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Immunolocalization of the G Protein α Subunit Encoded by the *GPA1* Gene in Arabidopsis

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Heterotrimeric GTP binding proteins (G proteins) are important signal transducers in lower eukaryotes and in animal cells. In plants, the occurrence of GTP binding proteins has been reported, but their biological function remains unclear. Two genes coding for G protein α subunits have been cloned: *GPA1* in Arabidopsis and *TGA1* in tomato. To gain some insights into the function of *GPA1*, we describe an extensive immunolocalization of GPα1, the gene product of *GPA1*, during Arabidopsis development. Our results show that GPα1 is present through all stages of development and in all organs examined, with the exception of mature seeds. It is expressed in roots, floral stem, rosette leaves, cauline leaves, flowers, and seedpods. Interestingly, the level of GPα1 protein is higher in immature organs than in mature organs. GPα1 is present at a high level in the root meristem and elongation zone, in the shoot and floral meristems, and in the leaf primordium and floral organ (sepal, petal, stamen, and gynoecium) primordia. During flower development, dividing microspores, but not mature pollen, show high levels of GPα1. During pollination, GPα1 is present in the growing pollen tubes. The protein is also present in nectaries and developing ovules and, after fertilization, in developing embryos. In mature tissue, GPα1 is preferentially found in the vascular system but is also present in other cell types. The complexity of the GPα1 localization pattern suggests that GPα1 might be involved in different signaling pathways depending on the developmental stage.

INTRODUCTION

Classically, heterotrimeric GTP binding proteins are involved in transmitting extracellular signals to specific effectors and have been implicated in a variety of signaling pathways in animals and lower eukaryotes. In mammals, heterotrimeric G proteins are important for metabolic, hormonal, and growth factor regulation of adenylyl cyclase activity, ion channels, and phospholipases (for reviews, see Gilman, 1987; Simon et al., 1991; Gupta et al., 1992), for transmission of visual, olfactory, and taste stimuli (Stryer, 1986; Jones and Reed, 1989; McLaughlin et al., 1992), and for cell differentiation (Wang et al., 1992; Watkins et al., 1992). Recently, some heterotrimeric G proteins have been implicated in intracellular vesicular transport (Donaldson et al., 1991; Stow et al., 1991; Colombo et al., 1992; Ktistakis et al., 1992; Pimplikar and Simons, 1993). In yeast, heterotrimeric G proteins are involved in mating (Dietzel and Kurjan, 1987; Miyajima et al., 1987; Obara et al., 1991) and in sensing nutrition (Isshiki et al., 1992). In the slime mold Dictyostelium, G proteins are required for aggregation (Firtel et al., 1989) and for multicellular development (Hadwiger and Firtel, 1992). Finally, in Drosophila, a G protein is involved in embryo development (Parks and Weischaus, 1991). Clearly, the number of different pathways that involve heterotrimeric G proteins in many different organisms is quite large and continues to increase with new discoveries.

Heterotrimeric G proteins are composed of α, β, and γ subunits that are associated in an inactive GDP-bound form. Activation by a ligand of a cell surface receptor causes replacement of GDP by GTP and dissociation of the GTP α subunit complex from the βγ dimer and stimulation of its downstream effector. The α subunit is thought to confer the specificity of the interaction with the receptor and the effector, but there is accumulating evidence for a role of the βγ dimer in determining the specificity of G protein function (for review, see Birnbaumer, 1992). Termination of the signal occurs when GTP bound by the α subunit of the G protein is hydrolyzed to GDP. The α subunit then reassociates with the βγ complex.

Functionally, G proteins have often been identified by their susceptibility to the bacterial pertussis and cholera toxins. Through ADP ribosylation at specific sites, the pertussis toxin uncouples the receptor from its G protein and thus blocks signal transduction, while the cholera toxin blocks the GTPase activity of the α subunit and locks it in an activated form. Gα subunits can be ADP ribosylated by one or both or none of the toxins (for reviews, see Kaziro et al., 1991; Simon et al., 1991).

Little is known about signal transduction involving G proteins in plants; however, there is evidence for the presence of GTP binding proteins in plant extracts (Hasunuma and Funadera, 1987; Drobak et al., 1988). Antibodies directed against a conserved motif of the α subunit of animal G proteins have detected proteins of similar size in the microsomal

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fractions of both monocotyledons and dicotyledons (Blum et al., 1988). Several fractions of GTP binding proteins isolated from pea have been shown to be ADP ribosylated by pertussis toxin (Hasunuma et al., 1987b). However, none of these studies has shown the involvement of GTP binding proteins in specific signal transduction in plants.

Other studies, based mainly on the fact that binding of the G protein α subunit with a nonhydrolyzable analog of GTP, GTPγS, could be affected by extracellular stimuli, have provided a link between G protein and signal transduction. For example, red and far-red light inhibit GTPγS binding by GTP binding proteins in *Lemna* (Hasunuma et al., 1987a), auxin enhances GTPγS binding in rice (Zaina et al., 1990), and blue light activates GTPγS binding in the plasma membranes of etiolated peas (Warpeha et al., 1991). Furthermore, GTPγS has been shown to stimulate the release of inositol phosphate derivatives from membrane isolated from *Acer* (Dillenschneider et al., 1986) and can also mimic the swelling induced by red light in etiolated wheat protoplasts (Bossen et al., 1990). In broad bean, GTPγS reduces the inward K^+ current in guard cells (Fairley-Grenot and Assmann, 1991) and the outward K^+ current in the mesophyll cells (Li and Assmann, 1993). It has been suggested that a 45-kD GTP binding protein from cultured soybean cells is involved in the elicitation of defense responses (Legendre et al., 1992), and in *Avena* seedlings and in soybean cell cultures, pertussis and/or cholera toxin–susceptible GTP binding protein(s) seems to be involved in phytochrome-mediated gene activation (Romero et al., 1991; Romero and Lam, 1993). Recently, Neuhaus et al. (1993) demonstrated that in tomato seedlings, phytochrome phototransduction involved the activation of one or more G proteins.

In animals, at least 17 genes coding for Gα subunits have been isolated to date. Many are ubiquitously expressed, but some are present in only very specific cell types (for review, see Simon et al., 1991). In plants, however, only two genes coding for a G protein α subunit have been isolated: *GPA1* from Arabidopsis (Ma et al., 1990) and its homolog from tomato, *TGA1* (Ma et al., 1991). This apparent uniqueness might suggest that these genes perform important functions in plants.

To gain more insight into the function of G proteins in plants, we conducted an extensive protein gel blot analysis and immunolocalization study using specific antibodies directed against a peptide from the C-terminal region of GPα1, the gene product of *GPA1*. We present results showing that GPα1 is widely distributed throughout development. Interestingly, we found that the GPα1 level is higher in immature organs (i.e., growing leaves) compared to mature organs (i.e., full-size leaves). In mature organs, GPα1 is present primarily in the vascular tissue and mesophyll cells. In developing organs, GPα1 is present at high levels in the root meristem and elongation zone, in the shoot and floral meristems, in the leaf and floral organ primordia, in developing embryos, and in growing pollen tubes and nectaries. The implications of the GPα1 localization pattern in terms of its possible roles in plant signal transduction will be discussed.

RESULTS

Protein Gel Blot Analysis

To estimate the relative levels of GPα1 during development, we performed protein gel blot analysis. The antiserum used in this study was a gift from P. A. Millner (University of Leeds, U.K.). It was raised against a synthetic oligopeptide, DETLRRRWLLFAGLL, that corresponds to the C terminus of GPα1. This sequence is not conserved among G protein α subunits. Total protein was extracted from various plant organs at different stages of development, the analysis of which is described below. The antibodies detected a band at 45 kD, as shown in Figure 1, which corresponds to the predicted size of the product of the *GPA1* gene (Ma et al., 1990) and which is absent when the blot is treated with the serum depleted of the anti-GPα1 antibodies by incubation with the antigenic synthetic peptide (data not shown).

GPα1 Protein Is Present Early during Seed Germination

GPα1 is not present in mature dried seeds (Figure 1A, lane 1). However, as early as 1 day after the beginning of germination, the level of the protein rises dramatically and continues to increase during the first week of growth (Figure 1A, lanes 2 to 5). Accumulation of GPα1 starting at the onset of germination suggests a role for GPα1 in early plant development. The 30-kD cross-reacting band is only detected in extracts from the seed and the very early seedlings; it appears to be unrelated to GPα1 because unlike GPα1, it is still present when the blot is incubated with the serum depleted of the anti-GPα1 antibodies (data not shown).

