Petunia actin-depolymerizing factor is mainly accumulated in vascular tissue and its gene expression is enhanced by the first intron

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Abstract

Actin-depolymerizing factor (ADF) is one of the actin cytoskeleton-modulating proteins. We have characterized the accumulation pattern of petunia ADF proteins. PhADF proteins are accumulated in every petunia organ and their accumulation is differentially regulated by developmental signals. Their cellular localization is vascular tissue-preferential in vegetative organs, whereas somewhat different in reproductive organs. In reproductive organs, PhADFs are present in outer integument, endocarp of ovary wall, transmitting tissue of style, and epidermis and endothecium of young anther. From a petunia genomic library, we have isolated a genomic clone encoding PhADF1. Comparison to complementary DNA sequence revealed that the coding region of PhADF1 gene consists of three exons and two introns. Analysis of chimeric gene expression using β-glucuronidase as a reporter gene in transgenic Arabidopsis revealed that PhADF1 was strongly expressed in every vegetative tissue except petal. In addition, expression of the gene was highly enhanced by its first intron. These results suggest that PhADF1 gene of petunia is mainly expressed in vascular tissues and its expression is regulated by intron-mediated enhancement mechanism. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Actin-depolymerizing factor; Vascular tissue; Gene expression; Intron-mediated enhancement

1. Introduction

Actin microfilaments are essential cytoskeletal elements in eukaryotic cells. The remodeling of actin microfilaments in response to internal or external signals is involved in cell locomotion, cell shape change, cytoplasmic streaming, cell division, cell elongation, and development (Bamburg et al., 1999; Meagher et al., 1999; Meagher et al., 1999; Baluška et al., 2001). The reorganization of the actin network in the cell is modulated by several kinds of actin-binding proteins, such as cross-linking proteins, severing proteins, capping proteins, and depolymerizing proteins (Ayscough, 1998; Carlier, 1998). In plants, actin-depolymerizing factor (ADF) and profilin have been identified and characterized (Staiger et al., 1997). Plant ADF is a low molecular weight (ca. 16 kD) actin-binding protein of the ADF/cofilin group that includes destrin, cofilin, actophorin, and depactin (Staiger et al., 1997; Lappalainen et al., 1998). The interaction between actin and ADF is regulated by phosphorylation, pH, and phosphoinositide (Lopez et al., 1996; Gungabissoon et al., 1998; Smertenko et al., 1998; Mun et al., 2000; Allwood et al., 2001). In addition, ADF increases the turnover rate of actin filaments (Carlier et al., 1997) and acts synergistically with profilin (Didry et al., 1998). ADF is encoded by small multigene families in plants (Lopez et al., 1996; Mun et al., 2000; Allwood et al., 2001). In monocots, each family can be divided into two classes that differ in their expression patterns: the pollen-specific and vegetative classes of ADF proteins (Kim et al., 1993; Lopez et al., 1996). However, although monocot ADF proteins are functionally distinct (Smertenko et al., 2001), the functional divergence of dicot ADF proteins is uncertain (Mun et al., 2000). In petunia and Arabidopsis, expression patterns of vegetative class genes were reported (Mun et al., 2000; Dong et al., 2001).

Nuclear genes in plants are primarily regulated at the transcriptional level, and their promoters consist of multiple

Abbreviations: ADF, actin-depolymerizing factor; CTAB, hexadecyl trimethylammonium bromide; GUS, β-glucuronidase; IME, intron-mediated enhancement; PCR, polymerase chain reaction; UTR, untranslated region

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sequence modules that respond to ubiquitous, developmentally regulated, or inducible transcription factors (Donath et al., 1995). The regulatory elements are commonly located in the promoter and 5'-untranslated region (UTR). However, recent studies have demonstrated that some introns have a high enhancement effect on gene expression in plants. Usually, the magnitude of intron-mediated enhancement is greater in monocots than dicots. Although it is not certain how introns positively affect gene expressions, some evidences suggest that introns increase messenger RNA (mRNA) accumulation by a posttranscriptional mechanism, such as facilitating mRNA maturation or by enhancing the stability of transcripts (Rose and Beliakoff, 2000).

