Distinct Roles of the First Introns on the Expression of Arabidopsis Profilin Gene Family Members[1]

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Profilin is a small actin-binding protein that regulates cellular dynamics of the actin cytoskeleton. In Arabidopsis (*Arabidopsis thaliana*), five profilins were identified. The vegetative class profilins, *PRF1*, *PRF2*, and *PRF3*, are expressed in vegetative organs. The reproductive class profilins, *PRF4* and *PRF5*, are mainly expressed in pollen. In this study, we examined the role of the first intron in the expression of the Arabidopsis profilin gene family using transgenic plants and a transient expression system. In transgenic plants, we examined *PRF2* and *PRF5*, which represent vegetative and reproductive profilins. The expression of the *PRF2* promoter fused with the β-glucuronidase (*GUS*) gene was observed in the vascular bundles, but transgenic plants carrying the *PRF2* promoter-GUS with its first intron showed constitutive expression throughout the vegetative tissues. However, the first intron of *PRF5* had little effect on the reporter gene expression pattern. Transgenic plants containing *PRF5* promoter-GUS fusion with or without its first intron showed reproductive tissue-specific expression. To further investigate the different roles of the first two introns on gene expression, the first introns were exchanged between *PRF2* and *PRF5*. The first intron of *PRF5* had no apparent effect on the expression pattern of the *PRF2* promoter. But, unlike the intron of *PRF5*, the first intron of *PRF2* greatly affected the reproductive tissue-specific expression of the *PRF5* promoter, confirming a different role for these introns. The results of a transient expression assay indicated that the first intron of *PRF1* and *PRF2* enhances gene expression, whereas *PRF4* and *PRF5* do not. These results suggest that the first introns of profilin genes are functionally distinctive and the first introns are required for the strong and constitutive gene expression of *PRF1* and *PRF2* in vegetative tissues.

Diverse actin-binding proteins regulate cellular dynamics of the actin cytoskeleton to perform various biological processes, such as locomotion, elongation, shape change, cytoplasmic streaming, division, and development of the cell (Bamburg et al., 1999; Kost et al., 1999; Meagher et al., 1999; Balŭska et al., 2001). Among these proteins, the small actin-binding protein, profilin (12–15 kD), plays an important role in actin dynamics (Gibbon and Staiger, 2000; Kovar et al., 2000; McKinney et al., 2001). In plants, profilin was originally identified in birch pollen as a potent allergen (Valenta et al., 1991), and it has been isolated and characterized in various plants, such as maize (*Zea mays*), tobacco (*Nicotiana tabacum*), bean (*Phaseolus vulgaris*), Arabidopsis (*Arabidopsis thaliana*), tomato (*Lycopersicon esculentum*), and castor bean (*Ricinus communis*; Staiger et al., 1993; Mittermann et al., 1995; Vidali et al., 1995; Christensen et al., 1996; Huang et al., 1996; Yu et al., 1998; Guillen et al., 1999; Schobert et al., 2000). Plant profilins have been shown to bind G-actin in vitro, and they are biochemically similar to nonplant profilins (Valenta et al., 1993; Guillen et al., 1999; Kovar et al., 2000). Arabidopsis profilin complemented both a budding yeast (*Saccharomyces cerevisiae*) profilin deletion mutant and a fission yeast (*Schizosaccharomyces pombe*) cdc3-124/profilin mutant, indicating that plant profilin is functionally equivalent with yeast profilin in vivo (Christensen et al., 1996). Like many other plant cytoskeletal genes, profilins exist as a multigene family and fall into either vegetative or reproductive classes, as shown in maize and Arabidopsis (Staiger et al., 1993; Christensen et al., 1996; Huang et al., 1996). In Arabidopsis, three vegetative and two reproductive profilins have been isolated and characterized. Profilins expressed in vegetative tissues are encoded by *PRF1*, *PRF2*, and *PRF3*. Both *PRF4* and *PRF5* encode reproductive class profilins that are preferentially expressed in pollen (Christensen et al., 1996; Huang et al., 1996). Analysis of transgenic plants harboring *PRF1* and *PRF2* promoter-β-glucuronidase (GUS) fusion constructs showed that these two profilins are mainly expressed in the vascular bundles of vegetative tissues (Christensen et al., 1996; Ramachandran et al., 2000). Recently, Kandasamy et al. (2002) investigated the spatial and developmental expression of profilins in Arabidopsis using vegetative and reproductive profilin-specific antibodies. They examined the root tip, sepal, and pistil using a monoclonal antibody that

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reacts strongly with PRF1 and PRF2, and weakly with PRF3. Based on the results of biochemical and structural localization analysis, the authors concluded that vegetative profilins are expressed in all vegetative organs and tissues. The discrepancy with previous work on profilin gene expression may reflect the presence of regulatory sequences in profilin genes. However, such regulatory elements have not yet been identified in profilin genes. Recently, it has been shown that some introns are involved in the regulation of spatial or temporal expression in potato (*Solanum tuberosum*) *Sus3* (Fu et al., 1995), bean *PsaD* (Bolle et al., 1996), tobacco *Ubi.U4* (Plesse et al., 2001), petunia (*Petunia hybrida*) *PhADF1* (Mun et al., 2002), and Arabidopsis *PAT1* (Rose and Last, 1997), *AGAMOUS* (Deyholos and Sieburth, 2000), and *FLC* (Sheldon et al., 2002). Particularly, in the case of genes that are known to express constitutively, such as *PAT1*, *PhADF1*, and *Ubi.U4*, introns were required for their strong and constitutive expression (Rose and Last, 1997; Plesse et al., 2001; Mun et al., 2002).

In this study, to identify the regulatory elements controlling profilin gene expression, we examined the effect of an intron of the profilin genes. Although genes encoding profilins are interrupted by two introns, we only tested the first intron, because introns that regulate gene expression are generally located near the 5′ region of the gene (Clancy and Hannah, 2002; Rose, 2002), and the first intron was about 4 times longer in length than the second intron. Our results show that the first intron of vegetative profilins (*PRF1* and *PRF2*) plays an important role in strong and constitutive expression in vegetative tissues both in the transgenic and/or transient assay and enhances expression both at the mRNA and at the protein level. We also tested the first intron of reproductive profilins (*PRF4* and *PRF5*) to determine whether a regulatory role of introns is conserved in reproductive profilin. In contrast with *PRF1* and *PRF2*, the first intron of *PRF4* and *PRF5* did not alter expression patterns significantly. Furthermore, the first intron of *PRF2* was able to alter spatial expression patterns of both *PRF2* and *PRF5* promoters.

RESULTS

The First Intron of *PRF2* Is Required for Strong and Constitutive *PRF2* Expression in Vegetative Tissues

Although introns are removed during the mRNA maturation process, some introns are known to enhance or regulate gene expression in various ways. To characterize the role of introns in vegetative profilin gene expression, we have cloned the *PRF2* gene and generated two *PRF2* promoter-GUS fusion constructs. The first construct, pPRF2-5′, includes the 1.6-kb promoter, 5′-untranslated region (UTR), and the start codon fused with the *GUS* gene in the pBI101 vector. The second construct, pPRF2e-p2i, contains the promoter, the entire first exon including 5′-UTR, the first intron, and 24 bp of the second exon (Fig. 1A). The short second exon was included for proper intron splicing. Three independent transgenic plants carrying single copies of the pPRF2-5′ or pPRF2e-p2i constructs were examined to analyze the quantitative effect of the first intron of *PRF2* on gene expression.

