, www.plantcell.org

The Arabidopsis *AMP1* Gene Encodes a Putative Glutamate Carboxypeptidase

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Arabidopsis *amp1* mutants show pleiotropic phenotypes, including altered shoot apical meristems, increased cell proliferation, polycotyly, constitutive photomorphogenesis, early flowering time, increased levels of endogenous cytokinin, and increased cyclin *cycD3* expression. We have isolated the *AMP1* gene by map-based cloning. The *AMP1* cDNA encodes a 706–amino acid polypeptide with significant similarity to glutamate carboxypeptidases. The *AMP1* mRNA was expressed in all tissues examined, with higher expression in roots, stems, inflorescences, and siliques. Microarray analysis identified four mRNA species with altered expression in two alleles of *amp1*, including upregulation of CYP78A5, which has been shown to mark the shoot apical meristem boundary. The similarity of the AMP1 protein to glutamate carboxypeptidases, and in particular to *N*-acetyl α-linked acidic dipeptidases, suggests that the AMP1 gene product modulates the level of a small signaling molecule that acts to regulate a number of aspects of plant development, in particular the size of the apical meristem.

INTRODUCTION

Plant meristems are a driving force of growth and development, with profound influences on the rate of growth, the developmental fate, and the architecture of plants. Plant hormones are defined as substances that exert regulatory effects on growth and development, and much plant hormone action is on meristematic tissue. Classically, cytokinins, auxins, gibberellins, abscisic acid, and ethylene were characterized as plant hormones. More recently, it has become clear that other signaling molecules exist in plants, including brassinosteroids (Altmann, 1998), jasmonic acid (Reymond and Farmer, 1998), and peptides (Bisseling, 1999).

The *amp1-1* (*altered meristem program*) mutant was isolated on the basis of increased cotyledon number and an increased rate of leaf initiation (Chaudhury et al., 1993). Other alleles of *amp1* have been isolated, including *pt* (*primordia timing*; Mordhorst et al., 1998), *cop2* (*constitutively photomorphogenic*; Hou et al., 1993), and *hpt* (*hauptling*; Jurgens et al., 1991). Although originally isolated as a mutant with altered embryonic and postembryonic meristem programs, *amp1* was shown to have many different phenotypes, such as transformation of leaves to cotyledons (Conway and Poethig, 1997), altered flowering time and photomorphogenesis (Chaudhury et al., 1993), and an increased level of cyclin D3 (Riou-Khamlichi et al., 1999; Nogué et al., 2000a). One explanation for this developmental pleiotropy is that the level of or sensitivity to one or more plant hormones is altered in *amp1*. Consistent with this hypothesis, the *amp1* mutant was found to have an increased level of cytokinin biosynthesis (Chaudhury et al., 1993; Chin-Atkins et al. 1996; Nogué et al., 2000b).

The addition of cytokinin can mimic some of the phenotypes of the *amp1* mutant, which include photomorphogenesis (Chin-Atkins et al., 1996), increased cyclin D3 expression (Soni et al., 1995; Riou-Khamlichi et al., 1999), and increased anthocyanin accumulation (Deikman and Hammer, 1995). However, other phenotypes of *amp1*, including polycotyly and day-neutral flowering time, have not been mimicked by cytokinin application. Because the *amp1* mutant has larger meristems (Nogué et al., 2000a), a possible site of cytokinin biosynthesis, it is possible that the high cytokinin level in *amp1* is caused by the increased size of the meristems. The failure to mimic all of the *amp1* phenotypes by an application of cytokinin suggests that the primary effect of the *amp1* mutation may be something other than a simple increase of cytokinin.

We have isolated the *AMP1* gene by map-based cloning. In this article, we demonstrate that *AMP1* encodes a glutamate carboxypeptidase with significant similarity to those that cleave small signaling peptides and similar molecules in other eukaryotes. We postulate that the primary role of AMP1 is to modulate the levels of one or more small signaling molecules in the plant. This signaling molecule acts to

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control a number of developmental processes, including the size of the apical meristem and cytokinin biosynthesis.

RESULTS

Isolation of the *AMP1* Gene

The *AMP1* gene was isolated using a map-based cloning strategy. Mapping populations consisting of 64 recombinant events between the *amp1* allele *pt* and *CSR1* to the north and 97 recombinant events between *amp1-1* and *cer7* to the south were used to map the *AMP1* gene to a single bacterial artificial chromosome (BAC), F22D14 (Figure 1). Nucleotide polymorphism markers derived from sequences at the end of this BAC showed that in the mapping population there were two crossover events between the north end of this BAC and *AMP1* and one crossover event between the south end of the BAC and *AMP1*. Markers from within F22D14 all comapped with *AMP1*. F22D14 lies on the lower arm of chromosome III, 21.1 Mb from the north end of the chromosome.

A complementation test was then used to identify the *AMP1* gene. The *pt* allele was transformed with subclones of F22D14 spanning the entire BAC. A 10-kb subclone from the extreme southern end of F22D14 (Figure 1) complemented the *amp1* mutant phenotype of the *pt* allele (Figure 2); this clone corresponds to bases 26,888 to 36,862 of T5N23. Most of the other constructs tested failed to give transformants; in general, only those constructs complementing the mutant phenotype gave rise to plants that survived on the selection medium. The presence of the transgene in the complemented plants was confirmed by polymerase chain reaction analysis using a primer from within the insert fragment and a M13 reverse sequencing primer. This primer pair gives a product only when the transgene is present. The progeny of the primary transformants segregated for wild-type and *amp1* mutant phenotypes; the wild-type plants all contained the transgene, whereas plants showing the *amp1* phenotype did not (data not shown). This finding confirmed that the 10-kb fragment contained the *AMP1* gene.

