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Developmental Regulation of Pyrroline-5-Carboxylate Reductase Gene Expression in Arabidopsis[1]

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***At-P5R*, a gene encoding the last enzyme of the proline (Pro) biosynthetic pathway in *Arabidopsis thaliana*, is developmentally regulated. To characterize the *cis* elements responsible for this developmental regulation, a series of 5′ deletions of the *At-P5R* promoter were transcriptionally fused to a β-glucuronidase (GUS)-coding region and transformed into Arabidopsis. The complete promoter of *At-P5R* directs strong GUS activity in root tips, the shoot meristem, guard cells, hydathodes, pollen grains, ovules, and developing seeds, all of which contain rapidly dividing cells and/or are undergoing changes in osmotic potential. This expression pattern is consistent with the function of Pro as an energy, nitrogen, and carbon source and as an osmoticum in response to dehydration. Promoters longer than 212 base pairs (bp) showed the same expression pattern, whereas those shorter than 143 bp did not direct any detectable GUS activity in any organs. This suggests that a 69-bp promoter region located between −212 and −143 bp is necessary to establish the tissue-specific expression of *At-P5R* during development. The Pro content measured in different organs suggests that, in addition to transcriptional control of the biosynthetic pathway, the transport of Pro may play a role in its distribution within Arabidopsis. Several aspects of the relationship between Pro metabolism and plant physiology are discussed.**

The accumulation of Pro is a striking metabolic response to osmotic and other stresses by a large number of organisms from bacteria to higher plants and is thought to play a pivotal role in osmotic stress tolerance (Kishor et al., 1995). Pro has been suggested to function as an osmoticum (Wyn Jones et al., 1977), as an energy or reducing power sink (Blum and Ebercon, 1976; Walton et al., 1991), as a nitrogen storage compound (Ahmad and Hellebust, 1988), as a hydroxyl-radical scavenger (Smirnoff and Cumbes, 1989), as a compatible solute that protects enzymes (Schobert and Tschesche, 1978; Paleg et al., 1981, 1984), as a means of reducing acidity (Venekamp et al., 1989), and as a way to regulate cellular redox potentials (Saradhi and Saradhi, 1991).

During plant development in the absence of stress, levels of free Pro vary considerably among different plant organs (Chiang and Dandekar, 1995). Furthermore, the ratio between free Pro and total free amino acids is not constant between different organs in different developmental stages (Venekamp and Koot, 1984), indicating that variations in free Pro content are not related to fluctuations in the size of total amino acid pool. In *Arabidopsis thaliana*, Pro preferentially accumulates in tissues that experience dehydration, such as pollen and seeds (Chiang and Dandekar, 1995). Moreover, the level of free Pro is dependent on leaf part, age, and position (Amberger-Ochsenbauer and Obendorfer, 1988; Madan et al., 1994). These observations prompted suggestions of a potential role of free Pro in flower development (Vansuyt et al., 1979; Mutters et al., 1989), and the high Pro concentration in pollen cells has been proposed to function as a readily accessible energy source (Holden, 1973; Dashek and Harwood, 1974).

In higher plants Pro is synthesized via two routes. Both routes involve glutamic-γ-semialdehyde, which is synthesized from glutamate by P5CS or from Orn by Orn-δ-aminotransferase. Glutamic-γ-semialdehyde cyclizes spontaneously to pyrroline-5-carboxylate, which is further reduced to Pro by P5CR (Adams and Frank, 1980; Stewart, 1981; Delauney and Verma, 1990; Hu et al., 1992). During stress the biosynthesis of Pro from glutamate is enhanced (Boggess et al., 1976a, 1976b; Buhl and Stewart, 1983; Rhodes et al., 1986), whereas the Orn pathway seems to be inhibited (Delauney et al., 1993).

Most studies of the Pro biosynthetic pathway in plants have been concerned with its activity during stresses. Genes encoding P5CS and P5CR and the corresponding cDNAs have been cloned and characterized in *Vigna aconitifolia* and Arabidopsis (Delauney and Verma, 1990; Hu et al., 1992; Verbruggen et al., 1993; Savouré et al., 1995; Yoshiba et al., 1995). P5CS has been shown to catalyze the limiting step in Pro accumulation in response to osmotic stresses (Kishor et al., 1995).

The study of Pro biosynthesis during development is important; not only will it help us to reveal the site of Pro biosynthesis and to elucidate the roles of Pro during development (Delauney and Verma, 1993) but it also could indicate the relationships between development and stress signal transduction pathways underlying Pro biosynthesis.

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Abbreviations: CaMV, cauliflower mosaic virus; P5CR, pyrroline-5-carboxylate reductase; P5CS, pyrroline-5-carboxylate synthase.

Previous studies concerning Pro accumulation during development have been largely physiological, which may suffer from experimental limitations in directly assaying Pro in specific tissues. We addressed this problem by studying the expression of the genes encoding the enzymes on the pathway.

P5CR lies at the converging point of both routes of Pro biosynthesis. Although P5CR is not the rate-limiting enzyme for Pro accumulation (Szoke et al., 1992), it is differentially regulated during development and induced during salt stress (Verbruggen et al., 1993).