GPα1 Protein Levels Vary with Age in Vegetative Tissue

In roots and cotyledons of 2-week-old seedlings (Figure 1B, lanes 1 and 2), GPα1 is present at a slightly higher level than in roots and leaves of 3- to 4-week-old plants (before bolting; Figure 1B, lanes 3 and 4). In 5-week-old plants (after bolting or flowering), the level of GPα1 in roots and rosette leaves (Figure 1B, lanes 5 and 6) is quite low compared to younger organs (Figure 1B, lanes 3 and 4). As the roots and leaves mature, the level of GPα1 decreases. In the aerial parts of 5-week-old plants, the floral stem contains quite a high level of GPα1 (Figure 1B, lane 7) compared to roots and rosette leaves (Figure 1B, lanes 5 and 6). The oldest cauline leaves of the plant, which are collected near the base of the inflorescence stem (Figure 1B, lane 8), have a lower level of GPα1 than younger, not fully expanded, cauline leaves from near the top of the inflorescence (Figure 1B, lane 9). In each case, as the different organs mature,

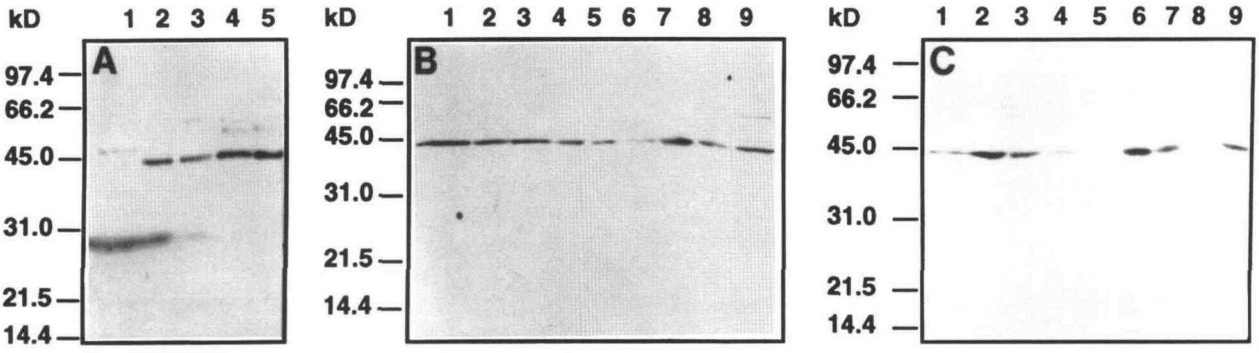

Figure 1. Protein Gel Blot Analysis of GPα1.

GPα1 protein was detected in total protein extracts of tissues collected at different times of plant development. Immunoblotting was performed with a polyclonal serum directed against a peptide sequence unique to the C terminus of GPα1 from Arabidopsis, followed by a horseradish peroxidase–conjugated second antibody detected by chemiluminescence. The serum reacts specifically with a band at 45 kD, corresponding to the *GPA1* gene product.

(A) Seedling development: lane 1, seeds; lane 2, seedlings 1 day after beginning of germination; lane 3, seedlings 3 days after beginning of germination; lane 4, seedlings 5 days after beginning of germination; lane 5, seedlings 7 days after beginning of germination.

(B) Vegetative tissue development: lane 1, roots and lane 2, cotyledons from 15-day-old plants; lane 3, roots and lane 4, rosette leaves from 4-week-old plants (before bolting); lane 5, roots and lane 6, rosette leaves from 6-week-old plants (after bolting); lane 7, floral stem; lane 8, cauline leaves collected at the base of the floral stem; lane 9, cauline leaves collected near the top of the floral stem.

(C) Flower development: lane 1, pedicel of mature flowers; lane 2, floral buds before stage 13; lane 3, floral buds after stage 13; lane 4, petals and lane 5, stamens from stage 15 flowers; lane 6, seedpods 5 days after pollination; lane 7, seedpods 10 days after pollination; lane 8, carpel walls; lane 9, seeds 10 days after pollination.

the level of GPα1 decreases, indicating that the expression of GPα1 is developmentally regulated. There is also a small but constant difference of GPα1 level between the roots and the leaves, the latter having a lower level of the protein (Figure 1B, lanes 1 and 2, 3 and 4, and 5 and 6).

GPα1 Protein Is Present in the Different Flower Parts at Different Levels

To examine GPα1 in flowers, we compared various floral organs at different stages of floral development. GPα1 is present in the pedicel of the flowers (Figure 1C, lane 1), at a relatively low level. Young floral buds before stages 13 (floral stages according to Smyth et al., 1990), when anthesis occurs, have more GPα1 than mature flowers from stages 13 to 15 (Figure 1C, lanes 2 and 3). In individual parts of a stage 15 flower, little GPα1 is present in the petals, and even less, if any, is present in mature stamens (anthers and filaments) (Figure 1C, lanes 4 and 5). In seedpods 5 to 6 days after pollination, GPα1 is at a relatively high level, which is reduced in more mature seedpods collected 8 to 10 days after pollination (Figure 1C, lanes 6 and 7). If we dissect such a mature seedpod, separating the seeds from the carpel wall, we see that the carpel wall contains most of the GPα1 protein in the seedpod at that stage of development (Figure 1C, lane 8). The green seeds (containing mature embryos) have a lower level of protein (Figure 1C, lane 9).

Our results from protein gel blot analysis indicated that GPα1 is present in all tissues examined, with the exception of seeds. GPα1 appears as early as 1 day after germination. Furthermore, the level of GPα1 protein is higher in young developing organs: young roots, leaves, and flowers as well as in growing seedpods. As organs age, the level of GPα1 decreases, suggesting a role for GPα1 in development.

Immunolocalization Analysis

To further investigate the distribution of GPα1, we performed immunolocalization studies on sections of various Arabidopsis organs at different stages of development, as discussed below. For these studies, young seedlings, roots, cauline leaves, floral stems, flowers, and seedpods were fixed and embedded in wax, as described in Methods. Sections of these different organs were treated with the primary antibodies, which were visualized using secondary antibodies coupled to alkaline phosphatase.

Localization of GPα1 during Vegetative Growth

In 1-week-old seedlings, GPα1-specific staining appears most dramatically in the shoot apical meristem and the leaf primordia, as shown in Figures 2A to 2C. Staining is also present in the vascular system and, to a lower degree, in the ground