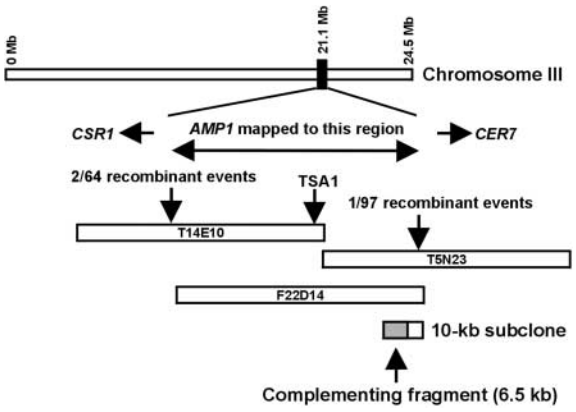

Figure 1. Map-Based Isolation of the *AMP1* Gene.

Two populations of plants with recombination events between the *PT* and *CSR* loci or *AMP1* and *CER7* were used to map the *AMP* gene. Markers from the ends of BAC F22D14 mapped the *AMP1* gene to this BAC, with two recombinant events between *CSR1* and *AMP1* at the north end of the BAC and one recombinant event between *CER7* and *AMP1* at the south end of the BAC. This region is covered by the sequences of BACs T14E10 and T5N23. The marker TSA1 (tryptophan synthase) located at 21.1 Mb on chromosome III is located at the southern end of T14E10. A 10-kb subclone of F22D14 in pBin19 complemented the *pt* allele. A 6.5-kb fragment of the 10-kb subclone complemented the *pt* allele. The diagram is not to scale.

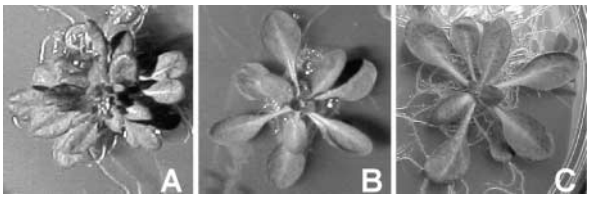

Figure 2. Complementation of the *pt* Allele.

(A) Untransformed *pt* plant.
(B) *pt* transformed with the 10-kb clone.
(C) *pt* transformed with the 6.5-kb clone.

The 10-kb clone contains two predicted genes. To define which one was the *AMP1* gene, the 10-kb clone was further subcloned into two fragments of 6.5 kb (bases 26,888 to 33,366 of T5N23) and 6.2 kb (bases 30,745 to 36,862 of T5N23), each containing one of the two predicted genes. Only the 6.5-kb clone complemented the *pt* allele.

To confirm that the gene in the 6.5-kb fragment encodes the AMP1 gene product, the sequence of the predicted coding region was determined from *amp1-1*, *pt*, and the corresponding wild-type ecotypes (Columbia and Landsberg *erecta*, respectively). In the *amp1-1* mutant, a G-to-A base change gives rise to a stop codon, and in the *pt* allele, a G-to-A base change leads to a glutamate-to-lysine change in the predicted amino acid sequence (Figure 3). We concluded that the gene on the 6.5-kb fragment encodes the AMP1 gene product.

An *AMP1* cDNA of 2.2 kb and encoding a polypeptide of 705 amino acids was isolated. Comparison with genomic sequence shows that the *AMP1* gene contains 10 exons (Figure 3). A putative TATA box is located 60 bp upstream of the translation start site in the genomic sequence. The translation start site also agrees with the annotation of the Arabidopsis genome sequence. Eight copies of a CT repeat are present 4 bp upstream of the translation start site in Columbia; in Landsberg *erecta*, only seven copies are present.

The *AMP1* cDNA we isolated starts 12 bp upstream of the ATG within this repeat region; there are no 3′ intron splice sites in this region. Therefore, the cDNA must contain the complete open reading frame because there would be a frameshift leading to a different polypeptide in one of the ecotypes if the repeat region were translated.

AMP1 Encodes a Putative Glutamate Carboxypeptidase

The AMP1 amino acid sequence was used to search the GenBank database. The proteins of known function most closely related to AMP1 are *N*-acetyl α-linked acidic dipeptidases (NAALADases) of the glutamate carboxypeptidase II protein family. The glutamate carboxypeptidases are ubiquitous across the eukaryotes. Figure 4 shows an alignment of three proteins from the human NAALADase family (Pangalos et al., 1999): NAALADaseI (also known as FOLH1 or prostate-specific membrane antigen), NAALADaseII, and NAALADaseL.

The deduced AMP1 protein sequence has significant similarity to the human NAALADases, with 26/48% amino acid identity/similarity to NAALADaseI, 28/48% identity/similarity to NAALADaseII, and 27/47% identity/similarity to NAALADaseL. A number of zinc binding residues have been identified in the NAALADases (Rawlings and Barrett, 1997); these residues are all conserved in the AMP1 protein (Figure 4), as is the glutamate that acts as the nucleophile in catalysis (Gingras et al., 1999; Pangalos et al., 1999). In the *pt* allele of *amp1*, a single base change leads to the nonconservative substitution of a zinc binding glutamate with a lysine. The *AMP1* gene has 10 exons, whereas NAALADaseI has 19 exons (O'Keefe et al., 1998), and the intron positions are not conserved.

In Arabidopsis, there are genes encoding two putative proteins with similarity to AMP1 (Figure 4). The first is located on chromosome IV BAC F10A2; the predicted protein is shorter than AMP1 (280 amino acids compared with 705). The second is located on chromosome V BAC T29J13; this predicted protein (670 amino acids) is of similar length and shows 39/56% amino acid identity/similarity to AMP1. This protein has a shorter N terminus than AMP1 and also shows less similarity to the human NAALADases than AMP1, the most similar being NAALADaseII, with 14/21% identity/similarity. The two related Arabidopsis proteins have four of the five conserved residues for zinc binding and the catalytic glutamate residue. Database searches also identified expressed sequence tags with similarity to *AMP1* from various plant species, including monocots and dicots, indicating that the *AMP1* gene is likely to be ubiquitous in plants.