We analyzed the expression of the *PRF2* promoter-GUS fusion construct in various developmental stages of transgenic Arabidopsis. In 3-d-old seedlings containing pPRF2-5′, GUS staining was mainly observed in the vascular bundles of cotyledons. Some faint staining was detected near the vascular bundles, and there was no expression in the shoot apical meristem region (Fig. 1B). Most parts of the root showed GUS expression, except the root tip (Fig. 1C), and relatively strong staining was observed in the vascular bundles of the root (data not shown in detail). This preferential expression in vascular bundles was also maintained in 15-d-old plants. In this case, GUS expression was detected in the vascular bundles of rosette leaves and petioles (Fig. 1, D and E). Most of the roots were stained, but no GUS expression was observed in the root tips. The older expanded leaves showed stronger expression than smaller ones (Fig. 1D). In flowers, the filaments of stamens and the veins of sepals were stained. No GUS staining was observed in anthers, pollen, carpels, or petals (Fig. 1F). In young siliques, staining was detected in the receptacle (Fig. 1G). These expression patterns were largely in agreement with previous reports (Christensen et al., 1996). However, expression of the *PRF2* promoter-GUS was completely changed by the inclusion of the first intron within the construct. In 3-d-old seedlings containing pPRF2e-p2i, strong GUS staining was detected throughout the plant. Most parts of the seedlings, including the root hairs, were deeply stained. Shoot apical meristems and root tips also showed GUS expression (Fig. 1, H and I). These strong and constitutive GUS expressions were observed also in 15-d-old plants. The deep blue staining was detected in the rosette leaves, petioles, roots, root hairs, and trichomes (Fig. 1, J and K). Almost every part of the floral organs, such as the petals, sepals, anthers, filaments, and stigmas, were stained (Fig. 1L). In young developing siliques, the stigma region and receptacle were stained (Fig. 1M). Although these results suggest a significant role of the intron in *PRF2* gene expression, they cannot rule out the effect of coding regions on gene expression because pPRF2e-p2i has a full length of the first exon encoding 41 amino acids. Therefore, to examine the effect of the coding region, a pPRF2e construct that harbors the promoter region and the first exon fused with the *GUS* gene was generated, and the expression patterns of this construct were analyzed in 23 lines of T1 transgenic plants. Because of copy number variations of the transgene or positional effects, there were some variations, but most of the plants showed similar GUS expression patterns with pPRF2-5′ (data not shown), suggesting that the first exon of *PRF2* has no significant effect on the gene expression pattern.

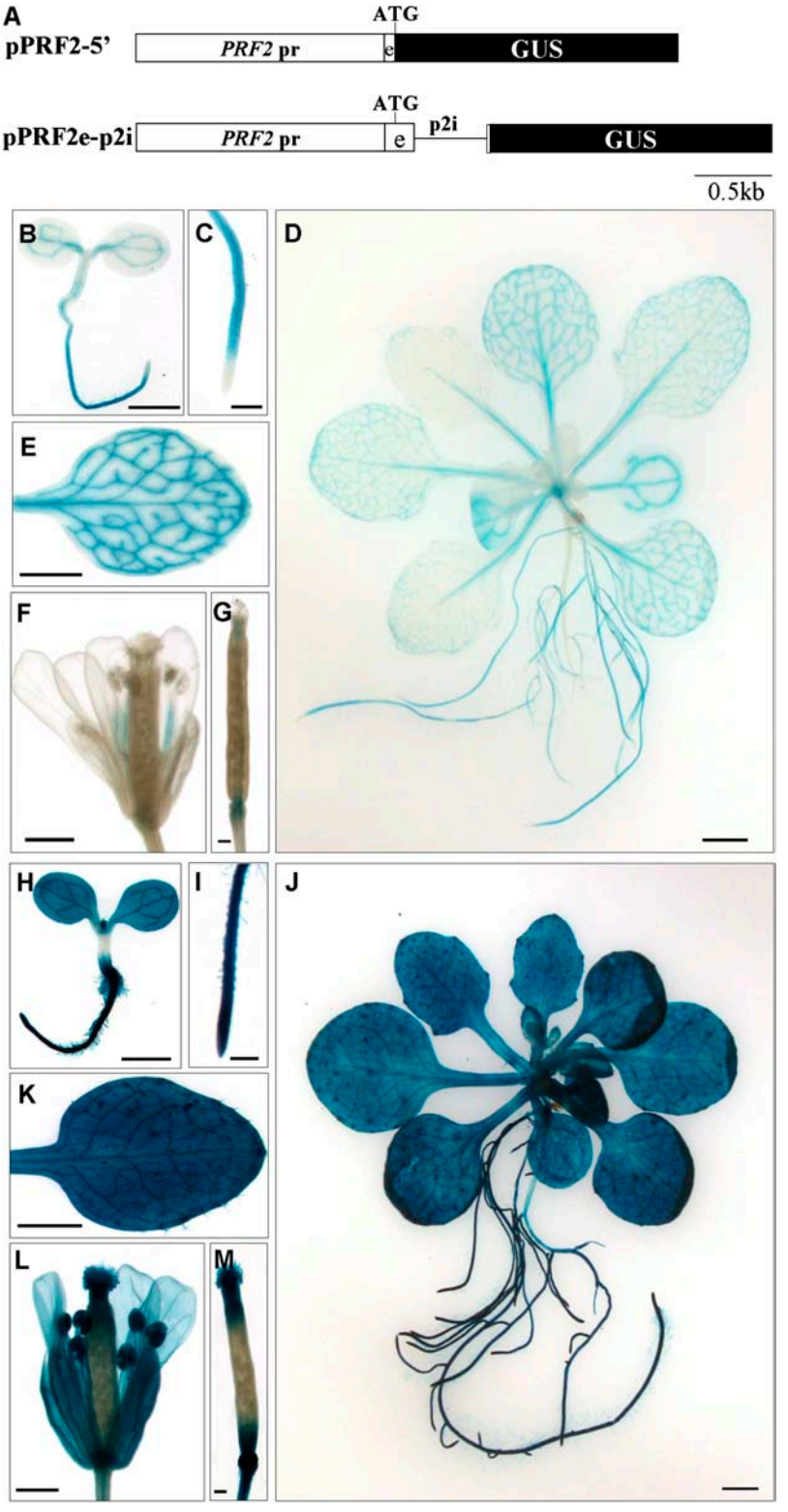

Figure 1. Histochemical analysis of GUS expression in transgenic Arabidopsis containing pPRF2-5′ and pPRF2e-p2i constructs. A, Schematic representations of *PRF2* promoter-GUS fusion constructs. The box with *PRF2* pr is the promoter region of *PRF2*; the box with e is the first exon of *PRF2*; the black box with GUS is the GUS coding region; the line with p2i is the first intron of *PRF2*; ATG is the start codon. B to G, From pPRF2-5′; H to M, from pPRF2e-p2i; B and H, 3-d-old seedlings; C and I, roots of 3-d-old seedlings; D and J, 15-d-old plants; E and K, rosette leaves; F and L, flowers from 5-week-old plants; G and M, young siliques. GUS staining was performed for 12 h. Scale bars, 1 mm (B, D, E, H, J, and K), 0.2 mm (C, G, I, and M), and 0.5 mm (F and L).

Furthermore, in a transient assay, the entire open reading frame (ORF) in the pPRF2ORFGP construct showed no positive effect on GUS expression and the expression level of the pPRF2geGP that contains the entire *PRF2* gene except the 3′-UTR was similar to that of the pPRF2e-p2iGP (Fig. 7B). These results support the fact that the sequences of *PRF2* mRNA or amino acids fused to the *GUS* gene do not affect enhanced gene expression. Therefore, these data demonstrate that the first intron of *PRF2* is responsible for the strong

and constitutive expression of *PRF2* throughout plant tissues.

The First Intron of *PRF2* Increases Both mRNA and Protein Levels

To investigate the basis for different expression of pPRF2-5′ and pPRF2e-p2i constructs, levels of mRNA and protein were analyzed. The accumulation of *GUS* transcripts was analyzed by reverse transcription (RT)-PCR analyses. One microgram of total RNA from roots, leaves, stems, and flowers was used for RT, and PCR was performed for 25 cycles to prevent saturation of PCR products. Sequencing of RT-PCR products of pPRF2e-p2i indicated that splicing occurred precisely (data not shown). In all tissues, the transcript level of transgenic plants carrying pPRF2e-p2i was increased when compared to pPRF2-5′ (Fig. 2), suggesting that different expression occurred at the transcription level. The increased mRNA levels were particularly apparent in the leaf and stem. To examine whether the increase in transcript level is fully reflected in the protein level, protein accumulation in the leaves was analyzed by western-blot analysis and GUS assay (Fig. 3). Twenty micrograms of total soluble proteins from the leaves of 4-week-old plants were used for immunoblot analysis with the GUS antibody. Similar to the transcript accumulation pattern, the levels of protein were much higher in pPRF2e-p2i than pPRF2-5′ plants. Results of fluorometric GUS assay indicated that pPRF2e-p2i gene expression was enhanced by about 15-fold relative to that of pPRF2-5′ (Fig. 3). Thus, the increase in transcript accumulation induced by the first intron was reflected in the GUS protein level. Therefore, strong expression of pPRF2e-p2i is correlated with increased accumulation of the *GUS* transcript. These results suggest that the role of the first intron of *PRF2* is closely related to increasing steady-state mRNA levels.

