Expression analysis of two gene subfamilies encoding the plasma membrane H+-ATPase in *Nicotiana plumbaginifolia* reveals the major transport functions of this enzyme

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Summary
The plasma membrane H+-ATPase couples ATP hydrolysis to proton transport, thereby establishing the driving force for solute transport across the plasma membrane. In *Nicotiana plumbaginifolia*, this enzyme is encoded by at least nine *pma* (plasma membrane H+-ATPase) genes. Four of these are classified into two gene subfamilies, *pma1-2-3* and *pma4*, which are the most highly expressed in plant species. We have isolated genomic clones for *pma2* and *pma4*. Mapping of their transcript 5’ end revealed the presence of a long leader that contained small open reading frames, regulatory features typical of other *pma* genes. The *gusA* reporter gene was then used to determine the expression of *pma2*, *pma3* and *pma4* in *N. tabacum*. These data, together with those obtained previously for *pma1*, led to the following conclusions. (i) The four *pma-gusA* genes were all expressed in root, stem, leaf and flower organs, but each in a cell-type specific manner. Expression in these organs was confirmed at the protein level, using subfamily-specific antibodies. (ii) *pma4-gusA* was expressed in many cell types and notably in root hair and epidermis, in companion cells, and in guard cells, indicating that in *N. plumbaginifolia* the same H+-ATPase isoform might be involved in mineral nutrition, phloem loading and control of stomata aperture. (iii) The second gene subfamily is composed, in *N. plumbaginifolia*, of a single gene (*pma4*) with a wide expression pattern and, in *Arabidopsis thaliana*, of three genes (*aha1*, *aha2*, *aha3*), at least two of them having a more restrictive expression pattern. (iv) Some cell types expressed *pma2* and *pma4* at the same time, which encode H+-ATPases with different enzymatic properties.

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Introduction
The plasma membrane H+-ATPase actively pumps protons from the cytoplasm to the apoplasm and generates an electrochemical potential difference across the membrane. This, in turn, activates solute transport through a variety of secondary transporters and channels. Consequently, the proton pump is thought to play a key role in many physiological processes (Michelet and Boutry, 1995; Palmgren, 1998; Serrano, 1989; Sussman, 1994).

In roots, for instance, where mineral nutrition takes place and gravity is perceived, immunological studies have revealed H+-ATPase accumulation in the root cap, the epidermis and hairs, and also the central cylinder region (DeWitt and Sussman, 1995; Parets-Soler et al., 1990; Samuels et al., 1992). In many plant species, the few plasmodesmata between the sieve tube–companion cell complex and mesophyll cells suggest an apoplasmic sucrose loading (for a review see Frommer and Sonnewald, 1995). H+/sucrose symporters have been localized in phloem tissues, and might be fuelled by H+-ATPase found in abundance in the plasmalemma of the companion cells (Bouché-Pillon et al., 1994; DeWitt and Sussman, 1995). H+-ATPase energizes ion transport when the guard cells are swelling, and is thus involved in stomata aperture and regulation of gas exchange between the plant and its environment (Becker et al., 1993; Nakajima et al., 1995). According to the acid-growth theory of cell elongation, auxin causes the cells to excrete protons in the apoplasm (for a review see Rayle and Cleland, 1992), causing wall loosening and cell expansion. The H+-translocating ATPase might therefore be a major target of the phytohormone to accomplish this extracellular acidification.

Considering these various physiological processes in which H+-ATPase plays a role, we may question whether this diverse activity results from a single enzyme or whether it requires several isoforms, each adapted for a different function and/or regulation. In all the species analysed so far, the H+-ATPase is encoded by a multigene family (Ewing and Bennett, 1994; Harper et al., 1994; Perez et al., 1992). In *Nicotiana plumbaginifolia*, the H+-ATPase gene family contains at least nine members, called *pma* (plasma membrane H+-ATPase) (M. Arango, M. Oufattole and M. Boutry, unpublished results). Four of these have been sequenced and classified into two gene subfamilies according to their nucleotide sequence identity: *pma1*, *pma2* and *pma3* share 95–97% amino acid identity, while *pma4* is only 79–82% identical to the other three (Moriau...
et al., 1993; Perez et al., 1992). This division into two subfamilies was confirmed in other plant species belonging to monocots and dicots (Ewing and Bennett, 1994; Frias et al., 1996; Harms et al., 1994; Harper et al., 1994; Jin and Bennetzen, 1994; Nakajima et al., 1995).