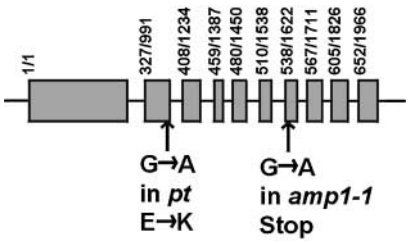

Figure 3. Structure of the *AMP1* Gene.

Exons are shown as boxes, with the positions of point mutations in *amp1* alleles indicated by arrows. The amino acid/nucleotide positions (relative to the start codon) at the 5′ end of each exon are shown above the sequence.

AMP1 Is Expressed throughout the Plant

The expression pattern of the *AMP1* mRNA was examined by ribonuclease protection assay on total RNA isolated from various plant parts (Figure 5). *AMP1* is expressed in all parts of the plant. The expression was higher in the bolt stem, inflorescence, root, and silique than in rosette or cauline leaves. The lowest level of AMP1 mRNA expression was in leaves. Because the *amp1* mutant has increased endogenous cytokinin, the abundance of *AMP1* mRNA was examined in 12-day-old seedlings grown with and without 10 μM zeatin; there was no significant variation in the abundance of the *AMP1* mRNA between these treatments.

CYP78A5 Expression Is Induced in the *amp1* Mutant

To further understand the effects of the *amp1* mutation on Arabidopsis development, microarray analysis was performed. We reasoned that because the *amp1* phenotype affects many aspects of plant growth and development and *AMP1* is expressed throughout the plant, at least some of the changes in gene expression attributable to the *amp1* mutation are likely to be detectable in RNA isolated from whole seedlings. We compared gene expression in two *amp1* alleles with that in the corresponding wild types using microarrays representing more than 7000 unique clones.

The microarray data were analyzed according to Schenk et al. (2000). The replication of experiments and the stringency of the cutoff criteria for significant changes in gene expression, particularly for genes with signal intensities close to background level, means that any genes identified are likely to have significant changes in expression between the two samples.

The microarray data were analyzed initially by combining the results from the four replicate comparisons of *amp1-1*: Columbia and *pt*:Landsberg *erecta*. To meet the cutoff criteria, a gene had to show an induction or repression of at least 2.0 in all four replicates of the experiment. The results are shown in Table 1. This analysis identified four genes induced and four genes downregulated in *amp1-1* compared with the Columbia wild type and nine genes induced in *pt* compared with the Landsberg *erecta* wild type. Other than genes with no similarity to genes of previously defined function, the genes that were differentially regulated in the *amp1*

