Characterization of Arabidopsis Acid Phosphatase Promoter and Regulation of Acid Phosphatase Expression

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The expression and secretion of acid phosphatase (APase) was investigated in Indian mustard (*Brassica juncea* L. Czern.) plants using sensitive in vitro and activity gel assays. Phosphorus (P) starvation induced two APases in Indian mustard roots, only one of which was secreted. Northern-blot analysis indicated transcriptional regulation of APase expression. Polymerase chain reaction and Southern-blot analyses revealed two APase homologs in Indian mustard, whereas in Arabidopsis, only one APase homolog was detected. The Arabidopsis APase promoter region was cloned and fused to the β-glucuronidase (GUS) and green fluorescent protein (GFP) reporter genes. GUS expression was first evident in leaves of the P-starved Arabidopsis plants. In P-starved roots, the expression of GUS initiated in lateral root meristems followed by generalized expression throughout the root. GUS expression diminished with the addition of P to the medium. Expression of GFP in P-starved roots also initiated in the lateral root meristems and the recombinant GFP with the APase signal peptide was secreted by the roots into the medium. The APase promoter was specifically activated by low P levels. The removal of other essential elements or the addition of salicylic or jasmonic acids, known inducers of gene expression, did not activate the APase promoter. This novel APase promoter may be used as a plant-inducible gene expression system for the production of recombinant proteins and as a tool to study P metabolism in plants.

Simple environmental signals profoundly alter gene expression by interacting with the regulatory DNA sequences. Characterizing and cloning of these sequences provide genetic tools for regulating the expression of useful recombinant genes in plants. One of the most important, yet least available mineral nutrients for plant growth is phosphorus (P). It plays a vital role in energy transfer and metabolic regulation and is a component of many biological molecules, such as DNA and RNA. Consequently, P assimilation, storage, and metabolism are critical to plant growth and development (Duff et al., 1994). Plants respond to low levels of bioavailable P by increased root growth, higher expression of P-transporters (Muchhal et al., 1996; Leggewie et al., 1997; Liu et al., 1998; Burleigh and Harrison, 1999), and by alterations in metabolism including the induction of RNAses (Beriola et al., 1994). In addition, secretion of acid phosphatases from roots (APases; EC 3.1.3.2) is a notable consequence of P deficiency (Goldstein et al., 1988; Duff et al., 1991; Li and Tadano, 1996). The levels of induction of APase production and secretion in roots can be dramatic. Major increases of APase released from P-starved roots were demonstrated for various plant species (Ascencio, 1997). For example, under P-deficient conditions, the secretion of APase from lupine roots increased up to 20 times compared with the P-sufficient conditions (Tadano and Sakai, 1991), with large amounts of APase detected in soil surrounding the roots (Li et al., 1997). APase release was also observed in P-starved tissue cultures of the oilseed rape (Carswell et al., 1997) and in *Brassica nigra* cell cultures where increased APase activity was associated with the P-regulated de novo synthesis of the enzyme (Duff et al., 1991). Trull et al. (1997) reported that several APase isozymes were present in roots and shoots of Arabidopsis, but only a subset of these isozymes was induced by P stress.

The degree and magnitude to which APase gene expression is regulated by P levels prompted us to investigate the possibility that an APase promoter might be used to effectively regulate the expression of transgenes in plants by simple alteration in P fertilization. The ability to regulate recombinant gene expression with simple, cost effective, and environmentally safe stimuli is an important target of plant biotechnology. It allows the on-demand regulation of the transgenic traits only at times when their expression is needed, thus avoiding energetically wasteful and environmentally undesirable constitutive expression. Various methods of chemically regulating transgene expression in plants have been reported (for review, see Ayoma and Chua, 1997; Gatz, 1997). Cloned salicylic acid (SA)- and 2.6-dichloroisonicotinic acid-inducible promoters were successfully employed for regulating transgene expression, in spite of phytotoxicity problems associated with their use. Benzol(1, 2, 3) thiadiazole-7-cabothiotic acid *S*-methyl ester, was also an effective and relatively safe inducer of this promoter (Uknes et al., 1993; Friedrich et al., 1996; Lawton et al., 1996). Other promoters responsive to heavy metals (Gatz, 1997) such as copper

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(Mett et al., 1993, 1996; McKenzie et al., 1998) were cloned as well as others responsive to tetracycline (Gatz et al., 1992; Gossen and Bujard, 1992; Baron et al., 1997; Thompson and Myatt, 1997) and glucocorticoid (Aoyama and Chua, 1997). The wide utilization of these promoters may be hampered by the negative environmental effects of the induction molecules. The recently identified ethanol-inducible *alc* gene expression system (Caddick et al., 1998; Salter et al., 1998), which utilizes regulatory sequence domains from the cauliflower mosaic virus 35S promoter, has the potential to be both safe and cost effective.

Sensitive in vitro and activity gel assays developed for this work confirmed the induction of APase production and secretion from roots of hydroponically cultivated Indian mustard (*Brassica juncea)* plants in response to P-deficiency. The cloned APase promoter and signal sequences conferred specific P-inducibility onto β-glucuronidase (GUS) and the green fluorescent protein (GFP) reporter genes. This work elucidated the pattern of spatial and temporal expression of the APase gene in P-deficient plants and demonstrated that the APase promoter can be used to regulate the expression of transgenes in Arabidopsis. This work also provides molecular tools to study responses to P-deficiency and general P metabolism in plants.

RESULTS

Induction of APase in P-Deficient Indian Mustard Roots

Proteins secreted from Indian mustard roots grown in aseptic hydroponic medium supplemented with various P concentrations were analyzed for APase activity over time. The level of APase activity secreted from the roots of P-starved plants increased daily and was much greater in plants grown in lower P concentrations than in plants grown in higher P concentrations (Fig. 1). To further analyze APase induction under P starvation, extracted and secreted proteins were separated on SDS gel and renatured for the detection of APase activity (Fig. 2). At least two proteins with APase activities (42 and 84 kD) were observed in Indian mustard roots grown in 1 mM P and only one, which corresponded to a protein of 84 kD, was found to be secreted at the amounts just above the detection limits used for the assay (Fig. 2, lanes 1 and 2, respectively). Both activities greatly increased in P-starved roots, but only the 84-kD APase isoform was secreted in large amounts (Fig. 2, lanes 3 and 4, respectively). The pH optimum of the secreted APase activity was found to be 4.6 (data not shown).