We have previously reported the expression characteristics of two complementary DNAs (cDNAs) encoding petunia ADF. These genes are abundantly expressed in every plant organ except pollen. In addition, they are highly active in mature vegetative tissue (petal, leaf, and stem) and their expression is differentially regulated by developmental signals (Mun et al., 2000). In this study, we report the localization patterns of PhADF proteins in petunia and the isolation and characterization of the petunia ADF gene, PhADF1. We have used immunocytochemistry to identify the cellular localization of PhADF proteins. In addition, we have analyzed β-glucuronidase (GUS) expression in transgenic Arabidopsis plant carrying various kinds of PhADF1::GUS chimeric genes to verify the expression pattern of PhADF1 gene. From this analysis, we have demonstrated that the PhADF1 protein is mainly accumulated in vascular tissues and its expression is strongly enhanced by the first intron.

2. Materials and methods

2.1. Plant material

The self-fertile tetraploid Petunia hybrida Vilm. cv. Comench (2n = 4x = 28), which was regenerated from diploid (2n = 14) calluses derived from mesophyll protoplasts, was grown under standard greenhouse conditions (Oh and Kim, 1988). Plant samples were harvested at the flowering stage and stored at −80°C.

2.2. Polyclonal antibody preparation and Immunoblot analysis

Expression and purification of recombinant-PhADF proteins were performed according to Mun et al. (2000). Polyclonal antisera were raised in mice with 100 μg of recombinant PhADF proteins, suspended in Freund’s incomplete adjuvant (Sigma), at 14-day intervals. Total protein extracts from various petunia tissues were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Hybond PVDF, Amersham). Blots were incubated with anti-PhADF polyclonal antibodies at a dilution of 1:1000 and then incubated with secondary antibody (Sigma) at a dilution of 1:5000. The membranes were assayed using ECL-Plus Reagent (Amersham).

2.3. Immunofluorescence assay

Tissue samples of petunia roots, stems, leaves, and flowers were prepared according to Cox and Goldberg (1988). Tissue sections (10 μm thick) blocked in 2.5% (w/v) non-fat dry milk (Sigma) were incubated with anti-PhADF polyclonal antibodies at a dilution of 1:100 for 1 h and then incubated with FITC-conjugated secondary antibody (Sigma) at a dilution of 1:40 for 1 h. Mounted slides were examined for immunofluorescence under a confocal scanning laser microscope (MRC-1024, Bio-Rad). The fluorescent signal was obtained and pseudocolored in green. Final figures were composed using Adobe Photoshop version 5.0 (Adobe System Inc.).

2.4. Genomic library construction and isolation of the genomic clone

For construction of a genomic library, genomic DNA from leaves of petunia was isolated by CTAB extraction and further purified by CsCl/EtBr ultracentrifugation. Purified DNA was partially digested with Sau3AI, size-fractionated (15–23 kb) on sucrose gradients, and then cloned into the XhoI site of pFIXII vector (Stratagene) according to the manufacturer’s directions.

About 6 × 105 recombinant phages were screened using a 32P-labeled petunia PhADF1 cDNA clone. After a second round of screening, genomic DNA inserts from positive clones were mapped with restriction enzymes and subcloned into the pBluescript SK vector (Stratagene). Overlapping subclones were generated using the Nested Deletion Kit (Pharmacia). Manual nucleotide sequencing was performed by the dyeoxy-nucleotide chain termination method on double-stranded plasmid DNA using the Sequenase (version 2.0), DNA-Sequencing Kit (USB), and [α-32P] dATP (Amersham) as a label. Sequence data were analyzed with programs in the DNASTAR package (DNASTAR, Madison, WI) and internet PLACE site (http://www.dna.affrc.go.jp).