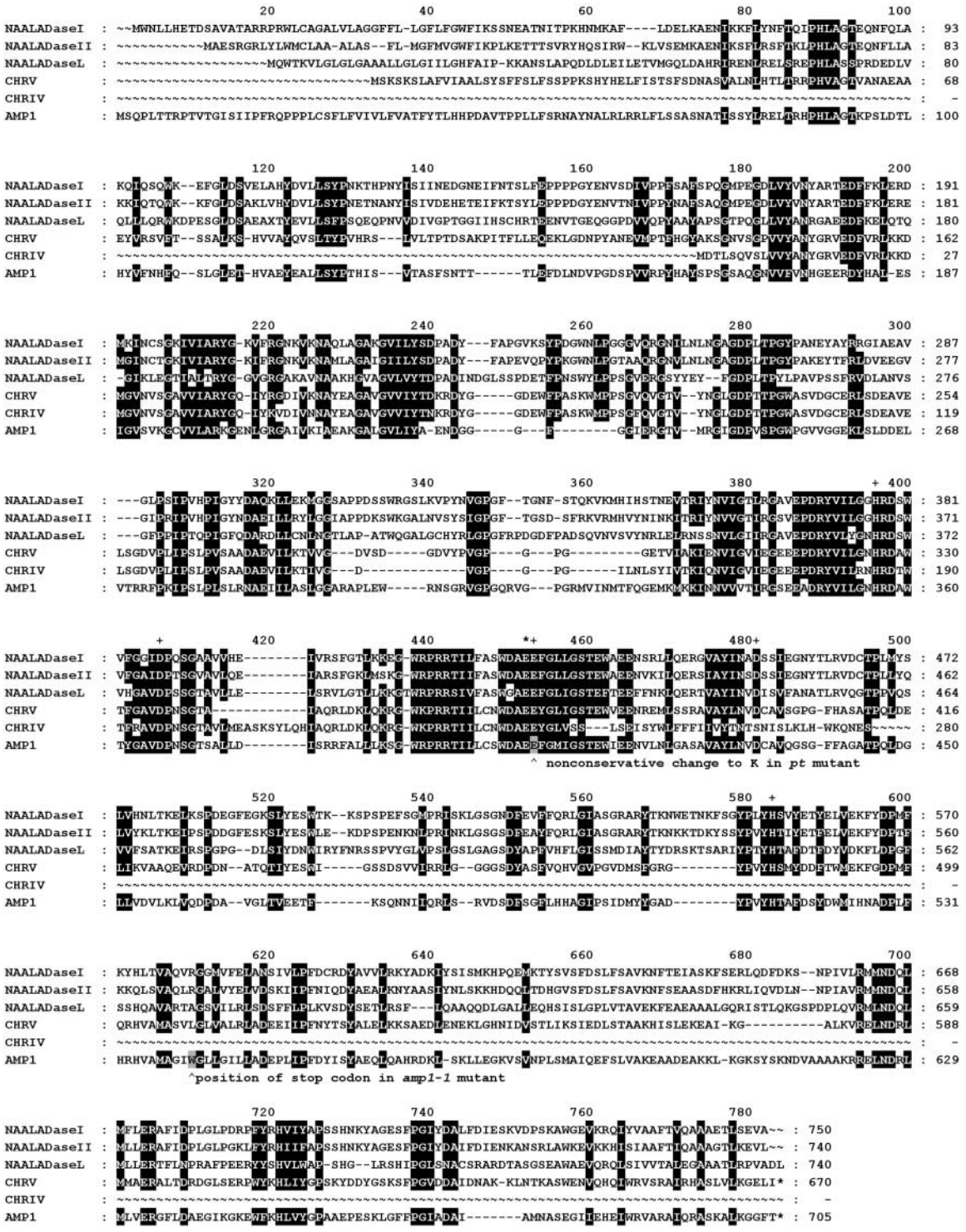

Figure 4. Alignments of AMP Peptide Sequences.

An alignment of AMP1 with three human glutamate carboxypeptidases (Pangalos et al., 1999): NAALADaseI (also FOLH1 or prostate-specific membrane antigen; Israeli et al., 1993), NAALADaseII, and NAALADaseL, and two Arabidopsis proteins with similarity to AMP1: chromosome IV BAC 10A2 (CHRIV) and chromosome V BAC T29J13 (CHRV). The positions of the E-to-K substitution in the *pt* allele of *amp1* and the stop codon in *amp1-1* are shown. Putative zinc binding residues (Gingras et al., 1999; Pangalos et al., 1999) are indicated by plus signs. The nucleophile thought to be important in catalysis (Gingras et al., 1999; Pangalos et al., 1999) is marked with an asterisk. The sequence alignment was produced using the Pileup program in GCG (Genetics Computer Group, Madison, WI); highlighted residues are conserved amino acids (including conservative substitutions) in five of the protein sequences.

mutants have putative metabolic functions (e.g., pyruvate decarboxylase and β-amylase). A gene encoding a cytochrome P450 of unknown function, CYP78A5 (Zondlo and Irish, 1999), was upregulated in both mutants. The cDNA for this gene was represented by three clones on the array; hence, the replication for this gene was greater than 4.0 for both experiments.

The data from the microarray experiments also were analyzed by combining the *amp1-1* and *pt* experiments to identify genes regulated in both *amp1-1* and *pt*. It is likely that changes in expression that are common to both *amp1* alleles are specific to the *amp1* mutation rather than a combinatorial effect of the *amp1* mutation and the background ecotype. Two analyses were performed. The first analysis was with all eight experiments combined; this identified four genes induced in *amp1*/*pt* alleles (Table 1). These genes encode β-amylase, a putative peroxidase, pyruvate decarboxylase, and CYP78A5. A second combined analysis was performed omitting two of the arrays from the *pt*:Landsberg *erecta* comparison that had higher background signals than the other arrays. The results of this analysis identified three genes upregulated in *amp1*/*pt* alleles: β-amylase, pyruvate decarboxylase, and CYP78A5 (these were the same genes identified in the eight-array comparison). Two genes were identified as downregulated: a putative GDSL motif lipase/hydrolase protein and a 12S cruciferin.

The results from the six-experiment comparison were confirmed by high-stringency RNA gel blotting (Figure 6). The 12S cruciferin was not detected by RNA gel blot analysis. The signal intensity on the microarray for this gene was of the same order of magnitude as the other differentially expressed genes so it is likely that the changes seen in the microarray experiment for this gene were the result of cross-hybridization of a related sequence. Consistent with this finding, strong hybridization was observed on RNA gel blots with the 12S cruciferin probe before treatment with RNaseA. The RNA gel blot analysis confirmed the microarray results for the CYP78A5, pyruvate decarboxylase, GDSL motif lipase, and β-amylase genes. All four mRNAs show strong induction or repression in *amp1-1* and *pt* alleles with the exception of the GDSL motif lipase, which was only weakly downregulated in *pt*. The identification of only strongly regulated genes is the result of the stringency of our data analysis. It is likely that the expression levels of other genes also are altered in *amp1* mutants that we are not able to detect by microarray analysis at present because either the expression level is too low or changes in mRNA expression are too small to meet the cutoff criteria.

Cytokinin Regulates the Expression of Two Genes Induced in *amp1*

Because *amp1* mutants have six times the wild-type level of endogenous zeatin (Chin-Atkins et al., 1996), changes in gene expression in *amp1* could be attributable to cytokinin regulation of gene expression. To investigate this possibility, RNA gel blot analysis was performed to examine whether any of the genes identified in the microarray analysis also were regulated by applied cytokinin. The results of this analysis (Figure 6) showed that both β-amylase and pyruvate decarboxylase mRNA contents are strongly upregulated in plants grown on high cytokinin medium, suggesting that the changes in expression of these genes in *amp1* mutants are a consequence of the increased level of cytokinin. In contrast, CYP78A5 and the putative GDSL motif lipase gene were not cytokinin regulated, suggesting that the regulation of their expression in the *amp1* mutant is not caused by the increase of cytokinin.