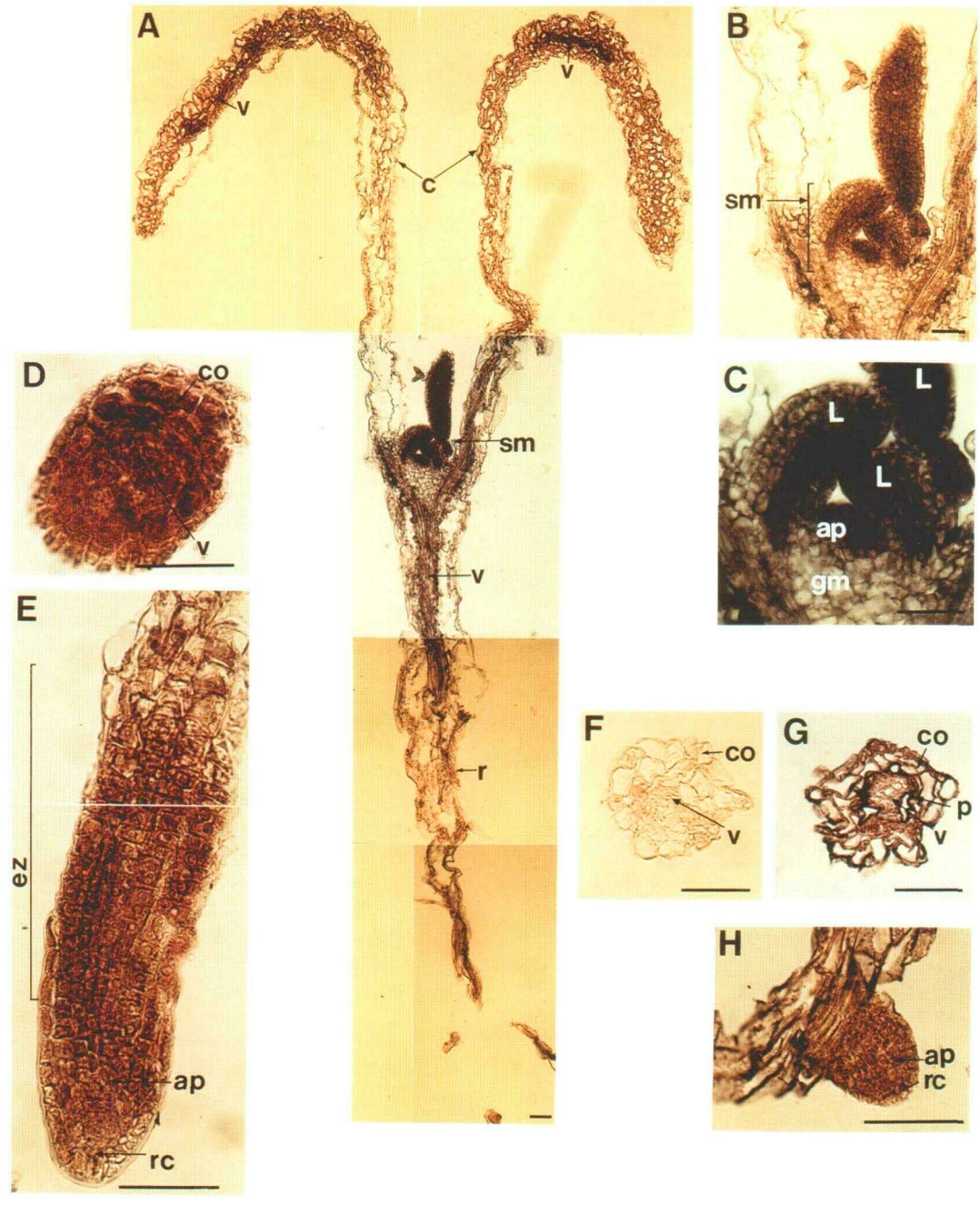

Figure 2. Distribution of GPα1 in 1-Week-Old Arabidopsis Seedlings.

(A) to **(H)** Immunolocalization of GPα1 with antibodies directed against a C-terminal peptide of GPα1. Specific staining for GPα1 is present in all tissues and all organs of the seedling, but stronger staining is seen in the apical meristems (shoot and root), in the root elongation zone, and in the leaf primordia. **(F)** The control section. The antiserum was depleted of antibodies directed against the GPα1 protein by incubating it with 20 μg/mL peptide at 37°C for 30 min.

(A) Longitudinal section through a 1-week-old seedling.

(B) and **(C)** Show **(A)** in more detail. Visible are the shoot apical meristem with the apical meristem, ground meristem, and leaf primordia.

(D) Cross-section of a root in the elongation zone.

(E) Longitudinal section of a root tip apex showing the root cap, the root apical meristem, and the elongation zone.

(F) and **(G)** Cross-sections of a young root.

(H) Longitudinal section of a developing lateral root.

ap, apical meristem; c, cotyledon; co, cortex; ez, elongation zone; gm, ground meristem; L, leaf primordia; p, pericycle; r, root; rc, root cap; sm, shoot meristem; v, vascular tissue. Bars = 50 μm.

tissue of the root and the cotyledon (Figure 2A). The apical meristem, shown in detail in Figures 2B and 2C, is composed of actively dividing meristematic cells that stained heavily. By contrast, very low staining is observed in the ground or rib meristem, which gives rise to the pith. These cells are already large and vacuolated and in an early stage of differentiation. Three leaf primordia at different stages of development can be seen and all three are heavily stained (Figures 2B and 2C). At this early stage of leaf formation, the primordia appear long and narrow because most of the cell divisions accompanied by a coordinated amount of cell expansion are periclinal.

In roots, the most striking staining appears in the root tip of both the main root (Figure 2E) and lateral roots (Figure 2H). The staining is seen in the root cap, the root apical meristem, and in the elongation zone in which the cells have divided and are elongating and in which the vascular tissue has begun to differentiate (Figures 2E and 2D). In the older portion of the root in which the vessel elements are mature, the GPα1 protein is in all cell types, but at a lower level than in the root tip (Figure 2G). GPα1 is present in the vessel elements and in the cortex and perhaps at a higher level in the pericycle. The pericycle is potentially meristematic in young roots and is the site of lateral root initiation. No staining is seen in comparable sections incubated with antiserum depleted of the GPα1 antibodies (root shown in Figure 2F; other data not shown). Although GPα1 is present in all tissues of the root and the shoot in young seedlings, its level is higher in the cells of the shoot, root, and lateral root meristems, which are actively dividing, undifferentiated cells and in the leaf primordia cells, which are dividing and elongating but not yet fully differentiated.

Localization of GPα1 in Vegetative Organs of Flowering Plants

In plants that have reached a reproductive phase (after bolting), GPα1 staining in young cauline leaves (collected near the top of the inflorescence) appears mainly in the vascular system and in the mesophyll cells, as shown in Figure 3A. A greater magnification of the section (Figure 3C) shows staining in the xylem and the phloem, with perhaps more staining in the latter. Both the spongy and the palisade mesophyll cells are stained, whereas the epidermal cells appear unstained or stained to a much lower level. No staining is observed when the section is incubated with the antiserum depleted of the antibody against GPα1 (Figure 3B). In the floral stem (Figures 3E and 3G), the staining is concentrated in the cortex of the stem, in the mesophyll cells, and in the vascular bundle, with little or no GPα1 protein detected in the pith (parenchyma cells). Higher magnification of a vascular bundle shows the localization of GPα1 in the phloem, the xylem, and the cambium, which is a meristematic tissue that can initiate secondary tissue (Figure 3H). The negative control sections show no staining (Figures 3D and 3F). Therefore, from these results, we can conclude that in organs that are differentiated and photosynthetic (cauline leaves and floral stem), GPα1 is present in the vascular tissue and the mesophyll cells but not, or to a much lower level, in the epidermis or the pith. One clear difference between these two groups of tissues is that the former is involved in energy metabolism and transport, whereas the latter is not.

In the mature roots (Figure 3I), secondary thickening has taken place, and the vascular cambium has given rise to secondary phloem and secondary xylem, composed of xylem vessel fibers and parenchyma cells. The epidermis and the cortex have been replaced by cork cells and by a secondary cortex (the phelloderm), respectively. The level of GPα1 protein is low in this organ, but as in the stem, a higher level of staining is observed in the vascular cambium (Figure 3J). There is also some staining in the parenchyma cells of the secondary xylem and in the developing cork (Figure 3J). Much lower staining is observed in mature roots (Figures 3I and 3J) compared to young roots (Figure 2G), similar to the results from protein gel blotting experiments. In mature roots, GPα1 is restricted to meristematic cells (in the cambia) and to a lower degree to vascular tissue.

Localization of GPα1 during Early Flower Development

During early flower development, GPα1 is present in the apical meristem and in young developing buds of early floral stages, as shown in Figure 4A. At a higher magnification (Figure 4B), we see heavy staining of the inflorescence meristem, as well as the flower buttress that arises from the flank of the meristem (stage 1 flower), and the floral meristem of a stage 2 flower. In a stage 3 flower (sepal primordia arise) and in a stage 4 flower (sepals do not cover the bud yet; Figure 4C), GPα1 is still found in the floral meristem and in the rapidly growing sepals. In a stage 6 flower (Figure 4D), when the sepals enclose the bud, staining is visible in the stamen primordia and in the petal primordia that are barely initiated, as well as in the central dome that will give rise to the gynoecium. There is little staining remaining in the sepals. In a stage 7 flower (Figure 3E), the central dome, the stamens that become stalked at their base, and the petal primordia are stained, but the sepals are no longer stained. In a stage 8 flower (Figure 4F), the central cylinder is stained, as are the stamens, where pockets of sporogenous tissue are visible. The petals, which are still quite small, are also stained. Therefore, in young flowers as in young seedlings, the floral meristem as well as young developing organs display high-level staining for GPα1. It is interesting to note that as the first floral organs, the sepals, become fully formed, they stop exhibiting GPα1 staining.

