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A Plant Plasma Membrane Proton-ATPase Gene Is Regulated by Development and Environment and Shows Signs of a Translational Regulation

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A proton-pumping ATPase is present in the plasma membrane of plant cells where it sustains transport-related functions. This enzyme is encoded by a family of genes that shows signs of both transcriptional and post-transcriptional regulation. The regulation of *pma1*, one of the *Nicotiana plumbaginifolia* H^+-ATPase genes, was characterized with the help of the β-glucuronidase (*gusA*) reporter gene in transgenic plants. *pma1* is active in the root epidermis, the stem cortex, and guard cells. This activity depends on developmental and growth conditions. For instance, *pma1* activity in guard cells was strongly enhanced when the plant material (young seedlings or mature leaves) was incubated in liquid growth medium. *pma1* is also expressed in several tissues of the reproductive organs where active transport is thought to occur but where scarcely any ATPase activity has been identified, namely in the tapetum, the pollen, the transmitting tissue, and the ovules. Several *pma* genes have a long 5′ untranslated region (leader sequence) containing an upstream open reading frame (URF). Analysis of translational and transcriptional fusions with *gusA* in transgenic plants suggests that the *pma1* leader sequence might activate translation of the main open reading frame, even though the URF is translated by a large majority of the scanning ribosomes. As confirmation, transient expression experiments showed that the *pma1* leader causes a fourfold post-transcriptional increase of main open reading frame expression. Deletion of the URF by site-directed mutagenesis stimulated the main open reading frame translation 2.7-fold in an in vitro translational assay. These results are consistent with a regulatory mechanism involving translation reinitiation. Altogether, they suggest a fine, multilevel regulation of H^+-ATPase activity in the plant.

INTRODUCTION

In plants and fungi, an ATPase is present in the plasma membrane where it pumps protons out of the cell. The resulting electrochemical proton gradient is the primary force driving the transport of ions and solutes across the plasma membrane. Plant H^+-ATPase is thought to play an important role in several aspects of plant physiology: nutrient uptake, loading of phloem, stomata opening, cell elongation, intracellular pH regulation, and salinity tolerance (for reviews, see Serrano, 1989; Sussman and Harper, 1989).

The molecular study of plant H^+-ATPase has shown that it is encoded by a multigene family: the *aha* (Arabidopsis H^+-ATPase) genes in Arabidopsis (Harper et al., 1989, 1990; Pardo and Serrano, 1989; Houlné and Boutry, 1994), the *lha* (*Lycopersicon* H^+-ATPase) genes in tomato (Ewing et al., 1990), and the *pma* (plasma membrane H^+-ATPase) genes in *Nicotiana plumbaginifolia* (Boutry et al., 1989; Perez et al., 1992; Moriau et al., 1993). All H^+-ATPase genes reported to date are transcribed. It is thus imaginable that the different genes or isozymes of a plant are specialized and function in different cells and tissues or at different metabolic states and under different environmental conditions. Differential transcriptional regulation of the H^+-ATPase genes between various organs has indeed been demonstrated by RNA gel blot hybridization (Ewing et al., 1990; Harper et al., 1990) and S1 nuclease mapping (Perez et al., 1992; Houlné and Boutry, 1994). Moreover, one gene of Arabidopsis (*aha3*) has been fully studied by the reporter gene technique and shown to be selectively active in the conductive vessels of the plant, in pollen, and in ovules (DeWitt et al., 1991).

Furthermore, the structure of the *N. plumbaginifolia pma* mRNAs suggests regulation at a post-transcriptional level (Perez et al., 1992): the mRNAs of *pma1* and *pma3* each have a long 5′ region upstream from the main open reading frame. This leader region is ~260 nucleotides long, whereas the predominant size for plant genes is 40 to 80 nucleotides (Joshi, 1987). The leader sequence contains an additional upstream open reading frame (URF) of 10 (*pma1*) or six (*pma3*) codons. A URF is also present in the leaders of *pma4* (*N. plumbaginifolia*),

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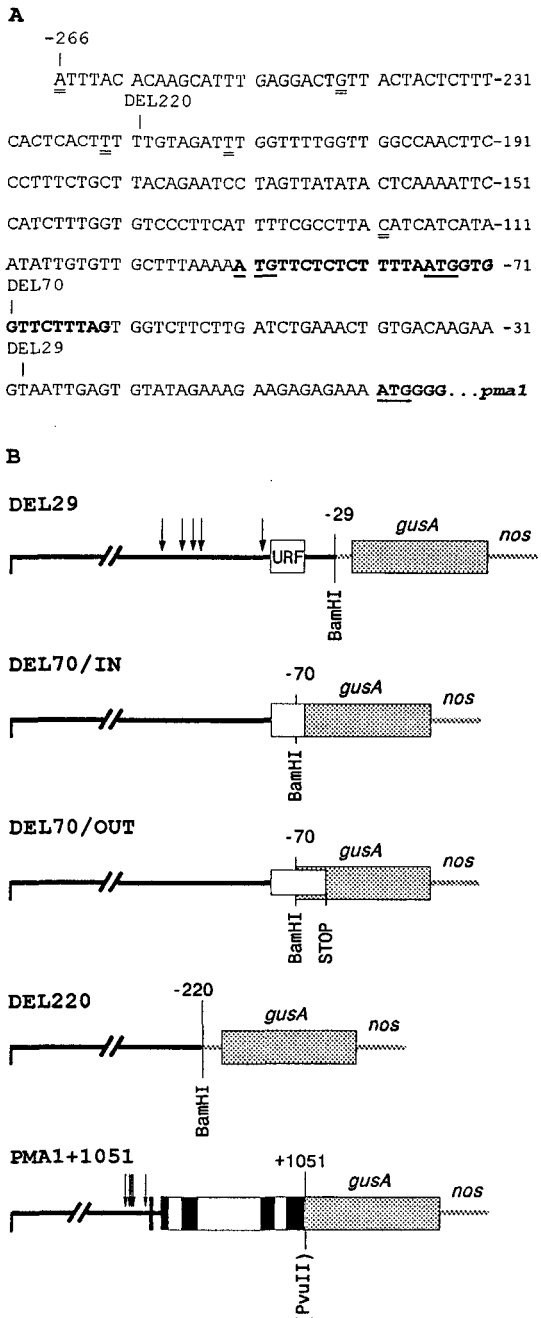

Figure 1. Sequence of the Deletions Made in the Leader of *pma1* and the *pma1–gusA* Constructs Introduced into *N. tabacum*.

(A) Nucleotide sequence of the leader of *pma1* and positions of the deletions selected to make the *pma1–gusA* constructs. The deletions are in pBluescript KS+. In the 5′ direction, they extend to 2300 nucleotides upstream from the ATG of *pma1* and end with a BamHI linker at the 3′ end for subsequent cloning. Double-underlined nucleotides are the 5′ ends of the *pma1* mRNA that were detected by S1 nuclease mapping (Perez et al., 1992). Boldface characters indicate open reading frames, and the ATG codons are underlined.

lha1 (tomato), and *aha2* (Arabidopsis). Long leaders containing one or more URFs are commonly found in genes whose regulation has to be tightly controlled, for example, protooncogenes, transcription factor genes, and viral genes (reviewed in Kozak, 1991). It is thus intriguing to find these features in the plant plasma membrane H^+-ATPase genes.

Here, we use the reporter gene technique to demonstrate the cell-specific expression of one of the *N. plumbaginifolia pma* genes, namely *pma1*. This gene is expressed in cells and tissues involved in active nutrient transport, such as root epidermal cells and reproductive tissues. The *pma1* gene is also expressed in guard cells, where an H^+-ATPase is known to be involved in stomata opening. We further show that the *pma1* URF is efficiently translated and that the *pma1* leader sequence affects reporter gene translation.

RESULTS

Tissue- and Cell-Specific Expression of *pma1*

The plasma membrane H^+-ATPase of *N. plumbaginifolia* is encoded by a multigene family. To characterize the cell-specific expression of a single *pma* gene, we fused the 2.3-kb DNA fragment lying directly upstream from the *pma1* coding region with the *gusA* reporter gene (Jefferson et al., 1987) and introduced this construct into the closely related species *N. tabacum* by transformation with Agrobacterium. The reporter gene encodes a β-glucuronidase (GUS), which can easily be assayed and located by histochemistry in transgenic plants.