Michelet et al. (1994) previously reported on the cellular localization of pma1 gene expression, using the gusA reporter gene. Here, we describe the expression pattern of pma2, pma3, and pma4 and thus obtain a full picture of the two divergent gene subfamilies. All together, expression of the four N. plumbaginifolia H+-ATPase genes accounts for most of the transport occurrences known in the plant. Our results also indicate that the expression pattern of the four genes partly overlaps. The presence of more than one H+-ATPase isoform in a single cell type at the same developmental stage thus raises the possibility of distinct modes of activity and/or regulation for the H+-ATPase subfamilies.

Results

Genomic cloning

S1 nuclease protection assays have been performed previously to quantify transcripts of pma1, pma2, pma3 (first subfamily) and pma4 (second subfamily). They are all expressed in roots, stems, leaves and flowers, although at various levels depending upon the gene and the organ (Moriau et al., 1993; Perez et al., 1992). A more detailed analysis was performed for pma1, using the gusA reporter gene. This revealed that pma1 was expressed in specific cell types according to developmental regulation (Michelet et al., 1994). To extend this approach to pma2, pma3, and pma4, and thus obtain the complete expression pattern of these two pma gene subfamilies, we searched for genomic clones containing the putative promoter region of pma2 and pma4 (that of pma3 was already available; Perez et al., 1992). A N. plumbaginifolia genomic library was screened with probes derived from pma2 and pma4 cDNA clones. The longest clones obtained for pma2 (clone 2-10) and pma4 (clone 4-2) contained ~5 kb and 2.3 kb, respectively, upstream from the translation start. Restriction maps of both genomic clones were in agreement with cDNA and partial genomic sequences already available (Moriau et al., 1993; Perez et al., 1992) and confirmed, together with Southern blot analysis carried out on genomic DNA with specific probes (Moriau et al., 1993), that neither subfamily contains any gene other than pma1, pma2, and pma3, and pma4, respectively.

5’ transcript mapping

Primer extension experiments were conducted to find the approximate localization of the pma2 and pma4 transcript start sites. In both cases, the size of the predicted messenger leader was much larger (~350 nt and ~230 nt for pma2 and pma4, respectively; results not shown) than the mean size for a plant leader sequence (40–80 nt; Joshi, 1987). The 5’ terminus of the pma2 and pma4 transcript was then determined by S1 nuclease mapping. The S1 mapping performed for pma2 (Figure 1) confirmed the primer extension data, as a single band indicated a 358-nt long leader. A length of 230 nt was found for pma4 (Figure 1), thus also confirming primer extension results. A more diffuse band, 12 nt shorter, was also observed, probably resulting from an RNA:DNA heteroduplex loosening in this region composed of nine A:T nucleotide pairs.

It has been reported previously that pma1 and pma3 have an unusually long leader (5’ untranslated transcribed region) that contains a small open reading frame (uORF) of 10 and six codons, respectively (Perez et al., 1992). Transcript 5’ mapping of pma2 and pma4 revealed similar features. The pma2 transcript had a 358-nt long leader containing two non-overlapping uORF of 13 codons (Figure 2a). In addition, comparison of the genomic and cDNA sequences revealed an intron of 114 nucleotides. As for pma4, the leader was 230 nt long and contained an uORF of six codons (Figure 2b). Regulatory properties of the pma1 and pma3 leaders and uORF have been discussed (Lukaszewicz et al., 1998; Michelet et al., 1994) and might thus be applied to pma2 and pma4.

Expression of pma as monitored by the gusA reporter gene

To follow the expression of each pma gene in situ, we relied on the gusA reporter gene. Convenient restriction
sites were found 14 (pma2) or 19 (pma4) nucleotides downstream, or 64 nucleotides upstream (pma3) from the PMA initiator codon. pma–gusA constructs were introduced into tobacco, using an Agrobacterium tumefaciens T-DNA-derived vector. Over 40 independent transgenic lines were obtained for each construct. F1 or F2 generations were subjected to histochemical analysis and enzymatic assays. As expected, due to the position effect, the expression was quantitatively variable (data not shown). However, within each set of plants, most GUS-positive plants displayed a similar expression pattern. In general, pma3–gusA was by far less expressed. In the following sections, we will only mention its expression in the few cell types where GUS activity was detected.