Analysis of APase DNA and RNA in Indian Mustard and Arabidopsis

For our analyses we used available sequence information to further characterize the APase genes in

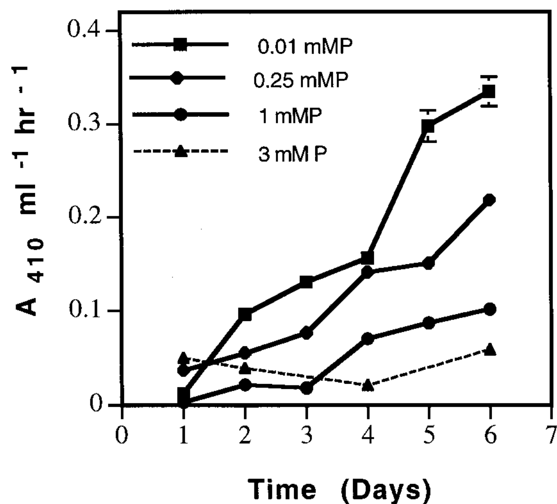

Figure 1. Time course of APase activity exuded from Indian mustard roots grown in sterile media containing 3, 1, 0.25, and 0.01 mM P. Each point is a mean of six replicates ± SE. The experiment was repeated three times with similar results.

Indian mustard and Arabidopsis. Because these plants are genetically related (both are the members of the Brassicaceae family), we utilized the genomic DNA sequence encoding a precursor of the Arabidopsis secreted APase as deposited by Patel et al. (1996) in the GenBank. We designed a set of four oligomers (21 bp) from the APase coding region, 568U, 608U, 1383L, and 1561L (see "Materials and Methods") to be used as primers for the amplification of the APase gene fragments. Two simultaneous PCR reactions were performed using Arabidopsis DNA as a template. The first contained the 568U upper primer and the 1561L lower primer and the second contained the 608U upper primer and the 1383L lower primer. Each of the PCR reactions resulted in only one product of 1,100 and 800 bp, respectively, as expected from the Arabidopsis APase sequence information.

The primers 608U and 1383L were used to amplify the APase sequence from Indian mustard DNA. This PCR reaction resulted in two fragments of about 1,000 and 900 bp (data not shown). These fragments were sequenced and showed high homology, 64% and 70% respectively, to the Arabidopsis APase gene, suggesting that the Indian mustard genome may contain two different APase genes. Southern-blot analysis of the Arabidopsis and Indian mustard DNA with the Arabidopsis APase gene as a probe revealed one signal in the Arabidopsis DNA and at least two strong signals in the Indian mustard DNA (Fig. 3). These results indicate that Arabidopsis has only one APase gene, whereas Indian mustard has at least two different APases genes in its amphidiploid genome. Northern-blot analysis using the Indian mustard 900-bp APase fragment as a probe was performed on mRNAs extracted from Indian mustard roots grown in low and high P concentrations for 5 d (Fig. 4). The size of the expressed APase mRNA was found to be

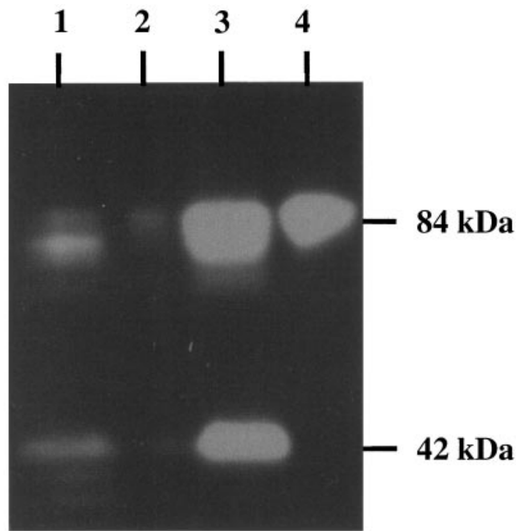

Figure 2. Activity-gel detection of APase exuded from Indian mustard roots. Lanes 1 and 3 contained 50 μg of root-extracted proteins; lanes 2 and 4 contained 5 μg of root-secreted proteins, renatured following their separation by SDS-PAGE. Lanes 1 and 2, Proteins produced after 9 d of normal fertilization conditions (1 mM P); lanes 3 and 4, proteins produced after 9 d of P starvation (0.01 mM P).

1,380 bp corresponding to a 46-kD protein precursor and consistent with the hypothesis that the secreted form of the Indian mustard APase is a homodimer. The gradual increase of APase mRNA in the P-starved roots versus no expression in P-sufficient roots is consistent with the transcriptional regulation of the APase gene by P.

Cloning the APase Promoter Region from Arabidopsis

The Southern-blot analyses and the PCR results suggested the existence of at least two DNA regions with high homology to the APase gene in the Indian mustard genome and only one APase gene in the Arabidopsis genome. Therefore we decided to clone the APase promoter and its signal peptide sequences from Arabidopsis. GenomeWalker libraries were prepared from Arabidopsis DNA using the Universal Genome Walker Kit (CLONTECH, Palo Alto, CA), and gene specific primers GSP1 and GSP2 (609L and 562L, respectively) were designed using the Arabidopsis APase gene sequence (see "Materials and Methods"). This procedure led to the cloning of three overlapping fragments (approximately 700; 1,100; and 1,300 bp) of the Arabidopsis APase promoter region (data not shown). These fragments were sequenced and the longest 5′ sequence upstream from the APase gene was analyzed for regulatory sequences and promoter-like elements (Fig. 5).

To study the tissue specificity of the APase promoter and to elucidate the spatial and temporal patterns of the APase expression in roots, two plant transformation vectors were constructed: APase promoter (Pr)-GUS, which contained the 1,300-bp promoter region fused to the GUS reporter gene and PS-GFP, in which the APase promoter and its signal peptide were fused to the GFP protein. Arabidopsis plants were transformed with these constructs and transformed T2 plants were used for further analysis.

Expression of GUS in Arabidopsis Plants Transformed with the Pr-GUS Construct

Arabidopsis plants containing the Pr-GUS construct were grown in sterile liquid 0.25× Hoagland medium for 14 d and thereafter transferred to the medium with or without P. The first signs of GUS expression appeared in the leaves of P-starved Arabidopsis plants after 3 to 5 d of P starvation. The expression of GUS in the roots of P-starved plants was first detected in the lateral root primordium (Fig. 6, A and B) and in the emerging lateral root meristems (LRM; Fig. 6C). Following 14 d of P starvation, high expression of GUS was found in young lateral root tips, but not in the apical root tip meristem (Fig. 6D). After 17 d of hydroponic cultivation, GUS expression was still the strongest in the meristematic regions of lateral roots (Fig. 6E), eventually spreading throughout the hydroponically-grown P-starved roots (data not shown). In seedlings germinated and grown in washed sand lacking P, strong GUS expression was evident in both the roots and shoots 10 d after sowing (Fig. 6H). In the roots of sand-grown seedlings, P-starvation induced GUS expression in vascular tissue soon after germination (data not shown) and within 10 d extended throughout most of the root tissue (except in the epidermal layer and root hairs; Fig. 6F). Transformed plants grown in sand or in hydroponic medium supplemented with 0.25× Hoagland solution containing 0.25 mM P showed almost no induction of GUS expression (Fig. 6G).