2.5. Primer extension analysis

The transcription start sites of the genomic clones were determined by primer extension. 32P-end labeled 28 base synthetic oligonucleotide primer, PhADF1-PE (Table 1) complementary to nucleotides +57– +84 of the PhADF1 gene, was hybridized to 10 μg of total RNA prepared from petunia leaf. The first strand cDNAs were synthesized using Primer Extension System (Promega). The products from the primer extension and sequence reaction were separated through a 6% denaturing polyacrylamide gel.

2.6. Construction of the chimeric genes

Schematic diagrams of chimeric constructs are repre-
sent in Figs. 4 and 6. Promoter fragments of PhADF1 were generated by polymerase chain reaction (PCR). Primers used in PCR reactions are represented in Table 1. For construction of PhADF1 promoter fusions, the 1.7 kb promoter region with 5'-UTR (PA101) and the 3.3 kb promoter with intron 1 (PA102) were amplified using the sense primer PA101-F and the antisense primers PA101-R or PA102-R, respectively. The PCR products were cloned into the pGEM-T easy vector (Promega) and verified by sequencing. The resulting vectors were digested with BamHI, and DNA fragments were then inserted into pBI101 (Clontech).

For construction of PA102.1, PA105, and PAC1, BamHI sites were generated at both ends of the fragment containing intron 1 of PhADF1 by PCR, using the sense primer PA105-F and the antisense primer PA102-R. The amplified and then cloned PCR fragment was digested with BamHI and inserted into PA101, pBI101, and pBI121 (Clontech), respectively. The orientation of the intron in PA102.1, PA105, and PAC1 was verified by restriction enzyme digestion. Similarly, PA103 and PA104 were constructed by PCR using the sense primers PA101-F or PA104-F and the antisense primer PA103-R. The amplified fragments were cloned into BamHI site of pBI101 and PA102, respectively. The PhADF promoter::GUS fusion constructs were transformed into Agrobacterium tumefaciens C58C1Rif" by the freeze-thaw method (An et al., 1988).

2.7. Plant transformation and histochemical analysis

For plant transformation, Arabidopsis ecotype Columbia was used. Plants were grown on rock-wool bricks watered with MS solution under constant white-fluorescent light at 22°C. Arabidopsis plants with 2–3 inflorescences were transformed using the floral-dip method (Clough and Bent, 1998). Transgenic lines were selected on 0.5 × MS/0.8% tissue culture agar plate with 50 mg/l kanamycin. The T1 transgenic plants were self-fertilized, and T2 seeds were screened for segregation on plates containing kanamycin. Candidates with single T-DNA insertion were verified by Southern blot analysis, and homozygous T2 seeds from several independent transformant plants were used for further characterization.

Histochemical analysis of GUS activity was performed as described by Stomp (1992) at 37°C for 12 h. Tissues were cleared of chlorophyll in ethanol. Photographs were taken using a stereomicroscope. Fluorometric analysis was performed as described by Jefferson (1987) using 4-methylumbelliferyl-β-glucuronide. GUS values were expressed as pmol 4-methylumbelliferone min⁻¹ mg soluble protein⁻¹.

3. Results

3.1. PhADF proteins are mainly accumulated in vascular tissues of petunia

To characterize the tissue distribution patterns of PhADF proteins, we raised polyclonal antibodies against PhADF1 and PhADF2, respectively, and performed immunoblot analysis. However, anti-PhADF1 and anti-PhADF2 polyclonal antibodies were able to cross-hybridize not only to PhADF1 but also to PhADF2 protein (data not shown). Western blotting of tissue extracts showed that the PhADF proteins were expressed at comparable levels in every tissue of mature petunia organs analyzed except anthers (Fig. 1A). The accumulation patterns of PhADF proteins in reproductive (anther) and vegetative tissues (petal and leaf) during petunia organ development were examined in detail (Fig. 1B). Flower development was categorized according to maturity as stages 1–8. During floral development, the PhADF proteins in anther accumulated at comparable levels in the young bud stages (anther 1, 2). After anther opening, PhADF proteins were not detected. However, as petals developed, PhADF protein levels were high in the elongated bud stage (petal 5) and persisted to the expanded stage (petal 7). Interestingly, a minor band of 20 kD was detected in petals, which reflects the existence of an isotype. Similar expression patterns were observed during leaf development. Leaves were categorized by their position from the shoot apex (leaf 1–8). PhADF proteins were expressed early in leaf development and gradually increased, being present at high levels in fully expanded leaves (leaf 6), and then diminished in old leaves. The protein accumulation patterns were in agreement with the tissue distribution of mRNAs that we have previously reported (Mun et al., 2000). This result