Seed histochemistry

Mature seeds were collected from transformed plants and kept for 3 days in a moist environment prior to histochemical GUS staining. For pma2– and pma3–gusA seeds, GUS was active in the hilum, where the funiculus was attached during fruit maturation (shown for pma4–gusA in Figure 3a). When embryos were expelled from seeds by pressure, blue-staining of the entire pma4–gusA embryo (i.e. cotyledons and radicle) was observed (Figure 3b), unlike pma2– and pma3–gusA-transformed embryos (shown for pma3–gusA in Figure 3c). Later, when the taproot emerged, both pma2– and pma3–gusA-transformed lines exhibited GUS expression in the cotyledons, still confined inside the seed coat, on the side that was in close contact with the endosperm (the seed nutrient reserve) (Figure 3d,e). This type of expression might be related to the observation that Vicia faba embryo epidermal cells in contact with the endosperm accumulate high levels of H+-ATPase (Bouche-Pillon et al., 1994). In addition to cotyledons, GUS staining was also found in the root cap cells of emerging pma4–gusA seedlings (Figure 3e).

Two to four leaves seedling histochemistry

In seedling roots, both pma2 and pma4 genes were expressed in the root cap. In pma2–gusA seedlings, GUS staining was restricted to the meristem proximal statocyte storeys (Figure 4a), but in pma4–gusA seedlings, staining was observed in all cap cell layers (Figure 4b). In the root elongation zone, GUS staining was found for pma4–gusA plants only (Figure 4b). Above this region, all three pma–gusA genes were expressed. pma3–gusA expression was restricted to the root central cylinder (data not shown). pma2– and pma4–gusA expression was found in the cortex parenchyma cells and the central cylinder region (shown in Figure 4c for pma4–gusA). Furthermore, in pma4–gusA transgenic seedlings, GUS staining was also observed in root hairs (Figure 4d; note that, in this case, permeabilization of the seedling was omitted because hair staining was sensitive to this treatment) and in root primordia (Figure 4e), especially abundant in the root–shoot transition region (crown).

In cotyledons and leaves, GUS was detected in both mesophyll cells and conducting tissues in pma2–gusA transgenic seedlings (Figure 4f) and was more restricted to conducting tissues in pma4–gusA seedlings (Figure 4g).

Longer exposure also revealed GUS expression in the mesophyll of pma4–gusA plants. Moreover, peeling the epidermis uncovered GUS staining for pma2–gusA and pma4–gusA in the guard cells and the subsidiary cells around them (shown for pma4–gusA in Figure 4h, and in detail in Figure 4i).

At the tip, the meristematic region and the developing leaf were found to express pma4–gusA highly (Figure 4j).
served in the same cell types for a given pma-gusA transgenic plant. In roots, primary meristems were stained in pma2-gusA and pma4-gusA plants (not shown) as for the seedlings. Root primordia were also highly stained (shown in Figure 5a for pma2-gusA). The central cylinder and most of the cortex were highly stained in pma4-gusA plants (Figure 5b). Cross-section (Figure 5c) revealed staining of most of the cell types (conducting tissues, pericycle, endodermis, cortex). A similar, but lower, expression was found for pma2-gusA plants (Figure 5d).

More particularly, the endodermis was hardly stained and, in the cortex, only the most external layer was active. In pma3-gusA plants, expression was more abundant in the epidermis and in the most external cortical cell layer (Figure 5e).

**Stem histochemistry**

Solanaceous plant species are characterized by a bicolateral vascular bundle. In the stem, the vascular tissues are
composed of continuous rings of xylem and external phloem, separated from each other by the procambium (or cambium) and the internal phloem, which lies as discrete strands around the periphery of the pith next to the primary xylem.

\[ \text{pma2–gusA} \] expression was detected in both the internal and external phloem, the cortex parenchyma cells and in the epidermis (Figure 6a,b). \[ \text{pma4–gusA} \] was differentially expressed along the stem. In the lowest region (Figure 6c,d), GUS was expressed as for \[ \text{pma2–gusA} \], and also in

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**Figure 5.** GUS histochemical detection in roots. (a) \[ \text{pma2–gusA} \] and (b) \[ \text{pma4–gusA} \]: longitudinal view. (c) \[ \text{pma4–gusA} \]: cross-section (ph: phloem tissue; xv: xylem vessel; p: pericycle; en: endodermis). (d) \[ \text{pma2–gusA} \]: cross-section (c: cortex; e: epidermis; p: phloem tissue; en: endodermis). (e) \[ \text{pma2–gusA} \]: cross-section (e: epidermis; c: cortex).