Localization of GPα1 during Late Flower Development

During late stages of flower development, GPα1 is present at high levels in specific cells of stamens and gynoecium. In the stamens of stage 9 flowers, the staining corresponding to GPα1 is very intense in the tetrads of microspores, as shown in Figure

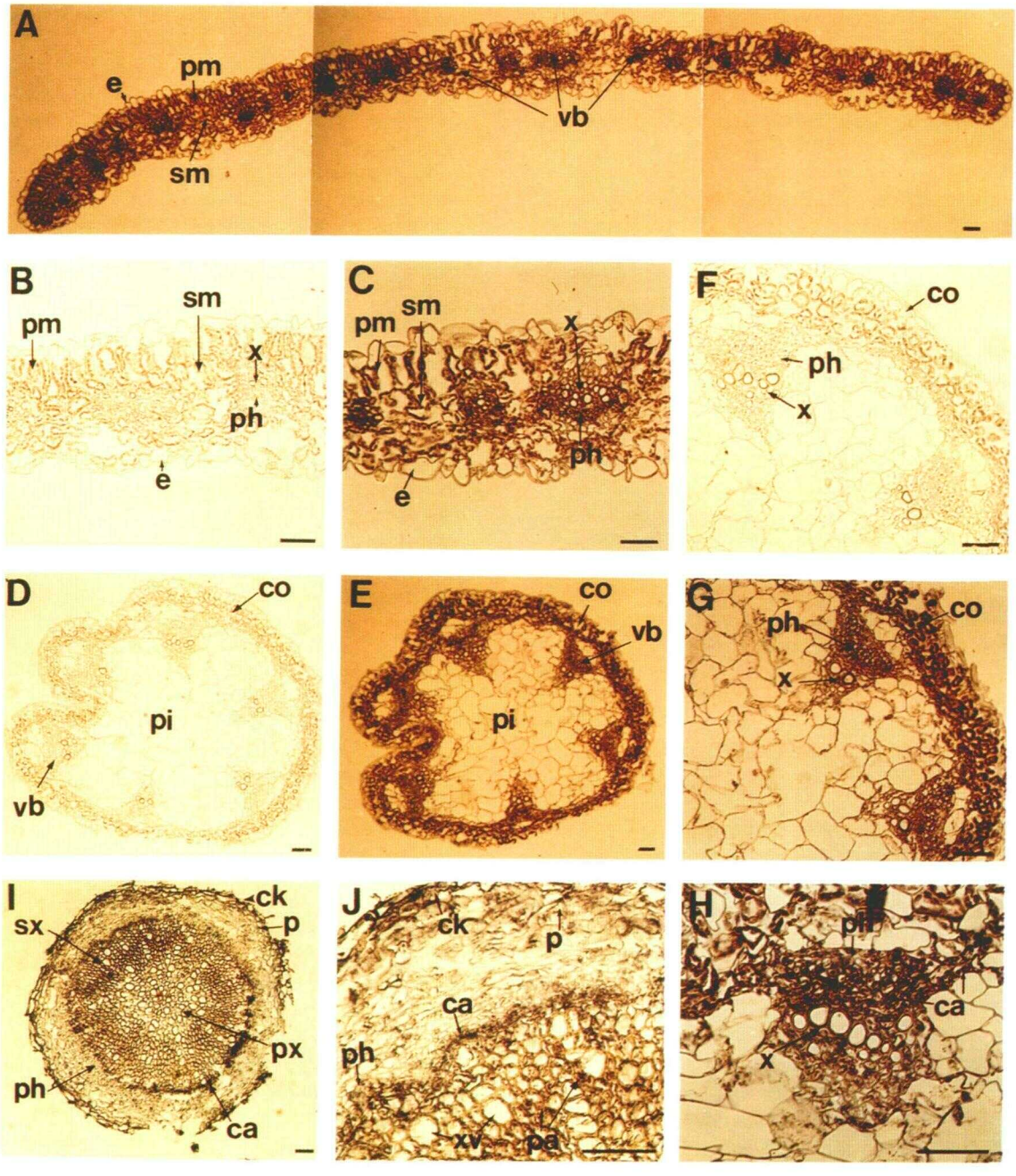

Figure 3. Distribution of GPα1 in the Vegetative Organs of 6-Week-Old Plants.

(A), **(C)**, **(E)**, and **(G)** to **(J)** Immunolocalization of GPα1. Staining for GPα1 is quite high in the vascular tissue and the mesophyll cells of the leaf and stem and is much lower in the mature root.

(B), **(D)**, and **(F)** Control sections treated as given in the legend to Figure 2.

(A) Cross-section of a young cauline leaf. The main and the lateral vascular bundles are clearly visible.

(B) and **(C)** Detailed views of **(A)** showing the epidermis, the palisade mesophyll cells, the spongy mesophyll cells, the xylem, and the phloem.

(D) and **(E)** Cross-sections of the floral stem.

(F) and **(G)** Detailed views of **(D)** and **(E)** showing the cortex, phloem, and xylem.

(H) Detailed view of **(G)** showing a vascular bundle where the vascular cambium is clearly visualized.

(I) Cross-section of a mature root where secondary thickening has occurred.

(J) Detailed view of **(I)** showing the secondary structures of the root. Cork and phelloderm have arisen from the phellogen. Secondary xylem (composed of xylem vessels and parenchyma cells and fibers) and secondary phloem have arisen from the vascular cambium.

ca, cambium; ck, cork; co, cortex; e, epidermis; p, phelloderm; pa, parenchyma cells and fibers; ph, phloem; pi, pith; pm, palisade mesophyll; px, primary xylem; sm, spongy mesophyll; sx, secondary xylem; vb, vascular bundle; x, xylem; xv, xylem vessel. Bars = 50 μm.

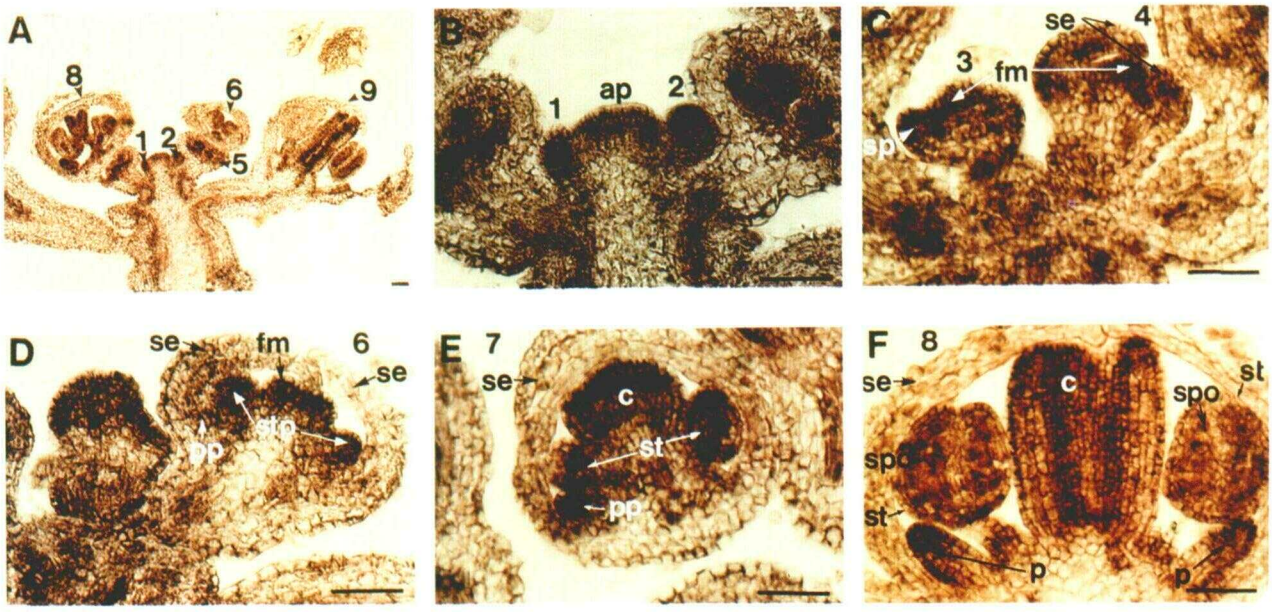

Figure 4. Distribution of GPα1 during Early Flower Development.

Specific staining for GPα1 appears in all the floral organ primordia from stages 1 to 8. The stages reached by each bud are indicated by numbers.
(A) Longitudinal section through the inflorescence meristem showing stages 1, 2, 5, 6, 8, and 9. (See Figure 5 for a discussion of stage 9.)
(B) Longitudinal section through the apical meristem and floral primordia at stages 1 and 2 of development.
(C) Longitudinal section through a stage 3 floral primordium, when sepal primordia arise, and a stage 4 floral primordium.
(D) Longitudinal section through a stage 6 floral primordium. The sepals enclose the bud, and the petal and stamen primordia have arisen.
(E) Longitudinal section through a stage 7 floral primordium. The gynoecium begins to differentiate and form a cylinder.
(F) Longitudinal section through a stage 8 flower. The petals are still very small, and sporogenous tissue is evident in the pollen sac.
ap, apical meristem; c, gynoecium; fm, floral meristem; p, petal; pp, petal primordia; se, sepal; sp, sepal primordia; spo, sporogenous tissue; st, stamen; stp, stamen primordia. Bars = 50 μm.