The leader region of the *pma1* mRNA shows unusual features, and the corresponding DNA region (displayed in Figure 1A) was therefore included in our construct (construct DEL29, Figure 1B). In such a construct, *gusA* should undergo the same transcriptional and translational regulation as *pma1*. However, we cannot exclude that some other *pma1* sequences (e.g., within or downstream from the transcribed region) not found in construct DEL29 affect *pma1* expression as well. Eighteen primary transformants produced viable seed after self-

(B) Schematic representation of the *pma1–gusA* constructs. The arrows indicate the mRNA 5′ ends. The boxes indicate open reading frames. In the DEL29 construct, *gusA* is under the control of the promoter and leader of *pma1*. The DEL70/IN and DEL70/OUT constructs are translational fusions between the URF of *pma1* and *gusA*. In construct DEL70/IN, the URF and *gusA* are in the same reading frame, whereas in the DEL70/OUT construct, they are out of frame. In the latter case, the URF is prolonged to a stop codon downstream from the *gusA* ATG (see Methods). Construct DEL220 results from transcriptional fusion between the promoter of *pma1* and *gusA*. Most of the leader of *pma1* is absent. Construct PMA1+1051 is a translational fusion; black boxes are exons; white boxes, introns. *nos*, 3′ untranslated sequence of the nopaline synthase gene. The diagrams are not drawn to scale.

pollination. In 13 of these F_1 generation plants (DEL29 plants, Figure 2), we observed above-background GUS activity. The activity level varied among the transgenic plants. This is typically observed with ectopic genes and probably reflects the quantitative influence of the genetic environment of the position in which the insertion occurred.

We analyzed the expression of *gusA* by histochemistry in 10 of these plants and consistently obtained the results shown in Figures 3 and 4, where blue coloration indicates the presence of GUS and thus reflects expression of *pma1.* In young seedlings grown on agar, we found *gusA* expressed at the border of the cotyledons and in root epidermal cells in the absorbing region of the root or the maturation zone where the root hairs elongate (Figure 3A). When the plantlets were grown in liquid medium without sucrose, the pattern of expression of *gusA* surprisingly changed: activity was found in the guard cells of the cotyledons and often in those of the stem as well (Figure 3B). Expression in the roots was lost, but expression at the border of the cotyledons was retained. In liquid medium, plantlets possibly take up nutrients via their cotyledons rather than through their roots, which do not develop under such conditions. This argues in favor of *pma1* being expressed in the absorbing or feeding part of the plant. Our observations further show that *pma1* expression is environmentally regulated. GUS-specific activity in plants grown in liquid was ~20 times higher than in plants of the same size grown on agar (result not shown).

In larger plants grown in vitro, activity was detected in guard cells only in the cotyledons and leaves of the most active plants, especially when there was no sucrose in the growth medium (results not shown). When plants were grown in soil, only the activity at the border of the leaves was observed. This activity was restricted to the parenchyma and was not associated with dividing cells. Interestingly, when leaves were incubated during 8 hr in water, GUS expression was induced (Figure 3C) but was still restricted to the border of the blade and clearly appeared in guard cells (insert of Figure 3C). Incubation of leaves in a nutritive solution resulted in a still stronger histochemical staining (result not shown).

At a later developmental stage, *gusA* expression was detected in the anthers of floral buds (Figures 3D and 3E, small and big buds). This activity, which appears already in 3-mm-long buds, was restricted to the single layer of cells that feeds the development of microspores, namely the tapetum (see Figures 3F and 3G for a detailed view). This tissue degenerates during pollen maturation, and GUS activity was then found only in the pollen grains (Figure 3H). To avoid artifactual staining (Mascarenhas and Hamilton, 1992), the pollen was incubated in the absence of any other tissue.

Mature pollen grains may eventually land on the stigma of a flower where they will germinate and grow a pollen tube to fertilize the ovules. The rapid growth of the pollen tubes is sustained by nutrients provided by the transmitting tissue, the core of the pistil through which they grow. The cells surrounding the transmitting tissue showed marked GUS activity, and the transmitting tissue itself showed some activity (Figures 4A and

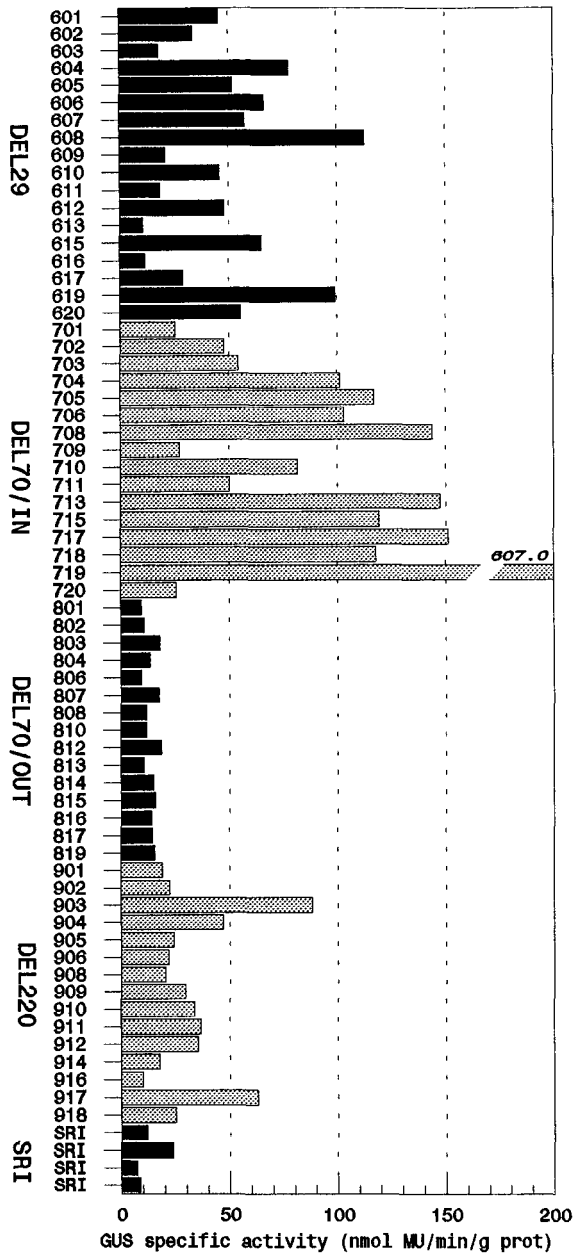

Figure 2. GUS Activity Measured in F_1 Seedlings of the Different *pma1–gusA* Transformants.

Three 8-day-old kanamycin-resistant plantlets per primary transformant were ground together, and GUS activity was measured in the mixture by fluorometry. Plants are grouped according to the construct they contain, as given in Figure 1. SR1 is the *N. tabacum* cultivar that was transformed. SR1 plantlets were grown on a medium without kanamycin, and their GUS activity constitutes the background activity. All measurements were done with four time points and had a linear regression coefficient exceeding 0.99. GUS activity is given in nanomoles of 4-methylumbelliferone per minute per gram of protein. The broken column indicates that activity of plant 719 (607 units) goes beyond the maximum of the graph.