**Figure 6.** GUS histochemical detection in stems. (a) \[ \text{pma2–gusA} \]: cross-section at the stem lower level. (b): detail of (a) (ep: external phloem; ip: internal phloem). (c) \[ \text{pma4–gusA} \]: cross-section at the stem lower level. (d) Detail of (c). (e) \[ \text{pma4–gusA} \]: detail of the conducting tissues (ip: internal phloem; xp: xylem parenchyma; xv: xylem vessels). (f) \[ \text{pma4–gusA} \]: cross-section at the middle part of the stem. (g) \[ \text{pma3–gusA} \]: cross-section at the node level.
the pith parenchyma cells. More detailed observation revealed strong expression in the xylem parenchyma cells, and the cells between the phloem and the xylem tissues, especially in leaf traces (Figure 6e). In the middle and upper part of the stem, GUS expression was localized to the inner conducting tissues only (Figure 6f).
Expression of the H\textsuperscript{+}-ATPase gene family

**Figure 9.** Western blot analysis of the PMA1,2,3 and PMA4 subfamilies.

Western blot analysis

To confirm the expression of the pma genes at protein level, we developed an immunological approach. The purpose was not to duplicate an exhaustive analysis at cell level but to check the contribution made by the two pma subfamilies analysed in various organs. As the three members of the pma1, pma2, pma3 subfamily share a high identity (over 96%) throughout the predicted amino acid sequence, it was not possible to design regions against which specific antibodies could be obtained. On the other hand, the much lower identity between the two subfamilies (79–82%) led us to synthesize two sets of peptides corresponding to the PMA1,2,3 or PMA4 sequence. This was performed for two H\textsuperscript{+}-ATPase regions, either in the large loop or in the C-terminal region. Rabbit polyclonal antibodies were obtained and those against the peptides of the large loop region were found to be the strongest and were affinity-purified. Their specificity was checked by detection of either PMA2 or PMA4 individually expressed in the yeast Saccharomyces cerevisiae (de Kerchove et al., 1995; Luo et al., 1999). There was no cross-reaction between them (Figure 9).

Immunodetection was performed on a microsomal fraction prepared from several organs of *N. tabacum* and *N. plumbaginifolia*. The latter was analysed as the *PMA1* gene promoter region.

**Immunodetection**

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**Discussion**

We have described the expression pattern of three genes encoding isoforms of the plasma membrane H\textsuperscript{+}-ATPase of *N. plumbaginifolia* (pma2, pma3 and pma4). Together with a previous report on the *pma1* expression (Michelet et al., 1994), we now have the expression pattern of two

**Leaf histochemistry**

In leaves, both pma2 and pma4 promoters induced GUS expression in the mesophyll and vein tissues (upper view for pma4-gusA in Figure 7a, and cross-section for pma2-gusA in Figure 7b). A closer inspection of the conducting tissues of the main vein (Figure 7c for pma2-gusA and Figure 7d for pma4-gusA) revealed the blue pigment in the internal and external phloem (the latter was more pronounced for pma4), the cells between the phloem and xylem vessels, and in the xylem parenchyma. In addition, the starch sheath was clearly stained. Section along a minor vein enabled localization of the GUS reaction product to the companion cell (Figure 7e).

Furthermore, pma2-gusA and pma4-gusA gene expression pattern also involved guard cells and their subsidiary cells, as in seedlings (Figure 7f, g, respectively) and, particularly for pma4-gusA, the long trichomes or glandular hairs (Figure 7h). The pma3 promoter controlled gusA expression in leaf major veins (Figure 7i), especially in the xylem parenchyma (Figure 7j).

**Flower histochemistry**

Flowers from transgenic plants were stained for GUS at various bud lengths. In floral buds from 0.5 cm long, pma2-gusA expression was restricted to the anthers (Figure 8a), as in pma1 gene expression (Michelet et al., 1994). However, 4-cm long buds expressed GUS in other floral parts, i.e. the conducting tissues of the receptacle and placenta, the nectaries, the ovary, and the ovules (results not shown).

The pma3 gene expression pattern in flower organs was similar to that of pma1 (Michelet et al., 1994): the tapetum cell layer of young floral buds, mature pollen grains, style transmitting tissue, and ovules (results not shown).

In pma4-gusA plants, GUS expression was found in the pollen grains (Figure 8b), the style transmitting tissues (Figure 8c) and in most of the fruit organs (Figure 8d).