CYP78A5 Overexpression in *amp1* Is Confined to the Apical Region

We have shown that the cytochrome P450 CYP78A5 is upregulated in *amp1* mutants. Zondlo and Irish (1999) identified this cytochrome P450; in wild-type plants, its expression is confined to meristematic regions. In the vegetative shoot apical meristem, CYP78A5 is expressed in a zone flanking the meristem. To determine whether the increased CYP78A5 expression in *amp1* mutants was caused by increased expression in the meristem region or by ectopic expression in other parts of the plant, we analyzed CYP78A5 expression in leaves and shoot tips of mutant and wild-type plants using RNA gel blotting (Figure 7A). There was no detectable CYP78A5 in RNA isolated from leaves of 12-day-old *amp1* mutant or wild-type plants; however, there was a large increase in CYP78A5 mRNA abundance in RNA isolated from shoot tips of *amp1* mutants compared with wild type. Because *amp1* has an enlarged apical meristem, it is likely that the enlarged meristem gives rise to more cells

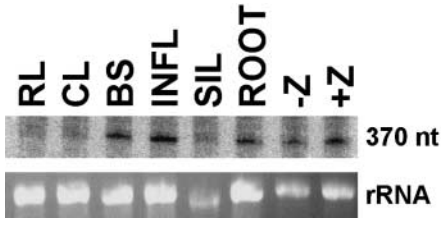

Figure 5. Expression of the AMP mRNA.

Ribonuclease protection assay using an antisense RNA probe made from an 800-bp region of *AMP* spanning the 3′ 370 bp of exon 1 and 242 bp of exon 2. Samples were from mature C24 plants (35 days old). RL, rosette leaf; CL, cauline leaf; BS, bolt stem; INFL, inflorescence; SIL, mature siliques; ROOT, root; −Z, 12-day-old light-grown seedlings (Columbia); +Z, 12-day-old light-grown seedlings with 10 μM zeatin (Columbia). Note that the silique sample is underloaded compared with the other lanes. nt, nucleotides.

Table 1. Results of Microarray Analysis of Gene Expression in *amp1* Mutants[a]

Gene Annotation	GenBank Accession Number	Induction[b] (fold)	Replication[c]
amp1-1:Columbia			
Putative proline-rich protein	U78721	5.20	4
CYP78A5	AC016662	4.60	12
Pyruvate decarboxylase	U71121	4.17	4
Putative protein	AL163817	2.29	4
Putative aldehyde decarboxylase (CER1)	D64155	−2.67	4
Lipase/acyl hydrolase	AP000600	−3.3	4
12S cruciferin (ATCRU3)	U66916	−4.93	4
Putative endochitinase	AC002333	−5.63	4
pt:Landsberg *erecta*			
CYP78A5	AC016662	6.75	8
Putative peroxidase	AL161813	7.34	4
β-Amylase	M73467	6.42	4
Putative protein	AC007260	5.59	4
Glutathione *S*-transferase 1	L12057	3.92	12
Putative glutathione *S*-transferase	AF326903	2.94	4
Putative 1,3-β-glucanase	AL161543	2.90	4
Unknown protein	AC021640	2.88	4
Hypothetical protein	AF077407	2.74	12
Combined results			
β-Amylase (6, 8)[d]	M73467	6.90	7
Putative peroxidase (8)	AL161813	6.18	6
Pyruvate decarboxylase (6, 8)	U71121	5.82	7
CYP78A5 (6, 8)	AC016662	5.28	23
Putative protein with GDSL motif (6)	AC016829	−4.25	5
12S cruciferin/ATCRU3 (6)	U66916	−5.72	6

[a]Gene expression changes in 12-day-old seedlings of *amp1-1* and *pt* mutants compared with wild type for individual mutants and combined results for both mutants are shown.

[b] Induction is the fold induction of the *amp1* mutant compared with wild type.

[c] Replication is the number of experiments in which the cutoff criteria were met; these experiments were used to calculate the average intensity for each channel to determine the induction. Where the replication value is greater than the number of experiments performed, there were multiple elements for the gene on the array.

[d] Numbers refers to whether the gene was identified in analysis of six experiments (four *amp1-1*:Columbia and two *pt*:Landsberg *erecta*) or of eight experiments (four *amp1-1*:Columbia and four *pt*:Landsberg *erecta*).

expressing CYP78A5. The *clv1* and *clv3* mutants both have enlarged apical meristems, although not to the same extent as *amp1* (Mordhorst et al., 1998). In 12-day-old seedlings of *clv1* and *clv3*, there is increased CYP78A5 mRNA compared with wild-type plants (Figure 7B). Conversely, in homozygous *stm1* plants lacking an apical meristem, we could not detect CYP78A5. Therefore, the abundance of CYP78A5 positively correlated with the size of the meristem.

DISCUSSION

We used map-based cloning to isolate a gene encoding a putative glutamate carboxypeptidase that complemented an *amp1* mutation. We confirmed that this was the *AMP1* gene by showing that it has point mutations in the gene sequence in two *amp1* mutant alleles. The *AMP1* mRNA appears to be expressed throughout the plant, with higher abundance in stems, inflorescences, roots, and siliques.

Biochemical Function of the AMP1 Gene Product

The *AMP1* gene product encodes a protein with similarity throughout its sequence to the human NAALADase proteins (Pangalos et al., 1999). These proteins have an N-terminal membrane-spanning domain, with the majority of the protein being extracellular. NAALADase is a chloride-dependent zinc metalloproteinase, requiring a divalent ion cofactor

(Robinson et al., 1987). The human NAALADases have differing activities: NAALADaseI and NAALADaseII hydrolyze the neuropeptide *N*-acetyl-L-aspartyl-L-glutamate (NAAG), releasing glutamate, whereas all three show the general peptide metabolic dipeptidyl peptidase IV activity (Pangalos et al., 1999). The NAALADases also hydrolyze folate polyglutamate (Pinto et al., 1996; Tiffany et al., 1999) and probably are involved in dietary folate uptake. NAALADaseI is most highly expressed in prostate tissue, NAALADaseII in ovary, testis, and discrete brain areas, and NAALADaseL in small intestine, spleen, and testis (Pangalos et al., 1999).