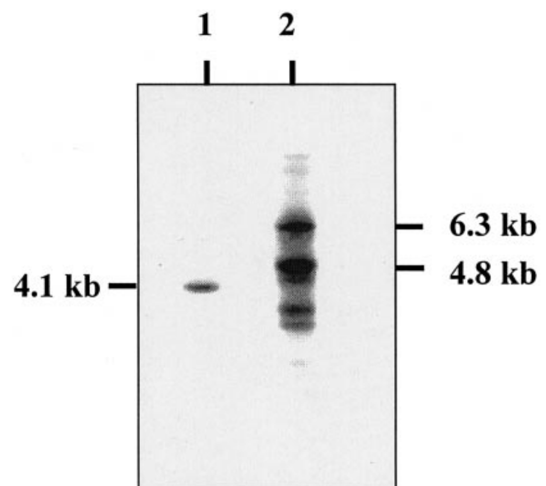

Figure 3. Southern-blot analysis of *Eco*RI-digested DNA using the Arabidopsis APase gene fragment as a probe. Lane 1, 5 μg of *Eco*RI digested Arabidopsis DNA. Lane 2, 10 μg of *Eco*RI-digested Indian mustard DNA.

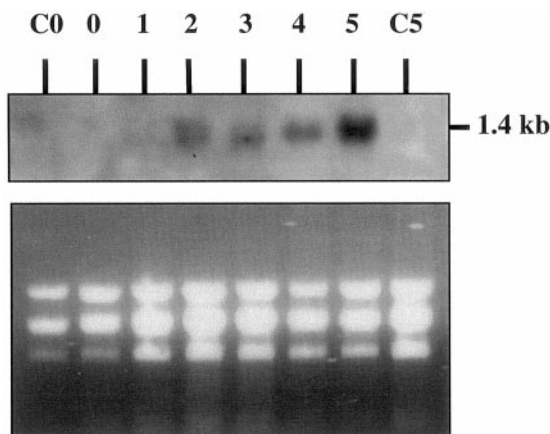

Figure 4. Northern-blot analysis of APase expression in P-starved Indian mustard roots using the Indian mustard 900-bp APase gene fragment as a probe. The size of the expressed APase mRNA was 1.4 kb (upper panel). The lower panel shows amounts of total RNA loaded on each lane. Lane C0, A control plant at d 0 grown in standard (1 mM) P concentration. Lanes 0 to 5, Plants grown in low (0.01 mM) P for 0 to 5 d, respectively. Lane C5, Control plant at d 5 grown in standard (1 mM P) concentration.

GUS expression in the P-starved plants cultivated in both systems diminished after the plants were fertilized with 0.25× Hoagland medium containing 0.25 mM P. Twelve out of 14 GUS-APase seedlings growing in sand culture showed visible GUS staining 2 d after fertilization. This number decreased to nine after 6 d and to three after 9 d.

Expression of GFP in Arabidopsis Plants Containing the APase Promoter/Signal Peptide-GFP (PS-GFP) Construct

Arabidopsis plants transformed with the construct containing the PS-GFP were grown in the hydroponic system as described above. Just as in the Pr-GUS plants, the expression of GFP in the roots of P-starved plants was first evident in the lateral root primordium (data not shown) and in emerging LRM (Fig. 7B). At the early stages of induction GFP was not expressed in the apical root meristems (ARM) indicated by the arrow (Fig. 7A). P-starvation induced not only GFP accumulation in roots, but also the secretion of GFP from roots into the medium, as evident from the bright green-blue fluorescence of the hydroponic medium after exposure to UV light (Fig. 7F). This was expected since in contrast to the Pr-GUS transformants PS-GFP transformed plants contained the APase promoter and its secretory signal peptide. No fluorescence was observed in the roots (Fig. 7D) or in the hydroponic medium of the non-transformed control plants (Fig. 7F).

Specificity of APase Promoter Induction

Pr-GUS transformants, germinated and grown in sand culture were used to test the specificity of the APase promoter induction by nutritional deficiencies other than P-limitation or by known chemical inducers of gene expression such as SA (Ward et al., 1991) and jasmonic acid (JA; Staswick, et al., 1991; Creelman et al., 1992; Tables I and II). Both chemical inducers were applied at concentrations capable of strongly inducing a variety of plant genes. These treatments were compared with the effects of P removal (Table I) or the addition of Man (Table II). Man is known to induce P deficiency in plant cells because of its ability to bind cellular phosphates as Man 6-phosphate (Watkins and Frenkel, 1987). Therefore Man-induced P-starvation can be used as a tool to study P metabolism and utilization in plant tissues (Herold and Lewis, 1977). GUS activity was strongly and consistently induced only in P-starved seedlings or in seedlings treated with Man, which had toxic effects at highest concentrations (50 mM). Monitoring