We cloned a gene encoding P5CR in Arabidopsis (Verbruggen et al., 1993), *At-P5R*, previously named *At-P5C1*, which includes a 2574-bp 5′ flanking region (GenBank accession no. Y08951). Promoters of different lengths were made by successive 5′ deletions and fused to the GUS reporter gene (*gus*). Their activities were analyzed in transgenic Arabidopsis at different developmental stages. Analysis of the transgenic plants showed that a proximal promoter of 212 bp is sufficient to direct strong *gus* expression in root tips, apical meristems, guard cells, pollen, and developing seeds. The relationship between *gus* expression and the Pro content in different organs during development is discussed.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh C24 were surface-sterilized by treatment with 5% sodium hypochlorite for 15 min and then rinsed six times in sterile, distilled water. Germination was carried out on K1 agar medium containing Murashige-Skoog salts, one-tenth-strength vitamins in B5 medium, 0.5 g/L Mes, pH 5.7, 1% Suc, and 0.8% agar at 22°C, with a 16-h/8-h day/night photoperiod and 60% RH. Ten-day-old seedlings were transferred from K1 agar medium to soil. The seedlings were grown to maturity in a greenhouse at 22°C with 16-h/8-h day/night photoperiod (at a light intensity of 125 μE m^{-2} s^{-1}).

Construction of the *At-P5R* Promoter-*gus* Fusion

For the construction of the promoter-*gus* fusion the pGUS1 (a kind gift of Plant Genetic Systems, Gent, Belgium) was used as a vector; pGUS1 contained a promoterless *gus*-coding region, the region surrounding the translation start site that was modified to maximize its expression in the plant, followed by the terminator of the octopine synthase gene. A 1.2-kb 5′ regulatory region of *At-P5R*, including a 127-bp untranslated leader (Verbruggen et al., 1993), was amplified by PCR with primers ATCTGCAGCTCGCCGTCTTCAATC (including a *Pst*I site), from −1086 to −1063 bp, and GAATCTCCATGGGGAAAATTTAAC (including a *Nco*I site), from +114 to +137 bp, with respect to the transcription start site. The amplified fragment was digested with *Pst*I and *Nco*I and cloned into the *Pst*I and *Nco*I sites of pGUS1 immediately upstream of the *gus*-coding region, resulting in plasmid pGUS1086.

pGUS1086 was then used as starting material to generate successive 5′ deletions of the promoter using a double-stranded nested deletion kit (Pharmacia) according to the manufacturer's instructions. pGUS1086 was linearized at two close restriction sites, *Sph*I and *Xho*I, located in pGUS1 and the 5′ end of the *At-P5R* promoter, respectively. A series of *gus* fusions was obtained with a deleted promoter of *At-P5R* of 847, 727, 560, 435, 343, 212, and 143 bp in length with respect to the transcriptional initiation site, designated pGUS847, pGUS727, pGUS560, pGUS435, pGUS343, pGUS212, and pGUS143, respectively. These constructs were confirmed by sequencing.

To construct the *gus* fusion with the entire 2574-bp 5′ flanking region of *At-P5R*, an *Sma*I-*Nsi*I fragment ranging from −2573 to −284 bp was cloned into the *Pst*I (blunt-ended with T4 DNA polymerase) and *Nsi*I sites of pGUS1086. The recombinant plasmid was designated pGUS2574. A construct containing a *gus* fusion with a 0.8-kb CaMV 35S promoter was also made as a positive control in the transformation of Arabidopsis.

The binary vector pGSV4 (Plant Genetic Systems) was used in the transformation of Arabidopsis. The *Hin*dIII-*Sma*I fragments from different deletion constructs containing the chimeric *gus* gene and the *nos* terminator were cloned into the *Xba*I (filled in with Klenow) and *Hin*dIII sites of pGSV4. The resulting binary vectors were correspondingly designated pTiGUS2574, pTiGUS1086, pTiGUS847, pTiGUS727, pTiGUS560, pTiGUS435, pTiGUS343, pTiGUS212, and pTiGUS143.

Transformation of Arabidopsis

The above-mentioned binary vectors containing *gus* fusion constructs were mobilized from *Escherichia coli* DH5 into *Agrobacterium tumefaciens* C58C1Rif^R via triparental mating with a helper *E. coli* strain containing the mobilization plasmid pRK2013. Transformation of root explants of *A. thaliana* C24 was performed as described previously (Valvekens et al., 1988). Seeds of primary transgenic plants were collected individually. Analysis was carried out in the T_2 generations.

Enzymatic Assay of GUS Activity

Ten plants were analyzed from each construct described above (the transgenic lines were designated correspondingly with the names of the constructs listed above). GUS assays were carried out using 10-d-old seedlings or 4-week-old mature plants. Histochemical assays for GUS activity were performed as described by Jefferson et al. (1987), with some modifications. Organs of mature plants or entire seedlings were treated first with cold (−20°C) 90% acetone for 30 min at room temperature, washed three times with 0.1 M sodium phosphate buffer, pH 7.2, and incubated for 4 h (or as specified in the figure legends) in staining buffer (0.5–1 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-8-D-glucuronide, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 100 mM sodium phosphate, pH 7.2) at 37°C in the dark.