Table 1

Oligonucleotides used in PCR, primer extension and GUS fusion construction

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Location</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhADF1-PE</td>
<td>+57–+ 84 of PhADF1</td>
<td>Primer extension</td>
<td></td>
</tr>
<tr>
<td>PA101-R</td>
<td>−1581–1560 of PhADF1</td>
<td>Construction of</td>
<td></td>
</tr>
<tr>
<td>PA101-R</td>
<td>+60–+ 87 of PhADF1</td>
<td>PA101, PA102, PA102.1</td>
<td></td>
</tr>
<tr>
<td>PA102-R</td>
<td>+1748–+ 1764 of PhADF1</td>
<td>PA103, PA104, PA105</td>
<td></td>
</tr>
<tr>
<td>PAC1</td>
<td>+2090–+ 2114 of PhADF1 and PAC1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA104-F</td>
<td>+1999–+ 2024 of PhADF1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA105-F</td>
<td>+58–+ 84 of PhADF1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*’F’ and ‘R’ designate forward and reverse orientation oligonucleotides, respectively. ‘+’ and ‘−’ represent upstream and downstream from the transcription initiation site. The sequences in italic were synthetic extensions or modified sites for restriction enzyme digestion.
suggestions that ADF gene expression in petunia is regulated at the transcriptional level.

For analysis of the spatial distribution of the PhADF proteins in petunia tissues, immunocytochemical studies were performed (Fig. 2). In vegetative organs, high levels of PhADF accumulation could be observed mainly in the vascular tissue, particularly in xylem and phloem (Figs. 2E–G). Weak signals were detected in cortex and mesophyll cells. However, in reproductive organs, PhADF protein accumulation patterns were complex. In petals, PhADFs were detected in vascular tissue at high levels (Fig. 2H). In pistils, PhADFs were expressed in the outer integument of the ovule and endocarp of the ovary wall (Fig. 2O), the transmitting tissue of style (Fig. 2R), and the stigma (Fig. 2S). In young anthers, strong signals were observed in the epidermis, endothecium, and pollen (Fig. 2T). However, in mature anthers, no signal was observed (data not shown). Weak expression was also detected in the vascular bundles of the style and anther.

3.2. Isolation of the PhADF1 gene and characterization of its structure

A genomic library was constructed with λFIXII phage and partially Sau3AI-digested DNA fragments prepared from petunia. A total of 6 × 10⁵ independent clones were screened by plaque hybridization with the PhADF1 cDNA as a probe and five positive clones were isolated. Based on restriction enzyme digestion and Southern hybridization, one clone was finally selected for further analysis. A 5.5 kb SalI fragment of the clone was subcloned into pBlue-scriptII SK (Fig. 3A). This clone contained a portion of ADF gene which covers the entire PhADF1 cDNA sequences.