Finally, GUS expression was found for the three genes in fruit pods, fertilized ovules, placenta and receptacle reproductive tissues, as well as the funiculus (results not shown).

pm3–gusA expression was restricted to the conducting tissues of leaf traces (Figure 6g).

complete gene subfamilies. Genes from both monocot as well as dicot species have been shown to belong to either subfamily, which suggests a gene duplication event predating plant class separation (Moriau et al., 1993). All the cDNA clones encoding H^+\text{-ATPase} in various monocot as well as dicot species can be grouped into one or the other subfamily. This observation, together with the data reported here, strongly suggests that, as a whole, these two subfamilies contain the major H^+\text{-ATPases} expressed in plants. Four other *N. plumbaginifolia* *pma* genes have been isolated as genomic clones but no corresponding clone was found in root or leaf cDNA libraries (M. Oufattole, M. Arango and M. Boutry, unpublished data), suggesting that their expression might be restricted to specific cell types or repressed under normal growth conditions.

The choice of using *N. tabacum* as a host plant for *pma* expression analysis instead of *N. plumbaginifolia* should not lead to major errors, as both species are closely related. Moreover, numerous examples have been reported where genes, isolated from various dicot species, were properly regulated and expressed in tobacco. In addition, northern blotting with gene-specific probes (Moriau et al., 1993; Perez et al., 1992) and western blotting with subfamily-specific antibodies (Figure 9) have confirmed expression of the two subfamilies in all *N. plumbaginifolia* organs. We have, however, to consider the hypothesis that the *gusA* fusions did not contain all the sequences necessary for the appropriate expression of the *pma* genes. At least 2 kb of the upstream region was included in each case but it is possible that sequences further upstream or downstream, possibly in introns, play a role in gene transcription. *In situ* hybridization with *pma*-specific probes or *in situ* immunodetection of tagged H^+\text{-ATPase} isoforms (DeWitt and Sussman, 1995) have confirmed expression of the two subfamilies in all *N. plumbaginifolia* organs. We have, however, to consider the hypothesis that the *gusA* fusions did not contain all the sequences necessary for the appropriate expression of the *pma* genes. At least 2 kb of the upstream region was included in each case but it is possible that sequences further upstream or downstream, possibly in introns, play a role in gene transcription. *In situ* hybridization with *pma*-specific probes or *in situ* immunodetection of tagged H^+\text{-ATPase} isoforms (DeWitt and Sussman, 1995) have confirmed expression of the two subfamilies in all *N. plumbaginifolia* organs. We have, however, to consider the hypothesis that the *gusA* fusions did not contain all the sequences necessary for the appropriate expression of the *pma* genes. At least 2 kb of the upstream region was included in each case but it is possible that sequences further upstream or downstream, possibly in introns, play a role in gene transcription. *In situ* hybridization with *pma*-specific probes or *in situ* immunodetection of tagged H^+\text{-ATPase} isoforms (DeWitt and Sussman, 1995) have confirmed expression of the two subfamilies in all *N. plumbaginifolia* organs. We have, however, to consider the hypothesis that the *gusA* fusions did not contain all the sequences necessary for the appropriate expression of the *pma* genes. At least 2 kb of the upstream region was included in each case but it is possible that sequences further upstream or downstream, possibly in introns, play a role in gene transcription. *In situ* hybridization with *pma*-specific probes or *in situ* immunodetection of tagged H^+\text{-ATPase} isoforms (DeWitt and Sussman, 1995) have confirmed expression of the two subfamilies in all *N. plumbaginifolia* organs. We have, however, to consider the hypothesis that the *gusA* fusions did not contain all the sequences necessary for the appropriate expression of the *pma* genes. At least 2 kb of the upstream region was included in each case but it is possible that sequences further upstream or downstream, possibly in introns, play a role in gene transcription. *In situ* hybridization with *pma*-specific probes or "in situ" immunodetection of tagged H^+\text{-ATPase} isoforms (DeWitt and Sussman, 1995) would be required to address this problem fully. However, these approaches do not have the flexibility of the *gusA* reporter gene to follow expression of several genes in all tissues at all developmental stages, and might be better used to address more specific questions.

The large palette of expression of the four *pma* genes considered together represents most of the cell types where H^+\text{-ATPase} is expected to play a role. In general, meristematic regions and fast developing flower organs strongly expressed *pma*–*gusA*. We could include in these sink tissues the starch sheath around the conducting vessels.

In young seedlings, grown *in vitro*, high H^+\text{-ATPase} expression was found in the root hairs, suggesting that mineral nutrition might mainly occur at this location. In adult plants, root hairs of plants grown in the soil or hydroponically (not shown in the latter case) were much less stained. Instead, *pma*–*gusA* expression was found to varying levels, according to the gene, in the epidermis, the cortex and the endoderm. This might point to the involvement of these cells in mineral nutrition.