The First Intron of *PRF5* Has Little Effect on *PRF5* Gene Expression

We demonstrated the important role of the first intron in gene expression of *PRF2*, the vegetative profilin. To determine whether the role of first introns is conserved in a reproductive profilin, we examined the first intron of *PRF5*. About 1.5 kb of the promoter region of *PRF5* and the first exon were translationally fused with the *GUS* reporter gene in a pPRF5e construct that tests promoter activity. To analyze the intron's role, a pPRF5e-p5i construct, which includes all parts of pPRF5e as well as the first intron of *PRF5* with 24 bp of the second exon, was generated. The schematic structures of pPRF5e and pPRF5e-p5i are depicted in Figure 4A. Because we mainly focused on the effect of the intron on the *PRF5* spatial expression pattern, we did not isolate the single-copy lines, and more than 10 lines of T1 plants harboring pPRF5e and pPRF5e-p5i constructs were examined for the analysis. GUS histochemical analysis of transgenic plants harboring pPRF5e showed strong expression in pollen. Staining was also observed in the stigmas and anthers. Very weak staining was detected in the upper part of filaments and petals (Table I; Fig. 4D). GUS expression was not detected in 3-d-old seedlings and 15-d-old plants in most lines that were examined (Fig. 4, B and C). Although it is not clear whether GUS staining in other floral organs is an actual expression or just diffusion from pollen, GUS expression of pPRF5e was predominant in pollen. A pollen-specific expression pattern was in agreement with a previous report (Christensen et al., 1996). pPRF5e-p5i, which includes its own first intron within the construct, showed almost identical expression with pPRF5e. GUS staining was not observed in the vegetative tissues of nearly all transgenic plants (Fig. 4, E and F). Like pPRF5e, GUS staining in flowers was mainly detected in the pollen and anthers (Table I; Fig. 4G). These results suggest that the first intron of *PRF5* has no significant effect on the expression pattern of the *PRF5* gene.

The Role of Introns under the Control of Heterogeneous Promoters

The analysis of transgenic plants carrying *PRF2* and *PRF5* promoter-GUS fusion constructs revealed that the first intron of *PRF2* plays an important role in gene expression, while the first intron of *PRF5* has little effect. However, it is unclear whether only the intron itself is responsible for these differences. Other factors,

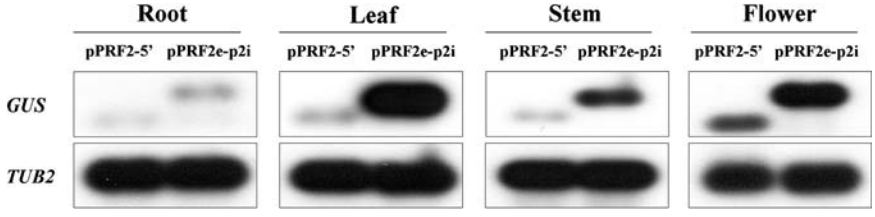

Figure 2. RT-PCR analysis of transcript expression of transgenic Arabidopsis containing pPRF2-5′ and pPRF2e-p2i constructs in various tissues. One microgram of total RNA from each organ was used for RT. *TUBULIN2* (*TUB2*) amplified with TUB-F (5′-CTCAAGAGGTTCTCAGCAGTA-3′) and TUB-R (5′-CTCAAGAGGTTCTCAGCAGTT-3′) primers was used as a control. After electrophoresis of PCR products, it was transferred to the nylon membrane and hybridized with ^{32}P-labeled *GUS* or *TUB2* probes.

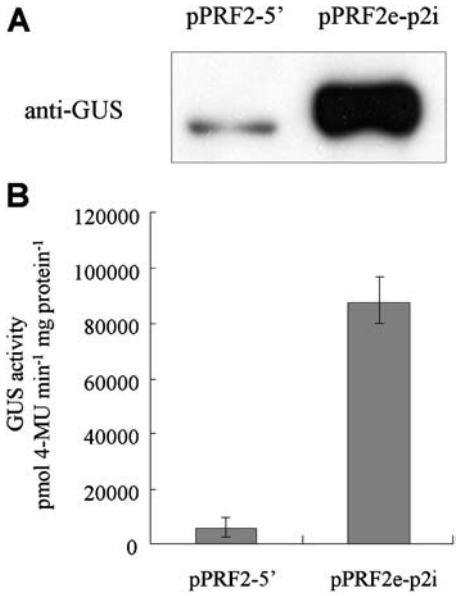

Figure 3. Western-blot analysis and determination of GUS activity in transgenic Arabidopsis containing pPRF2-5′ and pPRF2e-p2i. A, Total soluble protein (20 μg) from mature leaves was loaded per lane and analyzed using monoclonal GUS antibody. B, GUS activity is expressed as pmol 4-methyl-umbelliferone min^{-1} mg^{-1} protein. Values are means ± SD (n = 3).

such as interaction between intron and promoter, could be involved in profilin gene expression. Thus, to investigate the roles of introns in detail, introns were exchanged between *PRF2* and *PRF5*. For the construction of pPRF2e-p5i, the first intron of *PRF5* was inserted at the 3′ end of the first exon in the pPRF2e construct. In the same way, the first intron of *PRF2* was introduced into the pPRF5e construct, thus giving pPRF5e-p2i (Fig. 5A). For efficient splicing, both introns were accompanied with short flanking exons. The resulting constructs were transformed into Arabidopsis, and the expression pattern was analyzed in more than 20 independent T1 plants. In 3-d-old seedlings of pPRF2-p5i, expression was mainly observed in cotyledons and roots (Fig. 5B). This vascular bundle-specific expression was also observed in leaves, petioles, and roots of 15-d-old plants (Fig. 5C). In flowers, expression was observed in the filaments and sepals. No GUS staining was detected in pollen or in anthers (Table I; Fig. 5D). The overall expression patterns of pPRF2-p5i plants were very similar to those of pPRF2-5′ (Fig. 1, B–G). These data indicate that the first intron of *PRF5* has no significant effect on the expression of the *PRF2* promoter. However, unlike the first intron of *PRF5*, the first intron of *PRF2* had significant influence on the expression of the *PRF5* promoter. GUS histochemical analysis of pPRF5e-p2i showed strong expression throughout the plant. Deep blue staining was observed in nearly every part of the plant, including root hairs and tips of 3-d-old seedlings (Fig. 5E). The expression pattern was totally different from that of pPRF5e or pPRF5e-p5i (Table I; Fig. 4, B–G). This strong expression was also observed in 15-d-old plants (Fig. 5F). Most of the floral organs, including stigmas, anthers, pollen, filaments, petals, and sepals, also showed GUS expression (Table I; Fig. 5G). This constitutive expression pattern was quite similar to that of pPRF2e-p2i (Fig. 1, H–M). Thus, the properties of the two introns were maintained under heterogeneous promoters, and this confirmed the functional distinctiveness of the two introns. Particularly, the first intron of *PRF2* had a significant effect on the expression pattern of both the *PRF2* and *PRF5* promoters, and this demonstrates that the first intron of *PRF2* is responsible for the constitutive expression of pPRF2e-p2i and pPRF5e-p2i.

The First Intron of *PRF2* Alters the Spatial Expression Pattern of *PRF* Genes

The expression of pPRF2-5′ was mainly observed in the vascular bundles of vegetative tissues. But faint GUS staining was detected in nonvascular tissues. In the pPRF2e-p2i plants, GUS staining is slightly stronger in the vascular tissues. In addition, increased expression induced by the intron was observed in the RT-PCR and western-blot analysis. Therefore, it is possible that strong expression of pPRF2e-p2i could be a result of a quantitative increase in the expression pattern of pPRF2-5′. However, the result with pPRF5-p2i suggested that the intron alters the spatial expression pattern of the *PRF5* promoter. To determine the role of the intron in detail, GUS staining was performed in 3-d-old seedlings at various incubation times (Fig. 6). In pPRF2-5′, GUS staining was observed to occur weakly in the vascular bundles of cotyledons after 1-h staining. This vascular expression pattern was maintained and staining intensity was increased according to the staining time. After 12-h staining, weak staining was observed in the vascular bundles and nearby tissues. In pPRF2e-p2i plants, no vascular patterning was observed in the cotyledons after 1-h incubation. The overall parts of the cotyledons were evenly stained. This pattern was maintained after staining for 4, 8, and 12 h. In the roots, a different pattern was observed and maintained in both samples from the beginning. If the intron just increased expression, the early stage of the staining pattern of pPRF2e-p2i would be similar to that of pPRF2-5′. Therefore, these results suggest that the intron does not just increase or enhance gene expression, but instead is also involved in regulating spatial gene expression patterns.