5A. As shown in more detail in Figure 5O, the microspores themselves are highly stained as is the callose wall that unites them, whereas the tapetum and the anther wall appear unstained. In a control longitudinal section of an anther (incubated with the depleted serum; Figure 5N), no staining is observed in the anther wall, the tapetum, or the pollen mother cells, which are surrounded by a callose wall. When the microspores become separated from each other (stage 10), no staining is visible in the anthers or the microspores (Figure 5B). In the developing carpel of the flower, some low staining can be seen in the arising ovule primordia (stage 9; Figure 5A), which becomes more intense by stage 10. After stigmatic tissue appears (stage 11; Figure 5C), the most noticeable change in the flower development is the formation of the ovules. The nucellus containing the megaspore mother cell and the inner and the outer integuments become visible at stage 12 (Figures 5E and 5F), and, by the end of stage 12, the outer integument recovers the inner integument and the nucellus (Figures 5G and 5H). At the time of fertilization (stage 14; Figure 5K), the anthers contain mature pollen grains and the gynoecium has generated a short style. The integuments nearly completely envelop the embryo sac, leaving only a small aperture at the apex of the ovule, the micropyle, through which the pollen tube will enter prior to fertilization (Figure 5L). The nucellus has degenerated and the inner integument is in close contact with the embryo sac (Figure 5M). During all of these phases of development, ovules show intense staining for GPα1 (Figures 5C to 5E, 5G, 5J, and 5K). The funiculus (the stalk attaching the ovule to the placenta), the nucellus, and the integuments stain as they appear at stage 12 (Figure 5F). As ovules develop, the level of GPα1 in the outer integuments decreases (Figure 5H, in a late stage 12 flower; Figure 5L, in a stage 14 flower), while staining increases in the inner integument (Figures 5L and 5M). The embryo sac is not stained (Figure 5M).

The carpel walls are lightly stained from stage 11 through stage 14 (Figures 5B to 5K), with a decrease of GPα1 level toward the later stages. The outer layer of the false septum (a structure arising from the placenta) is also stained when it appears at stage 11 (Figure 5B) and during stage 12 (Figures 5E to 5H). The cross-section of a stage 12 flower shows staining in the vascular tissue of the petals and the filaments and in the epidermis of the petal (Figure 5D). A longitudinal

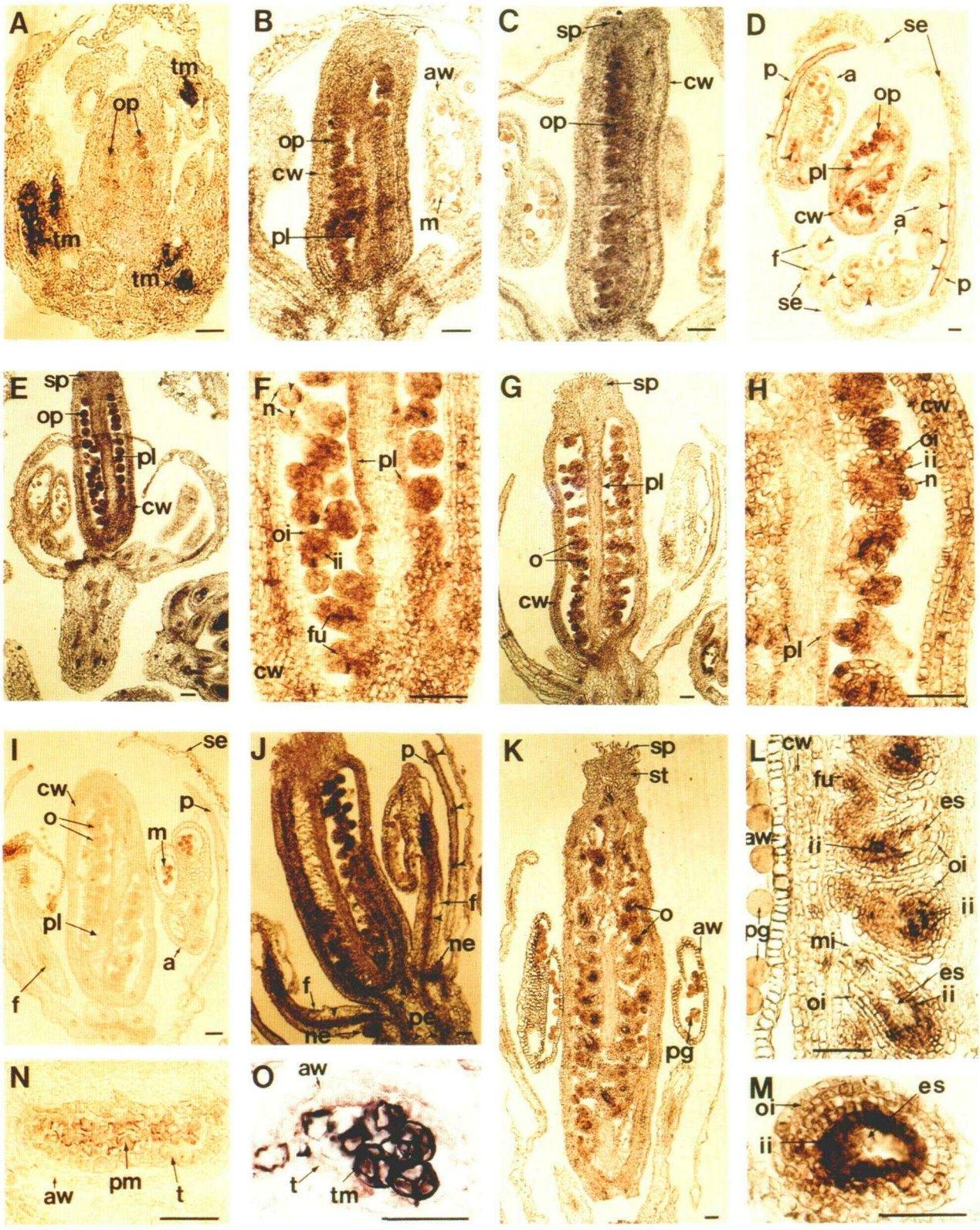

Figure 5. Distribution of GPα1 in Flower during Stages 9 to 12 and 14.

section at the same stage (Figure 5J) also shows staining in the vascular tissue of the different flower organs (petal, filament, pedicel, and carpel), as well as in small structures at the basis of the filament called the nectaries, which are sugar secreting glands (Figure 5J). A similar section, incubated with the serum depleted of GPα1 antibodies, does not exhibit any staining (Figure 5I). During late flower development, GPα1 is present in dividing cells, such as tetrads of microspores, ovule primordia, and ovules. In the more mature flower organs (petals, stamens, and pedicel), the GPα1 level is low but is principally present in the vascular system.

Localization of GPα1 during and after Pollination

During pollination, after contact with a receptive stigma, the mature pollen grain germinates, as shown in Figures 6A and 6C. It then penetrates the style through the transmitting tissue (Figure 6B) to reach the ovule and fertilize the embryo sac (Figure 6D). The mature pollen grain does not stain for GPα1, whereas the growing pollen tube is heavily stained, indicating that GPα1 protein is present at a high level in this developing structure. The stigmatic papillae and the transmitting tissue itself are not stained.

Shortly after fertilization in a globular stage embryo, strong staining is seen in the embryo proper, and weaker staining is observed in the suspensor, the seed coat, and the cellular endoderm (Figure 6E). The suspensor is made of cells that are vacuolated and are differentiated, whereas the embryo proper is composed of small dividing cells. Heavier staining is seen in the most inner layer of the inner integument that differentiated into an integumentory tapetum or endothelium. However, staining in the endothelium does not appear to be due to GPα1 (see below). In a late globular stage embryo (3 to 4 days after fertilization), the same pattern as in the earlier stage is seen; staining is strong not only in the embryo but also in the cellular endoderm (Figure 6F). At this stage, the three principal tissues of the plant (dermal, vascular, and fundamental) have been initiated. In a seed with an embryo at the heart stage (4 to 5 days after pollination), the cotyledons appear as a result of localized concentration of growth on both sides of the shoot meristem. The endoderm, which has started to form cell walls, and the embryo are stained (Figure 6G). This pattern continues in a linear cotyledon stage embryo (6 days after fertilization), at which time the radicle (the embryonic root), the hypocotyl, and the central cylinder begin to differentiate (Figure 6H). In the curled embryo (6 to 8 days after pollination), strong staining remains in the embryo while it decreases in the endoderm and the seed coat (Figure 6I). From the protein gel blot experiments, we learned that the GPα1 antibodies react in a nonspecific manner with a protein present in seeds and in very young seedlings. Figures 6J to 6L show seedpod sections containing seeds at various stages of development that were treated with the serum depleted of the GPα1 antibodies. The embryo is unstained but the endothelium is stained in a nonspecific manner. The most striking pattern of GPα1 staining during and after pollination is seen in the growing pollen tube and in the embryo. GPα1 staining in the embryo is uniform and does not reflect the appearance of any specific tissue.

Figure 5. (continued).

(A) to **(H)**, and **(J)** to **(O)** Immunolocalization of GPα1. Strong staining for GPα1 is observed mainly in the tetrads of microspores and in the developing ovules. **(I)** and **(N)** The control sections treated as described in the legend to Figure 2.