The complete nucleotide sequences of the genomic clone was determined. The transcribed region of PhADF1 (GenBank Accession No. AY038063) has a length of 2432 bp, and the 5'-, 3'UTRs and 82 bp and 191 bp long, respectively. The coding region of PhADF1 is divided into three exons interrupted by two introns (Fig. 3A). Sequence comparison revealed that the gene structures between PhADF1 and Arabidopsis AtADF1 are conserved. The 1659 bp first intron was located immediately downstream of the start codon, and the 80 bp second intron split codon 90. The consensus splicing sequence at the 5' exon/intron junction, Ggt, and 3' to the end of intron, agG, were found (Hanley and Schuler, 1988). The AT contents of the first and second introns are 64 and 74%, respectively. The transcription start site of PhADF1 gene was determined by primer extension (Fig. 3B). Sequences similar to cis-regulatory elements from eukaryotic promoters are found in the 5' flanking region. The putative TATA box (~30–25) is found at position 30 bp upstream from the transcription initiation site. Thirty-nine nucleotides upstream from the TATA, a putative CAAT box (~71–66) is found (Fig. 3C). Several potential transcription factor-binding sites and hormone response domains were detected (data not shown). This finding suggests that transcription of the PhADF1 gene may be influenced by a variety of genetic elements. A putative poly(A) addition signal consisting of an AATGAAA sequence was located at 42 bp downstream from the stop codon.

Examination of the nucleotide composition of PhADF1 gene in the 5'-UTR and upstream region of the first intron revealed many stretches rich in C/T (sequences (Fig. 3C). In the actin genes of soybean and rice, the C/T repeat sequences play an important role in controlling actin gene expression (Huang et al., 1997). The first intron immediately 3' to the initiation codon has also been observed in the Arabidopsis AtADF1 gene. Based on position, the intron is similar to the leader introns in several plant genes. The
leader introns of polyubiquitin genes and the rice Act1 actin gene enhanced gene expression in transgenic plants (Zhang et al., 1991; Garbarino et al., 1995). This result suggests that the first intron of PhADF1 gene may have an important role in regulating ADF gene expression.

3.3. Expression of PhADF1 in transgenic Arabidopsis

To examine the cellular expression of PhADF1 gene, we fused GUS coding region to the PhADF1 promoter. Two kinds of fusion constructs linking each promoter to the GUS
gene were created to verify the influence of the first intron on expression (Fig. 4). These two constructs differ in the amount of the PhADF1 transcribed region included. A 1.7 kb fragment terminating just before start codon was used for PA101, while a 3.3 kb fragment including all of exon 1, intron 1, and five codons of exon 2 was used for PA102. Fusion constructs were introduced into wild-type Arabidopsis by A. tumefaciens-mediated transformation, and the GUS expression patterns were determined by histochemical staining. Many lines were obtained for each construct, and five independent transformed lines with a single T-DNA insertion were examined to characterize each promoter- or promoter/intron-GUS construct.

In PA101, the PhADF1 promoter was strongly active in the vascular tissues. GUS staining was detected in the vascular bundle of cotyledons in 2-day old seedlings (Fig. 5A). In 5-day old seedlings, GUS expression was also detected in vascular bundles of the hypocotyl and root.

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**Fig. 3.** Genomic sequence and organization of the PhADF1 gene. (A) Restriction map of petunia PhADF1 gene carried by the λFIXII clone. Exons are represented by black boxes. (B) Determination of the PhADF1 promoter transcription start site by primer extension. Primer extension reaction was carried out as described in Section 2. The transcription start sites in the corresponding plus strand DNA are indicated by arrows. (C) Nucleotide sequence around the transcription start site of the PhADF1 gene. The transcription start site is designated as +1. A putative TATA box and CAAT box motifs are boxed. C/T rich sequences are underlined. The dotted-arrow indicates the primer site of primer extension analysis.

**Fig. 4.** Structures of chimeric genes carrying promoter and intron of PhADF1 gene. The filled boxes indicate the first exon of PhADF1 gene. Thin lines represent promoter region and the first intron of PhADF1 gene. The open box is the GUS coding region. NOS signifies the nopaline synthase terminator.
(Fig. 5B), and the level of GUS persisted in mature plants (Figs. 5C–E). In mature plants, leaf primordia were stained, but the emerging leaf showed little GUS staining (Fig. 5C). GUS staining was mainly seen in the vasculature of older rosette leaves, and weak staining was also detected in leaf blades (Figs. 5C,D). In mature roots, the vascular cylinder above the root meristem expressed GUS (Fig. 5E). In flowers, GUS activity was found in the vascular tissues of the filaments of the stamens and sepals (Fig. 5F), whereas weak staining was detected in the receptacles of flowers (Fig. 5G). No staining was observed in petals (Fig. 5F) and pollen (data not shown).