The First Intron of *PRF2* Affects Gene Expression in a Position-Dependent Manner

The results above demonstrate that the first intron of *PRF2* enhances gene expression and can induce ectopic expression under the control of a tissue-specific

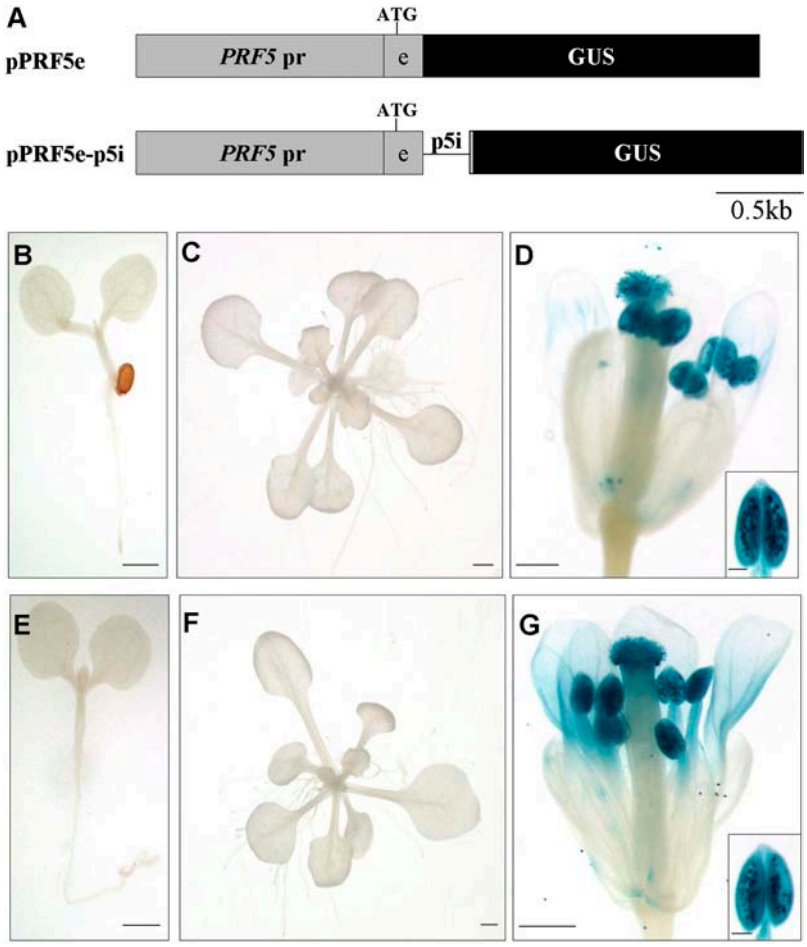

Figure 4. Histochemical analysis of GUS expression in transgenic Arabidopsis containing pPRF5e and pPRF5e-p5i constructs. A, Schematic representations of *PRF5* promoter-GUS fusion constructs. The gray box with *PRF5* pr is the promoter region of *PRF5*; the gray box with e is the first exon of *PRF5*; the black box with GUS is the *GUS* coding region; the line with p5i is the first intron of *PRF5*; ATG is the start codon. B to D, From pPRF5e; E to G, from pPRF5e-p5i; B and E, 3-d-old seedlings; C and F, 15-d-old plants; D and G, flowers. Scale bars, 0.5 mm (B, D, E, and G), 1 mm (C and F), and 0.1 mm (anthers in small box).

promoter. To determine whether controlling elements exist within the intron, three intron deletion constructs were generated (Fig. 7A). The resulting constructs were transiently expressed in Arabidopsis leaf mesophyll protoplasts, and normalized GUS activity with cotransfected luciferase activity was taken as a measure of promoter activity. Among the three intron deletions, the intron deletion d1 showed decreased GUS expression when compared to the full-length intron (Fig. 7B). The intron deletion analysis indicates that the enhancing elements would exist within the intron.

To examine the possibility that the first intron of *PRF2* acts as an enhancer, the first intron of *PRF2* was cloned upstream of the *PRF2* promoter-GUS fusion construct in a forward or reverse direction (Fig. 7A). The resulting constructs were transiently expressed in Arabidopsis leaf mesophyll protoplasts. Although both pp2iF-PRF2eGP and pp2iR-PRF2eGP constructs have the intron upstream of the promoter region, their relative GUS activity was similar to that of the pPRF2eGP construct (Fig. 7B). This suggested that the first intron of *PRF2* is not a classical enhancer. To further confirm this result, the intron was cloned upstream of the cauliflower mosaic virus 35S minimal promoter-GUS fusion construct (Fig. 7A). There was no detectable enhancer activity under the control of the minimal promoter (Fig. 7B). Therefore, these results demonstrate that the intron is not a classical transcriptional enhancer and it enhances gene expression in a position-dependent manner.

Table I. *Summary of expression patterns of PRF constructs in reproductive organs*

GUS staining intensity in various tissues was indicated by a minus (−), negative or weakly detectable; plus (+), light blue; ++, blue; +++, dark blue. The flower stalks were cut and incubated in GUS staining solution for 12 h. The ratio of the number of transgenic plants that show GUS staining to the total number of independent transgenic plants is indicated below the staining intensity.

Construct	Stigma	Anther	Filament	Petal	Sepal	Cut Edge
pPRF2e	− (0/23)	− (0/23)	++ (21/23)	− (0/23)	++ (16/23)	+ (16/23)
pPRF2e-p5i	− (0/23)	− (0/23)	++ (14/23)	− (0/23)	+ (7/23)	− (2/23)
pPRF5e	++ (12/12)	+++ (12/12)	− (2/12)	− (1/12)	− (0/12)	− (0/12)
pPRF5e-p5i	++ (10/13)	+++ (13/13)	− (1/13)	− (1/13)	− (0/13)	− (0/13)
pPRF5e-p2i	++ (15/24)	+++ (21/24)	++ (19/24)	+ (8/24)	++ (15/24)	+++ (23/24)

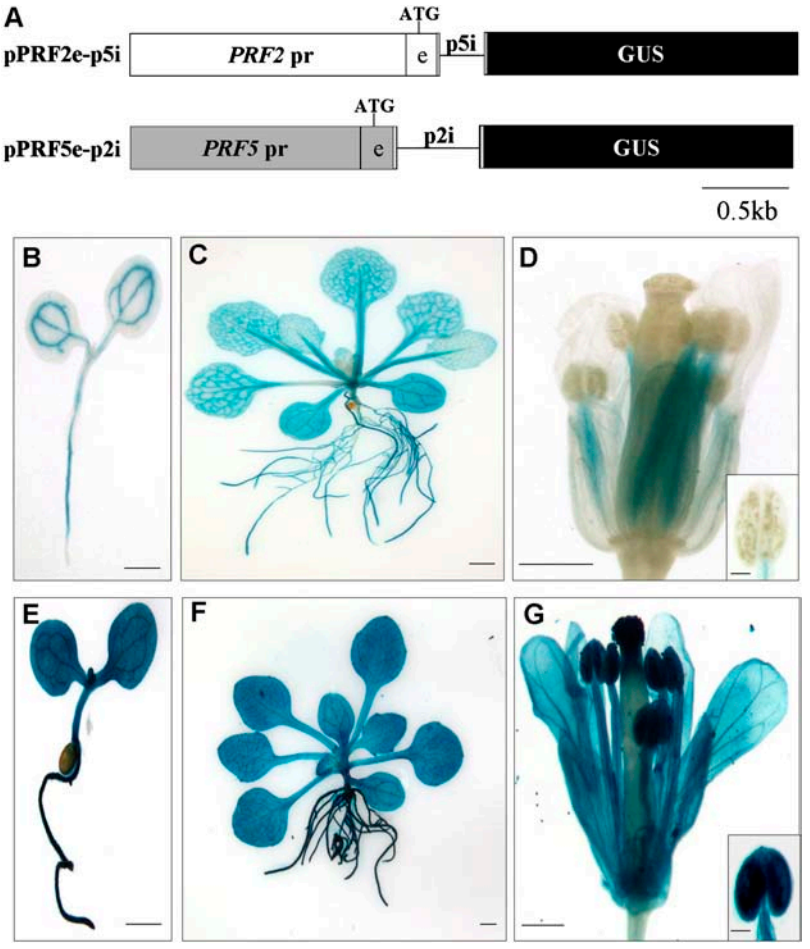

Figure 5. Histochemical analysis of GUS expression in transgenic Arabidopsis containing pPRF2e-p5i and pPRF5e-p2i. A, Schematic representation of promoter-GUS fusion constructs. The white box with *PRF2* pr is the promoter region of *PRF2*; the white box with e is the first exon of *PRF2*; the line with p2i is the first intron of *PRF2*; the gray box with *PRF5* pr is the promoter region of *PRF5*; the gray box with e is the first exon of *PRF5*; the line with p5i is the first intron of *PRF5*; the black box with GUS is the *GUS* coding region; ATG is the start codon. B to D, From pPRF2e-p5i; E to G, from pPRF5e-p2i; B and E, 3-d-old seedlings; C and F, 15-d-old plants; D and G, flowers. Scale bars, 0.5 mm (B, D, E, and G), 1 mm (C and F), and 0.1 mm (anthers in small box).