(A) Longitudinal section through a stage 9 floral primordium. The microspore mother cells divide to form a tetrad and the ovule primordia arise.

(B) Longitudinal section through a stage 10 floral primordium. The microspores are free in the pollen sac.

(C) Longitudinal section through a stage 11 flower. The stigmatic papillae appear.

(D) Cross-section through a stage 12 flower. Arrows point to the vascular tissue in the filament and the petals.

(E) Longitudinal section through a midstage 12 flower.

(F) More detailed view of **(E)**. Arrows point to the highly vacuolated megaspore inside the nucellus.

(G) Longitudinal section through a late stage 12 flower. The ovules show anatropous orientation.

(H) More detailed view of **(G)**. The outer integument is beginning to cover the inner integument and the nucellus.

(I) Control longitudinal section through a stage 12 flower.

(J) Longitudinal section through a late stage 12 flower showing the vasculature in the filament of the stamens, in the carpel, in the petals, and in the pedicel (arrows). The nectaries are also indicated.

(K) Longitudinal section through a stage 14 flower at the time of fertilization. The anthers contain mature pollen grains. The upper part of the gynoecium has differentiated a short style with a sharp boundary from the stigmatic papillae.

(L) More detailed view of **(K)**. The integuments nearly completely envelop the embryo sac, leaving only a small aperture, the micropyle.

(M) Longitudinal section through an ovule at the stage of fertilization. The embryo is in close contact with the inner integument, while the nucellus has degenerated.

(N) Control longitudinal section through an anther of a bud at stage 9 of floral development. The pollen mother cells are evident.

(O) More detailed view of **(A)**. Tetrads of microspores in the pollen sac. The tapetum is visible.

a, anther; aw, anther wall; cw, carpel wall; es, embryo sac; f, filament; fu, funiculus; ii, inner integument; m, microspores; mi, micropyle; n, nucellus; ne, nectary; o, ovule; oi, outer integument; op, ovule primordia; p, petal; pe, pedicel; pg, pollen grain; pl, false septum arising from the placenta; pm, pollen mother cells; se, sepal; sp, stigmatic papillae; st, style; t, tapetum; tm, tetrads of microspores. Bars = 50 μm.

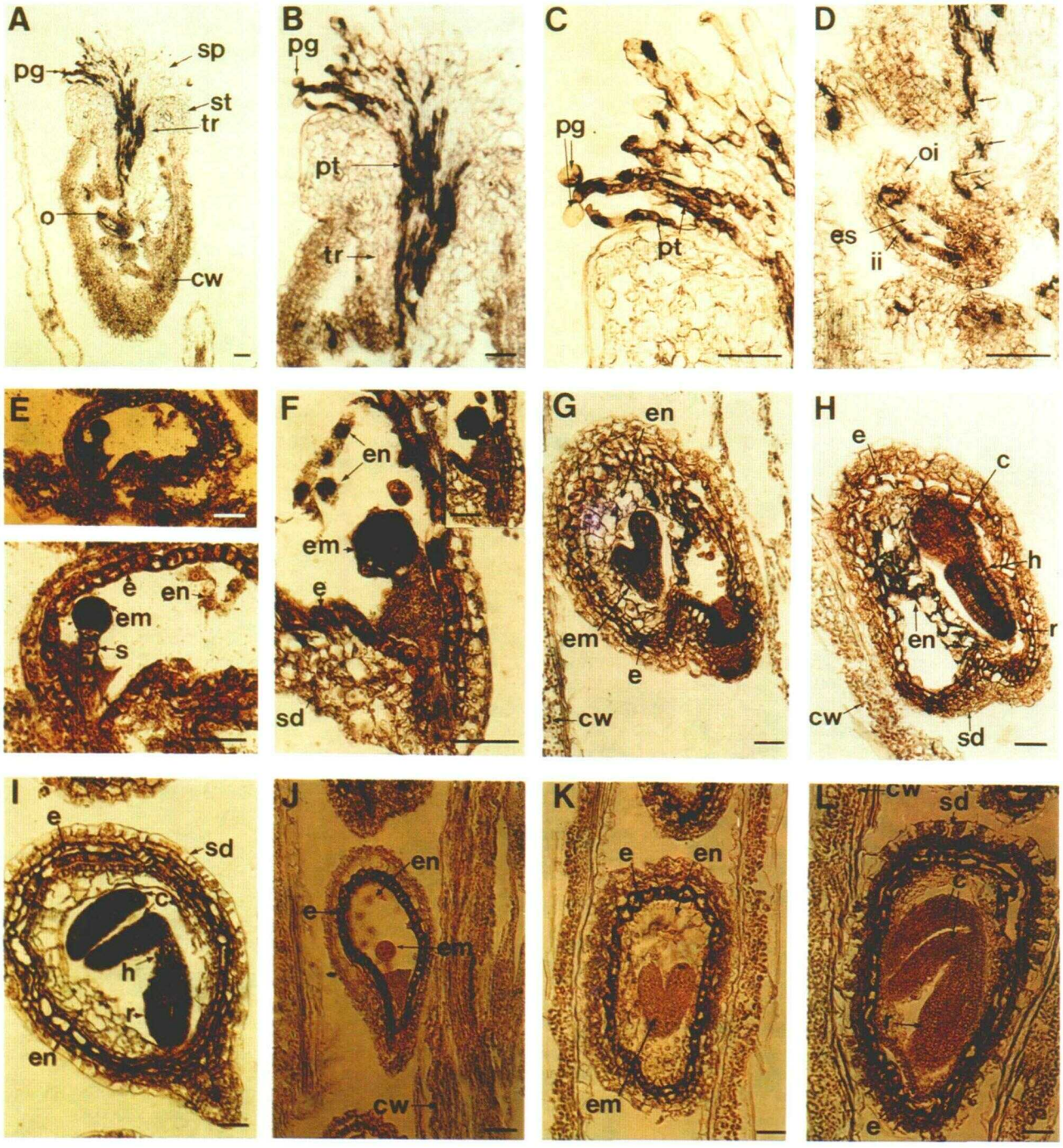

Figure 6. Distribution of GPα1 in Flower during and after Fertilization.

The GPα1 staining appears essentially in the pollen tubes and in the growing embryos of developing seeds.

(A) Longitudinal section through a carpel at stage 14 when fertilization occurs. The pollen grains have germinated at the surface of the stigma, and the pollen tubes are growing through the transmitting tissue.

(B) More detail of **(A)** is shown.

(C) A more detailed view of **(B)**.

(D) More detailed view of **(A)**. The arrows point to the pollen tube growing on the side of the ovule.

GPA1 Expression Pattern during Embryo Development

Protein gel blot analysis shows that serum used in this study reacted nonspecifically to a seed protein and nonspecific antibody staining was evident in the endothelium. Although the control staining during embryogenesis did not show any nonspecific staining in the embryo itself, we wanted to ascertain the reliability of our observations. Therefore, we analyzed transgenic plants containing a translational fusion between *GPA1* and the *uidA* reporter gene from *Escherichia coli* (encoding β-glucuronidase [GUS]) for expression of the *GPA1* gene during late embryogenesis. From the early heart stage (4 to 5 days after pollination) to the mature cotyledon stage of development (8 to 10 days after pollination), the embryo exhibits a high level of GUS staining, as shown in Figures 7A to 7E. During the desiccation stage (12 to 14 days after pollination; Figure 7F), the staining begins to decrease, and in the embryo of a mature seed (14 to 15 days after pollination; Figure 7G), the staining is absent. The GUS staining results are in agreement with our antibody results. GPα1 is present at a high level in the embryo during embryogenesis but is not present in the mature seed.

DISCUSSION

Plants respond to extracellular signals, such as hormones, light, temperature, gravity, and "touch" (particles in the air, water, or soil and attack by pests), but how these signals are transduced into the cell is poorly understood. The fact that G protein α subunits have been shown to be present in plants (Ma et al., 1990, 1991) suggests that a G protein signal transducing system similar to the ones present in animals might also play a role in plants.