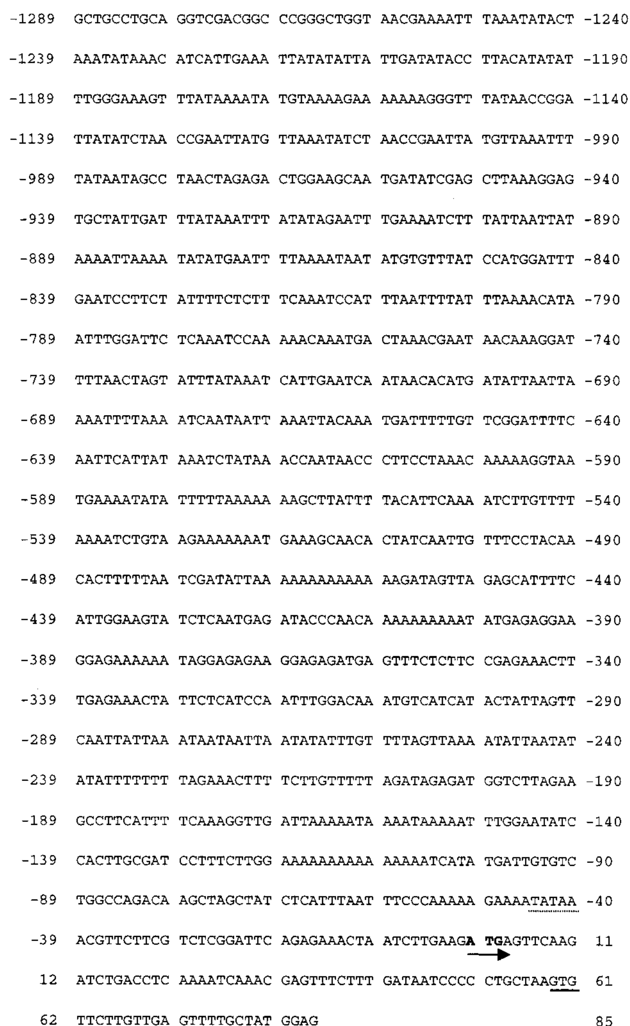

Figure 5. Arabidopsis APase promoter sequence. The putative TATA box is indicated by a dotted line, the ATG start codon is indicated by an arrow, and the position of the 562L primer (used in cloning the promoter) is underlined.

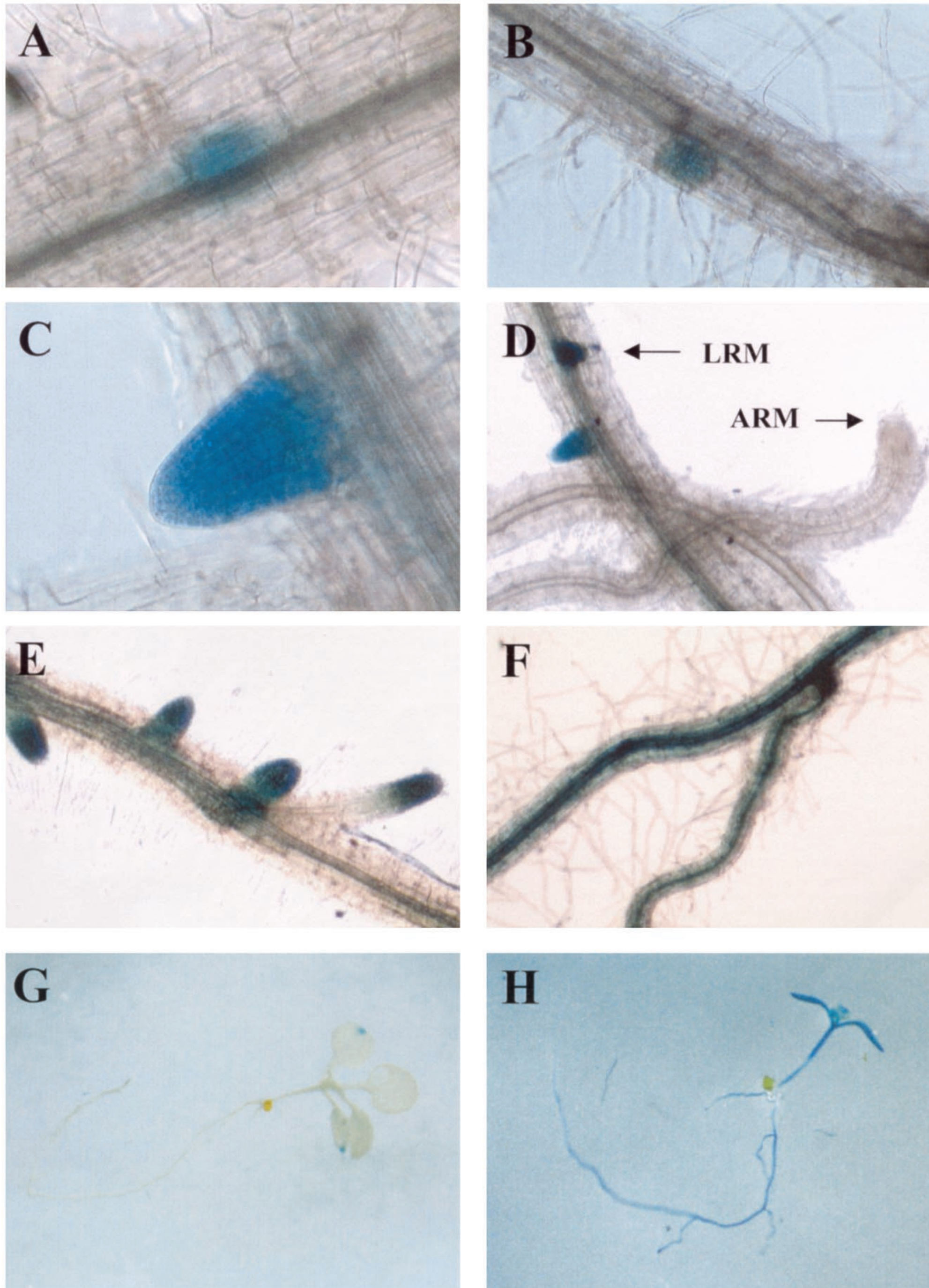

Figure 6. GUS expression in Arabidopsis plants transformed with the Pr-GUS construct. Plants were grown for 14 d under normal fertilization and then transferred to a hydroponic system lacking P for 14 (A–D) or 17 d (E). Plants were germinated in sand cultures fertilized with Hoagland solutions, with or without P, and stained after 10 d (F–G). A, Initiation of a lateral root primordium with localized expression (×200). B, Lateral root primordium (×100) showing expression in several cell layers. C, Emerging LRM (×200). D, Portion of root showing LRM and an ARM (×40). E, Root after 17 d in hydroponic system without P (×40). F, Roots after germination in sand cultures fertilized without P (×40). G, Ten-day-old seedling from a normally fertilized sand culture. H, Ten-day-old seedling from sand culture lacking P.

the spatial and temporal patterns of GUS expression in sand-grown Arabidopsis seedlings deprived of P revealed an initially strong induction in the apical meristem, approximately 5 d after sowing, followed by expression in the shoots, and lastly, after approximately 9 d, the expression in the roots, with the whole seedling expressing GUS at that time. The experiment was terminated 17 d after sowing be-