The Role of the First Intron in the Profilin Gene Family

To examine whether the role of the first intron observed in *PRF2* and *PRF5* gene expression is also applicable to the entire Arabidopsis profilin gene family members, the promoter-GUS fusion constructs with or without their first introns were generated and their expression levels were compared in the Arabidopsis protoplast transient expression system (Fig. 8). The promoter-GUS fusion constructs of two vegetative profilins, *PRF1* and *PRF2*, showed comparable changes in the GUS expression level depending on the presence of their own first intron. This suggests that the first introns of *PRF1* and *PRF2* have similar effects on gene expression. On the contrary, the promoter-GUS fusion constructs of two reproductive profilins, *PRF4* and *PRF5*, showed a basal level GUS activity in leaf mesophyll protoplasts regardless of the presence or absence of their first intron. Because they are not expressed properly in vegetative tissues, it is uncertain whether their introns enhance gene expression or not. Thus, the role of the first introns of *PRF4* and *PRF5* is not clear in this system. Nonetheless, considering that the first

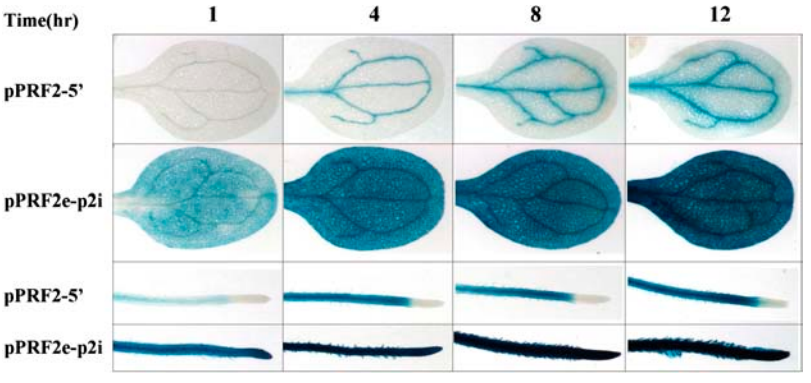

Figure 6. GUS staining patterns of transgenic Arabidopsis containing pPRF2-5′ and pPRF2e-p2i constructs at various incubation time points. Seedlings grown on Murashige and Skoog medium for 3 d were incubated for 1, 4, 8, and 12 h, respectively, and the reaction was stopped and the tissues cleared by incubation with 100% ethanol.

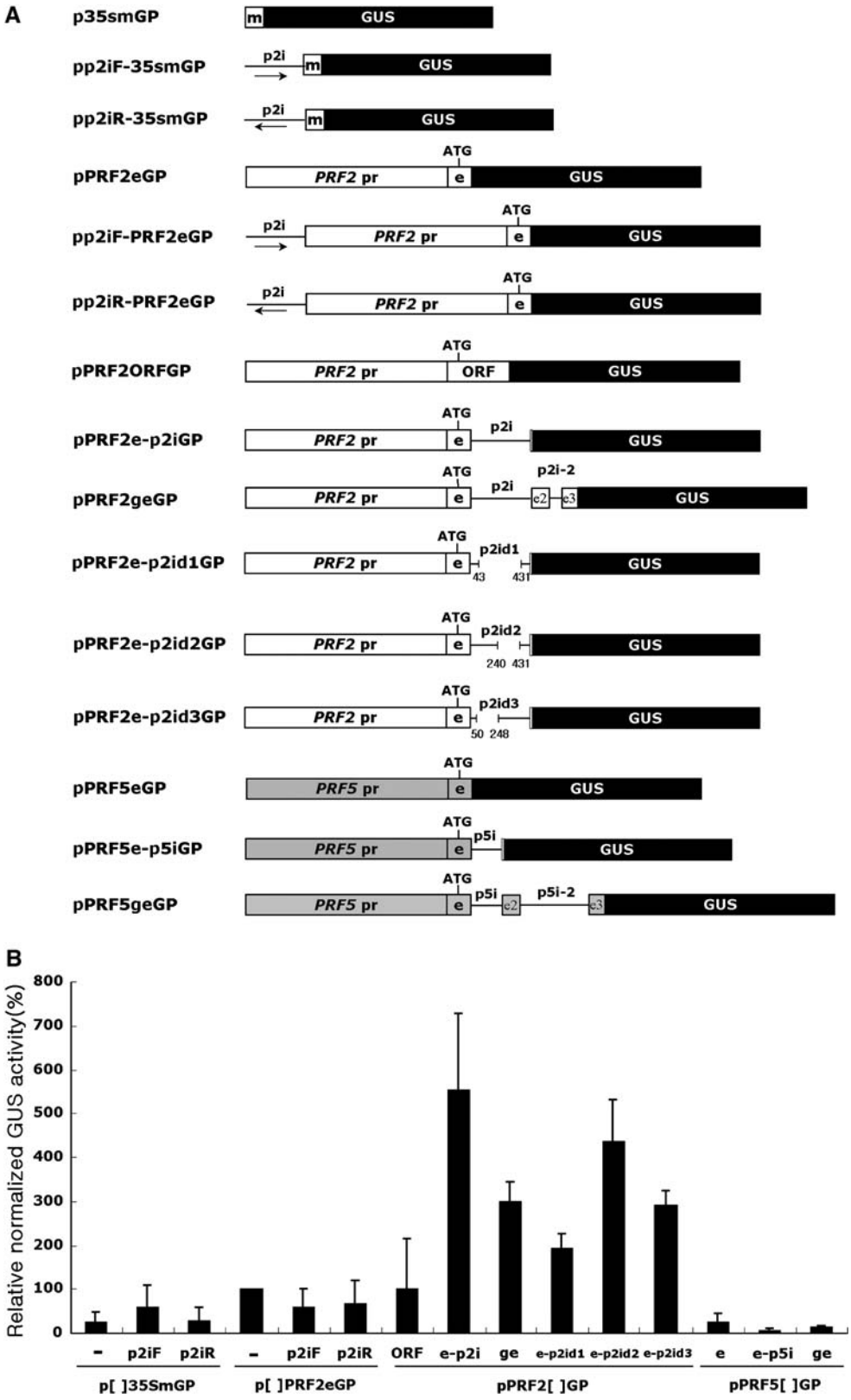

Figure 7. Transient expression analysis of various *PRF2* and *PRF5* promoter-GUS fusion constructs. A, Schematic representation of construction for transient expression. GP at the end of the construct name indicates a vector for transient expression. The white box with m is a 35S minimal promoter; the black box with GUS is the GUS ORF; the thin line with p2i is the first intron of *PRF2*; the white box with *PRF2* pr is the *PRF2* promoter region; the white box with e is the first exon of *PRF2*; ATG is the start codon; ORF is the ORF of *PRF2* cDNA; the white boxes with e2 and e3 are the second and third exons of *PRF2*; the line with p2i-2 is the

intron of the *PRF2* altered the expression pattern of the *PRF5* promoter (Fig. 5, E–G), our results indicate that the first introns of reproductive profilin genes are functionally different from those of *PRF1* and *PRF2*. Interestingly, although *PRF3* encodes a vegetative-type profilin, the *PRF3* promoter-GUS fusion construct was expressed at a basal level and a positive role of the first intron was not detected (Fig. 8B).