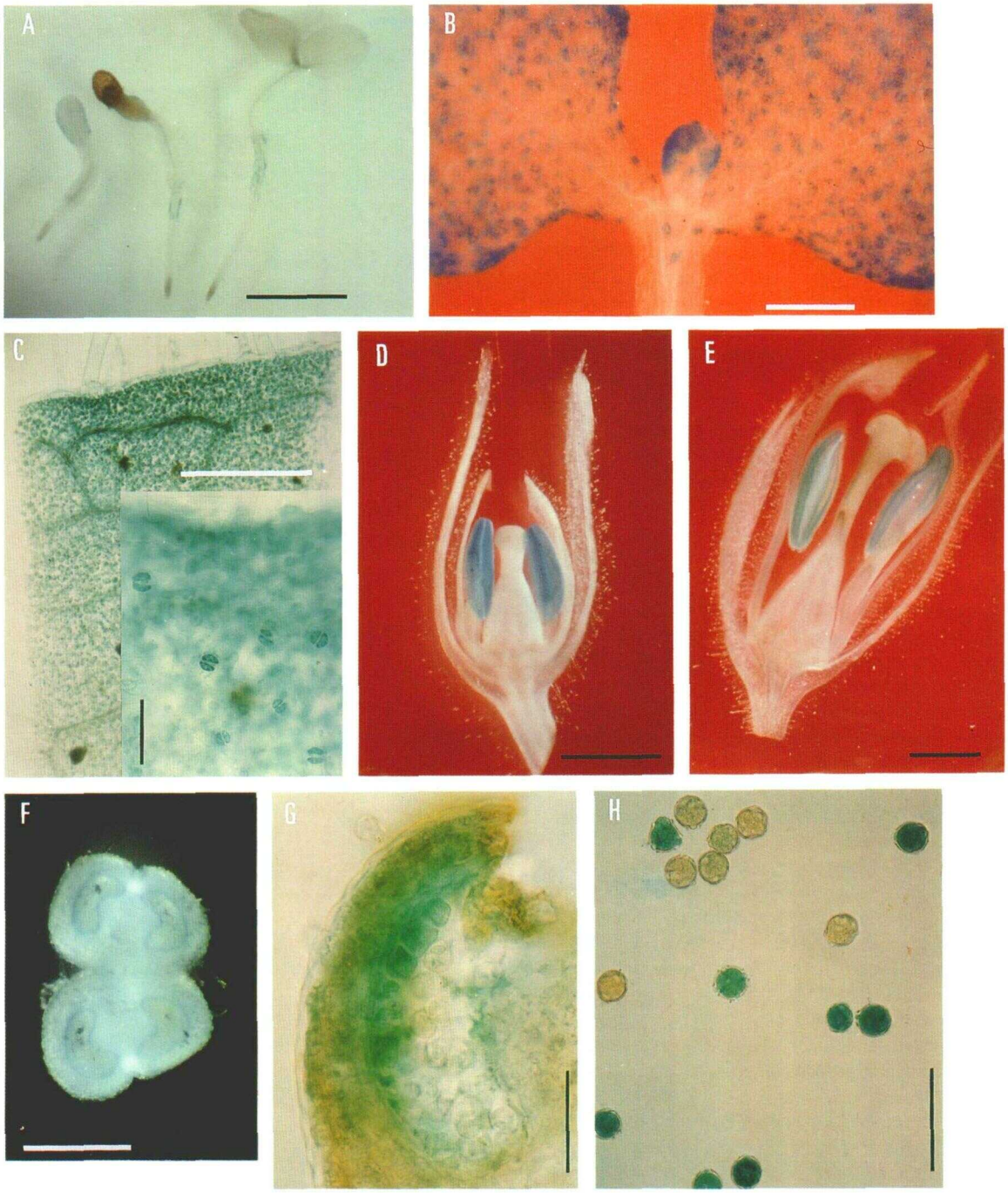

Figure 3. Histochemical Localization of GUS in Transgenic Plants Containing a *pma1–gusA* Construct.

Plant sections were incubated at 37°C for 15 to 24 hr in the presence of 1 mM X-gluc. The blue color reveals the presence of GUS. Plants containing the DEL70/IN construct (Figure 1) are shown in **(A)** and **(C)**. Plants containing the DEL29 construct are shown in **(B)**, **(D)**, **(E)**, **(F)**, **(G)**, and **(H)**.

(A) Young seedlings. GUS activity is seen at the border of the cotyledons and in the epidermis of the actively transporting region of the root.

(B) Plant grown in liquid medium. GUS activity is seen in the guard cells of the cotyledons and in part of the stem, at the border of the cotyledons, and in the young leaf.

(C) Transverse section of a leaf taken from a plant grown in soil and incubated in water for 8 hr. GUS activity was observed in the parenchyma at the limb margin and, under these conditions, in guard cells, as shown in the inset.

(D) and **(E)** Longitudinal sections of floral buds at two developmental stages. GUS activity is seen in the anthers.

(F) and **(G)** Cross-sections of anthers. GUS activity is restricted to the tapetum.

(H) Mature pollen grains.

White bars = 0.5 mm; black vertical bars = 0.1 mm; black horizontal bars = 2 mm.

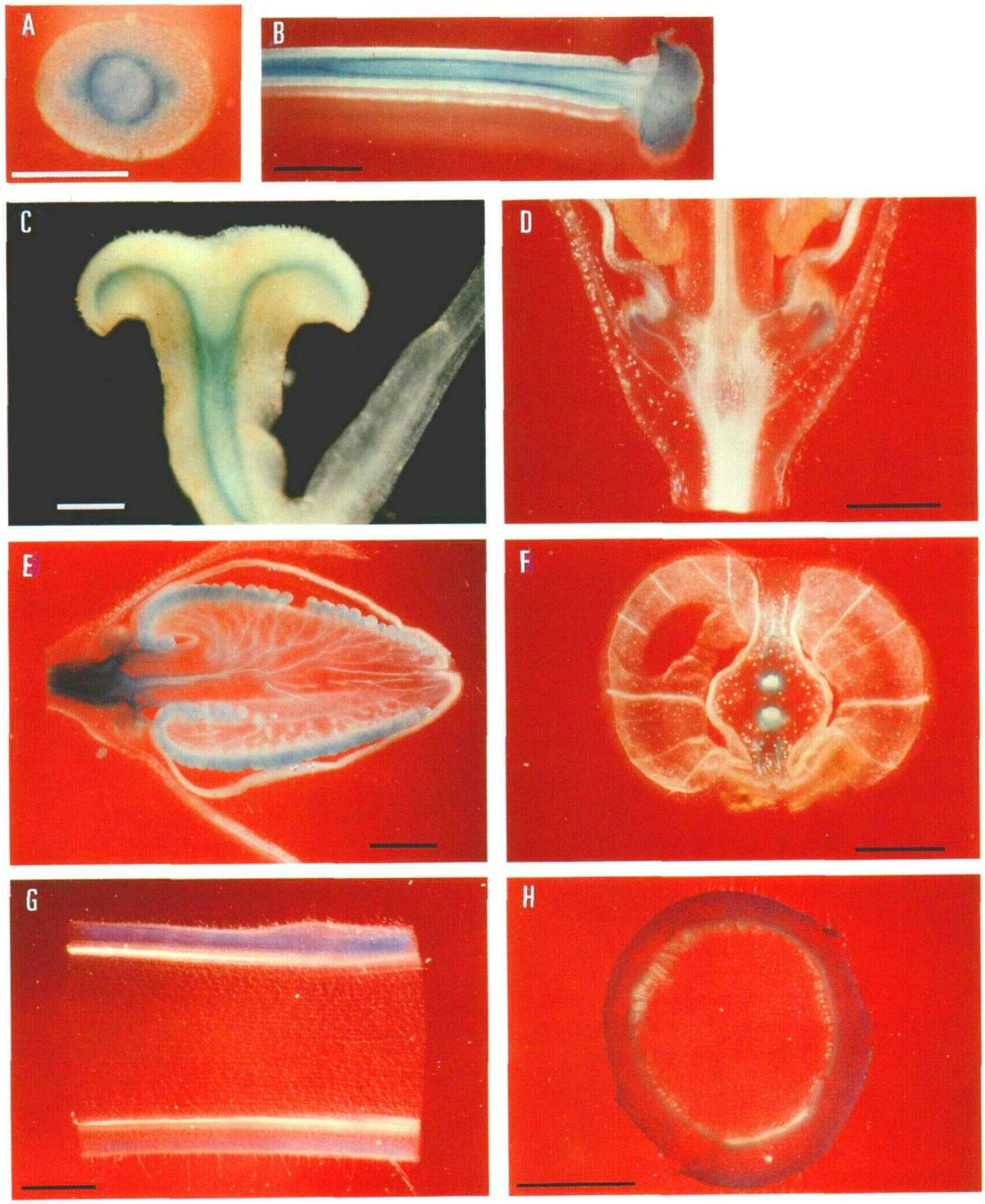

Figure 4. Histochemical Localization of GUS in Transgenic Plants Containing a *pma1–gusA* Construct.

Plant sections were incubated as given in Figure 3. Plants containing the DEL70/IN construct (Figure 1) are shown in **(C)** and **(F)**. Plants containing the DEL29 construct are shown in **(A)**, **(B)**, **(D)**, **(E)**, **(G)**, and **(H)**.

(A) Cross-section of a pistil after flower anthesis. GUS activity is seen mainly in the cells surrounding the transmitting tissue but also in the bulk of that tissue and in the conductive tissues.

(B) Longitudinal section of a pistil after flower anthesis. GUS activity is as shown in **(A)** but also occurs in the stigma and not in the conductive tissues.

(C) Longitudinal section of a pistil of a floral bud. GUS activity is seen only in the cells surrounding the transmitting tissue.

(D) and **(E)** Longitudinal sections of developing fruits. GUS activity is seen in the abscission zone of the corolla **(D)** or in the ovules and vascular tissues of the placenta, particularly at the base of the placenta and fruit.

(F) Cross-section of the lower part of a fruit. GUS activity is seen in the phloem.

(G) and **(H)** Longitudinal and cross-sections of stems. GUS activity is seen in the cortex of both cross-sections; only in **(H)** can GUS activity be detected in the epidermis. **(H)** shows a thin section cut from a 5-mm-thick section after histochemical staining.

White bars = 0.5 mm; black bars = 2 mm.

4B). Does activity in the transmitting tissue originate from the growing pollen tubes or exclusively from the maternal tissue? In the most active transgenic plants, activity can already be seen before anthesis (flower opening) when the pollen is still imprisoned in the stamens. But GUS activity is then found only in the layer of cells surrounding the transmitting tissue (Figure 4C), suggesting that the transmitting tissue itself might not express GUS.

