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# A Major Isoform of the Maize Plasma Membrane $H^+$-ATPase: Characterization and Induction by Auxin in Coleoptiles

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**The plasma membrane (PM) $H^+$-ATPase has been proposed to play important transport and regulatory roles in plant physiology, including its participation in auxin-induced acidification in coleoptile segments. This enzyme is encoded by a family of genes differing in tissue distribution, regulation, and expression level. A major expressed isoform of the maize PM $H^+$-ATPase (*MHA2*) has been characterized. RNA gel blot analysis indicated that *MHA2* is expressed in all maize organs, with highest levels being in the roots. In situ hybridization of sections from maize seedlings indicated enriched expression of *MHA2* in stomatal guard cells, phloem cells, and root epidermal cells. *MHA2* mRNA was induced threefold when nonvascular parts of the coleoptile segments were treated with auxin. This induction correlates with auxin-triggered proton extrusion by the same part of the segments. The PM $H^+$-ATPase in the vascular bundles does not contribute significantly to auxin-induced acidification, is not regulated by auxin, and masks the auxin effect in extracts of whole coleoptile segments. We conclude that auxin-induced acidification in coleoptile segments most often occurs in the nonvascular tissue and is mediated, at least in part, by increased levels of *MHA2*.**

## INTRODUCTION

The major plasma membrane ATPase of plant cells (PM $H^+$-ATPase) is an electrogenic proton pump, which has been proposed to play important bioenergetic and regulatory roles in plant physiology (Serrano, 1989; Briskin, 1990). For example, the electrochemical proton gradient generated by the enzyme may be the driving force for secondary active transport at the plasma membrane. In addition, the two components of the proton gradient, the electrical membrane potential and the pH gradient, may have regulatory effects on voltage-regulated membrane proteins and on pH-sensitive enzyme and transport systems, respectively.

Immunocytolocalization of the PM $H^+$-ATPase has shown enrichment in tissues specifically involved in active transport, such as stomatal guard cells, phloem cells, and root epidermal cells. This finding is in agreement with the proposed bioenergetic role of the enzyme (Serrano, 1993). Molecular cloning has demonstrated the existence of several genes expressing similar but distinct forms of the proton pump. Ten different PM $H^+$-ATPase genes have been identified in Arabidopsis (Sussman, 1994) and seven in tomato (Ewing and Bennett, 1994). Because these genes may have different functions, functional and tissue localization studies must be performed with isoform-specific methodologies, such as expression of cDNAs in heterologous systems (Palmgren and Christensen, 1994), expression of promoter–reporter fusions in transgenic plants (DeWitt et al., 1991), and in situ hybridization with isoform-specific probes (Coen et al., 1990; Jackson et al., 1991).

The plant PM $H^+$-ATPase seems to be regulated by all of the important factors controlling plant physiology: light, hormones, phytotoxins, and environmental stress (Serrano, 1989). The molecular mechanisms of these regulations, however, are only starting to be elucidated (Serrano, 1993; Sussman, 1994). One important aspect of PM $H^+$-ATPase regulation is its activation by auxin in elongating tissues, such as maize coleoptile segments. The "acid growth" theory states that when exposed to auxin, susceptible cells extrude protons into the wall at an enhanced rate via the PM $H^+$-ATPase, resulting in a decrease

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in apoplastic pH (Hager et al., 1971; Rayle and Cleland, 1977, 1992). The lowered pH then induces wall loosening, probably involving acid-activated xyloglucan endotransglycosylase (Fry et al., 1992) and "expansin" proteins that break wall hydrogen bonds (Shcherban et al., 1995). Although the detailed mechanisms are not known, it seems that auxin-induced wall acidification is an essential component of the elongation response and that it is mediated by increased activity of PM $H^+$-ATPase (Evans, 1985; Rayle and Cleland, 1992). The PM $H^+$-ATPase in elongating tissues may be regulated by auxin through changes in either catalytic activity or amount of enzyme. However, the controversy remains because the finding that auxin induces the synthesis of the PM $H^+$-ATPase (detected with a specific antibody) in maize coleoptile segments (Hager et al., 1991) could not be reproduced in hypocotyls from either tomato (Ewing and Bennett, 1994) or sunflower (Cho and Hong, 1995).