DISCUSSION

Profilin is a small actin-binding protein that regulates actin cytoskeleton dynamics and plays a role in cell elongation, cell shape, and flowering (Ramachandran et al., 2000). In Arabidopsis, there are five genes encoding three vegetative (PRF1, PRF2, and PRF3) and two reproductive (PRF4 and PRF5) profilins. All five members are very similar in amino acid sequences and genomic structures. All of these profilin genes are interrupted by two introns. The first intron of vegetative profilins is located between amino acids 41 and 42 (Gln and Leu) and that of reproductive profilins is located between amino acids 44 and 45 (Gln and Phe). Because *PRF4* and *PRF5* have three additional amino acids in the first exon, virtually all Arabidopsis profilin genes contain first introns at identical positions. In spite of conserved intron position, our results indicate that the roles of introns in gene expression are quite different between vegetative (*PRF1* and *PRF2*) and reproductive (*PRF4* and *PRF5*) profilin genes. GUS histochemical analyses of promoter-GUS fusions of *PRF1* and *PRF2* that encode major vegetative profilins indicate that their expressions are vascular bundle specific (Christensen et al., 1996; Ramachandran et al., 2000). These expression patterns were unexpected because vegetative profilin was assumed to show constitutive expression like vegetative actins. In our research, the expression of *PRF2* promoter-GUS fusions was nearly identical with previous results. However, when the first intron was included in the construct, the expression pattern was greatly changed (Fig. 1). Strong and constitutive GUS staining was observed in most tissues and at diverse developmental stages. In the seedlings, expression was detected in the cotyledons, shoot apical meristems, roots, root tips, and root hairs. In 15-d-old plants, rosette leaves, roots, root hairs, and trichomes were stained. In flowers, stigmas, anthers, filaments, petals, and sepals showed GUS expression. Different expression patterns of promoter-GUS fusion constructs, with or without introns, were also evident in mRNA and protein accumulation. The presence of the intron resulted in a 15-fold increase in GUS activity in leaves (Figs. 2 and 3). This constitutive expression pattern of pPRF2-p2i is mostly in good agreement, except in ovules with in situ immunolocalization of vegetative class profilins (*PRF1*, *PRF2*, and *PRF3*) in Arabidopsis (Kandasamy et al., 2002). Therefore, it is evident that expression of *PRF2* is not vascular bundle specific, and the first intron plays an important role in the strong and constitutive expression of *PRF2*.

To further investigate the role of profilin introns, we examined the first intron of the reproductive profilin *PRF5*. GUS staining with the pPRF5e construct was mainly detected in pollen and anthers. But, unlike the results obtained with the intron of *PRF2*, GUS expression patterns of the pPRF5e and pPRF5e-p5i constructs were nearly identical (Fig. 4; Table I), indicating that the first intron of *PRF5* has little effect on the gene expression pattern. Although the spliceable intron was placed downstream of the first exon of *PRF2* (pPRF2e-p5i), there was no significant alteration of expression pattern, confirming that the *PRF5* intron had little effect on the gene expression pattern.

The result of intron exchange between *PRF2* and *PRF5* suggests that the first intron of *PRF2* does not just enhance or increase the level of gene expression (Fig. 5). Both pPRF5e and pPRF5e-p5i showed reproductive tissue-specific expression. But, when the first intron was replaced with the intron of *PRF2*, strong GUS expression was observed throughout the plant body. This expression pattern of pPRF5e-p2i is quite surprising and demonstrates that the *PRF2* intron completely changes tissue-specific expression of the *PRF5* promoter. To determine whether this also occurred in the expression of *PRF2*, GUS staining patterns of plants harboring pPRF2-5′ and pPRF2e-p2i constructs were analyzed at various incubation times (Fig. 6). If the *PRF2* intron just increased the expression level, the expression pattern of pPRF2e-p2i in the early stage of staining would be similar to that of pPRF2-5′. Plants of pPRF2e-p2i incubated for 1 h were stained evenly in the cotyledons and roots, and the staining was obviously different from pPRF2-5′ (Fig. 6). Therefore, these results suggest that the first intron of *PRF2* is involved in regulating spatial gene expression.

Because the two first introns examined in this study maintained their own properties under the control of both the *PRF2* and *PRF5* promoters (Fig. 5), the different roles of these introns seem to be the properties

Figure 7. (*Continued.*)
second intron of *PRF2*; the gray box with *PRF5* pr is the *PRF5* promoter region; the gray boxes with e2 and e3 are the second and third exons of *PRF5*; the lines with p5i and p5i-2 are the first and second introns of *PRF5*. Arrows indicate the orientation of intron. The numbers in intron deletion constructs indicate the deletion points from the 5′ end of the intron. B, Relative normalized GUS activity in percentage. The full name of each construct is the addition of each insert name into the brackets of the vector name. For example, p[p2iF-]35SmGP is the pp2iF-35SmGP construct. Each transfection was performed at least three times. GUS activity was normalized with cotransfected luciferase activity and relative activity of pPRF2eGP was set to 100%. Bars are means ± SD.

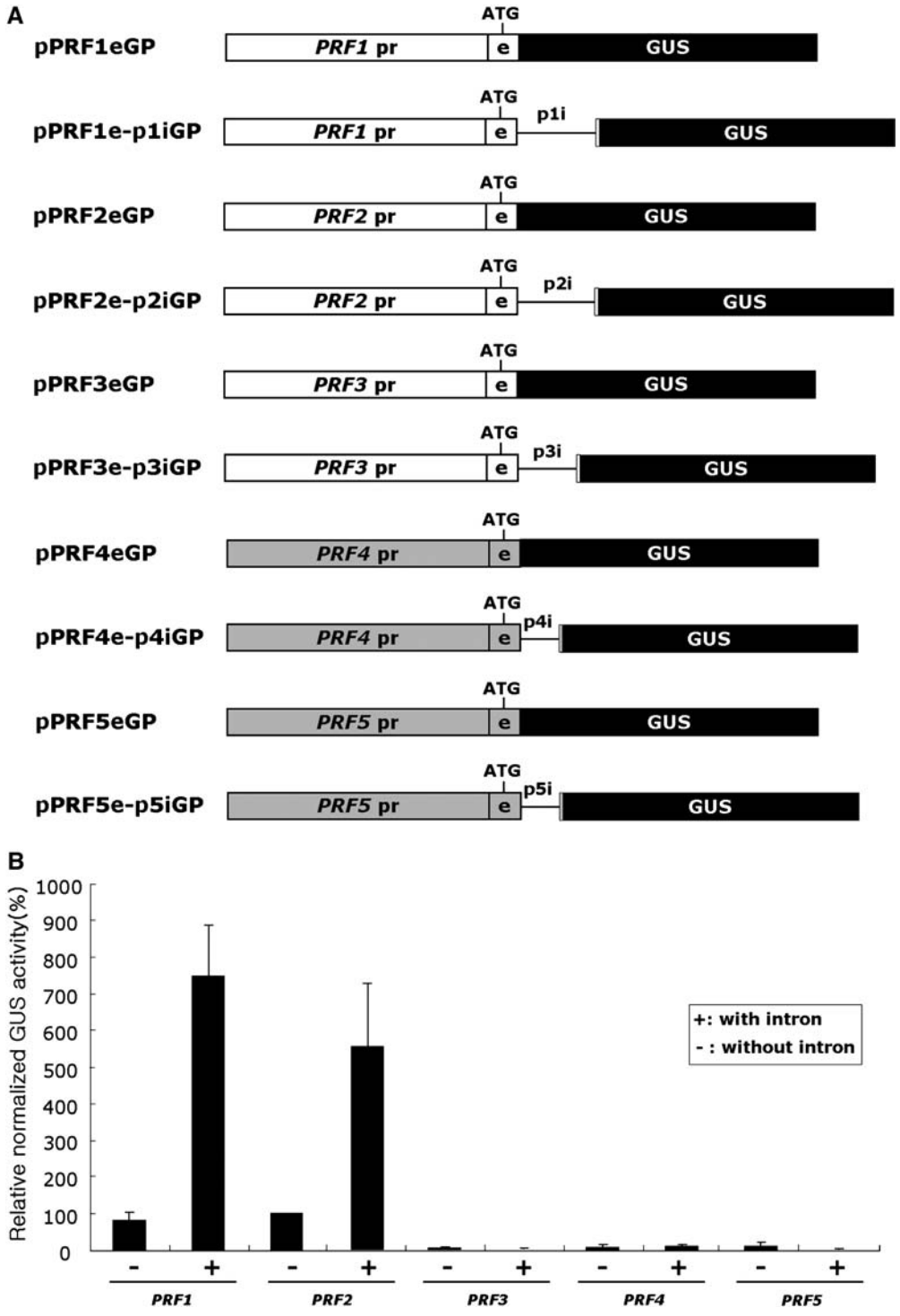

Figure 8. Effect of the first intron on the expression of the Arabidopsis profilin gene family. A, Schematic representation of construction for transient expression. GP at the end of the construct name indicates a vector for transient expression. The white box is vegetative genes; the gray box is reproductive genes; the white or gray box with e is the first exon of each gene; the black box with GUS is GUS ORF; the thin lines with p1i, p2i, p3i, p4i, and p5i are the first introns of *PRF1*, *PRF2*, *PRF3*, *PRF4* and *PRF5*, respectively; the white or gray box with gene name pr is the promoter region of each gene; ATG is the start codon. B, Relative normalized GUS activity in percentage. A plus (+) indicates the presence of an intron within the construct; a minus (−) indicates no intron. Each transfection was performed at least three times. GUS activity was normalized with cotransfected luciferase activity and relative activity of pPRF2eGP was set to 100%. Bars are means ± SD.