GUS-stained material was directly observed using bright-field microscopy. Some samples were further processed by embedding in London Resin White (London Resin, London, UK) and sectioning using dark-field optics on a microscope (Diaplan, Leitz, Heerbrugg, Switzerland).

Quantitative kinetic analysis of GUS activity was conducted using fluorometry in a computer-controlled microtiter plate reader according to the method of Breyne et al. (1993). The total protein concentration was determined using the Bradford protein assay (Bio-Rad). The GUS activity was expressed as units of GUS enzyme per microgram of total protein in each sample.

Measurement of Free Pro Content

Stems, rosette leaves, cauline leaves, roots, siliques, and flowers were collected separately from 4-week-old flowering transgenic plants and immediately frozen in liquid nitrogen. Samples of 100 mg were ground in liquid nitrogen and processed for free Pro measurement as described by Bates et al. (1973).

RESULTS

To reveal the domains responsible for the developmental regulation of *At-P5R* a series of 5′ promoter deletions starting from −1086 bp were fused with a *gus* reporter gene (Fig. 1). The activities of the deleted promoters and the entire 2574-bp 5′ flanking region were analyzed in transgenic Arabidopsis plants. The T_2 generations of 10 independent transformants for each construct were analyzed by histochemical and fluorometric GUS assay.

Pattern of GUS Expression Directed by the *At-P5R* Promoter in 10-d-Old Seedlings

The seedlings containing *gus* under the control of the promoters longer than 212 bp, GUS2574 to GUS212, showed the same patterns of GUS activity in histochemical assays, whereas plants containing the 143-bp promoter fragment, GUS143, had no detectable GUS activity.

Strong staining was observed in the root tips of plants showing GUS activity (Fig. 2, A and B) and in lateral root primordia (Fig. 2C) after 1 h of staining. An equally strong expression was observed in the shoot apical meristem, leaf primordia, and young leaves (Fig. 2, A and D). Less expression was detected in the root vascular cylinder (Fig. 2C) and cotyledons (Fig. 2A), whereas no expression was observed in the root cortex, epidermis of the differentiation zone, root hairs (Fig. 2B), hypocotyls (Fig. 2A), or stems (Fig. 2, A and D).

Longitudinal sections of root tips revealed that the observed strong expression was localized in the meristematic cells, with expression declining in the elongation zone (Fig. 2E). Longitudinal sections of the apical meristem showed strong expression in the meristematic cells, leaf primordia, and emerging leaves (Fig. 2F).

Ten-day-old seedlings grown under the conditions of our study usually had three pairs of leaves. In younger leaves (second or third pair) the GUS activity was detected throughout the leaf blade (Fig. 2G) and tended to be stronger at the tip, with high activity in guard cells and on the base of trichomes (Fig. 2G). In older leaves (first pair) the GUS activity was low and mainly restricted to the veins (Fig. 2H), guard cells (Fig. 2, H and I), hydathodes (Fig. 2H), and base of the trichomes (Fig. 2J).

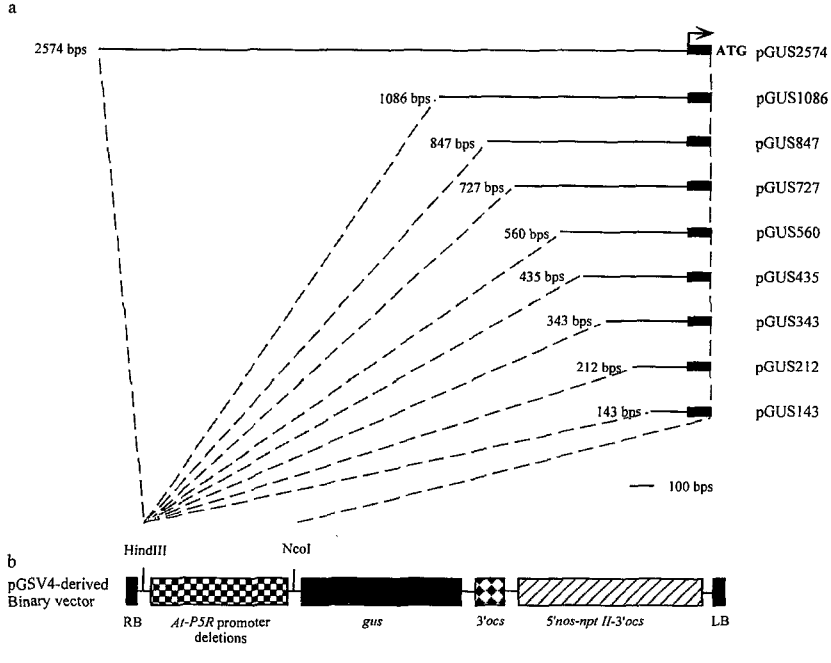

Figure 1. Structure of 5′ promoter deletion constructs. a, Schematic representation of the *At-P5R* promoter-*gus* constructs. Fusions between the 5′ flanking region of *At-P5R* and the *gus* gene were constructed as described in "Materials and Methods." The promoter length is indicated with respect to the transcription initiation site (shown by an arrow). The leader is represented with a solid box. ATG represents the translation initiation site of *At-P5R*. pGUS2574 to pGUS143 represent the names of transgenic lines containing different deletions of the promoter. b, The *At-P5R* promoter deletion constructs in the binary vector derived from pGSV4. *Hin*dIII and *Nco*I were used to introduce the different deletions. The marker gene *5′nos-nptII-3′ocs* encodes the neomycin phosphotransferase gene with the nopaline synthetase promoter and with the octopine synthetase terminator. RB and LB, Right and left borders, respectively, of the T-DNA.