When a wild-type tobacco (SR1) flower was fertilized by transgenic pollen, low GUS activity was observed in the transmitting tissue (result not shown), whereas above-background activity was measured in style extracts: the GUS-specific activity is 26 units (nanomoles of 4-methylumbelliferone per minute per gram of protein) for self-fertilized SR1 and 74 and 43 units when fertilized by pollen of plant 619 (DEL29) or plant 904 (DEL220, discussed below). Together, these results show that there is some activity in the pollen tubes. This somewhat contradicts the results of Obermeyer et al. (1992) who immunolocalized H^+-ATPase in the plasma membrane of *Lilium longiflorum* pollen grains and pollen tubes and found it absent or sparsely distributed in the pollen tubes. However, as we will discuss later, it is not known whether their monoclonal antibody recognizes all the H^+-ATPase isoforms. Furthermore, we also detected a higher signal in pollen grains than in tubes.

We observed a lower consistency for the histochemical staining of fruits than for that of other organs. Only half of the plants displaying GUS activity showed staining in the fruit. Stain can be found in the abscission area of the corolla or in the ovules and the conductive tissues at the base of the ovary (Figures 4D and 4E). A cross-section of a fruit showing activity in the conductive tissues (Figure 4F) suggests that the activity is restricted to the phloem. This was confirmed by the fact that the blue stain coincided with the fluorescence of a callose-specific fluorochrome, that is, alkaline discolored aniline blue (Eschrich and Currier, 1964; L. Waterkeyn, personal communication).

All the other plants discussed below and exhibiting above-background activity in Figure 2 showed the same profile of GUS expression as that reported above for DEL29 plants.

The validity of our histochemical analysis is substantiated by the GUS-specific activities measured in a series of organs and cell types as detailed in Table 1 for two transgenic plants. However, a striking discrepancy between quantitative assays in stems and histochemical analysis was noted. A clear activity was measured in stems, but we failed to detect any staining in hand-cut cross-sections. We surmised that the shape of the stem cells could be responsible for this: being very long, most of them might lose part of their cytoplasm upon sectioning. And indeed, histochemical analysis of longitudinal sections and of 5-mm-thick cross-sections revealed strong staining of the stem cortex (Figures 4G and 4H) and, in cross-sections only, of the epidermis. The activity in the cortex follows a gradient, which is higher in the outer cell layers. This resembles the gradient of activity observed in leaf parenchyma.

Table 1. GUS-Specific Activity in Two Typical *pma1–gusA* Transgenic Plants and in a Nontransformed SR1 Plant[a]

Plant Material	Plant 608[b]	Plant 903[c]	SR1
Limb border	224.1	30.0	2.2
Limb center	28.2	4.7	2.6
Stem	356.8	42.8	11.2
Anthers from 10- to 12-mm buds	149.6	86.5	9.7
Ovaries from 10- to 12-mm buds	16.1	4.2	5.5
Corolla	ND[d]	4.5	4.4
Pollen	811.6	214.2	9.7

[a] GUS activity is given in nanomoles of 4-methylumbelliferone per minute per gram of protein. Activity was measured by fluorometry at four time points.
[b] Plant 608 contains the DEL29 construct.
[c] Plant 903 contains the DEL220 construct.
[d] ND, not determined.

The *pma1* Leader and URF Affect GUS Expression in Transgenic Plants

The mRNA of *pma1* has a leader sequence with several 5′ boundaries extending from −120 to −266 nucleotides of *pma1* initiation codon. The leader contains a short URF (Perez et al., 1992). Several other *N. plumbaginifolia*, tomato, and Arabidopsis H^+-ATPase genes also have a URF and a leader sequence longer than those usually found in plants. To study the possible post-transcriptional regulation exerted by the leader of *pma1*, we prepared three deleted forms of this leader sequence. They were fused with *gusA*, yielding the constructs DEL29, DEL70/IN, DEL70/OUT, and DEL220 shown in Figure 1B. Positions of deletions are given with respect to the *pma1* initiation codon. Plants containing DEL220 expressed a *gusA* mRNA having a leader composed of the first 46 nucleotides of the longest leader of *pma1* plus 23 nucleotides from the BamHI linker to the ATG of *gusA*. Plants containing DEL29, however, expressed *gusA* under the control of both the promoter and leader sequence of *pma1*. By comparison with plants containing DEL220, they should reveal post-transcriptional regulatory properties of the leader sequence and URF. DEL70/IN is an in-frame fusion between the URF (seven of nine residues) and GUS. DEL70/OUT is identical to the former except for two additional nucleotides leading to an out-of-frame fusion. These constructs were introduced into *N. tabacum*, and GUS activity was measured in the F_1 seedlings (Figure 2). Both DEL29 and DEL220 plants produced above-background GUS activity. The activity varied considerably between different plants of the same series, as is usual for transgenic plants. Under all other conditions tested, whether GUS was measured directly or detected histochemically, the observed behavior of plants containing DEL29 and DEL220 was qualitatively identical, although DEL29 plants were more active than DEL220 plants. Table 2 summarizes the results of an experiment in which *gusA* mRNA concentration was measured together with GUS activity in a series of transgenic plants. It suggests that

Table 2. *gusA* mRNA Concentration and GUS Activity in *pma1–gusA* Transgenic Plants

Construct	Plant	*gusA* mRNA[a]	GUS[b]	mRNA Strength[c]
DEL29	605	34	14.9	0.44
	606	28	30.6	1.09
	608	21	135	6.43
Mean ± SD				2.65 ± 2.68
DEL70/IN	705	77	77.7	1.01
	708	33	115	3.48
	719	100	585	5.85
Mean ± SD				3.45 ± 1.98
DEL70/OUT	803	92	6.4	0.07
	806	20	3.4	0.17
	807	33	3.2	0.10
	808	24	2.6	0.11
Mean ± SD				0.11 ± 0.04
DEL220	903	48	35.5	0.74
	904	52	24.2	0.47
	917	73	28.6	0.39
	918	48	60.1	1.25
Mean ± SD				0.71 ± 0.34
PMA1 + 1051	1014	9	ND[d]	
	1015	89	ND	
	1016	93	ND	
	1017	84	ND	

[a] *gusA* RNA signal density divided by *atp2-1* RNA signal density. These values are expressed in the percentage of the value obtained with plant 719 to correct for variations between blots (see Methods).
[b] GUS-specific activity (nanomoles of 4-methylumbelliferone per minute per gram of protein) minus background measured on untransformed plants.
[c] GUS activity divided by *gusA* mRNA. The higher this value is for an mRNA, the better it is translated.
[d] ND, not determined.

the DEL29 and DEL70/IN mRNAs are at least as well translated as the DEL220 mRNA. Thus, the leader of *pma1* with its URF does not inhibit translation of a downstream open reading frame, even though the ribosomes do efficiently initiate translation at the URF, as shown by the results obtained with the DEL70/IN and DEL70/OUT plants.

DEL70/IN plants contain *gusA* in frame with the two sets of ATG codons of the URF (see Methods for sequence details). Any GUS activity measured in these plants ensues from initiation at either of these ATGs and at the ATG of *gusA*. In DEL70/OUT plants, where the ATG of *gusA* is not in frame with those of the URF, all the activity measured will reflect bypassing of the URF ATGs and initiation at the ATG of *gusA*. Figure 2 shows that there is high GUS activity in DEL70/IN plants and none at all in DEL70/OUT plants. As shown in Table 2, DEL70/IN and DEL70/OUT plants contain comparable levels of *gusA* mRNA. The GUS reading frame of DEL70/OUT mRNA is thus very poorly translated in comparison to the other mRNAs studied. Altogether, this implies that a vast majority if not all of the scanning ribosomes initiate translation at one of the two sets of ATG codons of the URF. The translation of the URF is then seemingly followed by that of the main open reading frame, presumably after translation reinitiation.

Figure 1B also shows construct PMA1+1051, which was designed to study possible effects on expression exerted by introns 1 and 2 of *pma1*. Plants transformed with this construct did not show any GUS activity (results not shown). Considering that these plants contain *gusA* mRNA (Table 2), it seems probable that the corresponding chimeric PMA1–GUS protein containing two putative transmembrane spanning domains of PMA1 has no glucuronidase activity. However, the presence of this protein remains to be shown in the transgenic plants.

The *pma1* Leader Stimulates GUS Expression in Protoplasts

To thoroughly and quantitatively study the effects of the leader and URF on translation, we switched to another experimental system: transient expression in tobacco protoplasts. The first reason for using transient expression assays is because GUS is a stable enzyme that accumulates over time in transgenic plants. This may well hide a temporary or fluctuating translational effect. The second reason is that quantitative comparisons of different constructs are difficult in stably transformed plants because of transgene expression variability. In contrast, transient expression assays reflect the average activity of a large population of independently transformed cells, and various constructs can be compared if a second gene is used as an internal control.