In AMP1, the N-terminal membrane span and the zinc binding and catalytic residues that have been identified in the NAALADases are present, suggesting a conservation of biochemical function. Therefore, two potential substrates of AMP1 are small acidic peptides and folate polyglutamate. The probable glutamate carboxypeptidase activity of AMP1 makes it unlikely that it has direct involvement in cytokinin biosynthesis. The findings that not all *amp1* phenotypes are obtained by cytokinin application and not all genes regulated by AMP1 are cytokinin regulated are consistent with this.

We tested whether the substrates of the human NAALADases, folate and NAAG, have any effect on the growth and development of wild-type or *amp1* mutant plants. There were no detectable phenotypic changes in seedlings grown on medium containing folic acid at concentrations ranging from 5 to 500 μM or seedlings given a daily application of 1 μg of NAAG for a 2-week period. There also were no changes in AMP1 mRNA levels in the NAAG-treated plants (data not shown). These experiments suggest that the substrate of AMP1 is not NAAG or a folate derivative. Given the amino acid conservation between AMP1 and the NAALADases, the substrate of AMP1 is likely to be a small peptide or similar molecule.

A number of peptides have been shown to regulate processes in plant development (reviewed by Bisseling, 1999; Ryan and Pearce, 2001), including phytosulfokines, ENOD40, systemin, and CLAVATA3. CLAVATA3 is a 96–amino acid peptide thought to be the ligand of the leucine-rich repeat receptor CLAVATA1 (Fletcher et al., 1999), which acts to control meristem size. Double mutants of *pt* and *clv3* show additive effects on meristem size (Mordhorst et al., 1998), suggesting that the two genes act in different pathways; therefore, CLAVATA3 is unlikely to be a substrate of AMP1. The smallest plant signal peptide identified so far is phytosulfokine (Yang et al., 1999), a 5–amino acid peptide (YIYTQ) produced by processing of an 89–amino acid precursor. This peptide is active in promoting cell division, and it is possible that AMP1 could process it to an inactive form, although the peptide does not contain acidic residues, unlike the acidic dipeptide substrates of mammalian NAALADases. At present, there is no known plant signaling peptide that is an obvious candidate substrate for AMP1.

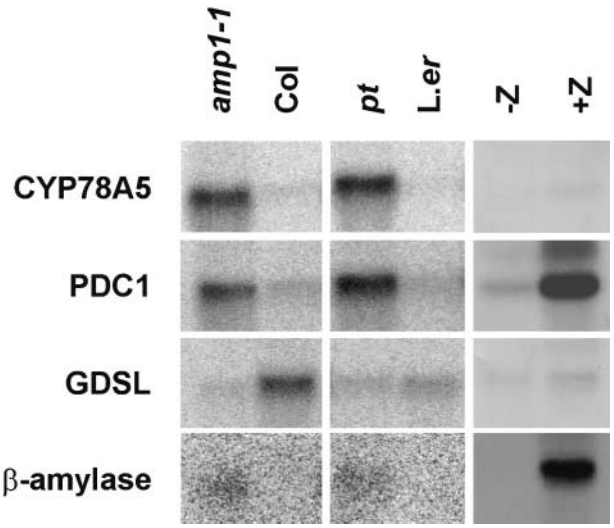

Figure 6. RNA Gel Blot Analysis of Genes Identified by Microarray Analysis.

Antisense RNA probes for CYP78A5, PDC1, GDSL, and β-amylase were hybridized to gel blots of total RNA isolated from 12-day-old seedlings of *amp1-1*, Columbia (Col), *pt*, Landsberg *erecta* (L.*er*), Columbia minus zeatin (−Z), and Columbia plus zeatin (+Z). Each lane was loaded with 20 μg of RNA. Blots were visualized by a PhosphorImager (*amp1-1*, Columbia, Landsberg *erecta*, and *pt*) or autoradiography (−Z and +Z).

Biological Role of AMP1

It is likely that the AMP1 protein exerts its effects on plant development via the concentration of either its substrate(s), the product of the breakdown of the substrate(s), or both. The *AMP1* gene is expressed in all parts of the plant we have examined. This fact, together with the pleiotropic nature of the *amp1* phenotype, suggests that AMP1 may have roles throughout the plant. When the substrate(s) of the AMP1 protein is defined, the mechanism by which AMP1 affects development should become clearer.

One of the major phenotypes of mutations in *AMP1* is an increase in the size of the vegetative apical meristem. Nogué et al. (2000a) reported a 2.5-fold increase in the diameter and an increase of 7-fold in the height of the meristem compared with wild type. We propose that AMP1 regulates the abundance of a signaling molecule that has a role in regulating meristem function. Another major phenotype of the *amp1* mutant is the increase of endogenous cytokinin; many of the *amp1* phenotypes can be attributed to the high level of endogenous cytokinin.

We used microarray analysis to identify differences in gene expression between *amp1* mutants and wild-type plants. We have shown that genes encoding the enzymes

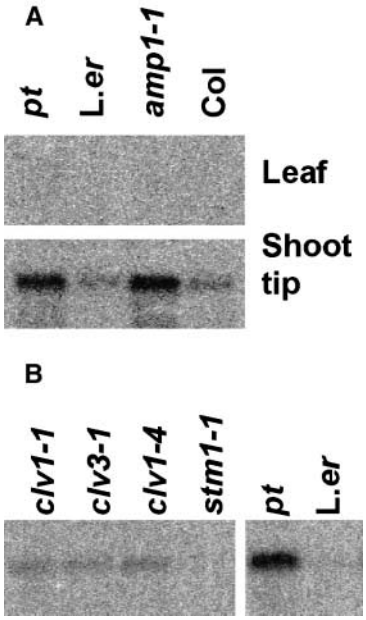

Figure 7. Expression Pattern of CYP78A5 in *amp1* and Meristem Mutants.