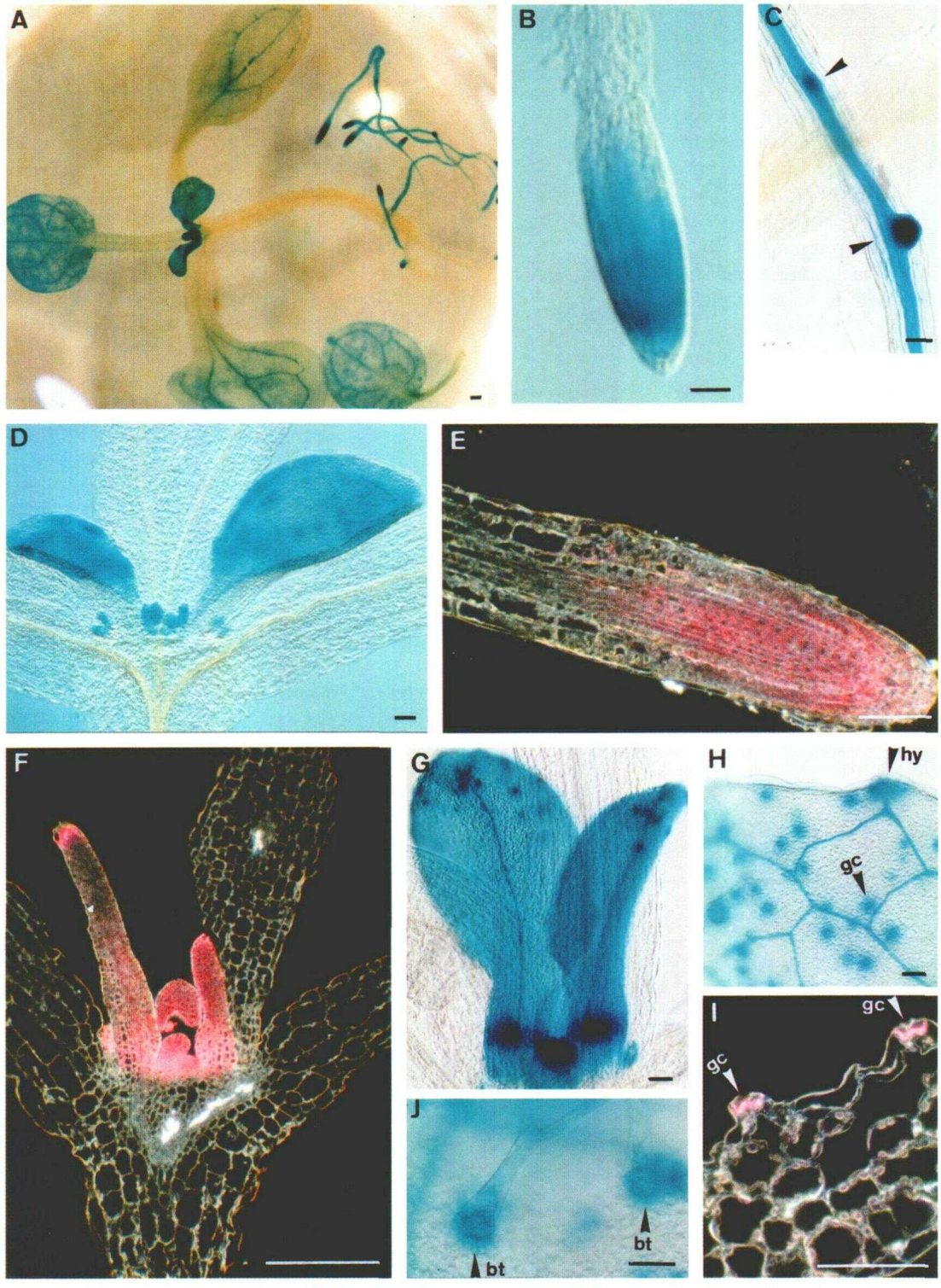

Figure 2. Histochemical localization of GUS activity directed by the *At-P5R* promoter in 10-d-old seedlings of transgenic Arabidopsis plants. A, Overview of GUS activity showing high activity in roots, shoots, and young leaves, stained overnight. B, Strong GUS activity in root tip. C, Strong GUS staining in lateral root primordia and vascular cylinder (arrowheads). D, GUS staining in shoot. E, Longitudinal root section through a root tip, stained overnight (the staining in the root cap might result from a diffusion). F, Longitudinal shoot section showing GUS activity in the apical meristem and leaf primordia. G, GUS staining in the third pair of leaves, stained for 6 h. H, GUS staining in the first pair of leaves, stained for 6 h. I, Transverse section of leaf showing GUS activity in guard cells. J, Leaf blade of the first leaf showing GUS activity at the base of trichomes (arrowheads), stained for 6 h. Staining was performed for 4 h unless otherwise indicated. bt, Base of trichome; gc, guard cell; hy, hydathode. Bars = 50 μm.