H^+\text{-ATPase} has long been suggested to fuel sugar loading into the phloem vessels. This is supported by the expression of *pma*–*gusA* (this work) and *Arabidopsis* *aha3* (DeWitt and Sussman, 1995) in the companion cells of the minor veins. However, other cell types in the conducting tissues were also strongly stained. This was especially the case for the xylem parenchyma cells and the cells between the xylem and the phloem in the major leaf vein and in the stem. These sites coincide with the location of transfer cells in Solanaceae species (Gunning et al., 1970). These cells might be involved in the exchange of nutrients between transpiration and assimilate streams. This allows, for instance, the developing tissues at the tip (young leaves, flowers, fruits), which have a reduced transpiration rate compared with mature leaves, to receive minerals through the phloem. Given the complexity of the conducting vessels, it is clear that a more detailed analysis is required, using for instance *in situ* immunolocalization and *in situ* RNA hybridization with specific probes. It will be of particular interest to follow H^+\text{-ATPase} expression in both the companion cells and the sieve elements. In fact, in potato, a Solanaceae species like tobacco, there is evidence that mRNA from a sucrose transporter gene (SiSUTi1) is transported from the companion cell to the sieve element where the corresponding protein is detected, suggesting that sucrose loading occurs directly in the sieve element (Kühn et al., 1997). Determining whether a similar transfer occurs for the tobacco *pma* transcripts will require a study of their expression without altering the RNA structure.

Guard cells are known to be active in transport and to express H^+\text{-ATPase} highly. This was confirmed by the expression of both *pma2*– and *pma4*–*gusA* in these cells. Subsidiary cells were stained as well. This observation suggests that these cells also undergo intense transport, possibly related to their function as ion storage for the guard cells.

At this stage, we have to be aware that this functional prediction based on the expression profile needs to be confirmed by other approaches. A very promising one consists in up- and downregulation of gene expression by genetic transformation, such as that performed for the *Arabidopsis* *aha3* gene, the overexpression of which led to acid tolerance in seedlings (Young et al., 1998).

We have confirmed the previous conclusion (Moriau et al., 1993) that *pma4* is the only gene belonging to the second subfamily. This contrasts with *Arabidopsis* in which this gene subfamily contains at least three representatives (*aha1, aha2* and *aha3*). The question is therefore whether a unique ancestral gene was duplicated twice within the *Arabidopsis* direct ancestor or whether two genes were lost in that of *Nicotiana*. Answering this question would require...
knowledge of the whole gene organization in other plant families, close to that of either *Nicotiana* or *Arabidopsis*. There is, however, another aspect that can already be addressed. If we speculate that the existence of gene subfamilies has a functional implication, conserved during evolution, we would expect the *pma4* expression to cover that of *aha1*, *aha2* and *aha3* together. With regard to the broad expression pattern of the *N. plumbaginifolia* *pma4*, expression of the *Arabidopsis* *aha1*, *aha2* and *aha3*, taken individually, seems more restricted. *AHA3* expression is restricted to companion cells and some floral organs (DeWitt and Sussman, 1995; DeWitt et al., 1991). *AHA2* is expressed in the root epidermis (J.F. Harper, personal communication). Although *AHA1* expression is still unknown, expression of the other two genes suggests that the role of *pma4* in *N. plumbaginifolia* is taken over by three genes in *Arabidopsis*. The maize *MHA2* H+-ATPase gene, like *pma4*, is expressed in guard cells, phloem cells and root epidermal cells (Frias et al., 1996). A further situation exists in *Vicia faba*, in which at least two H+-ATPase genes (*VHA1* and *VHA2*) belong to the same subfamily as *pma4*. They are expressed in guard cells and also in other cell types, but not in the vascular tissues (Hentzen et al., 1996), suggesting, again, that gene organization and expression might have evolved differently in different species.

An important observation of this study was that two different *pma* genes might be expressed in the same cell at the same developmental stage. According to the *gusA* reporter gene, *pma2* and *pma4* genes have overlapping expression patterns, e.g. in some cell types of the conducting tissues and in guard cells. Although this might just be considered as a way to obtain a higher expression, this redundancy might also have functional and/or regulatory implications. Given the central role of H+-ATPase in transport, its expression might be regulated by a large variety of signalling pathways. Two genes instead of one might therefore increase possibilities of regulation. At the protein level, it might also be speculated that isoforms encoded by distinct *pma* have different kinetics or regulatory properties. This speculation received support recently with the observation that PMA2 and PMA4, when expressed in the yeast *S. cerevisiae*, display distinct kinetics and confer differential pH sensitivity of yeast growth (Luo et al., 1999). Although these differences will have to be confirmed in the plant, we can already speculate that the simultaneous expression of *pma2* and *pma4* in certain cell types might cause a tighter control of this high ATP consumer.