(A) RNA gel blots of total RNA isolated from leaves and shoot tips of 12-day-old seedlings of *pt*, Landsberg *erecta* (L.*er*), *amp1-1*, and Columbia (Col). Each lane contained 10 μg of RNA.
(B) RNA gel blots of total RNA from whole 12-day-old seedlings of *clv1-1*, *clv3-1*, *clv1-4*, *stm1-1*, *pt*, and Landsberg *erecta* (L.*er*). Each lane contained 10 μg of RNA.
Blots were visualized by a PhosphorImager.

β-amylase and pyruvate decarboxylase are induced in *amp1* mutants and by the application of cytokinin. It is likely that the induction of these genes in *amp1* is a consequence of the increased levels of cytokinin in the mutant. Pyruvate decarboxylase is induced under conditions of abiotic stress, such as hypoxia, and in seed (de Bruxelles et al., 1996). A consequence of the increased growth rate of *amp1* could be that tissues are growing under hypoxic conditions or increased metabolic demand, hence the induction of pyruvate decarboxylase. Similarly, β-amylase may be induced to support the higher growth rate of *amp1*. Sucrose synthase also is induced by cytokinin (Soni et al., 1995), which likewise could be a consequence of the increased growth rate.

The microarray analysis also showed that *amp1* mutants have an increased abundance of the mRNA encoding CYP78A5. The level of this mRNA is not affected by cytokinin application, so the upregulation in *amp1* is not attributable to increased cytokinin, suggesting that CYP78A5 may act upstream of cytokinin in AMP1-controlled pathways. The increased expression of the CYP78A5 mRNA in *amp1* mutants is confined to the shoot tip region. CYP78A5 normally is expressed in a ring around the shoot meristem (Zondlo and Irish, 1999); the enlarged dimensions of the shoot meristem in *amp1* (Nogué et al., 2000a, 2000b) could account for the fivefold increase in CYP78A5 expression in the microarray experiments.

CYP78A5 was isolated originally from cauliflower florets; it is highly expressed in cauliflower heads but not in leaves (Zondlo and Irish, 1999). Cytochrome P450s have roles in many aspects of secondary metabolism; therefore, CYP78A5 is likely to catalyze a reaction in a secondary metabolic pathway. One possibility is that CYP78A5 encodes a step of the cytokinin biosynthesis pathway. The hydroxylation steps from N^6-(Δ^2-isopentenyl)adenosine to 9-ribosylzeatin and from N^6-(Δ^2-isopentenyl)adenine to zeatin have been shown to be catalyzed by cauliflower floret microsomal cytochrome P450s (Chen and Leisner, 1984). Because CYP78A5 is highly expressed in cauliflower florets, it is possible that it encodes a cytokinin biosynthetic cytochrome P450. If CYP78A5 does catalyze a cytokinin biosynthesis step, it could explain the sixfold increase of endogenous zeatin in *amp1* (Chin-Atkins et al., 1996).

The CYP78A subfamily of cytochrome P450 has six members in Arabidopsis, including CYP78A5. Four of these (CYP78A6, -7, -8, and -10) have not been characterized; however, overexpression of CYP78A9 results in partial parthenocarpy (Ito and Meyerowitz, 2000). None of the other members of the CYP78A subfamily of cytochrome P450 was represented on the microarray used in our expression analysis, so we do not know if any of these are upregulated in *amp1*. In general, members of the same plant cytochrome P450 subfamily catalyze the same or similar reactions (Choe et al., 1998; Helliwell et al., 2001), so the other members of the CYP78A subfamily may catalyze the same reaction in different parts of the plant.

Some of the pleiotropic phenotypes of *amp1* may be caused by the increased CYP78A5 expression. Zondlo and Irish (1999) reported that overexpression of CYP78A5 directed by the 35S promoter caused pleiotropic defects, including abnormal stem epidermal cell shapes, stem twisting, delayed flower opening with stunted stamens and petals, reduced seed set, and carpel elongation, with more severe phenotypes in later flowers and expansion of vascular bundles. The 35S::78A5 plants are not directly comparable to *amp1* mutants because increased CYP78A5 expression in *amp1* is confined to the shoot tip. However, there are some phenotypes common to *amp1* and 35S::78A5: delayed flower opening, similar alterations to stem cross-section, and reduction in hypocotyl stem length.

The question of why the *amp1* shoot meristem is enlarged remains to be answered. We suggest two simple potential mechanisms for this. The first is that AMP1 has a primary effect on meristem size via its substrate, and in *amp1*, the enlarged meristem leads to an increase in the expression of meristem-specific genes, including CYP78A5. CYP78A5 or another upregulated enzyme then increases the rate of cytokinin biosynthesis. The second is that cytokinin biosynthesis is increased in the *amp1* mutant, perhaps by upregulation of

CYP78A5, and the resulting increased cytokinin content leads to the increase in the size of the meristem. Our finding that CYP78A5 also is upregulated in *clv* mutants that have larger meristems than wild type and that appear to be defective in a different developmental pathway to *amp1* (Mordhorst et al., 1998) suggests that the first mechanism is more likely. Defining the mechanism by which AMP1-mediated processes control the size of the meristem should provide new insights into its development and function.