Analysis of the Organ-Specific Activity of the *At-P5R* Promoter in Mature Plants

Transgenic Arabidopsis plants containing the *gus* fusion with *At-P5R* promoter fragments of different lengths were grown to maturity as described in "Materials and Methods." Quantitative fluorometric GUS assays were performed in different tissues of flowering plants.

The results of the fluorometric assay are shown in Figure 3. Examination of the overall level of GUS activity driven by different lengths of promoter revealed no clear trend of increase or decrease. GUS2574 had very low GUS activities in all tissues tested, which were merely one-tenth of those in GUS1086. The GUS activities of GUS1086 tended to decrease as the length of the promoter shortened, but this trend was reversed in GUS560, which showed GUS activities as high as those in GUS1086. Thereafter, the GUS activities declined all the way down to GUS343. GUS212 only marginally increased the GUS activity relative to GUS343. The GUS activity in GUS143 was very low and almost undetectable in leaves and reproductive organs.

The levels of GUS activity in different tissues varied significantly. However, for most of the constructs the GUS activities decreased, in the order: roots > siliques > flowers > rosette leaves > cauline leaves > stems, except GUS2574 and GUS212, in which stems (both constructs) and flowers (GUS212 only) showed higher GUS activities than leaves and siliques, respectively. Transgenic plants containing a CaMV 35S promoter-*gus* fusion exhibited 100-fold higher GUS activity in all of the tissues tested compared with all of the above transgenic plants (data not shown).

Expression Pattern in Mature Leaves

In a histochemical assay the GUS activity in rosette leaves decreased with the age of the leaves. In young leaves the staining was almost uniform over the entire leaf blade. Strong expression was detected in hydathodes at the edge and tip of the leaves (Fig. 4A) and in guard cells (Fig. 4B). In older leaves GUS activity was very weak and was visible only in hydathodes. The GUS activity in cauline leaves was also stronger in younger ones.

Expression Pattern in Roots

GUS staining in roots of mature plants was slightly weaker than those in the seedlings, but the expression pattern remained the same. Intense staining could be seen in root tips and lateral root primordia and less staining was observed in the root vascular cylinder (Fig. 4C).

Expression Pattern in Flowers and Developing Seeds

The strongest GUS staining in mature plants occurred in flowers. In closed, unfertilized flowers, GUS activity was seen in the central septum of the carpels in the ovules, stigma, and pollen grains (Fig. 4D). In open, fertilized flowers expression in the outer wall of the carpels and stigma decreased compared with that in developing seeds, pollen grains, and the central septum of siliques (Fig. 4E). In sections of anthers strong GUS staining was located inside the pollen grains, whereas the tapetum remained unstained (Fig. 4F). In the central septum staining was seen only in the outer cell layer, whereas in developing seeds more intense staining appeared in the inner integument and embryos (Fig. 4E). Weak staining was observed in the tips of the petals (data not shown) and in the vascular bundles of the filaments (Fig. 4G). During seed ripening GUS activity generally decreased, and when the siliques reached 1 cm in length (still green), no staining could be observed. To eliminate the possibility that the hardening testa in late seed development impeded the substrate uptake, siliques and seeds were stained after they had been cut apart to facilitate the penetration of 5-bromo-4-chloro-3-indolyl-8-D-glucuronide; however, no staining was observed.

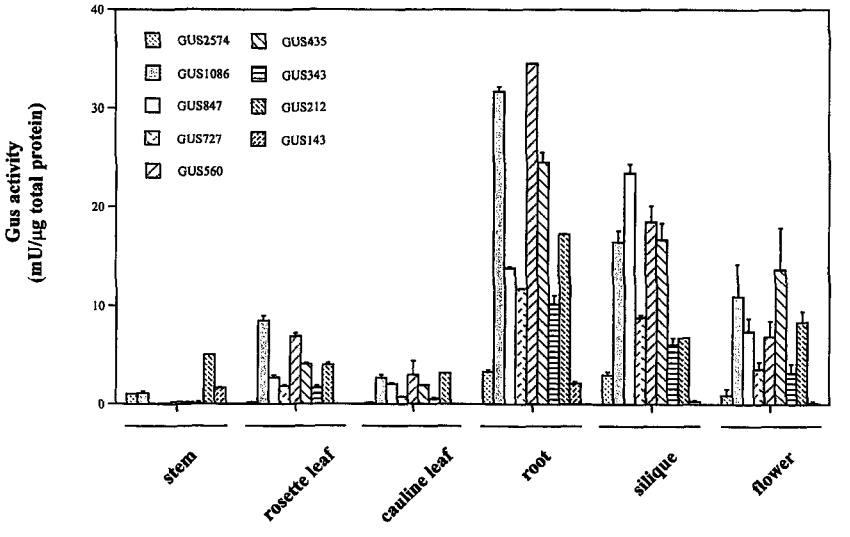

Figure 3. GUS activity in different organs of 4-week-old flowering Arabidopsis plants. Results are means ± SE. mU, Milliunits.