As transformation efficiency is low for protoplasts, the *pma1* promoter probably would have been too weak for our assays. Therefore, we used the cauliflower mosaic virus (CaMV) 35S strong transcription promoter to yield the constructs represented in Figure 5. Construct 35S–GUS is a control giving rise to an mRNA with a 41-nucleotide-long leader. Construct 35S–L–GUS has the leader of *pma1* cloned between the 35S promoter and *gusA*; this leader is 299 nucleotides long. The first, second, or both ATGs of the URF were mutated to obtain the 35S–LΔ1–GUS, 35S–LΔ2–GUS, and 35S–LΔ12–GUS constructs. Figure 6 shows the alignment of the leader sequences of *pma1*, the 35S–GUS, and the 35S–L–GUS constructs.

These five constructs were separately introduced by electroporation into tobacco leaf protoplasts together with a chloramphenicol acetyltransferase gene (*cat*) used as an internal control of transformation efficiency (35S–CAT, Figure 5). Table 3 summarizes the results obtained. GUS activity was increased four times by the leader of *pma1*. This shows that in tobacco protoplasts the leader of *pma1* enhances expression of a downstream gene. GUS activity barely increased when the first or second ATG was mutated separately, but mutation of both ATGs (there is no URF anymore) led to a further 57% increase in GUS activity. This suggests that the URF is

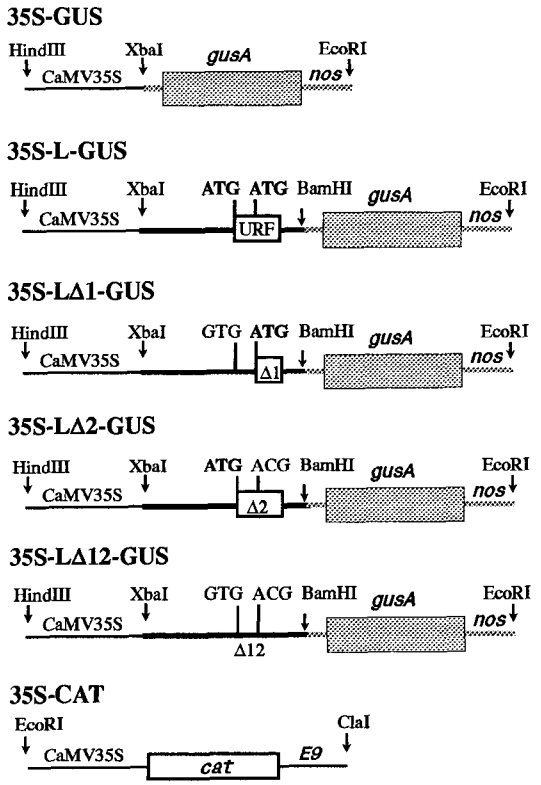

Figure 5. Constructs Used in Transient Expression Experiments.

All the *gusA* constructs are in pTZ19U (Genescribe-Z) and are transcriptional fusions with the CaMV 35S promoter. They contain the 3′ untranslated sequence of the *nos* gene. Open reading frames are boxed. In the 35S–L–GUS constructs, the *pma1* leader sequence was placed between the CaMV 35S promoter and the *gusA* gene (see Figure 6 for details). 35S–LΔ1–GUS, 35S–LΔ2–GUS and 35S–LΔ12–GUS are similar to 35S–L–GUS, except that the first, second, or both ATG codons, respectively, of the URF were mutagenized as indicated. The 35S–CAT construct is in pUC12 and was used as an internal control of electroporation efficiency; it has CAT between the CaMV 35S promoter and the 3′ untranslated region of the *rbcs-E9* gene (*E9*). The diagrams are not drawn to scale.

recognized by at least a fraction of the scanning ribosomes, a point that was clearly demonstrated in stably transformed plants.

The difference in mRNA strength or capacity to be translated observed in plants DEL29, DEL70, and DEL220 (Table 2) suggests that the leader of *pma1* regulates the expression of the main open reading frame during translation. To test this point further, we synthesized *gusA* mRNAs with various versions of the *pma1* leader in vitro and introduced them into protoplasts by electroporation, thus avoiding any possible transcriptional effect of the leader. Constructs used for this experiment were obtained from 35S–GUS, 35S–L–GUS, and 35S–LΔ12–GUS simply by removing the 35S promoter and by replacing the nopaline synthase gene (*nos*) terminator by a poly(A) stretch of 117 A residues. These constructs were called GUSpA, L–GUSpA, and LΔ12–GUSpA. By in vitro transcription, they give rise to *gusA* mRNAs similar to those obtained from in vivo transcription of the 35S–GUS, 35S–L–GUS, and 35S–LΔ12–GUS constructs. The first eight nucleotides of the leader sequence are changed as indicated in Figure 6, the *nos* 3′ untranslated region is removed, and the poly(A) region ends with the sequence 5′-GGAATT-3′ introduced by an EcoRI restriction site (see Methods).

Four independent in vitro transcriptions were performed and each was used for two protoplast electroporations. Table 4 summarizes the quantity of GUS produced from these mRNAs. Here again, the leader of *pma1* stimulates fourfold the expression of GUS, and this effect is now shown to be post-transcriptional. The mutagenesis of the URF might stimulate the expression of GUS somewhat as well, but the effect is less than that observed with transiently expressed plasmids.

The *pma1* URF Inhibits Translation in a Wheat Germ System

Finally, we have tested the effect of the *pma1* leader on in vitro translation of the synthetic mRNAs described above. Four independent in vitro transcriptions were carried for the GUSpA, L–GUSpA, and LΔ12–GUSpA constructs. They were used for translation in wheat germ extracts. Figure 7 shows the quantity of GUS synthesized from these transcripts. The *pma1* leader with its intact URF (L–GUSpA) inhibits by 30% translation of *gusA* when compared to the 41-nucleotide-long leader present in GUSpA. But the most dramatic effect is exerted by the suppression of the URF (LΔ12–GUSpA): it stimulates 2.7 times

Table 3. GUS Activity Measured after Transient Expression in Protoplasts of Plasmid-Carried Fusions of the *pma1* Leader to *gusA*[a]

Exp.	Reps.	35S–GUS	35S–L–GUS	35S–LΔ1–GUS	35S–LΔ2–GUS	35S–LΔ12–GUS
1	3	ND[b]	100	120.2	93.0	172.8
2	2	25.7	100	ND	ND	164.7
3	2	32.1	100	ND	ND	128.0
4	2	17.3	100	117.4	105.3	148.2
5	2	14.0	100	97.1	122.7	161.2
Weighted average[c]		22.3 ± 7.1	100	112.8 ± 10.0	105.0 ± 12.3	156.6 ± 15.8

[a] Activity was measured by fluorometry at four time points and was corrected for electroporation efficiency by CAT activity measured from the same samples. All the values are expressed as the percentage of the specific activity measured for construct 35S–L–GUS.
[b] ND, not determined.
[c] Average is weighted according to the number of repetitions.

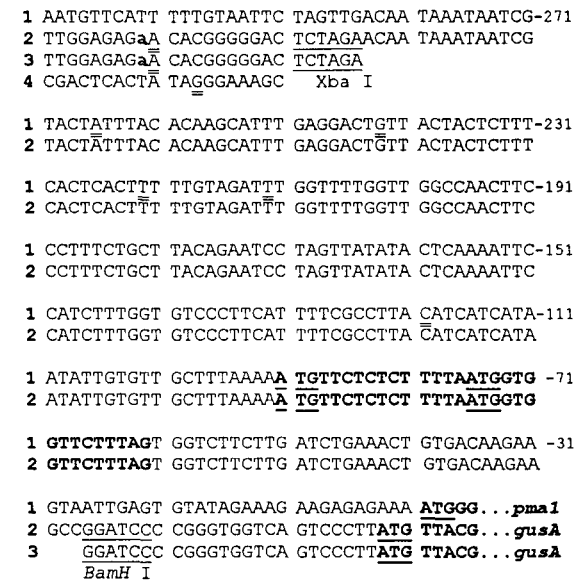

Figure 6. Comparison of the Leader Sequences of the Constructs Used in Transient Expression Experiments with that of *pma1*.