A limited number of signals, receptors, and effectors suggested to be involved in a G protein signaling system have been documented in plants. Signals such as light (Hasunuma et al., 1987b; Bossen et al., 1990; Warpeha et al., 1991; Romero and Lam, 1993), the phytohormone auxin (Zaina et al., 1990), and touch (Legendre et al., 1992) have been implicated in functioning through GTP binding proteins. The involvement of G protein(s) in phytochrome-dependent cell responses has been reported (Neuhaus et al., 1993; Romero and Lam, 1993); however, information on receptors for extracellular signals is still scarce in plants. Somewhat more is known about the possible cytosolic effectors involved in signal transduction in plants, but they are not well characterized. Evidence for the presence of cAMP in plants is still controversial, but Lusini et al. (1991) have presented evidence that the formation of cAMP by adenylyl cyclase from castor bean is stimulated by GTP. The phosphatidylinositol turnover pathway has been documented in plants (for a review, see Morse et al., 1989); thus, phospholipases might be possible effectors. Furthermore, downstream of G protein activation, calcium and calmodulin have been demonstrated to participate in the activation of gene expression and chloroplast development in tomato seedlings (Neuhaus et al., 1993). Ion channels that are important effectors in animals might also play such a role in plants, because ion channels interacting with toxin-sensitive G protein(s) in fava bean have been reported recently (Fairley-Grenot and Assmann, 1991; Li and Assmann, 1993). Only one gene coding for a G protein α subunit, *GPA1*, has been isolated in Arabidopsis, in spite of numerous efforts to clone new G protein genes (H. Huang and H. Ma, unpublished data). This fact might indicate that the *GPA1* gene product plays an important and unique role in plants.

The wide distribution of GPα1 suggests that it functions in many cells. This is reminiscent of the well-known G_s and G_{i2-3} and the more recently identified G_q and G_{11-13}, which are expressed in all tissues tested (Simon et al., 1990, Kaziro et al., 1991). G_s, G_i, and G_q interact with numerous receptors for hormones and growth factors and are involved in regulation of adenylyl cyclase, phospholipase C, Ca^{2+}, and K^+ channels, leading to cellular responses as diverse as lipolysis, glycogenolysis, gluconeogenesis, mitogenesis, and differentiation (Iyengar and Birnbaumer, 1990; LaMorte et al., 1993). The wide distribution of GPα1 throughout development is in agreement with the results obtained from transgenic plants containing a *GPA1–uidA* fusion (Huang et al., 1994), which yielded information about *GPA1* expression at the organ level. Using the

Figure 6. (continued).

(E) Longitudinal sections through a seed with a globular stage embryo. The most inner layer of the inner integument differentiates into an integumentory tapetum or endothelium. The lower picture is an enlargement of the upper one.

(F) Longitudinal section through a seed with a late globular stage embryo. The inset is at the same magnification as the upper portion of **(E)** and **(G)** to **(L)**.

(G) Longitudinal section through a seed with a heart stage embryo.

(H) Longitudinal section through a seed with a linear cotyledon stage embryo.

(I) Longitudinal section through a seed with a curled cotyledon stage embryo.

(J) to **(L)** Control sections with embryos at a globular stage, heart stage, and curled cotyledon stage, respectively, incubated with antiserum depleted of anti-GPα1 antibodies.

c, cotyledon; cw, carpel wall; e, endothelium; em, embryo; en, endosperm; es, embryo sac; h, hypocotyl; ii, inner integument; o, ovule; oi, outer integument; pg, pollen grain; pt, pollen tube; r, radicle; s, suspensor; sd, seed coat; sp, stigmatic papillae; st, style; tr, transmitting tissue. Bars = 50 μm.

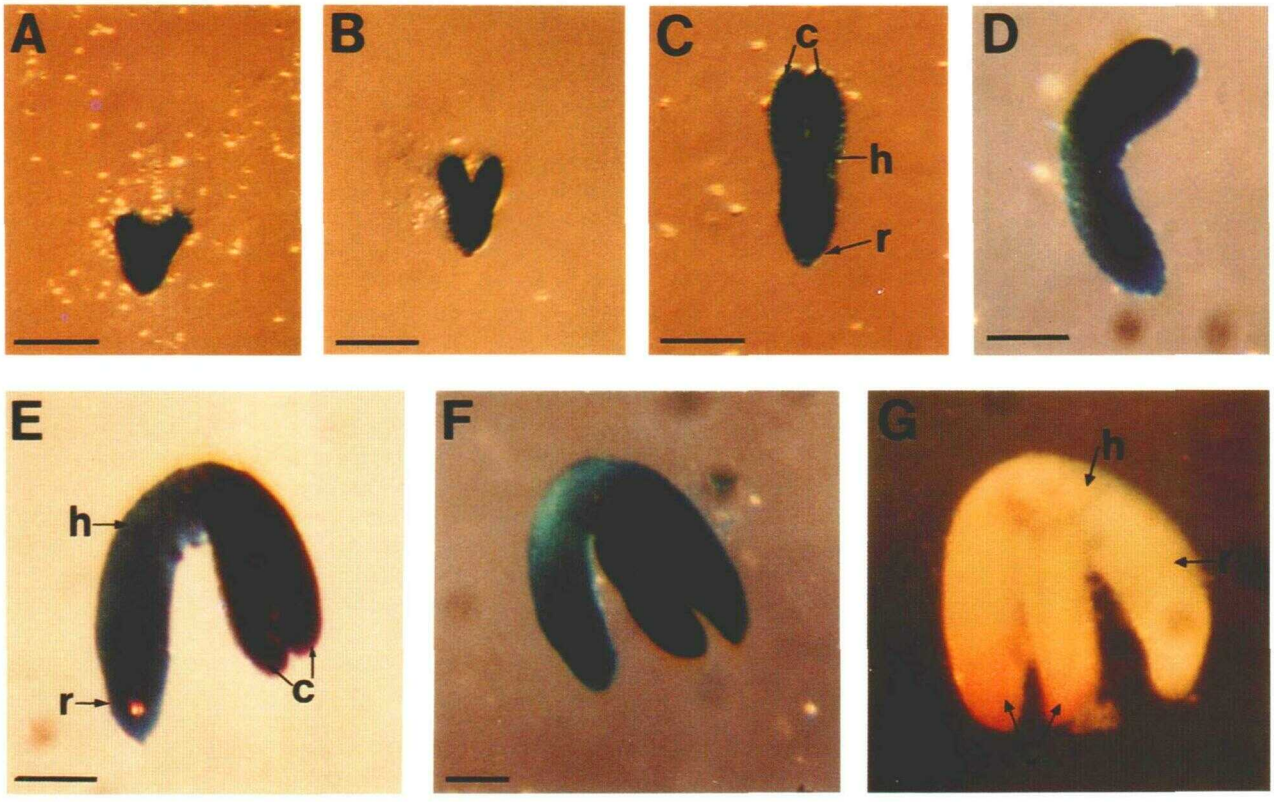

Figure 7. Analysis of the *GPA1–uidA* Fusion in Transgenic Arabidopsis during Late Embryogenesis.

(A) Early heart stage embryo.
(B) Heart stage embryo.
(C) Linear cotyledon stage embryo.
(D) Early curved cotyledon stage embryo.
(E) Mature cotyledon stage embryo.
(F) Embryo at a desiccation developmental stage.
(G) Embryo from a mature seed.
c, cotyledon; h, hypocotyl; r, radicle. Bars = 100 μm.

immunolocalization approach, we obtained more details of the expression pattern, especially during seedling and flower development. GPα1 is expressed throughout development from the onset of germination to the development of the embryo in different cell types and at different levels. It is possible that GPα1 mediates a common signaling pathway in these cells. Alternatively, GPα1 may be involved in different pathways in different cells, and these pathways share receptors or effectors that interact with GPα1. The GPα1 accumulation and localization pattern suggests at least three general growth phenomena that may require GPα1: cell division/elongation, cell nutrient accumulation/transport, and cell differentiation.

GPα1 and Cell Division/Elongation

Immunolocalization studies showed accumulation of GPα1 in primary vegetative meristems (apical root and shoot meristems) and secondary meristems (root and stem cambium) as well as in the reproductive inflorescence meristem. All of these structures are composed of small, rapidly dividing cells. The meristems are uniformly stained and do not show any restriction or preferential pattern of cell localization among cells whose fate is to become epidermal cells, cells of the vascular tissue, or parenchyma cells, indicating that if GPα1 has a role in meristem function, it is probably not involved in specifying cell fate. However, GPα1 distribution is not restricted to meristems. It is also present in the root elongation zone, in leaf primordia, and in floral organ primordia, which have already acquired their fate and are composed of cells that are still dividing, as well as differentiating, elongating, and expanding. Interestingly, the localization of GPα1 coincides to some extent with the expression in meristems and primordia of an Arabidopsis homolog (*CDC2*; Martinez et al., 1992) of the *Schizosaccharomyces pombe cdc2* (cell-division-cycle 2) gene, encoding the $p34^{cdc2}$ protein kinase, a key component of the eukaryotic cell cycle

pathway required for the entry into mitosis. The main differences between GPα1 and the Arabidopsis CDC2 localization patterns are in the inflorescence and floral meristems, and in the root quiescent center in which expression of *CDC2* is low. The GPα1 localization pattern and its similarity to that of CDC2 raise the possibility of the involvement of GPα1 in cell division. In humans, constitutively active mutants of G_s and G_{i2} α subunits have been identified in tumors (Landis et al., 1989; Lyons et al., 1990), indicating that some active Gα subunits promote cell division. Furthermore, certain growth factors are known to transmit their signal via G protein–coupled receptors, implicating G proteins in the control of cell proliferation (for review, see Pouysségur and Seuwen, 1992).