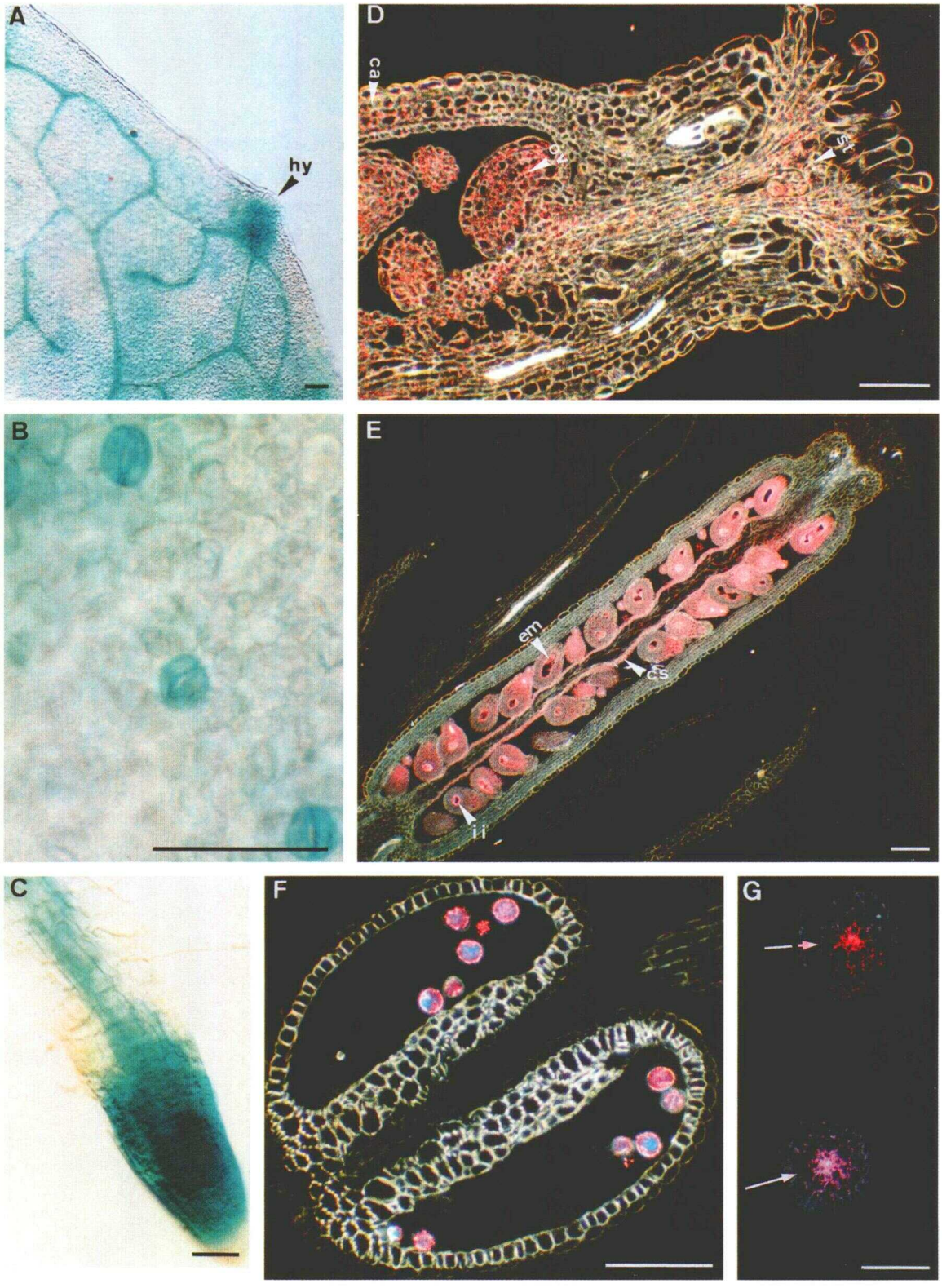

Figure 4. Histochemical localization of GUS activity in 4-week-old flowering transgenic Arabidopsis plants containing *At-P5R* promoter-*gus* fusions. A, Rosette leaf. B, Guard cells of opened stomata in a rosette leaf. C, Root tip. D to F, Longitudinal sections of a young flower bud (D), the silique of an opened flower showing developing seeds (E), and of the anthers and pollen grains (F). G, Transverse section of the filament. The staining was performed for 4 h. ca, Carpel; cs, central septum; em, embryo; ii, interintegument; hy, hydathode; ov, ovule; st, stigma. Bars = 50 μm.

Pro Content in Mature Plants

Pro content was determined in different tissues of mature plants. In plants grown under the conditions of the present study, a decreasing Pro content was found in the following order: flowers > siliques ≥ rosette leaves ≈ cauline leaves > stems > roots (Fig. 5).

DISCUSSION

Developmental and Spatial Regulation of the *At-P5R* Promoter

We describe the developmental and spatial activity of the *At-P5R* promoter using *gus* as a reporter gene. A histochemical GUS assay showed that, in general, the *At-P5R* promoter is actively expressed in young tissues, particularly in meristematic cells of the root tips, shoot apex, lateral root primordia, and leaf primordia of seedlings, as well as in young leaves, flower buds, and developing seeds of adult plants. In old tissues the activity of the *At-P5R* promoter was mainly restricted to certain types of cells, such as the vascular bundles of roots and leaves, guard cells, the base of trichomes, hydathodes, and pollen grains, where osmotic adjustment is likely to occur.