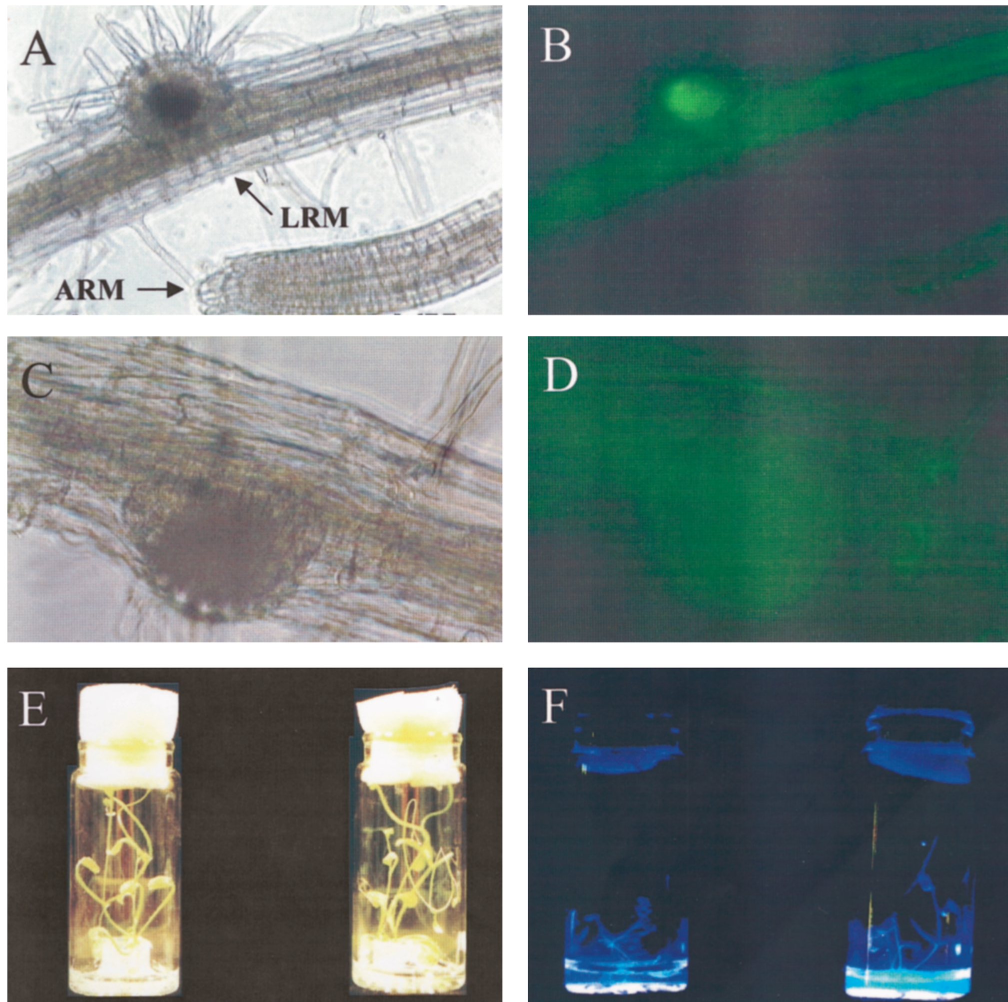

Figure 7. Expression of GFP in Arabidopsis plants transformed with the PS-GFP construct, which contains the APase promoter and signal peptide, fused to the GFP gene. Plants were grown for 14 d with P and then transferred to a hydroponic solution lacking P for 14 d. Wild-type plants grown under the same conditions served as controls. A, Light microscopy of PS-GFP transformed roots showing emerging LRM and ARM (×100). B, Fluorescence microscopy image of the roots shown in A (×100). C, Light microscopy of an emerging lateral meristem of the wild-type root (×200). D, Fluorescence microscopy image of the wild-type root shown in C (×200). E, Arabidopsis wild-type plant (left) and T2 PS-GFP transformed plant (right), grown in a hydroponic system. Photograph taken with normal light. F, Visualization of GFP secretion into the medium from the roots of transgenic Arabidopsis plant (right) and wild type (left) under UV illumination (same plants as in Fig. 7E photographed under UV light illumination). Seedlings were aseptically transferred into 20-mL glass vials containing 2 mL of hydroponic medium with or without P (see "Materials and Methods"). Photograph was taken after 5 d of incubation.

cause of the deterioration and death of the nutrient starved plants.

DISCUSSION

The ability to study temporal and spatial responses to P and other macronutrients in plants is often limited by the lack of appropriate molecular tools. Bioavailable P, which is critical to plant growth, is often present in limited amounts in soils. When plants have an adequate supply of P and/or absorb it at rates that exceed demand, P is usually stored in organic compounds (e.g. phytic acid) in the vacuoles or in the cytoplasm of the leaf cells (Lee and Ratcliffe, 1993; Schachtman et al., 1998). Plants usually absorb P from soil in the form of soluble orthophosphate anion (Pi, $H_2PO_4^-$, or HPO_4^{-2}), which is often present in limited amounts. In contrast, soils often contain large amounts of insoluble organic and mineral P compounds (Goldstein et al., 1987). One of the most profound responses of plants to P deficiency is the induction of APases capable of extracting inorganic phosphate from the organic compounds. We investigated the possibility of using this induction mechanism for regulating transgene expression in plants by simple alteration in P fertilization.

As expected, decreasing the concentrations of P in hydroponic medium caused progressively higher se-

Table I. *Specificity of APase promoter induction and nutrient deprivation*[a]

The experiment was repeated twice with similar results. For each treatment and sampling time 10 T2 seedlings that were the progeny of the GUS6 transformant T1 line were evaluated. Little variation in GUS expression was observed within each treatment and sampling time.

Days after Planting	Relative GUS Expression[b]				
	+P	−P	−N	−K	−Fe
7	−	++	−	−	−
9	−	+++	−	−	−
11	−	+++	−	−	−
13	−	+++	−	−	−
15	−	+++	−	−	−
17	−	+++	−	+−	+

[a] Arabidopsis seeds were germinated in washed sand watered with 0.25× Hoagland solution with elements indicated by − next to their chemical symbol (P, N, K, or Fe) removed from the nutrient solution at the beginning of the experiment (see "Materials and Methods"). +P indicates fertilization with complete Hoagland solution. [b] GUS expression was visually evaluated under the dissecting microscope and rated as +++ if the large part of the seedling turned blue, as ++ if only a small part of the seedling turned light blue, as + if any blue color was present in the seedlings, as +− if only hints of possible expression were detected, and as − if no GUS expression was detected in the seedling.

cretion of APase activity from Indian mustard roots. The gel activity staining technique we developed showed that at least two proteins with APase activities (42 and 84 kD) were observed in Indian mustard roots grown under P-sufficient conditions. Both activities greatly increased in P-starved roots, but only one APase isoform of 84 kD was secreted in large amounts. This observation may indicate that the secreted form of the enzyme is a homodimer made from two 42-kD subunits. This is consistent with the observation of Basha (1983), who purified a 240-kD APase from peanut seeds that was found to be composed of six identical 42.5-kD subunits. APases secreted by tomato and lupine roots were purified and characterized by Li and Tadano (1996) and were found to be of 68 and 72 kD, respectively. Both of these secreted enzymes were homodimers consisting of two identical subunits. The first plant APase gene identified in a nematode resistant tomato encoded a 25-kD peptide that formed a homodimer of about 51 kD (Williamson and Colwell, 1991).