For PA102, strong GUS staining was found in almost all parts of the plant. The PhADF1 promoter with the first intron was strongly active in hypocotyls, roots, and cotyledon tips and relatively weakly expressed in cotyledons and cotyledon/hypocotyl junctions of 2-day old seedlings (Fig. 5H). As seedlings grew, strong GUS staining was also found in cotyledons (Fig. 5I). In adult stage plants, all vegetative organs were strongly stained, and staining is relatively strong in the vasculature and leaf margins of adult rosette leaves (Figs. 5J,K). In addition, all cells in roots expressed very high level GUS expression (Fig. 5L). In flowers, intense staining was seen in the filaments of stamens and in sepals along vasculature. The PhADF1 promoter was highly active in the stigma and style of the pistil and in the receptacle of flowers (Figs. 5M,N). Relatively weak signals were also detected in the replum, inflorescence stalk (Fig. 5N), and cauline leaf (data not shown). However, no GUS signal was detected in petals (Fig. 5M) and pollen (data not shown).

Two lines of evidence suggest that the difference in GUS activity observed in PA101 and PA102 transgenic plants may be quantitative rather than qualitative. First, those tissues that show GUS staining in the PA101 line, such as vascular tissues and the base of the immature silique (Figs. 5C,F,G), are the regions of most intense staining in the PA102 line (Figs. 5J,M,N). Second, the staining pattern of the PA102 line treated for 1 h with GUS substrate resembles the pattern of the PA101 line stained for 12 h (data not shown). Thus, PhADF1 showed gradually increased expression especially in vascular tissues during plant development, and its expression is highly enhanced by the first intron.

Fig. 5. Histochemical analysis of GUS expression in transgenic Arabidopsis carrying PA101 and PA102 constructs. (A–G) are from PA101 transgenic lines; (H–N) are from PA102 transgenic lines. (A, H) Seedling of 2-day after germination. (B, I) Seedling of 5-day after germination. (C, J) 2-week old plant. (D, K) Rosette leaf. (E, L) Root. (F, M) Flower. (G, N) Silique. GUS staining was performed for 12 h. Scale bars represent 100 μm in (E, G, L, N); 1 mm in (A, B, D, F, H, I, K, M); and 5 mm in (C, J).
3.4. The first intron of PhADF1 acts as an enhancing element in PhADF1 gene expression

To determine whether the other regions in the PhADF1 coding sequence have an effect on expression of the PhADF1 gene, we constructed various PhADF1::GUS fusion constructs that contained different parts of the intragenic region (Fig. 6). Lines that have single copy T-DNA insertion were identified by segregation and Southern blot hybridization (data not shown), and GUS activity in leaves was assessed by histochemical staining and enzyme assay. We compared the GUS expression levels resulting from these constructs in 2-week old transgenic Arabidopsis leaves. As shown in Fig. 6, the highest GUS activity was recorded in PA102, in which the first intron was located between the PhADF1 promoter and the GUS coding region. PA102 provided GUS activity 22 times greater than that of PA101. In contrast, when the first intron was inserted at the same site with antisense orientation, the enhancement effect was diminished. PA102.1 represented a low level of GUS activity, averaging 10.2% of the activity of PA101. In PA102.1, very weak histochemical staining was detected in vascular tissues (data not shown). When the first intron is substituted for the 5′-flanking region of PhADF1 (PA105), GUS activity was recorded as below the control level. These results indicated that the first intron is able to enhance the PhADF1 expression only with sense orientation, and it has neither a general enhancer function nor promoter function.