The objective of this work was to use isoform-specific probes to investigate the tissue localization of maize PM $H^+$-ATPase mRNAs and their possible regulation by auxin in coleoptile segments. The only PM $H^+$-ATPase gene that has been reported in maize (*MHA1*, for Maize $H^+$-ATPase gene 1) is expressed at a low level (Jin and Bennetzen, 1994), and it was identified as a DNA fragment captured by the *Bs1* retroelement (Jin and Bennetzen, 1994; Palmgren, 1994). We have characterized a major expressed isoform of the maize proton pump. This novel PM $H^+$-ATPase gene, which we designated *MHA2*, is very different from *MHA1* and is preferentially expressed in guard cells and phloem and root epidermal cells. *MHA2* mRNA accumulation is induced by auxin in the nonvascular part of coleoptile segments, the same tissue that contributes the most to auxin-triggered proton extrusion.

## RESULTS

### Cloning and Molecular Analysis of *MHA2*

The maize PM $H^+$-ATPase gene *MHA2* was isolated as three overlapping cDNA fragments (Figure 1). Sequencing of the overlapping regions indicated that the three clones correspond to the same PM $H^+$-ATPase gene. This gene was different from the *MHA1* sequence described by Jin and Bennetzen (1994) and was designated *MHA2*.

The nucleotide sequence of *MHA2* and the deduced amino acid sequence are shown in Figure 2. The *MHA2* PM $H^+$-ATPase contains all of the conserved motifs of ATPases with phosphorylated intermediate (Serrano, 1989). Comparison with other ATPase sequences (Figure 3) indicates that *MHA2* belongs to the subfamily of plant PM $H^+$-ATPases defined by the Arabidopsis *AHA1-3* (for Arabidopsis $H^+$-ATPase) isoforms (Harper et al., 1989; Pardo and Serrano, 1989), the tobacco *PMA4* (for PM ATPase) isoform (Moriau et al., 1993), and the bean *BHA1* (for Bean $H^+$-ATPase) isoform (F. Campos, J.R. Pérez-Castiñeira, and R. Serrano, unpublished data).

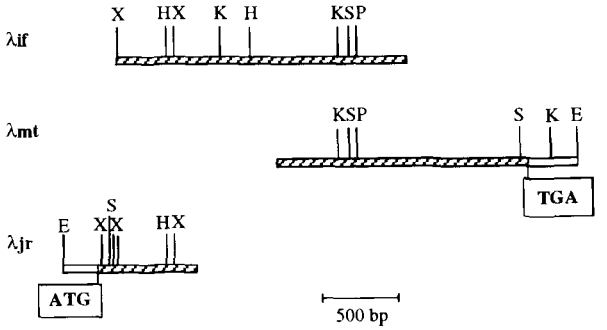


**Figure 1.** Cloned *MHA2* cDNA Fragments.

Restriction maps of three overlapping clones (λif, λmt, and λjr) comprising the complete cDNA sequence of *MHA2* are shown. Symbols for restriction sites are as follows: E, EcoRI; H, HindIII; K, KpnI; P, PstI; S, SacI; X, XhoI. The start (ATG) and stop (TGA) codons of the *MHA2* reading frame (striped bar) are indicated. The open regions correspond to the 5′ and 3′ noncoding parts of the cDNA.

The SacI-EcoRI fragment of the 3′ noncoding region of *MHA2* (Figure 1) exclusively recognized this gene, as shown by DNA gel blot analysis. This result suggests that *MHA2* is a single-copy gene. On the other hand, a less specific probe made from the λif insert (Figure 1) also recognized a related gene in the maize genome, especially when the hybridization and washes were performed at reduced stringency (data not shown).

*MHA2* inserts are well represented in cDNA libraries from maize seedlings. We have screened more than 200,000 plaques by using the λif insert (Figure 1) as a probe under low-stringency conditions. As a result, we have isolated 17 clones that all correspond to *MHA2* when sequenced. Therefore, other related genes are not well represented in the library, and *MHA2* is a major expressed isoform of maize PM $H^+$-ATPase. RNA gel blot analysis with the specific *MHA2* probe indicated that *MHA2* is expressed in all maize organs, although the expression level is higher in roots, followed by coleoptiles, flowers, and leaves (Figure 4).

### Cellular Distribution of *MHA2* mRNA

We have investigated the cellular distribution of *MHA2* transcripts by in situ hybridization of fixed sections from embryos and seedlings with a specific probe. The SacI-EcoRI fragment of the 3′ noncoding region of *MHA2* (Figure 1) exclusively recognizes this gene by DNA gel blot analysis (data not shown); therefore, sense and antisense digoxigenin-labeled riboprobes from this fragment were used.