Results of Southern-blot analysis indicated that Arabidopsis has only one APase gene, whereas Indian mustard has at least two different APase genes in its amphidiploid genome. Northern-blot analysis revealed the transcriptional regulation of the APase gene by the level of exogenous P. The size of the identified APase mRNA was 1.4 kb corresponding to a 46-kD protein, which was a likely precursor for the mature 42-kD APase monomer induced in the P-starved roots.

Since data suggest the existence of only one APase gene in the Arabidopsis genome with high homology to two APase genes in the Indian mustard genome, we chose to clone the APase promoter and its signal peptide sequences from Arabidopsis. To study tissue specificity and to elucidate the expression pattern of APase in roots, two plant transformation vectors were constructed: Pr-GUS, which contained the APase promoter region fused to GUS, and PS-GFP, in which the APase promoter and its signal peptide were fused to the GFP protein. Arabidopsis plants were transformed with these constructs and analyzed for transgene expression.

Observations from sand-grown Arabidopsis seedlings indicated that induction of APase expression in the apical meristem and leaves occurs before the induction in roots. Leaves of many plants store P in organic compounds such as phytic acid (Schachtman et al., 1998). One hypothesis is that before activating APase in roots to break down exogenous P-containing soil compounds, Arabidopsis plants strive to utilize endogenous P storage pools in the shoots via APase-mediated cleavage. The expression pattern of GUS in the roots in the early stages of P-starvation revealed strong induction of APase in developing lateral roots and in lateral root tips, but not in the apical root tip. We have also observed the increased lateral root initiation in Arabidopsis in low P-conditions compared with normally fertilized seedlings (data not shown). This increase was detected under hydroponic and soil cultivation. The influence of P deficiency on root architecture, which to a large extent confirms our observations, was summarized in Flores et al. 1997. Bonser et al. (1996) reported that P-efficient bean genotypes respond to low P availability by altering the gravitropism of basal roots so that the root system becomes shallower. In low-P soils, the P-efficient bean genotypes showed altered root growth angles and a shift of biomass allocation from basal roots to adventitious roots, which are diageotropic and thereby enhance

Table II. *Specificity of APase promoter induction and chemical induction*[a]

The experiment was repeated twice with similar results. For each treatment and sampling time 10 T2 seedlings that were the progeny of the GUS6 transformant T1 line were evaluated. Little variation in GUS expression was observed within each treatment and sampling time.

Days after Treatment	Relative GUS Expression[b]				
	SA	JA	MN	MN	MN
	100 μM		*5 mM*	*20 mM*	*50 mM*
1	−	−	−	+	++
3	+	−	−	+	+++
5	−	−	+	+	i[c]
7	+−	−	−	++	i

[a] Arabidopsis seeds were germinated in washed sand and fertilized with 0.25× Hoagland solution. SA, JA, or mannose (MN) at the indicated concentrations were gently sprayed onto the 14-d-old seedlings until run off and were added to the sand (concentrated solution to reach the same concentration in the sand solution as in the overhead spray). [b] GUS expression evaluated as in Table I. [c] Indicates that seedlings were too injured by treatment to be evaluated.

horizontal topsoil exploration (Lynch and Brown, 1998). Snapp et al. (1995) concluded that roots exploit high-P patches in low-P environments by increasing root branching specifically in those patches. In barley, lateral roots proliferated in nutrient patches (Drew, 1975). Stimulation of lateral root elongation in high-nitrate patches has also been found in Arabidopsis (Zhang and Forde, 1998). P-deficiency induced the development of clustered tertiary lateral roots (proteoid roots) in white lupine (Johnson et al., 1996). These roots synthesized increased amounts of phospho*enol*pyruvate carboxylase, which was transcriptionally regulated by P availability. Ribrioux et al. (1998) reported that when Arabidopsis was grown under low P, the primary root shortened and the lateral root growth increased, resulting in a shallower and wider root system. The high GUS expression directed by the APase promoter observed in lateral roots may serve as an efficient way for the plant to explore and use P pools in larger volumes of the soil. Arabidopsis plants may achieve this by enhanced lateral branching and increasing the APase expression and secretion from lateral roots. It is important that the GUS expression data indicate that the APase promoter is specifically induced by P-starvation and that is not a general stress promoter commonly found in plants (Tables I and II).

The expression of GFP in the roots of hydroponically-grown P-starved plants showed the same spatial and temporal expression pattern as found in the P-starved roots containing the Pr-GUS construct. However, P-starvation induced not only GFP accumulation in roots, but also the secretion of GFP from the roots into the hydroponic medium. This was expected, since in contrast to the Pr-GUS transformants, the PS-GFP transformed plants contained both the APase promoter and its secretory signal peptide. Secretion of GFP from roots of hydroponically grown tobacco plants was recently demonstrated by Borisjuk et al. (1999). The authors introduced a modified GFP variant that was fused to the ER signal peptide from calreticulin of *Nicotiana plumbaginifolia* into plants under the control of a strong root promoter. The secretion of GFP into the hydroponic medium was shown to be a result of a secretory process and not a result of passive protein leakage through the cell membranes or the consequence of cell breakage or sloughing off.