Other structures that showed high levels of GPα1 also primarily contain dividing and/or elongating cells. The tetrads of microspores arising from the mitosis and meiosis of the microspore mother cells showed intense staining, while the microspore mother cells and the pollen grains were not stained. Developing ovules are characterized by growth of the integuments, which contain dividing and expanding cells. After fertilization, the embryo is composed of rapidly dividing cells. Furthermore, the level of GPα1 decreases in these different organs as they differentiate and mature (see below).

Together, these results suggest the involvement of GPα1 in development and growth. One aspect of growth is a requirement of nutrients, and a common feature of these young organs (meristems, primordia, dividing microspores, developing ovules, and embryos) is that they can be considered as sinks for nutrients. Interestingly, the elongating pollen tubes and the sugar secreting nectaries can also be considered as sinks. Growth hormones, such as cytokinin and auxin, could be good candidates for signals mediated by GPα1 because they are involved in cell division and elongation. Cytokinins are also involved in sink–source interactions by enhancing the movement of sugar, amino acids, and other solutes from mature leaves into developing seeds and fruits. There is little information on the mode of action of cytokinins, but the binding of auxin to membrane-bound auxin binding proteins that stimulate cell elongation in rice coleoptiles has been shown to induce the activation of GTP binding proteins (Zaina et al., 1990), indicating that indeed the action of some hormones can be regulated through G proteins.

GPα1 and Nutrient Accumulation/Transport

The presence of GPα1 in the vascular tissue in all organs during development and in mesophyll cells, which are the photosynthetically active cells, suggests a role for GPα1 in nutrition, possibly through the regulation of nutrient accumulation or transport. In mammalian cells, G protein functions (G_s and G_i) were first discovered as mediators of hormonal regulation of glucose metabolism (Gilman, 1987). Recently, G_s and G_i have been implicated in modifying glucose transport through a cAMP-independent pathway (Honnor et al., 1992), and in yeast, *GPA2* (encoding a Gα subunit) is involved in sensing nutrition (Isshiki et al., 1992). As opposed to young organs that act as sinks, the photosynthetic cells and the vascular tissues are involved in producing and transporting energy, respectively. The relatively high levels of GPα1 in these cells suggest that GPα1 may be involved in a signaling pathway related to nutrient accumulation and/or transport. By extension, one aspect of GPα1 function could be in the control of cell division and growth through the regulation of nutrient transport.

GPα1 and Cell Differentiation

As organs mature, the level of GPα1 decreases. This is particularly clear for roots and leaves in the protein gel blot analysis. Immunolocalization results show that in the flower, as soon as the sepal primordia differentiate into sepals, the level of GPα1 drops dramatically. In petals and stamens, as the tissues (epidermal, vascular, fundamental, or ground tissue) differentiate, the level of GPα1 decreases in the ground tissue (mainly parenchyma cells) but not in the vascular tissue for both organs or in the epidermis of the petal (see below). In the gynoecium, the level of GPα1 also decreases after differentiation of this organ from the primordium, but the level remains detectable as the gynoecium continues its growth. Embryos have high levels of GPα1 throughout development but show a sharp decrease in the level of GPα1 during the desiccation phase. The fact that there is a high level of GPα1 in immature, not fully differentiated organs and that this level is reduced as the organs mature can be paralleled with the involvement of G proteins in cell differentiation in mammalian cells. Fibroblasts differentiate into adipocytes more rapidly when injected with antisense oligodeoxynucleotides to $G\alpha_s$ than do nontreated cells (Wang et al., 1992). Teratocarcinoma embryonic stem cells when induced to differentiate into primitive endoderm cells show a decrease in the steady state amount of $G\alpha_{i2}$. Further, an activated $G\alpha_{i2}$ mutant inhibits such differentiation, whereas decreasing $G\alpha_{i2}$ by antisense RNA promotes the differentiation (Watkins et al., 1992). Thus, in Arabidopsis, as in some mammalian cells, the level of the GPα1 subunit decreases as the cells differentiate, suggesting the involvement of GPα1 in cell differentiation.

As the plant ages, the localization of GPα1 becomes more cell specific. In the leaf, GPα1 is expressed in the vascular tissue and in the mesophyll cells (specialized parenchyma cells) but not in the epidermis. In the stem, again GPα1 is present in the vascular tissue and the cortex (mesophyll cells) but not in the pith (unspecialized parenchyma cells). In the mature petal, GPα1 is present in the vascular tissue and in the most outer layer of the petal, the epidermis. So this difference in cell type localization does not reflect a specialization in cell type but must reflect the particular role of GPα1 in a specific organ.

To investigate the possible function of GPα1, we have used protein gel blot analysis and immunolocalization to characterize the pattern of accumulation of the GPα1 protein in relation to plant development. The work presented in this paper

indicates the complexity of this pattern. The relatively high levels of GPα1 in dividing and fast growing cells and the presence of GPα1 in the vascular tissue and in the mesophyll cells suggest that it is involved in signaling in dividing/elongating cells, perhaps via the regulation of nutrient transport. The fact that GPα1 levels decrease in mature cells suggests that it might be involved in the regulation of cellular differentiation. Clearly, the suggested involvement of GPα1 in such important aspects of plant development indicates that GPα1 may have a very important and unique function. Further study of GPα1 will be required to elucidate its precise function and should provide insights into the relationship between signal transduction and growth and development of plants.

METHODS

Protein Gel Blot Analysis

Plant tissue was ground in buffer (0.1 M Tris, pH 8, 5 mM EDTA, 10 mM β-mercaptoethanol) and centrifuged at 8000*g* for 5 min. The pellet contained cell debris, which had no detectable GPα1 protein on the protein gel blot (data not shown). Thirty micrograms of total protein (measured using the Bio-Rad dye binding kit, based on the Bradford assay; Bradford, 1976) was separated on a 10% SDS–polyacrylamide gel, electroblotted onto a nitrocellulose membrane, and used for immunodetection. The polyclonal antiserum used in this study was a gift from P. A. Millner and was raised against a synthetic oligopeptide corresponding to the C terminus sequence of GPα1, the gene product of *GPA1*. The antibodies were visualized using a horseradish peroxidase–coupled donkey anti–rabbit serum and chemiluminescence (Enhanced chemiluminescence; Amersham).

Immunolocalization

Tissue was fixed in 3.7% formaldehyde, 5% acetic acid, and 50% ethanol. Fixed tissue was dehydrated with ethanol, cleared with xylene, and embedded in paraffin (Paraplast Plus; Oxford Labware, St. Louis, MO). Embedded tissue was sliced into 8-μm sections and placed onto slides coated with poly-L-lysine (Sigma). The sections were then dewaxed in xylene and rehydrated by passing through graded alcohols and rinsing in water. Blocking solution was applied to the sections for 10 to 20 min (TBS-T [30 mM Tris, pH 7.5, 37 mM NaCl, 0.5% Tween 80] with 10% dry milk). Antibody diluted at 1 part per 50 in blocking solution was added to the sections in a moist chamber for 1 to 2 hr. After three rinses of 5 min each in 50 mL of TBS-T, secondary antibody (donkey anti–rabbit antibody linked to alkaline phosphatase) at a dilution of 1 to 7500 was then added for 30 min to 1 hr in a moist chamber. After rinsing twice for 5 min in 50 mL of TBS-T and once for 5 min in 50 mL of TBS, the sections were ready for staining. The following were added to alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5): 0.66 mg/mL of nitro blue tetrazolium and 0.12 mg/mL of bromochloroindolyl phosphate. After the addition of the substrate to the slides (200 to 400 μL per slide), the color was allowed to develop at room temperature for ~30 min, and sections were mounted with an aqueous mounting medium and visualized by light microscopy.

GPA1-uidA Analysis

A translational fusion between a 4.1-kb HindIII *GPA1* genomic fragment (Ma et al., 1990) and the *Escherichia coli* reporter gene *uidA* (encoding β-glucuronidase [GUS]) was constructed by insertion of the fragment into the HindIII site of the binary vector pBI101.1 (Jefferson, 1987). The construct was introduced into Arabidopsis according to the method of Huang and Ma (1992), and histochemical staining with X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid [Sigma]) was performed essentially as described by Jefferson (1987).

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