Line 1, nucleotide sequence of *pma1* from position −310 from the main open reading frame ATG; line 2, sequence of the 35S–L–GUS construct from position −307 from the *gusA* ATG; line 3, sequence of the 35S–GUS construct from position −50 from the *gusA* ATG; line 4, junction sequence between the pTZ19U T7 promoter and the various GUS constructs (GUSpA, L–GUSpA, and LΔ12–GUSpA) used for in vitro transcription. Double-underlined nucleotides are the 5′ transcript boundaries of *pma1* (line 1), of the 35S transcript (lines 2 and 3), and of the T7 RNA promoter in pTZ19U (line 4). Nucleotides in boldface indicate open reading frames, and the ATGs are underlined. The single point mutation found in the 35S promoter used in the GUS system, which is just upstream of the transcription start site, is indicated as a boldface and lowercase letter (see Methods).

gusA translation, that is, much more than the 57% (DNA) or 20% (RNA) observed in vivo. This difference suggests that tobacco possesses some translation accessory somehow circumventing the inhibitory effect of the URF and conferring a translational stimulatory effect on the *pma1* leader. This translation accessory would allow a nonconventional translation of *pma1* in vivo but would be absent or poorly operating in the wheat germ system.

DISCUSSION

Tissue-Specific Expression of *pma1*

Differential expression of the genes encoding the plasma membrane H+-ATPase of *N. plumbaginifolia* was previously shown by quantifying the *pma1*, *pma2*, and *pma3* mRNAs in different organs (Perez et al., 1992). Whereas high levels of *pma1* mRNA were observed in the flowers and stem, this mRNA was present at much lower levels in roots and leaves.

In this study, we have conducted a more detailed study of the pattern of *pma1* expression in *N. tabacum* tissues. We can now explain the high level of mRNA in flowers by the fact that *pma1* is expressed in three different cell types, namely the tapetal cells of the anther, the pollen, and the cells delimiting the transmitting tissue. In stems, expression of *pma1* occurs in the cortex parenchyma. The low level of mRNA observed in leaves is also in agreement with the absence of histochemical staining in the leaves of our transgenic plants, except for the parenchyma at the border of the blade. The same is true for the roots, where the low level of mRNA observed reflects dilution of the *pma1* mRNA present in the epidermis of a short area of the root by all the other cells of the organ used to prepare the RNA.

False positive results have been reported for the histochemical staining of GUS in transgenic plants (De Block and Debrouwer, 1992; Mascarenhas and Hamilton, 1992; Uknes et al., 1993). Here, we point to possible false negative results in stems. With low expression promoters such as those of *pma1* analyzed here, it is necessary to prepare sections containing a majority of intact cells. Otherwise the loss of cytoplasm from the cut cells is sufficient to bring the staining to undetectable levels. Thus, very thick cross-sections or longitudinal sections are necessary for stems. It is also advisable to confirm histochemical results by GUS assays on protein extracts, which are less subject to artifacts.

Physiology and Tissue-Specific Expression

DeWitt et al. (1991) have used the *gusA* reporter gene to study *aha3*, an Arabidopsis gene encoding a plasma membrane H+-ATPase isoform. They report expression of *aha3* in phloem of all organs analyzed. *pma1* of *N. plumbaginifolia* is also expressed in the phloem, but of developing fruit only. Furthermore, *aha3* and *pma1* are expressed in pollen and ovules.

Table 4. GUS Activity Measured after Transient Expression in Protoplasts of Synthetic RNA-Carried Fusions of the *pma1* Leader to *gusA*[a]

Exp.	GUSpA	35S–GUSpA	35S–LΔ12–GUSpA
1	18	100	101
2	28	100	121
3	32	100	137
Average ± SD	26 ± 6	100	120 ± 15

[a] Activity was measured by fluorometry at four time points and is expressed as the percentage of specific activity obtained with 35S–L–GUSpA mRNA.

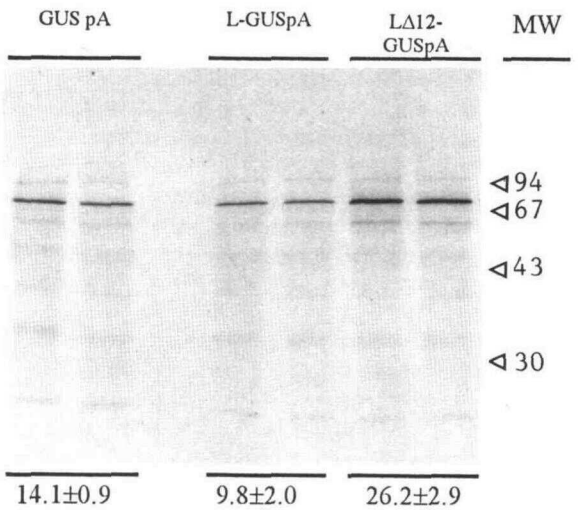

Figure 7. Translation of *gusA* under the Control of the *pma1* Leader in a Wheat Germ System.

The constructs are as described in Methods. Radiolabeled translation mixtures were resolved on a SDS–polyacrylamide gel. The numbers below the autoradiogram represent the average density of the GUS band from four independent transcription and translation experiments. MW, molecular weight markers ×1000.

Although this points to some resemblance between these two genes, *pma1* is expressed in more tissues than is *aha3*.

The presence of an H^+-ATPase in the epidermis of the roots and in guard cells, which is shown for PMA1 in this study, has been known for a long time and confirmed by immunolocalization (Villalba et al., 1991; Samuels et al., 1992). Also, H^+-ATPase–containing plasma membranes have been isolated from guard cells (Becker et al., 1993). These studies, however, made no distinction between single isoforms of the H^+-ATPase, and we are not certain whether the immunodetections revealed all the subfamilies of ATPases (as defined in Moriau et al., 1993). Therefore, a thorough understanding of the regulation of the expression of the H^+-ATPase genes in plants demands additional approaches, such as those described here or in situ hybridization.

pma1 is also expressed in tissues where the presence of an H^+-ATPase has scarcely or never been discussed. These include the stem cortex, the parenchyma at the leaf margin, the tapetum, the layer of cells surrounding the transmitting tissue, the pollen grains, and to a lesser extent, the pollen tubes. This observation confirms or suggests that these cells are involved in active transport processes, leading to the uptake or delivery of ions or nutrients (root epidermis, stem cortex, leaf parenchyma, pollen tubes, tapetal cells, cells delimiting the transmitting tissue, and phloem cells in the fruit) and allowing stomata opening (guard cells). It has further been suggested that H^+-ATPase is involved in cell elongation. This role could be partly sustained by *pma1* in the hair-developing cells of the root epidermis, in the stem cortex, and in the pollen tubes. Our results show that a single ATPase isoform is expressed in cells with distinct transport functions, all requiring the proton motive force. It will be interesting to see whether several H^+-ATPase isoforms work together in some cells. This might be the case because the four characterized *pma* genes of *N. plumbaginifolia* are highly expressed in flowers (Perez et al., 1992; Moriau et al., 1993). The combined expression of several isogenes in one cell could be a means of broadening the regulation range of enzyme concentration or allowing the occurrence of distinct PMA regulatory mechanisms in the same cell.

H^+-ATPase expression has been followed during pea flower development by immunolocalization (Parets-Soler et al., 1993). Interestingly, ATPase is detected in pollen sacs. However, we cannot exclude that expression of *gusA* observed in pollen in this study and in the study of *aha3* by DeWitt et al. (1991) was artifactual, as shown by Uknes et al. (1993).

It is also clear from our results that cell-specific expression of *pma1* does not necessarily mean constitutive expression in a particular cell type. For instance, *pma1* expression in guard cells is mainly found in the cotyledons and stems of young seedlings. Furthermore, this activity is modulated by the growth conditions. It is normally absent in mature leaves but appears after prolonged immersion in water and nutritive solutions. The activity of *pma1* in the phloem of the developing fruit only is another illustration of developmental regulation.

Post-Transcriptional Regulation of *pma1*

The leader sequence of the *pma1* transcript is unusual by its length and the presence of a URF. We have analyzed the effects of the leader sequence on the expression of the reporter protein GUS in three different systems: stable expression in transgenic plants, transient expression in protoplasts, and expression in an in vitro system. Two major conclusions can be drawn from these experiments. First, the whole leader stimulates GUS expression. Second, although the URF seems to be read by scanning ribosomes, it does not prevent the main open reading frame from being translated, implying the possibility of ribosome reinitiation. We will discuss these two conclusions successively.

The stimulation of GUS expression by the whole leader was observed both in stable and transient expression. Synthetic RNA gave similar results, thereby excluding a transcriptional effect. This enhanced expression requires a plant cell environment because in the in vitro translation system, the leader sequence was less effective in stimulating translation. We cannot strictly exclude an effect of the *pma1* leader on mRNA stability in the transient expression experiments, although such an effect does not seem very likely for a point mutation. Moreover, it is generally believed that the 3′ untranslated region, rather than the leader region, plays a critical role in mRNA stability (reviewed in Green, 1993; Jackson, 1993). Another

point to consider is whether the enhanced expression is a result of particular nucleotide stretches within the leader sequence or the increased length of the 5′ untranslated region. Deletion and site-directed mutagenesis analysis is required to address this question.