Our results provide interesting new information on APase and P biology while offering new molecular tools to study plant responses to P-deficiency. The data also indicate that the APase promoter can simply and effectively regulate the expression of transgenes in Arabidopsis, thus satisfying the following requirements for an effective inducible promoter: (a) It is responsive to a simple and environmentally benign chemical; (b) it can be up-regulated by lowering P levels and down-regulated by restoring P supply; (c) the basal level of expression is relatively low; (d) the induction occurs throughout the plant with the documented spatial and temporal kinetics; and (e) the induction is relatively strong, P-specific, and can possibly be made even stronger by future optimization. Further work, such as detailed promoter analysis, is still needed to maximize transgene expression using the APase promoter and to define the optimum conditions for turning its expression on and off. Nevertheless, the potentially slow response time of the APase promoter combined with difficulty of removing P from the root zone should impose limitations on its future use, particularly for soil-grown plants. However, the induction by Man as well as the potential utilization in hydroponic cultivation systems may still make the APase promoter practical in addition to its value as a research tool.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of Indian mustard (*Brassica juncea;* Raskin collection no. 426308) were germinated in Petri dishes on Gamborg's B-5 (LifeTechnologies-Gibco BRL, Grand Island, NY) agar medium and transferred to 20-mL vials containing one-quarter-strength (0.25×) Hoagland solution (Arnon and Hoagland, 1940). Plants were supported by slitted foam stoppers, which separated aseptically cultivated roots immersed in the sterile solution from shoots kept in the ambient air. Plants were grown on shakers (25 rpm), at 24°C under a 16-h photoperiod and 150 mE m^{-2} s^{-2} illumination provided by a mixture of fluorescent and incandescent lamps. After 10 d, plants were transferred into 125-mL flasks with 120 mL of 0.25× Hoagland solution containing 3, 1, 0.25, or 0.01 mM P (phosphate deficiency). To lower P concentration in the medium, $(NH_4)_2SO_4$ was substituted for $NH_4H_2PO_4$. The volume of medium in each flask was adjusted daily to 120 mL to compensate for transpiration and media sampling.

Determination of APase Activity in Hydroponic Solution

The hydroponic medium was sampled aseptically for chromogenic determination of APase activity and the flasks were refilled daily to the original volume. The activity of secreted APase in growth medium was determined using the substrate *p*-nitrophenyl-1-phosphate (Sigma, St. Louis) and 50 mM Na-acetate buffer pH 4.6 in a water bath at 37°C (Ascencio, 1997). The reaction was stopped after 1 hr with 1 M Na_2CO_3 solution and the formation of yellow *p*-nitrophenol was quantified at 410 nm in a spectrophotometer (DU 640, Beckman Instruments, Columbia, MD).

Activity Detection of APases after SDS-Gel Electrophoresis

For SDS-gel analysis, the hydroponic medium containing the secreted proteins was frozen, lyophilized, resuspended in a small volume of water, and dialyzed overnight

against 50 mM Na-acetate buffer, pH 4.6. Proteins were stored at −20°C until use. Soluble root proteins were extracted by homogenizing the root tissue in liquid nitrogen using a pestle and mortar. Na-acetate buffer at 0.1 M, pH 5.4 (1 mL per 2 g of tissue) was added and the tissue was extracted on ice for 15 min. The mixture was centrifuged at 10,000*g* for 30 min and the supernatant was collected and stored at −20°C until use. Protein concentration was determined as described by Bradford (1976) using a protein assay dye reagent (Bio-Rad, Hercules, CA) and bovine serum albumin as a protein standard.

Concentrated protein samples were resuspended in Laemmli buffer (Laemmli, 1970) without 2-mercaptoethanol and not boiled prior to loading. Proteins were separated by SDS-PAGE in 1.5-mm gels with 4% (w/v) acrylamide (stacking gel) and 10% (w/v) acrylamide (separating gel) in a protein electrophoresis cell (Mighty Small II, Hoefer Scientific Instruments, San Francisco). Enzymes were reactivated in situ by removing the SDS following the casein/EDTA procedure described by McGrew and Green (1990). After SDS removal, gels were washed four times using a 0.1 M Na-acetate buffer, pH 4.6. The substrate used for detection was 4-methylumbelliferyl-phosphate (M-8168, Sigma). APase hydrolyzes this compound, releasing fluorescent methylumbelliferone. Enzyme activity was detected by overlaying the gel with 300 μg/mL of 4-methylumbelliferyl-phosphate substrate in 100 mM Na-acetate, pH 4.6, mixed with 1% (w/v) low-melting-point agarose. The activity gels were viewed under UV light after a 1- to 10-min incubation with the substrate.

DNA and RNA Gel-Blot Analysis

DNA was isolated from Indian mustard and Arabidopsis plants using a plant DNA extraction kit (Phytopure, Nucleon Biosciences, Lancashire, UK). For Southern-blot analysis, DNA was digested by *Eco*RI, electrophoresed through 1% (w/v) agarose gel, and transferred to Hybond-N+ nucleic acid transfer membranes (Amersham, Piscataway, NJ). The 1,100-bp Arabidopsis APase probe was gel purified and radiolabeled with ^{32}P by random priming according to standard procedures (Sambrook et al., 1989). For northern-blot analysis, RNA was isolated from Indian mustard roots using the RNeasy plant mini kit (Qiagene, Valencia, CA), electrophoresed, and blotted according to manufacturer's instructions. The 900-bp Indian mustard APase probe was gel purified and radiolabeled with ^{32}P by random priming as described above.

Primer Design and PCR Amplification

A set of four 21-bp-long oligomers from the Arabidopsis APase coding region (Patel et al., 1996) was used as primers for the amplification of APase gene fragments. Two upper primers were synthesized: 568U (5′-TTGTTGAGTTTTGCTATGGAG-3′) starting at position 568, and 608U (5′-CAGAGGAAGTGATTTACCAGA-3′) starting at position 608. Two lower primers were also synthesized: 1383L (5′-TATCCCATCTATTGTTGTCGT-3′) starting at position 1,383, and 1561L (5′-ACGCCCTTTTGATGGAATACC-3′) starting at position 1,561. PCR reactions were performed using the GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA). DNA sequencing was performed with ABI 377 Dye-Terminator Cycle Sequencing at the Robert Wood Johnson Medical School DNA Synthesis and Sequencing Laboratory (Piscataway, NJ).