A quantitative GUS assay showed high *At-P5R* promoter activities in roots, siliques, and flowers and low activities in the other parts of the plant. The highest *At-P5R* promoter activity as measured by quantitative GUS assays was observed in roots (Fig. 3), which, remarkably, had the lowest free Pro content (Fig. 5). The total protein was lower in roots than in leaves, flowers, or siliques. Therefore, the *At-P5R* promoter activity in roots measured as GUS activity per microgram of total protein could be biased. Furthermore, in roots the level of *At-P5S* transcripts that encode the enzyme for the rate-limiting step in the Pro biosynthetic pathway from glutamate (Kishor et al., 1995) is also high (Savouré et al., 1995). Therefore, the roots appear to be a site of Pro biosynthesis, although free Pro does not accumulate. A likely explanation for the low level of free Pro in roots is the transport of Pro from roots to other parts of the plant. In accord with this hypothesis, two Pro transporter genes, *ProT1* and *ProT2*, have been shown to be actively expressed in roots of Arabidopsis (Rentsch et al., 1996).

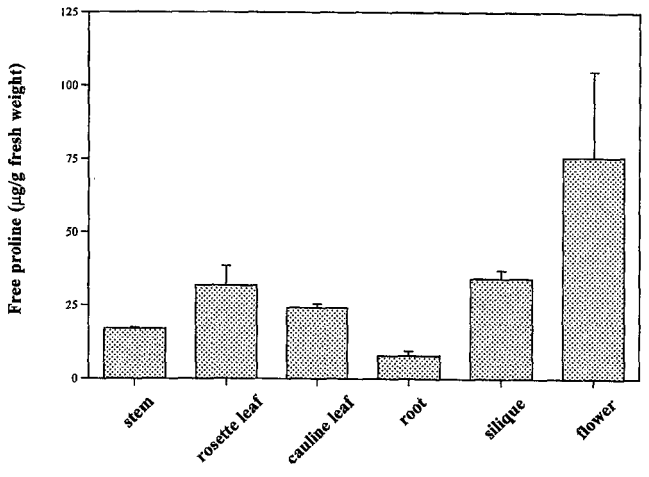

Figure 5. Free Pro content in different organs of 4-week-old flowering Arabidopsis plants. Results are means ± SE.

Our results also showed that in almost all transgenic lines the stem is the organ with the lowest *At-P5R* promoter activity but a higher level of free Pro content than roots. This observation might reflect the transport of Pro produced in roots or leaves via stem. *ProT1* is also strongly expressed in the vascular bundles of the stem (Rentsch et al., 1996), corroborating our hypothesis that the stem might be a Pro transport route rather than a site of biosynthesis.

The high Pro content in flowers found in the present study and in that of Chiang and Dandekar (1995) correlates well with the high *At-P5R* promoter activity. In particular, pollen grains of *Petunia hybrida* (Zhang et al., 1982) and ovules of *Vicia faba* (Venekamp and Koot, 1984), which are known to be Pro rich, also showed a high *At-P5R* promoter activity in a histochemical assay. Several genes related to Pro metabolism in Arabidopsis, such as the Pro biosynthesis genes *At-P5S* (Savouré et al., 1995) and *At-P5R* (Verbruggen et al., 1993), the Pro degradation gene *At-POX* (Verbruggen et al., 1996), and the Pro transporter gene *ProT1* (Rentsch et al., 1996), are actively expressed in flowers. These data suggest that there is a higher Pro turnover in flowers than in other organs of the plant, which is consistent with the function of Pro in providing energy and/or carbon and nitrogen sources for flower development.

The *At-P5R* promoter activity is stronger in young leaves than in older ones. This might be due either to a higher metabolic activity in young leaves, which have higher energy demands provided in part by higher Pro levels, or to the fact that young leaves are more suitable and active Pro sources for transport to the flowers.

During seed development the *At-P5R* promoter activity was initially strong in ovules of flower buds and during the early stages of seed development but decreased as seeds matured (data not shown). During the later stages of seed development (when the siliques were longer than 1 cm) the *At-P5R* promoter activity as measured by quantitative GUS assays in siliques increased. However, we failed to detect a corresponding level of the *At-P5R* promoter activity in the siliques of this stage in a histochemical assay. It is possible that the seed proteins that are not soluble in water inhibit substrate accessibility to GUS. A similar pattern for free Pro content was also found in different stages of seed development in *V. faba* (Venekamp and Koot, 1984). Signals that trigger the increase in *At-P5R* expression during later stages of seed development might come from processes associated with dehydration during seed maturation.

We also studied the cell-specific expression of *At-P5R*. In seedlings strong *At-P5R* promoter activity was observed in the rapidly dividing cells of the root and shoot apical meristem, in accord with propositions that Pro may be synthesized to fulfill energy requirements (Shetty et al., 1992). This result is consistent with the high levels of *P5CR* transcripts found by in situ hybridization in meristematic cells of the vascular cambium of the flower stem of Arabidopsis (Hare and Cress, 1996). The *At-P5R* promoter activity in guard cells may implicate the involvement of Pro

biosynthesis in the osmotic adjustment related to the opening of stomata.