A stimulatory effect on the expression of reporter genes has also been described, for instance, in the case of the tobacco and alfalfa mosaic virus leader sequences (Gallie et al., 1987; Gallie and Walbot, 1992; Datla et al., 1993). However, contrary to the latter, the *pma1* leader sequence contains a URF. It is thus surprising that in spite of the predicted inhibitory effect of the URF, the *pma1* leader sequence stimulates translation of the following open reading frame. In fact, several observations strongly suggest that the URF could be part of a translational regulation. The URF of the *pma1* transcript can be read by the majority of the ribosomes of a wheat germ extract because mutagenesis of the two URF AUGs increased 2.7-fold the translation of *gusA*. This is in agreement with the current model of eukaryotic mRNA translation (Kozak, 1989), which implies that an upstream AUG diverts the scanning ribosomes from recognizing a second. Additional evidence for the URF readability comes from transgenic plants containing DEL70/OUT. They synthesized *gusA* mRNA that was unable to direct the synthesis of GUS, but DEL70/IN plants synthesized high levels of GUS. This shows that the URF ATGs of these constructs are recognized by (nearly) all of the scanning ribosomes. However, the paradox is that massive translation of the URF does not preclude translation of the main open reading frame, as indicated by the GUS activity measured in DEL29 plants that have *gusA* under the control of the leader sequence and URF of *pma1*. In fact, URF suppression by mutagenesis of its two ATGs only led to a small increase in *gusA* expression in vivo, indicating either that in the intact leader, the URF is partly inaccessible to the scanning ribosomes or that an efficient mechanism of reinitiation takes place.

Replacement of the *pma1* coding sequence by that of *gusA* allowed us to tackle the effects of the leader directly in tobacco, a very homologous system in which the *pma1* product was already present. We should keep in mind that, just as the replacement of transcribed sequences by that of a reporter gene may alter the transcription regulation, the removal of a large part of the *pma1* transcript may affect the role of the leader sequence in a putative regulatory system. In our study, the presumably poor context of the *gusA* initiation codon did not modify the properties of the leader sequence, nor those of the URF, because *gusA* translational fusions retaining the whole *pma1* leader plus the first five nucleotides of its coding sequence gave similar results (results not shown). However, the results obtained with our experimental system will have to be validated by complementary approaches evaluating the importance of *pma1* coding and 3′ untranslated sequences in the role of the leader sequence.

In conclusion, recognition of the URF and reinitiation are possible targets for translational regulation. However, we cannot discard other hypotheses. For instance, there have been reports of direct entry of ribosomes at an internal site in viral and artificial mRNAs in eukaryotic cells (reviewed in Kozak, 1992). Another possibility raised in a recent report by Fütterer et al. (1993) shows that, in the CaMV 35S leader, a long sequence containing several open reading frames can be jumped over by the scanning ribosomes, which are transferred (shunted) from a 5′ "take-off" to a 3′ "landing" region. Whatever the mechanism of PMA1 translation is, a major task to prove a regulatory mechanism will consist in finding developmental or environmental conditions that modulate the recognition of the main open reading frame.

Physiology and Post-Transcriptional Regulation

Post-transcriptional regulation of genes has been studied in a number of cases. The *GCN4* and *CPA1* genes of *Saccharomyces cerevisiae* have become paradigms and their regulation has been amply discussed by others (for review, see for instance Hinnebusch, 1988; Müller and Trachsel, 1990).

Studies of the regulation of plant genes by their URFs and leaders are scarce. However, a thorough study of a plant virus transcript, namely the CaMV 35S transcript, has been conducted. This polycistronic transcript has a 600-nucleotide leader sequence containing eight URFs. Deletion analyses showed that the leader exerts both negative and positive regulatory effects (Fütterer et al., 1990). The negative effects come from the URFs, as in the case of the *pma1* leader. Fütterer and Hohn (1992) have suggested a possible physiological role for the URFs of the polycistronic 35S mRNA. In an artificial dicistronic mRNA derived from the 35S RNA, a URF was found to enhance translation of the second open reading frame mediated by a transactivator protein. The *pma1* mRNA carrying only one large open reading frame, the leaders of *pma1* and 35S should have different functions.

Another study by Lohmer et al. (1993) concerns a natural plant gene: *Opaque2* (*O2*) of maize, encoding a transcriptional activator. Similar to many other plant genes coding for transcription factors, *O2* has a long leader sequence and, in this case, three URFs. This study is particularly relevant because it enabled the authors to exclude any direct enzymatic role of the URF-encoded peptides. As was the case for *pma1*, mutagenesis of all the *O2* upstream ATGs increased translation of the main open reading frame. In contrast to what seems to occur in *pma1*, 40% of the ribosomes bypassed the *O2* URFs. This could point to a regulatory mechanism modulating the efficiency of URF recognition.

If indeed there is translational control of PMA1 synthesis, what could be its physiological role? So far, we have no clue; however, it is interesting to note that many other plasma membrane ATPase genes have one or several URFs. Indeed, a data base search showed that this is the case for most plant H^+-ATPases and also for most mammalian Ca^{2+}-ATPases, the stomach H^+,K^+-ATPase, a fish Na^+,K^+-ATPase, and a putative Cu^{2+}-ATPase (see Methods). Thus, translational control of

pma1, which requires further investigation, could illustrate a regulatory mechanism affecting many transport ATPases.

METHODS

Constructs

Plant Transformation

A genomic clone of *Nicotiana plumbaginifolia* containing 2.3 kb of the promoter region of *pma1* (Perez et al., 1992) and extending to 17 nucleotides downstream from the main AUG was cloned into the SmaI site of pBluescript KS+ (Stratagene). Progressive deletions from 3′ to 5′ were then obtained by the ExoIII–mung bean nuclease strategy after digestion with SstI and BamHI. BamHI linkers (GIBCO BRL; CCGGATCCGG) were then ligated to the deleted plasmids, which were subsequently introduced into *Escherichia coli* 7118 (Dente et al., 1983). Clones of interest were selected by sequencing single-stranded DNA (Sanger et al., 1977; Biggins et al., 1983). Inserts deleted to positions −29, −70, and −220 from the ATG were cut with SalI and BamHI, filled in (Klenow fragment of DNA polymerase I), and ligated into the SmaI site of pBI101-1 (constructs DEL70/IN and DEL220) or pBI101-3 (constructs DEL29 and DEL70/OUT; Jefferson et al., 1987). The junction between the upstream open reading frame (URF) and the β-glucuronidase reporter gene (*gusA*) in the DEL70 constructs reads as follows: DEL70/IN, AAA**ATG**TTCTCTCTTTTA**ATG**GTGGCCGGATC-GGGTGGTCAGTCCCT*TATG*; DEL70/OUT, AAA**ATG**TTCTCTCTTTT-A**ATG**GTGGCCGGATCGGGTACGGTCAGTCCCT*TATG*TTACGTCCTG-TAG (stop codon), where the URF ATGs are in bold characters, the remnants of the BamHI linker are underlined, and the *gusA* ATG is in italics. PMA1+1051 was obtained by cloning the EcoRI (−2299)-PvuII (+1051) fragment of *pma1* in the SmaI site of pBI101-2 after filling in with the Klenow fragment.

Protoplast Transformation

The 35S–CAT plasmid is as described in Morelli et al. (1985) and contains the chloramphenicol acetyltransferase (*cat*) gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter, with the ribulose bisphosphate carboxylase small subunit (*rbcs-E9*) polyadenylation site. The 35S–GUS plasmid is the pTZ19U plasmid (Genescribe-Z; U.S. Biochemical Corp.) containing the HindIII-EcoRI insert of pBI121 (Jefferson et al., 1987). The DEL29 plasmid (see above) was digested with HincII (−286 of ATG), phosphorylated BamHI linkers were added, and the insert corresponding to the long leader of *pma1* was subcloned after BamHI digestion into the BamHI site of 35S–GUS, giving rise to the 35S–L–GUS plasmid. The URF of the 35S–L–GUS plasmid was mutagenized by the method of Sambrook et al. (1989). The first ATG of the URF was mutated with the oligonucleotide 5′-GAGAACACT-TTTAAAGC-3′ and the second with the oligonucleotide 5′-AACCAC-CGTTAAAAG-3′; we used both oligonucleotides to completely destroy the URF. The mutated clones were sequenced on double-stranded DNA by the dideoxy termination method (Sanger et al., 1977) using T7 DNA polymerase (Pharmacia). Sequencing revealed that the 5′ BamHI site of the 35S–L–GUS plasmid was deleted as well as the first nucleotide of the remainder of the HincII site. Moreover, we discovered that the 35S promoter present in our pBI121 plasmid is mutated at position −1 of the major transcription start site (G residue in Guilley et al., 1982; A in pBI121; see Figure 6). After mutagenesis and complete sequencing, the mutated XbaI-BamHI inserts were cloned into 35S–GUS, yielding the 35S–LΔ1–GUS, 35S–LΔ2–GUS, and 35S–LΔ12–GUS plasmids. To obtain the template plasmids for in vitro transcription, the 35S promoter of the 35S–GUS, 35S–L–GUS, and 35S–LΔ12–GUS plasmids was removed by HindIII-XbaI digestion, filling in with the Klenow fragment, and ligation. Their nopaline synthase (*nos*) 3′ region was then replaced, after SacI-EcoRI digestion, by a SacI-EcoRI *cpma4* poly(A) fragment (Moriau et al., 1993) amplified by polymerase chain reaction to introduce a SacI restriction site at the 5′ end of the poly(A) fragment; an EcoRI site was already present at the 3′ end of the original clone. The primers used for the polymerase chain reaction were MLPMA4 and the universal primer. MLPMA4 reads 5′-CTATCAGCGAGCTCGCACAAA-3′, where the SacI site is underlined and the last three A residues mark the beginning of the *cpma4* poly(A) sequence, which is 117 nucleotides long.