Promoter Identification and Cloning

GenomeWalker libraries were prepared from Arabidopsis using the Universal Genome Walker Kit (previously called Promoter Finder Kit) by digesting the DNA with five blunt-ended enzymes. The genomic fragments were then ligated to specific adapters provided in the kit, resulting in five "Genome Walker libraries." Two gene specific primers (GSP1 and GSP2) were designed using the Arabidopsis APase gene sequence: 609L (used as GSP1) 5′-TGTCATCTGGTAAATCACTTCCTCT-3′ and 562L (used as GSP2): 5′-CTCCATAGCAAAACTCAACAAGAACAC-3′. Cloning the APase promoter region was facilitated by using these primers for amplification of the APase 5′ regions as detailed in the Universal Genome Walker Kit user manual (CLONTECH). The fragments resulting from the PCR reactions performed on the Genome Walker libraries were sequenced, 5′ sequences upstream of the APase gene were analyzed for regulatory sequences and promoter-like elements, and the signal peptide sequence was identified. To clone the DNA fragment containing the APase promoter region into the binary plant transformation vector PB101.1, which contained a promoterless GUS cassette (CLONTECH), a new primer, 504BamL, was synthesized. This primer was designed to amplify the promoter region and to create a *Bam*HI restriction site at the 3′ end of the amplified fragment (the *Bam*HI site is indicated by the underlined bases): 5′-GGATCCATCTTCAAGATTAGTTTCTCT-3′. Another primer, AP2-*Sal*I, was designed to create a *Sal*I site at the 5′ end of the amplified fragment: 5′-TCATAAGTCGACACTATAGGGCACGCGTGGT-3′. These primers were used to amplify the promoter region. The resulting fragment was digested with *Bam*HI and *Sal*I, gel purified, and cloned into the respective sites on the PB101.1 plant transformation vector. The resulting plasmid Pr-GUS was used to transform Arabidopsis plants.

To fuse the APase promoter and its signal peptide in-frame with the GFP sequence, a primer corresponding to 10 amino acids downstream of the cleavage site of the mature secreted APase was synthesized. This primer, 609BamL (5′-GGATCCTCTGGTAAATCACTTCCTCT-3′), was designed to create a *Bam*HI restriction site in the 3′ end of the amplified fragment. This primer and the AP2*Sal* I primer were used to amplify the promoter region with the signal peptide. The resulting fragment was digested with *Bam*HI and *Sal*I, gel purified, and cloned into the pEGFP vector (CLONTECH). The plasmid was digested with *Sal*I and *Stu*I (a blunt-ended restriction enzyme) and the fragment containing the promoter, signal peptide, and the GFP gene, was gel purified. The PB101.1 plasmid was digested using *Sal*I and *EcoI*CR I (a blunt-ended restriction enzyme) and

the GUS gene was separated from the vector by electrophoresis. The resulting vector was gel purified and the fragment containing the APase promoter, its signal peptide, and the GFP gene, was ligated into the vector. The resulting plant transformation vector, designated PS-GFP, was used to transform Arabidopsis plants.

Arabidopsis Transformation and Selection

Arabidopsis (Columbia ecotype) plants were transformed using the *Agrobacterium* vacuum infiltration method (Bechtold et al., 1993; Bent et al., 1994) and grown in pots containing Premier Promix (Quebec, Canada) for seed production. The seeds were plated on agar-Murashige and Skoog (MS) medium (Sigma, St. Louis) containing 10 g/L Suc, supplemented with 500 mg/L cefotaxime and 100 mg/L kanamycin. Eleven independent GFP transformants (T1) and fourteen independent GUS transformants (T1) showing strong expression of the corresponding proteins were grown to seeds in the greenhouse. T2 seeds produced from these plants were germinated on phytagel (Sigma) plates containing MS medium with 10 g/L Suc, and transferred after 5 to 7 d into LifeRaft membrane rafts in culture boxes (107×107×96 mm high; Gibco-BRL) containing 150 mL of liquid MS medium with 10 g/L Suc. The plants were grown on shakers under the conditions described above. Seedlings were tested for neomycin phosphotransferase II expression using the PathoScreen kit (Agdia, Elkhart, IN) after 7 d. T2 plants showing expression of the neomycin phosphotransferase II protein were transferred into a hydroponic system consisting of a glass vial containing sterile liquid 0.25× Hoagland medium with 0.25 mM P or without P. Plants were supported by the Eppendorf tube with the cut off tip, with roots immersed in 2 mL of the sterile solution.

Screening for GUS Expression under the APase-Inducible Promoter

A screening method was developed to evaluate GUS expression levels in the various transformed lines: T2 seedlings were germinated in pots containing fine granules of sand (40–100 mesh; Fisher Scientific, Pittsburgh). The sand was previously washed to remove any trace contaminants by soaking it in 0.1 M HCl for 45 min and then washing it extensively with ddH_2O until sand solution reached pH 6.5. Seedlings were germinated in pots containing the sand and then fertilized using 0.25× Hoagland with 0.25 mM P or without P. Phosphate starvation symptoms, retardation of growth, and shoot reddening became evident 8 d after sowing, and 2 d later the seedlings were stained with 5-bromo-4-chloro-3-indolyl-β-glucuronic acid to visualize GUS expression.

Promoter Specificity Analyses

The APase promoter response to nutrient deficiencies and known gene activators was determined using the sand experimental system as described above. Seedlings were germinated in pots containing the washed sand, and then fertilized with modified 0.25× Hoagland solutions lacking nitrogen, potassium, or iron. Nitrogen deficiency was achieved by substituting $NH_4H_2PO_4$ with K_2PO_4, substituting $Ca(NO_3)_2$ with $CaSO_4$, and substituting KNO_3 with K_2SO_4. Potassium deficiency was achieved by substituting KNO_3 with $NaNO_3$. Iron deficiency was achieved by removing FeEDTA from the medium. Aqueous solutions of SA and JA or Man were gently sprayed onto the 14-d-old seedlings until run off and added to the sand at the time of spraying (approximately 20 mL of 2.5× concentrated solution to reach the same concentration in the sand solution as in the overhead spray).

Histochemistry and Microscopy

GUS activity was determined according to the method of Jefferson et al. (1987). After staining, tissue was fixed in 1.5% (w/v) formaldehyde, 4% (w/v) acetic acid:30% (w/v) ethanol, for 30 min. The tissue was dehydrated through a graded ethanol series (50%, 95%, and 100% [w/v] for 20 min each step), incubated for 2 h in 100% (w/v) ethanol, rehydrated through a graded ethanol series (95% and 50% [w/v] for 20 min each step), and kept in water. GFP and GUS visualization and documentation were performed using a microscope (Eclipse TE200 Inverted, Nikon, Tokyo). The images were captured by 3- charge-coupled device 24-bit using Optronics DEI-750CE software. GFP detection was performed using Chloma, VT single-band filter sets 31039 JP1 with D470/20 nm exciter, D510/20 nm emitter, and 490dc/p beamsplitter. GUS detection was performed using a halogen lamp. Nikon objective lenses specifications were: magnification ×10 with a numerical aperture of 0.25 and a phase contrast Ph1; and magnification ×20 with a numerical aperture of 0.45 and a phase contrast Ph1. Pictures of the Arabidopsis seedlings were taken with a 35 mm Nikon 4–401S camera.

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