METHODS

Plant Material

The *Arabidopsis thaliana amp1-1* mutant was as described by Chaudhury et al. (1993). *pt* was obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK). *clv1-1*, *clv1-4*, *clv3-1*, and *stm1-1* were a gift from Professor David Smythe (Monash University, Melbourne, Australia). Transformation was by the floral dip method (Clough and Bent, 1998), and transformed plants were selected on Murashige and Skoog (1962) plates supplemented with kanamycin. Plants for RNA gel blot analysis were grown on Murashige and Skoog plates with the supplements indicated in Results, with the exception of C24 mature plants, which were grown on soil. Leaf material from 12-day-old seedlings was harvested by excising the cotyledons and the two to four partly expanded leaves on the seedling. Shoot tip was the material remaining after excision of the expanding leaf material with the roots and part of the hypocotyl removed.

Map-Based Cloning

A mapping population of plants with recombination events between *AMP1* and *CER7* was generated by crossing a double recessive *amp1-1 cer7* line (Chaudhury et al., 1993) in a Columbia/Landsberg *erecta* mixed background with a wild-type Nossens ecotype plant. The F1 was selfed to produce an F2 population that was screened for individuals showing an *amp1*/*CER7* (bushy, nonwaxy) phenotype; these plants contain a recombination event between the *AMP1* and *CER7* loci. A mapping population with recombinations between the dominant *csr1* (chlorsulfuron resistant) locus in the Columbia background (Haughn and Somerville, 1986) was generated by crossing *csr1* with the *amp1* allele *pt*. The resulting F1 plants were selfed, and the F2 plants were screened for individuals with *csr1* and *amp1* phenotypes (resistance to chlorsulfuron and bushy); these plants have a recombination event between the *CSR1* and *PT* (*AMP1*) loci. DNA was isolated from either F2 or F3 plants. *AMP1* was mapped to the bacterial artificial chromosome (BAC) F22D14 using a combination of restriction fragment length polymorphisms and sequence polymorphisms identified between the parents of the mapping populations. Genomic clones for complementation tests were from partial Sau3AI digests of F14D22 ligated into the pBin19 vector (Bevan, 1984) cut with BamHI.

The *AMP1* cDNA clone was isolated from a library made from mRNA isolated from developing siliques of the Landsberg *erecta* ecotype (Anna Koltunow, Commonwealth Scientific and Industrial Research Organization, Plant Industry) using a polymerase chain reaction fragment amplified from the 5′ end of the *AMP1* gene as a probe.

Microarray Analysis

The cDNA library used for the microarray was purchased from Mendel Biotechnology (Hayward, CA). The library comprises a nonredundant set of 5760 resequenced clones from the nonredundant set of Arabidopsis expressed sequence tags compiled by the Arabidopsis Biological Resource Center (Ohio State University, Columbus). The library was supplemented with 1452 cDNA clones from the Commonwealth Scientific and Industrial Research Organization, Plant Industry. The final array contained 7680 spots representing 7212 unique clones. Microarrays were produced as described by Schenk et al. (2000) except that printing was performed using an SDDC2 arrayer (Engineering Services Inc., Toronto, Canada) with Chipmaker 3 pins (TeleChem International, Sunnyvale, CA). Cy3- and Cy5-labeled probes were synthesized from 100 μg of total RNA as described by Schenk et al. (2000). Hybridization conditions were as described by Schenk et al. (2000) except that slides were scanned with a GenePix 4000 scanner (Axon Instruments, Foster City, CA) and image analysis was performed using GenePix 3.0 software (Axon Instruments).

Each experiment was repeated with four RNA samples isolated from four replicates of each genotype of plant (*amp1-1*, Columbia, *pt*, and Landsberg *erecta*). Plants were 12 days old at harvest. In two of the set of four replicates, the fluors were reversed to remove any effects caused by differential incorporation of the two labels.

The data from GenePix 3.0 was normalized and analyzed using custom Perl scripts (Schenk et al., 2000; http://cellwall.stanford.edu/scripts/index.shtml). Changes in gene expression data were identified as those with greater than twofold induction or repression in the mutant compared with the corresponding wild type in four of four experiments (when four slides were compared), five of six experiments (in comparisons of six slides), or six of eight experiments (when eight slides were compared).

RNA Methods

RNA was isolated using Trizol (Life Technologies, Rockville, MD). RNA for microarray analysis was further purified using Qiagen RNeasy columns (Qiagen, Melbourne, Australia). Ribonuclease protection assays were performed using the Hybspeed RPA kit (Ambion) with 5 μg of total RNA per sample according to the manufacturer's instructions. The antisense *AMP1* probe was made by transcribing an 800-bp fragment of the *AMP1* gene cloned into the pGEM T-Easy vector (Promega). RNA gel blots were probed with antisense probes generated by linearization of the cDNA clones used in the generation of the microarray at the 5′ end and transcribed from the 3′ end. RNA gel blots were treated with RNaseA for 15 min at room temperature and washed at high stringency (0.1 × SSC [1× SSC is 0.15 M NaCl and 0.015 M sodium citrate] and 0.1% SDS at 65°C for 20 min) before analysis using a PhosphorImager.

GenBank Accession Numbers

The GenBank accession numbers for the proteins described in this article are as follows: the 2.2-kb cDNA of *AMP1* (AF357217); BACs T14E10 (AL138656) and T5N23 (AL138650); human carboxypeptidases NAALADaseI (NM_004476), NAALADaseII (AJ012370), and

NAALADaseL (AJ012371); and Arabidopsis chromosome IV BAC 10A2 (AL161506) and chromosome V BAC T29J13 (AF296838).

ACKNOWLEDGMENTS

The authors thank Bjorg Shermann, Jane Pulford, Anna Wielopolska, Sue Allen, Trijntje Hughes, and Jun Yang for excellent technical assistance and Professor David Smyth for *clv* and *stm* seed. A.N.C.-A. was supported by an Australian Postgraduate Research Award and the Cooperative Research Centre for Plant Sciences, Australian National University.

Received April 13, 2001; accepted June 22, 2001.

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