Although not yet addressed extensively in this study, *pma* regulation during development might already be illustrated for *pma4* in phloem vessels. In 5–7-cm long plantlets, *pma4-gusA* was expressed in the outer phloem tissue of the stem (not shown). In adult plants, the expression was more complex. From the stem middle distance to the apex, the inner phloem tissue only was stained, while at the stem basal region both outer and inner phloems were active. It seems likely that in plantlets the outer phloem ensures bilateral movement of the sap to the root and developing leaves in a similar way as that observed for most plants species which have only the outer phloem. With the inner phloem development, specialization could occur, allowing major nutrient transport to the root and the aerial organs through the outer and inner phloem, respectively. A similar differential expression according to the stem position has been observed for a membrane intrinsic protein presumably involved in water transport (Jones and Mullet, 1995).

Using antibodies specific to each PMA subfamily, we were able to confirm the expression at protein level of both subfamilies, in all the organs analysed for both *N. tabacum* and *N. plumbaginifolia*. This has an important impact on biochemical approaches used to characterize H+-ATPases from plant membranes. Whatever the organ, there is no possibility to obtain and therefore characterize a membrane preparation containing a single isoform. It would be, of course, most interesting to develop *in situ* immunodetection to follow up the protein expression at cell level, although this would not allow distinction of the closely related PMA1, PMA2 or PMA3. And yet, a detailed comparison between protein and RNA expression would be of particular interest. We had, in fact, previously uncovered signs of translation regulation within the leader (transcript untranslated 5′ region) of *pma1* and *pma3* transcripts (Lukaszewicz et al., 1998; Michelet et al., 1994). This might also be the case for *pma2* and *pma4*, for which we have now identified their transcript 5′ end. Their leader also contains open reading frames of 4–14 codons. We recently showed for the *pma3* leader a mechanism of translation reinitiation: part of the ribosomes that have translated the leader small open reading frame are capable of initiating translation at the PMA reading frame level. We proposed that the rate of reinitiation might be subjected to regulation (Lukaszewicz et al., 1998). The presence of the same features in the four *pma* genes characterized so far suggests that we are dealing with a general translation mechanism, although the regulation modalities might differ according to the gene.

### Experimental procedures

**Genomic library construction**

*Nicotiana plumbaginifolia* genomic DNA extraction and purification were performed as described in Boutry and Chua (1985). DNA was partially digested with *Sau3A*; 15–20-kb fragments were isolated by centrifugation on a sucrose gradient and ligated to *XhoI*-generated arms of *λGEM™-11* vector. Prior to the ligation step, both the vector and insert ends were filled in, in the presence of dCTP and dTTP, to generate complementary sticky ends.
Library screening

After in vitro packaging, a library of ~500 000 independent clones was obtained and amplified in Escherichia coli K802. The genomic library was screened under low stringent conditions with two 32P-labelled probes corresponding to pma4 and pma2 cDNA.

S1 nuclease mapping of the transcript 5' end

The probe used for pma4 was prepared as follows. Ten pmoles of the LM1 primer (5' GCCATCTCGTCTC 3'), overlapping the PMA4 translational start, was radioactively labelled at the 5' end with T4 polynucleotide kinase and γ-32P-ATP. The labelled primer was used in combination with 10 pmoles of reverse primer to perform a PCR reaction on the pPRP4EX plasmid containing an insert extending from 280 nucleotides upstream from the PMA4 translational start to 24 nucleotides downstream. The labelled probe was separated by electrophoresis on a 5% polyacrylamide gel and purified by electroelution.

The probe used for pma2 was also obtained by PCR. The two primers used, SKP2-237 (5' CTGGTCTGCTCCTGTC 3') and SKP2-464 (5' GTGGCGTGTAAAGTTC 3'), were positioned upstream of the intron located in the pma2 5' untranslated region. Only the SKP2-237 primer was labelled at the 5' end with T4 polynucleotide kinase.