To test whether the second intron also affects PhADF1 expression, two different constructs (PA103 and PA104) were examined. In PA103, the GUS coding region was joined to 2.1 kb intragenic region containing exon 1, intron 1, exon 2, intron 2, and a portion of exon 3. This construct showed only 50% of the GUS enzyme activity observed with the construct carrying the first intron alone. The additional 105 amino acid residues at the N terminus of fusion protein encoded by PA103 construct may give rise to reduce GUS enzyme activity or stability. Otherwise, intron 2 may influence the PhADF1 promoter activity. Indeed, intron 2 alone reduced the GUS expression (PA104). This result indicates that the functions of the introns on PhADF1 expression are somewhat different, and the combined presence of the first and second intron does not support a synergistic effect on PhADF1 expression. A similar result was previously reported in rice OsTubA1 (Jeon et al., 2000).

It was observed in PAC1 that locating the first intron under the control of the 35S promoter resulted in a 1.2-fold increase in GUS expression level over that of the pBI121 control (Fig. 6). Although, the enhancement effect was not so high, this result also supports the concept that the first intron of PhADF1 can enhance gene expression. There-

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**GUS activity** | **Relative activity(%)**
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PA101 | 2538 ± 673 | 100
PA102 | 55828 ± 5381 | 2200
PA102.1 | 260 ± 67 | 10.2
PA103 | 1276 ± 227 | 50.3
PA104 | 550 ± 76 | 21.7
PA105 | 4 ± 1 | 0.2
pBI121 | 3492 ± 605 | 137.6
PAC1 | 4209 ± 343 | 165.8
WT | 3 ± 1 | 0.1

*Fig. 6. Schematic representations of fusion constructs and GUS activity in the leaves of transgenic Arabidopsis lines carrying them. The filled boxes indicate the exons of PhADF1 gene, and thin lines represent PhADF1 promoter region and introns. The open box is the GUS coding region. NOS signifies the nopaline synthase terminator. Arrows designate the orientation of the first intron. GUS activity is expressed as pmol methylumbelliferone min⁻¹ mg protein⁻¹. Values are means ± SE from five independent plants. Relative activity is expressed as a percentage of that observed for PA101.*
fore, only the first intron among the intragenic regions of PhADF1 gene has an enhancement effect on PhADF1 expression.

4. Discussion

The cellular expression of the PhADF proteins in petunia was characterized, using western blot analysis and immunolocalization to identify tissue-preferential expression of ADF genes. The anti-PhADF polyclonal antibodies detected a 16 kD protein in all tissues examined. In addition, a minor 20 kD protein was also detected in petals (Fig. 1). This protein expression pattern resembles the expression of PhADFs that we reported previously (Mun et al., 2000). Because the anti-PhADF antisera can cross-hybridize to PhADF1 and PhADF2 (data not shown), the 20 kD band represents the existence of other ADF isoform in petunia. In addition, immunolocalization analysis detected the ADF in pollen, where the PhADF1 and PhADF2 transcripts did not exist. Therefore, as previously proposed on the basis of genomic Southern hybridization, at least four different ADF proteins exist in petunia, and one of them may be the reproductive form. However, it is uncertain whether the reproductive form is expressed in pollen or is expressed outside of pollen and then transported into it. The accumulation patterns of PhADF proteins during petunia organ development were in concordance with mRNA expression. This result suggests that the expression of PhADF genes is regulated by developmental signal at the transcription level.

ADF is expected to be ubiquitously expressed in every cell for proper interaction with actin, which is a cytoskeletal component of all cells. It was therefore surprising to see the vascular bundle-preferential accumulation of PhADF proteins in vegetative plant organs. Although vascular bundle-specific expression was also reported in Arabidopsis phn2 (Christensen et al., 1996) and castor bean Re:PRO1 (Schober et al., 2000) profilins, we can not explain why the expression level of PhADF proteins is low in other cells. It is possible that expressions of PhADF genes are related to that of vascular bundle-specific actin or other PhADF isoforms might be expressed in all other cells or in all cells. To verify this hypothesis, further experiments are necessary.