of the introns themselves. Although sequences of introns are greatly variable in higher plants, AU richness is common in plant introns and is required for efficient splicing (Goodall and Filipowicz, 1989). Therefore, we analyzed the sequences of our two introns to determine whether there are significant differences in AU content. The AU content of the first intron was slightly lower in *PRF2* (64.4%) than in *PRF5* (67.7%), but both were similar to the average (67.5%) of Arabidopsis introns (Table II). Because AU content of both introns is above 60%, which is required for efficient splicing in dicot plants (Goodall and Filipowicz, 1989), it is likely that there is no significant difference in AU content. The most remarkable difference between two introns was in the length of them. The first introns of *PRF2* and *PRF5* are 478 and 260 bp in length, and they were 2.9 and 1.6 times longer than the average length of Arabidopsis introns (165.4 bp), respectively (Table II). Although the biological significance of long introns is not certain at present, long introns may serve as binding sites for various regulatory elements. The results of our intron deletion analysis suggest that the first intron of *PRF2* may have the binding motifs or regulatory elements and these are to be specified in further research.

Although the precise mechanism is not clear, the first intron of *PRF2* increased the corresponding mRNA level, and this was reflected in the protein level as well, as shown in Figures 2 and 3. In intron-mediated enhancement, generally the enhancement is related to increased steady-state mRNA accumulation (Callis et al., 1987; Rethmeier et al., 1997; Rose, 2002). Furthermore, the first intron of *PRF2* enhanced the gene expression in a position-dependent manner and could not induce the expression of the minimal promoter in a transient expression assay (Fig. 7). Therefore, it is supposed that the first intron of *PRF2* increases gene expression by the cotranscriptional or posttranscriptional mechanisms as discussed in the earlier reports on intron-mediated enhancement (Jeon et al., 2000; Bourdon et al., 2001; Clancy and Hannah, 2002; Rose, 2002, 2004).

Among the five profilins of Arabidopsis, we mainly examined the role of the first introns on the expression of *PRF2* and *PRF5* in transgenic plants. Genes encoding vegetative (PRF1 and PRF2) and reproductive (PRF4 and PRF5) profilin are highly similar to each other in many aspects, such as DNA and amino acid sequences, expression patterns, and, especially, the length of the first intron (Table II). These high degrees of similarity seem to be closely related to the evolutionary history of the profilin gene family. In the Arabidopsis genome, genes encoding *PRF1* and *PRF5* are located in chromosome 2, and *PRF2* and *PRF4* are located in chromosome 4 where they are arranged in tandem arrays. Interestingly, both of the *PRF1* and *PRF2* genes encoding vegetative profilins are flanked with a gene encoding a reproductive profilin. Considering that about 60% of the Arabidopsis genome was duplicated (Arabidopsis Genome Initiative, 2000), this suggests that one set of tandem arrayed profilin genes was duplicated to give two sets. Therefore, we expected that the role of the first intron on gene expression would also be similar. As expected, only the first introns of *PRF1* and *PRF2* increased reporter activity, while the first introns of *PRF4* and *PRF5* did not. These results indicate that different regulatory mechanisms by introns control gene expression in vegetative (except *PRF3*) and reproductive profilins. Considering that *PRF1* and *PRF2* are major vegetative profilins, our data demonstrate that their first introns are required for their strong and constitutive expression in vegetative tissues. In this respect, the *PRF3* gene is unique among profilin genes as shown in Table II. The *PRF3* gene is not coupled with other profilin genes in the Arabidopsis genome, and the length of the first intron was smaller than other vegetative profilins, *PRF1* or *PRF2*. The total number of *PRF3* expressed sequence tags (ESTs) found in the National Center for Biotechnology Information (NCBI) and The Institute for Genomic Research (TIGR) databases was only 25% of *PRF1* or *PRF2*, indicating that *PRF3* is not expressed as much as *PRF1* or *PRF2*. Our results indicating that *PRF3* promoter-GUS fusion constructs showed basal levels of expression in the transient expression system also support the above explanation (Fig. 8). At present, we do not know whether weak expression of *PRF3* is due to loss of intron-enhancing activity or significantly reduced promoter activity. Therefore, further examination of *PRF3*

Table II. *Arabidopsis Genome Initiative (AGI) identification, sequence properties of introns, and number of ESTs of Arabidopsis profilin genes*

The genomic distribution and structures of profilin genes were analyzed by NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/). AGI ID is the designation of the predicted genes that were given by the AGI. For example, the 1,976th annotated gene counting from the top of chromosome 2 is At2g19760. The number of ESTs was obtained from the NCBI Unigene (http://www.ncbi.nlm.nih.gov/UniGene) and TIGR (http://www.tigr.org) database.

Gene	AGI ID	Length		AU Content		No. of ESTs	
		Intron I	Intron II	Intron I	Intron II	NCBI	TIGR
		bp		%			
PRF1 (*PFN1*)	At2g19760	506	92	63.2	67.4	22	23
PRF2 (*PFN2*)	At4g29350	478	111	64.4	66.7	23	24
PRF3	At5g56600	367	66	68.7	69.7	5	6
PRF4 (*PFN3*)	At4g29340	256	85	70.3	67.1	2	4
PRF5 (*PFN4*)	At2g19770	260	575	67.7	70.2	0	2

gene expression would provide additional clues for understanding the importance of introns on expression of the profilin gene family.

Recently, the effect on gene expression of the leader intron in the Arabidopsis *ACT1* gene encoding reproductive actin was reported. *ACT1* is expressed most strongly in mature pollen, but it is also expressed in vegetative tissues, such as young vascular tissues and root tips. The leader intron was found to be required for the high-level expression of *ACT1* in reproductive tissues. In addition, substituting its leader intron with that of *ACT2* into an *ACT1* promoter-GUS fusion resulted in failure of expression in pollen (Vitale et al., 2003). Although only two introns under the control of a reproductive actin promoter were tested, at least these results suggest that introns play a role in actin gene expression. Therefore, it is possible that a role of introns in gene expression may have coevolved in the cytoskeletal genes.

In this study, we have demonstrated that the first introns of *PRF1* and *PRF2* play an important role in constitutive expression in most vegetative tissues and are functionally different from those of *PRF4* and *PRF5*. And we have discussed the possible importance of introns in the expression of profilin and actin genes. In future research, we will determine how the intron of the vegetative profilin genes affects gene expression and also examine the role of introns in the expression of various cytoskeletal genes.

MATERIALS AND METHODS

Plant Materials and Construction of Promoter-GUS Fusions for Transgenic Plants

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia was grown in a growth chamber (23°C, 16 h day/8 h night, 300 μE m^{-2}). For construction of *PRF2* promoter fusions, the 1.6-kb promoter region, including up to a 5′-UTR and the start codon (pPRF2-5′), the entire exon 1 (pPRF2e), and the first intron (pPRF2e-p2i), was amplified using P2-F (5′-GTCGACAATGTTCCACCACCTAC-3′), P2-R (5′-GGATCCCATCTTTCTTCTTCTCC-3′), P2e-R (5′-AGGAAGAAAA-ACGGATCCCTGAGGGAAAG-3′), and P2i-R (5′-GGATCCTGCTATCTCTGC-AGG-3′) primers, respectively. The PCR products were cloned into the pGEM-T easy vector (Promega) and confirmed by sequencing. The cloned fragments were digested with *Sal*I/*Bam*HI and ligated into the pBI101 vector (CLONTECH). Similar methods were used for construction of *PRF5* promoter fusions, pPRF5e and pPRF5e-p5i, using *PRF5*-specific primers: P5-F (5′-AAGCTTCTGGTCCTGTATTTGCCTAACCAAG-3′), P5e-R (5′-GGATC-CCTGAGGAAAATTAGCGCTCTGAG-3′), and P5i-R (5′-GGATCCTGTGAT-CTCTTGAGGTTTGAAC-3′). For intron exchange experiments, introns were amplified with P2int-F (5′-GGATCCGCTTTCCCTCAGGTTTTTCTTC-3′) and P2i-R for the first intron of *PRF2* and with P5int-F (5′-GGATCCAATT-TTCCTCAGGTATAATTAC-3′) and P5i-R for the intron of *PRF5*. Amplified introns were inserted into the *Bam*HI site of pPRF2e or pPRF5e. The resulting constructs were inserted into *Agrobacterium tumefaciens* strain C58C1Rif$^+$.