Plant total RNA (Moriau et al., 1993) and probe (5000–20 000 c.p.m.) were hybridized for 16 h at 42°C and 37°C (pma4 and pma2, respectively) in 80% (v/v) formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM PIPES (pH 6.4). S1 nuclease digestion was performed at 15°C for 1 h in 150 µl of 0.2 M NaCl, 50 mM sodium acetate (pH 4.6), 1 mM ZnSO4, 0.5% glycerol, 20 µg/ml herring sperm DNA and 600 U S1 nuclease. After ethanol precipitation, samples were electrophoresed in a denaturing 7 M urea–6% polyacrylamide gel.

gusA constructs

A 5-kb HindIII–Sau3A genomic fragment containing the putative promoter of the N. plumbaginifolia pma2 gene was inserted into the HindIII–Smal sites of the pBluescript SK+ plasmid (Stratagene). The Sau3A restriction site, localized 13 nucleotides downstream of the PMA2 initiator ATG, had its ends blunted, using T4 DNA polymerase. The genomic fragment was then excised from the Bluescript SK+ vector by a HindIII–BamHI digestion and ligated into the same restriction sites of pBl101.1 (Jefferson et al., 1987), yielding the pma2–gusA construct.

A 3-kb EcoRI genomic fragment extending to +198 from the pma2 transcription start site (or 64 nucleotides upstream of the translation start) was filled in with the Klenow fragment and ligated into the Smal site of pBl101.2.

Finally, a 2.3-kb BamHI–XhoI (the latter filled in with the Klenow fragment) genomic fragment containing the N. plumbaginifolia pma4 putative promoter region was inserted into the BamHI and Smal cloning sites of pBl101.1.

The junction of the promoter–gusA fusions was verified by DNA sequencing.

Plant transformation and GUS assays

The plasmids containing the three pma–gusA fusions were mobilized into Agrobacterium tumefaciens, strain LBA4404, by triparental mating. Tobacco (Nicotiana tabacum L. cv. Petit Havana SR1) was stably transformed using the leaf-disc method (Horsch et al., 1986).

Histochemical staining of β-glucuronidase (GUS) in seeds, embryos and seedlings required permeabilization for 20 min in 50 mM sodium phosphate buffer (pH 7.0) containing 20% ethanol, prior to incubation in 50 mM sodium phosphate buffer (pH 7.0) and 1 mM X-Gluc (Rose Scientific Ltd). Histochemical staining of hand-cut sections was performed in 100 mM sodium phosphate buffer (pH 7.0), 0.02% (w/v) NaN3, 0.1% (w/v) Triton-X-100, and 1 mM X-Gluc for 3–16 h at 37°C. When analysing to easily oxidize objects (i.e. fruits and flower organs), 10 mM β-mercaptoethanol and 10 mM EDTA were added to the incubation medium. When contiguous cell types (e.g. guard cells and subsidiary cells) were stained, a control was performed under oxidizing conditions (2 mM potassium ferricyanide, no β-mercaptoethanol) to rule out diffusion of the reaction product. After 3–16 h of incubation at 37°C, the GUS substrate solution was removed and the chlorophyll solubilized in 100% ethanol. Cleared samples were then preserved in 50% (w/v) glycerol.

Antiserum production

Two sets of peptides corresponding to two highly divergent regions between the two subfamilies were synthesized: AHNK- DIERR (PMA1,2,3) and NNSYRELSEIAE (PMA4) in the large loop, and TNFELNQLAE (PMA1, 2, 3) and NSYRELSEIAE (PMA4) in the C-terminal region of H+-ATPase. Peptides were coupled to keyhole limpet haemocyanin with glutaraldehyde and injected into rabbits. Antibodies were purified by affinity chromatography, using peptides coupled to ECH Sepharose 4B (Pharmacia).

Western blot analysis

Plasma membranes from yeast expressing either pma2 or pma4 were prepared as described by Luo et al. (1999). A microsomal fraction of plant organs was prepared as follows. Material (2 g) was homogenized in 8 ml of 250 mM sorbitol, 60 mM Tris, pH 8.0 (HCl), 2 mM EDTA, 0.6% polyvinylpyrrolidone, 5 mM DTT, 1 mM phenylmethysulphonyl fluoride and 2 μg/ml each of leupeptin, aprotinin, antipain and chymostatin. The homogenate was filtered and centrifuged in Eppendorf tubes at 10 000 g for 3 min. The supernatant was centrifuged further at 20 800 g for 60 min. The pellet was resuspended in 5 mM KH2PO4, 330 mM sucrose and 3 mM KCl.

Yeast and plant membrane fractions were analysed by SDS-PAGE and transferred onto a nitrocellulose membrane. H+-ATPase was detected using the antibodies described above and a secondary antibody revealed by chemiluminescence.

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