Actin is an important cytoskeletal protein that is expressed in all eukaryotic cells. Actins of higher plant are encoded by diverse gene families. For example, petunia possesses a superfamily of actin genes, which contains between 100 and 200 members that can be divided into at least six highly divergent subfamilies (McLean et al., 1990). In Arabidopsis, ten actin genes have been cloned, and eight genes that are functionally active were characterized in detail (Meagher et al., 1999). Based on phylogenetic analysis, mRNA accumulation pattern, and expression of GUS fusion constructs in transgenic Arabidopsis, three vegetative actins, ACT1, ACT3, ACT4, ACT11, and ACT12, were identified. The vegetative actins, ACT2 and ACT7, were expressed predominantly in vegetative tissues and some floral parts. Like vegetative actin expression patterns, PhADF1 was expressed in most vegetative tissues (such as germinating seeds, seedlings, and vegetative parts of mature plants) and in some floral tissues (such as sepals, filaments, and inflorescence stalks of transgenic Arabidopsis). PhADF1 activity was expressed more strongly in older tissues than in young tissues, suggesting an important role in regulating plant actin dynamics in mature vegetative tissues. Detailed examination of the expression of promoter::GUS fusions from PhADF1 gene revealed patterns that are quite similar to their possible potential counterparts, ACT2 and ACT7. In addition, promoter of PhADF1 gene has several hormone response elements. ACT7, the primary actin responding to plant hormone, also has the hormone response element on its 5'-flanking region (McDowell et al., 1996). These results strongly suggest that expression of actin and ADF in vegetative tissues may be correlated. Thus, we can propose that the evolution of the vegetative class of actin and ADF is concordant.

Plant genes have many regulatory elements which are necessary for proper gene expression. Recent research shows that not only promoters but also sequences downstream of the transcription start site can influence the level and pattern of plant gene expression. The members of downstream regulatory elements are 5'- or 3'-UTR, introns, and protein-coding regions (Rose and Last, 1997). The importance of introns on gene expression has been studied in a number of genes. Usually, introns enhance gene expression at the posttranscriptional level by increasing splicing efficiency of transcripts or by stabilizing mRNAs (Rose and Beliakoff, 2000). Like other plant genes, expression of PhADF1 gene was also affected by its intron. Through histochemical GUS staining, we have revealed that the first intron is very important for strong and constitutive promoter activity. In PhADF1::GUS, the promoter without the first intron expressed GUS only in vascular tissues. This phenomenon was also reported in Arabidopsis AtADF1 and AtADF6 (Dong et al., 2001). Instead, the promoter with the first intron induced strong GUS expression in every vegetative tissues (Fig. 5). However, the first introns did not function as transcriptional enhancers or regulatory elements for tissue-specific expression. We particularly note these results, because enhancement of gene expression by introns was also observed in plant cytoskeletal genes.

A rice α-tubulin gene, OsTubA1, showed a preferentially strong expression in actively dividing tissues which was mediated by the first intron (Jeon et al., 2000). Similarly enhanced expressions have been reported in actin genes. Plant actin genes have a 5'-leader intron and a C/T-rich repeat in the 5' non-coding region (Pearson and Meagher, 1990; Zhang et al., 1991). These C/T-rich sequence is also present at 5'-UTR and upstream of the first intron of PhADF1. The leader intron of the rice Act1 actin gene is
required for strong promoter activity in electroporated rice protoplasts. In addition, the promoter of the Arabidopsis ACT2 actin gene requires the leader intron for strong constitutive expression in vegetative tissues (An et al., 1996). This finding suggests a fundamental role of the introns and C/T-rich sequences in plant cytoskeletal gene expression. Expression of plant actin and ADF genes may be concordantly regulated by intron sequences. Further investigation of the regulatory sequences on the introns will provide information about correlated regulation of plant cytoskeletal gene expression. There is some disagreement between the ADF protein localization pattern in petunia and the promoter::GUS expression pattern in Arabidopsis. Probably, this may be due to differences in the regulatory systems controlling gene expression.

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