In leaves of mature plants a strong expression in hydathodes was observed. The hydathode is a secretory structure that removes water from the interior of a leaf and deposits it on the surface. Gene expression in hydathodes has been reported for the chitinase gene (Samac and Shah, 1991) and the *AthH2* gene that encodes a water-channel protein (Kaldenhoff et al., 1995). Because in some plants water is forced out of hydathodes by hydrostatic pressure, an osmolarity gradient may be established inside hydathodes, and Pro may play a role in this gradient. The *At-P5R* promoter is also active at the base of the trichome. In Arabidopsis the trichome is proposed to protect leaves from water loss and insect attack. The support cells at the base of trichomes have a general supporting function. In our experiments the *At-P5R* activity is not only restricted to the support cells but is more intense in the mesophyll cells under the trichomes.

Many functions of Pro have been proposed. From our results, it appears that the last step of Pro biosynthesis is differentially active in various plant tissues at different developmental stages, when Pro is likely to play different roles. In young, metabolically active tissues Pro may function as an energy and/or nitrogen and carbon source, whereas in old tissues the function of Pro seems to be mainly related to dehydration. In some tissues, such as flowers, Pro might fulfill both types of function.

Deletion Analysis of the *At-P5R* Promoter

The results of promoter deletion analysis showed the same specificity of GUS activity directed by promoters longer than 212 bp in different organs of mature plants and seedlings. The very low GUS activities in stem and roots directed by the 143-bp promoter in quantitative GUS assays is probably due to background, because they have never been detectable in histochemical assays. These data support the hypothesis that the 69-bp region between −212 bp and −143 bp with respect to the transcriptional start site is necessary to direct the tissue-specific expression of *At-P5R*.

The overall GUS activity fluctuated significantly when the promoter was shortened, suggesting that upstream elements (both enhancers and suppressors) may modulate the level of the *At-P5R* expression. As shown in Figure 3, the *At-P5R* promoter activities in leaves and roots showed the same tendency to increase or decrease. These data suggest that the regulatory elements responsible for the expression level in leaves and roots might be closely located.

An analysis of the 69-bp region of the *At-P5R* promoter revealed some interesting motifs. Sequence CAAAATAAG located from −210 to −202 resembles the *cis* elements responsible for the pollen-specific expression of the *NTP303* gene from tobacco (*Nicotiana tabacum*) (Weterings et al., 1995), although there is a one-base substitution to the hexamer core AAATGA. A TGA1b-binding site, TGACG, which is known to confer the root-specific expression of the CaMV 35S promoter, is present from −165 to −161 on the opposite strand. It has been reported that the tissue-specific expression pattern of promoters might be achieved by a combinatorial regulation model (Ondek et al., 1987; Benfey and Chua, 1990). Therefore, it is possible that *cis* elements responsible for the *At-P5R* expression in other tissues are also present in the 69-bp fragment. Further dissection is therefore needed to identify functional *cis* elements in this region.

The results presented here support previous reports that tissue-specific elements are maintained in proximal regions of the promoter. For example, in the osmotin promoter a fragment located at −248 to −108 upstream of the transcription start site is necessary for the same spatial, temporal, and developmental expression patterns as the full-length promoter. This fragment can also confer gene expression on a minimal CaMV 35S promoter in a transient assay (Raghothama et al., 1993; Liu et al., 1995). Pollen-specific elements are located at −84 to −55 in *LAT52* and at −88 to −69 in *LAT59* (Eyal et al., 1995).

Common Signaling during Development and during Osmotic Stresses?

Many osmotic stress-inducible genes have also been reported to be highly expressed in pollen or seeds, where developmentally programmed desiccation occurs; pollen- or seed-specific genes can also be induced by osmotic stresses. Osmotin, for example, is induced by many environmental stresses, including salt and drought, and is also highly expressed in pollen grains (Kononowicz et al., 1992). Wang and Cutler (1995) reported that the promoter of the low-temperature-inducible genes *kin1* and *cor6.6* also directed *gus* expression in early to middle stages of seed development and in pollen grains. *rd22*, a gene responsive to dehydration in Arabidopsis, is expressed in both seeds and drought-stressed leaves (Yamaguchi-Shinozaki and Shinozaki, 1993). Late-embryogenesis abundant (*lea*) genes (Dure, 1985, 1993) are also induced upon drought stress and application of exogenous ABA in vegetative tissues. These data suggest that common signals may exist to promote gene expression during developmentally programmed dehydration and environmentally induced osmotic stresses.

An interesting question is: which *cis* elements are responsible for the developmentally regulated expression of *At-P5R*? Because the expression of *At-P5R* promoter is detected in guard cells, hydathodes, and pollen grains, where osmoregulation might be implicated, and previous data have shown that *At-P5R* transcripts are enhanced by salt stress (Verbruggen et al., 1993), it would be interesting to determine whether the expression of *At-P5R* in developmental processes is regulated through the same elements conferring osmoregulation. Analysis of the *At-P5R* promoter activity during osmotic and heat stresses is in progress (X.-J. Hua, B. van de Cotte, M. Van Montagu, and N. Verbruggen, unpublished data).

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