Plant Transformation and GUS Histochemical Analysis

Transgenic Arabidopsis was generated by the floral-dip method (Clough and Bent, 1998). Transformed plants were selected on 0.5× Murashige and Skoog 0.8% (w/v) agar plates containing 50 mg L^{-1} kanamycin and 100 mg L^{-1} cefotaxim. For analysis of pPRF2-5′ and pPRF2e-p2i lines, T1 transgenic plants were self-pollinated, and single-copy lines were selected by Southern-blot analysis and tested for the segregation ratio of T2 seeds on agar plates containing kanamycin. The selected single home lines were used for further analysis. Histochemical and fluorometric analysis of GUS activity was performed as described previously (Mun et al., 2002). Images were obtained using either Nikon (Tokyo) Coolpix 4500 or Stemi SV11 (Zeiss) with photomatrix Coolsnap cf digital camera (Photomatrix).

RT-PCR Analysis

To investigate the expression patterns of the *GUS* transcripts in transgenic Arabidopsis, RT-PCR analyses were performed. Total RNA was purified from leaves of mature plants by Tri-Reagent (Molecular Research Center). One microgram of total RNA was used for RT in a volume of 25 μL and incubated at 42°C for 1 h with avian myeloblastosis virus reverse transcriptase and oligo(dT) primer (Promega), and inactivated by incubating for 5 min at 95°C. After completion of RT, 2 μL of RT products were amplified using P2RT-F (5′-AAACAGTCTCATCTCGCCGGAGAAG-3′) and GUSRT-R (5′-AAAGA-CTTCGCGCTGATACCAGAC-3′) primers. PCR was performed using Biotherm DNA polymerase (Genecraft) in a GeneAmp PCR system 9700 (Perkin-Elmer). The PCR condition was 5 min at 95°C, followed by 25 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, followed by 7 min at 72°C. The PCR products were run on agarose gel, transferred to Hybond XL membranes (Amersham), and hybridized. Probes were generated from *GUS* ORF from pBI101 and radiolabeled with [α-^{32}P]dCTP using the Rediprime II random prime system (Amersham). After high stringent washing, blots were exposed to the film. To confirm that correct splicing had occurred, RT-PCR products were cloned into the pGEM-T easy vector and sequenced using the ABI 3100 sequence analyzer (Applied Biosystems).

Western-Blot Analysis

Monoclonal GUS antibody (Molecular Probes) was used at a titer of 1:5,000 for western-blot analysis. Twenty micrograms of total soluble proteins from leaves were loaded per lane. Western-blot analysis was performed as described previously (Mun et al., 2000).

Sequence Analysis of Arabidopsis Profilin Genes

The genomic sequences of five profilin genes in Arabidopsis were obtained from Plant Genome Central at the NCBI (http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html). The total numbers of ESTs cloned were obtained from Unigene (http://www.ncbi.nlm.nih.gov/UniGene/) and TIGR (http://www.tigr.org). The average length and AU content of the Arabidopsis intron was calculated based on whole intron sequences obtained from The Arabidopsis Information Resource (TAIR; ftp://ftp.arabidopsis.org/home/tair/Sequences/blast_datasets/At_intron_20040301).

Transient Expression Analysis in Arabidopsis Leaf Mesophyll Protoplasts

For transient gene expression, the *Hin*dIII and *Eco*RI fragment from pBI101, which includes the *GUS* gene, was inserted into pUC19 and named as a pGP. The minimal promoter region of pBI121 was amplified with 35sm-F (5′-CTGCAGTCGACGCAAGACCCTTCCTCTATA-3′) and GUSRT-R primers and the *Pst*I/*Bam*HI fragment was ligated into pGP to generate p35smGP. For construction of pp2iF-35smGP and pp2iR-35smGP, the first intron of *PRF2*, which was amplified with P2int-sal-F (5′-GTCGACGCTTTCCCTCAGGTTTT-TCTTC-3′) and P2int-sal-R (5′-GTCGACTGCTATCTCTGCAGGCTTCAA-3′) primers, was inserted into the *Sal*I site of p35smGP. The *Sal*I/*Bam*HI fragments of *PRF2* and *PRF5* promoter-GUS fusion constructs in pBI101 were cloned into pGP (Fig. 8A). For construction of pp2iF-PRF2eGP and pp2iR-PRF2eGP, the first intron of *PRF2* was inserted into the *Sal*I site of pPRF2eGP. To make pP2ORFGP, the *Sal*I/*Bam*HI fragment of the pPRF2-5′ construct was ligated into pGP, thus generating pPRF2-5′GP, and the ORF of the *PRF2* gene was amplified with P2orf-F (5′-GGATCCATGTCGTGGCAATCATACGTC-3′) and P2orf-R (5′-GGATCCGAGACCAGACTCGATAAGGTAATC-3′) primers using cDNA made from total RNA and inserted into the *Bam*HI site of pPRF2-5′GP. The genomic region was amplified with P2-F and P2orf-R for pPRF2geGP and P5-F and P5orf-R (5′-GGATCCAAGACCCTGTTCGATCAAGTAATC-3′) for pPRF5geGP and ligated into pGP vector. For construction of intron deletion constructs, P2id1-R (5′-TTATTTGAGACAGAGGATCGGAAGAAAC-3′),

P2id1-F (5′-AGTCGGTTGAAGCTAATTGCCTACTTTG-3′), p2id2-R (5′-ACACCTATAGACAAGATTTGACTACTG-3′), and p2id3-F (5′-TTTGCTTGGCACAGAATCTTATCTCC-3′) were used. Schematic representation of these transient vectors is depicted in Figure 7A. For construction of *PRF1*, *PRF4*, and *PRF3* promoter-GUS fusions, genomic fragments were amplified with the following gene-specific primers: for *PRF1*, P1-F (5′-AAGCTTCATATACATACCAAAGCCATCATGGA-3′), P1e-R (5′-GGATCCCTGAGGAAATTTGGCGCTCTGAGC-3′), and P1i-R (5′-GGATCCATCGATTTCTTGAGGCTTCAAC-3′); for *PRF3*, P3-F (5′-AAGCTTCCCATAGAAAGCAATGTATATGCTC-3′), P3e-R (5′-GGATCCCTGAGGAAAATTGTTGCTCTGAGCC-3′), and P3i-R (5′-GGATCCCTGAATTTCCTCAGGCTTCACCTA-3′); and for *PRF4*, P4-F (5′-AAGCTTTTATCAGTCATACCATTTTTCTGGAC-3′), P4e-R (5′-GGATCCCTGAGGGAAGTTGGCGCTCTGAGCC-3′), and P4i-R (5′-GGATCCACTGAACTCCTGTCCCTTGAACTATATG-3′). They were inserted into the *Hin*dIII/*Bam*HI sites of pGP (Fig. 8A). For construction of p35SLucP, the internal control for transient expression analysis, the 35S promoter region from pBI121 and the gene encoding a firefly luciferase amplified with Luc-F (5′-GGATCCATGGAAGACGCCAAAAACATAAAG-3′) and Luc-R (5′-GAGCTCTTACACGGCGATCTTTCCGCCCT-3′) primers using pGL3-promoter vector (Promega) were introduced into the *Hin*dIII/*Sac*I sites of the pGP vector.

Arabidopsis mesophyll protoplasts from 3-week-old plants were transfected by the polyethylene glycol method (Sheen, 2002). Ten micrograms of plasmid DNA containing promoter-GUS fusion constructs were cotransfected with 10 μg of p35SLucP as an internal control. After transfection, protoplasts were incubated for 24 h at 24°C in the dark. Proteins were extracted by 1× cell culture lysis reagent buffer (Promega) and used for the GUS fluorometric and luciferase assay. Luciferase assay was performed according to the manufacturer's instructions (Promega) using the Victor2 multilabel microplate reader (Perkin-Elmer).

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