Plant Transformation and GUS Assay

The constructs were mobilized in *Agrobacterium tumefaciens* LBA4404 by triparental mating. The clones obtained were then used to transform *Nicotiana tabacum* cv SR1 by the method of Rogers et al. (1986). GUS activity was measured by fluorometry, essentially as described in Jefferson et al. (1987). Plant tissues were ground in 50 mM sodium phosphate, pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, and 0.1% Triton X-100. Flower tissues were very sensitive to oxidation and necessitated the addition of 20 mM dithiothreitol in the grinding buffer. After a 5-min centrifugation, the extract was frozen in liquid nitrogen, incubated for 15 min at 37°C, and centrifuged for 10 min. This yielded a clear supernatant. After taking an aliquot for the protein assay (Bradford, 1976), 4-methylumbelliferyl-β-D-glucuronide (Sigma) was added to the extract to a concentration of 1 mM, and the reaction was stopped in 50 to 100 μL aliquots by adding 1 mL of 0.2 M Na_2CO_3 after 0, 15, 30, and 45 min at 37°C. Fluorescence was measured on a Hoefer TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, CA). Histochemical staining was done on hand-cut sections at 37°C in 1 mM X-gluc (CHA salt; Biosynth AG, Staad, Switzerland), 50 mM $NaPO_4$, pH 7.0, 0.02% NaN_3 after a 2- to 5-min vacuum infiltration. For easily oxidizable objects (i.e., fruits, styles, and ovules), 10 mM β-mercaptoethanol was added. Where possible, results were confirmed by incubation with the same buffer containing 2 mM potassium ferricyanide to avoid possible artifacts (De Block and Debrouwer, 1992). For buds, flowers, and fruits, GUS activity observed in particular organs and tissues was always confirmed on isolated organs (i.e., anthers, pistils, pollen, and ovules). After a 15- to 24-hr incubation, chlorophyll was solubilized in 100% EtOH, and the objects were then preserved in 50% glycerol (w/w). Photographs were taken on Nikon SMZ-2T (Nikon Corporation, Tokyo, Japan), Makroskop Wild M400 (Wild, Heerbrugg, Switzerland), and Polyvar I (Reichert-Jung, Wien, Austria) microscopes.

In Vitro Cultures

Seed were sterilized for 2 min in 70% (v/v) ethanol, 20 min in 10% (v/v) bleach water, and washed three times in sterile distilled water. Culture media contained 4.7 g/L Murashige and Skoog salts (Murashige and Skoog, 1962), 0.7% agar, 3% sucrose, and 0.1 g/L kanamycin sulfate. The pH was adjusted to 5.6 before autoclaving. For liquid cultures, we used the above medium without agar and sucrose on a rotatory table (100 rpm). Growth was conducted at 24°C in dim light.

RNA Preparation and Slot Blot

Kanamycin-resistant F_1 plantlets cultured for 2 weeks in vitro were pooled to reach 100 mg of material (10 to 19 plantlets). Total RNA was prepared by the method of Verwoerd et al. (1989). At the same time, at least five plants from the same culture containers were pooled and used for a GUS fluorometric assay. Serial dilutions of RNA (10 to 0.0625 μg) of two plants were slot blotted on Hybond-N^+ membranes (Du Pont) together with two dilutions (8 and 1 μg) for all the other plants (Bio-Rad slot blot apparatus). Three membranes were necessary to accommodate all the samples, each sample being blotted on at least two membranes. The blots were hybridized with a ^{32}P-labeled *gusA* DNA probe of 1.88 kb (7.5 10^6 cpm/μg, 10^6 cpm/μL). After washing (last wash, 10 min in 1 × SSC [0.15 M NaCl, 0.015 M sodium citrate], 0.1% SDS at 65°C), the membranes were exposed to autoradiography for 17 days. The membranes were then washed in boiling 0.1% SDS and hybridized with an *atp2-1* (Boutry and Chua, 1985) ^{32}P-labeled DNA probe of 1 kb (20.6 10^6 cpm/μg). After washing (same conditions), the membranes were autoradiographed for 20 hr. Autoradiograms were analyzed on a Pharmacia LKB Imagemaster DTS densitometer. The signal density of the 1-μg slots were within the linear range for the *atp2* signal. Signals were weaker for *gusA*, and those obtained with the 8-μg slots were used for the analysis. Their density was corrected by dividing them by the *atp2-1* signal density and subtracting the background signal obtained with RNA from untransformed tobacco. The integrity of the RNA preparation was checked for four samples by RNA gel blot analysis with the *atp2-1* probe (result not shown).

In Vitro RNA Synthesis and Translation

EcoRI-linearized plasmid (1 μg) was used to synthesize RNA in a volume of 40 μL by the method described in Chaumont et al. (1990) without RNA column purification. An aliquot of the transcription reaction mixture (2 μL) was electrophoresed on a 1% agarose minigel during 10 min at 10 V/cm. The relative quantities of RNA synthesized were deduced from the densitometric analysis of the gel (see above) and did not vary significantly. Transcription reaction mixture (0.5 μL) was translated in vitro in a final volume of 6.25 μL with a wheat germ translation kit (Boehringer Mannheim; standard assay). The whole translation reaction mixture was analyzed by SDS-PAGE on a 10% gel following the procedure of Laemmli (1970). Proteins were then transferred to a nitrocellulose membrane, which was subsequently autoradiographed. The bands of interest were quantified by densitometry (see above).

Transient Gene Expression in Tobacco Protoplasts, GUS, and CAT Assays

Young leaves of *N. tabacum* cv SR1 from in vitro cultures were abraded on the underside with sandpaper No. 1200A and floated overnight on a digestion medium (0.125% [w/v] macerozyme R-10 [Onozuka; Yakult Pharmaceutical, Tokyo, Japan], 0.2% [w/v] cellulase R-10 [Onozuka; Yakult Pharmaceutical], 5 mM $CaCl_2$, 0.5 M sucrose, 0.1% BSA, pH 5.5, filter-sterilized) in a Petri dish. Protoplasts were then purified (Negrutiu, 1992) and their viability tested with fluorescein diacetate by fluorescence microscopy (Wildholm, 1972). The protoplasts were then electroporated (800-μL suspension in 0.4-cm gap cuvettes, 800 V/cm, 250 μF, Gene Pulser Transfection Apparatus; Bio-Rad) with 40 μg/mL plasmid purified on Diagen affinity columns (Diagen GmbH, Hilden, Germany) or 20 μL of transcription reaction mixture. The protoplasts were resuspended 24 hr later in 50 mM Tris-Cl, pH 8.0, 5.5 mM β-mercaptoethanol and sonicated for 5 sec (Virsonic Cell Disrupter 16-850; Virtis, Gardiner, NY). The protein concentration was measured according to Bradford (1976). GUS activity was measured by fluorometry (see above) and CAT activity by a phase extraction assay (Ausubel et al., 1989).

Data Base Search

The sequences of all the different plasma membrane ATPase genes were obtained by searching the Genbank EMBL Data Bank under the Enzyme Nomenclature codes EC 3.6.1.35 for H^+-ATPases, EC 3.6.1.36 for H^+,K^+-ATPases, EC 3.6.1.37 for Na^+,K^+-ATPase, and EC 3.6.1.38 for Ca^+-ATPase (specifying plasma membrane to avoid all the reticulum ATPases). The Cu^{2+}-ATPase has no proper code to date. Its sequence was obtained through its EMBL accession number L06133.

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