In maize embryos, the highest expression of *MHA2* was detected at the epidermis of the scutellum facing the endosperm (Figure 5B). In the aerial part of the seedlings, *MHA2* expression was enriched in phloem tissue of the vascular bundles of coleoptiles and leaves (Figures 5D and 5F) and in stomatal guard cells (Figure 5H). Control hybridizations with a sense

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GAATTCGGGGCGGTGGAACAGAGGAGGAGCCCGACTGAGGCTAGGCGAGCGGAGGCCTGGG      60
CGGACGGATCCATGAGAAGAAGCGGACCCTTCCTTTCTCGCACGGGAAGAGTAGCGAGGA
GGCGCGGGCCCCGTGCGGGTATGCCCGTGCCTGCCGTCCGCCTCTAGGACTAGGG     180
TCCGCTCCAGCGCCAGCCGTCCGCCATGGGTGGGCTCGAGGAGATCAAGAACGAAGCCGT
                          M  G  G  L  E  E  I  K  N  E  A  V
CGATCTGGAGAACATCCCCATCGAGGAGGTGTTCGAGCAGCTGAAATGCACCCGCGAGGG     300
 D  L  E  N  I  P  I  E  E  V  F  E  Q  L  K  C  T  R  E  G      31
GCTGAGCTCCAGCGAGGGCCAGCAGCGTCTCGAGATCTTTGGCCCCAACAGGCTCGAGGA
 L  S  S  S  E  G  Q  Q  R  L  E  I  F  G  P  N  R  L  E  E
GAAGAAGGAGAGCAAGGTCCTCAAGTTCCTTGGGTTCATGTGGAACCCGCTGTCATGGGT     420
 K  K  E  S  K  V  L  K  F  L  G  F  M  W  N  P  L  S  W  V      71
CATGGAGATGGCTGCCATCATGGCTATTGCACTCGCCAACAGTGGTGGCAAGCCCCCGGA
 M  E  M  A  A  I  M  A  I  A  L  A  N  S  G  G  K  P  P  D
CTGGCAGGACTTCGTCGGTATCATCGTGCTCTTGGTTATCAACTCCACCATCTCCTTCAT     540
 W  Q  D  F  V  G  I  I  V  L  L  V  I  N  S  T  I  S  F  I      111
TGAGGAGAACAATGCCGGCAACGCGGCCGCCGCACTCATGGCCAACCTTGCTCCAAAAAC
 E  E  N  N  A  G  N  A  A  A  A  L  M  A  N  L  A  P  K  T
CAAGGTTCTGAGAGATGGCCGATGGGGCGAGCAAGAGGCTGCAATCTTGGTTCCTGGTGA     660
 K  V  L  R  D  G  R  W  G  E  Q  E  A  A  I  L  V  P  G  D      151
CATCATCAGCATCAAGCTTGGCGATATCATCCCTGCCGACGCTCGTCTTCTCGAGGGTGA
 I  I  S  I  K  L  G  D  I  I  P  A  D  A  R  L  L  E  G  D
TCCACTCAAGGTTGACCAGTCTGCGCTTACTGGAGAATCCCTCCCGGTTACCAAGGGCCC     780
 P  L  K  V  D  Q  S  A  L  T  G  E  S  L  P  V  T  K  G  P      191
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 G  D  E  V  F  S  G  S  T  C  K  Q  G  E  I  E  A  V  V  I
TGCCACCGGAGTGCACACCTTCTTCGGCAAGGCCGCTCATCTTGTGGACAGCACCAACCA     900
 A  T  G  V  H  T  F  F  G  K  A  A  H  L  V  D  S  T  N  Q      231
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 V  G  H  F  Q  Q  V  L  T  A  I  G  N  F  C  I  C  S  I  G
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 V  G  I  L  V  E  I  I  V  M  F  P  I  Q  H  R  R  Y  R  S      271
CGGAATCGAGAACCTGTTGGTCCTCTTGATCGGTGGTATCCCAATCGCCATGCCTACCGT
 G  I  E  N  L  L  V  L  L  I  G  G  I  P  I  A  M  P  T  V
GCTTTCAGTTACCATGCCGATTGGTTCGCACAAGCTGTCACAGCAGGGTGCCATCACTAA    1140
 L  S  V  T  M  P  I  G  S  H  K  L  S  Q  Q  G  A  I  T  K      311
GAGGATGACTGCCATTGAGGAGATGGCTGGTATGGACGTGCTTTGCAGTGACAAAACCGG
 R  M  T  A  I  E  E  M  A  G  M  D  V  L  C  S  D* K  T  G
CACACTCACCCTCAACAAGCTTAGCGTCGATAAGAACCTCGTCGAGGTGTTCTGCAAAGG    1260
 T  L  T  L  N  K  L  S  V  D  K  N  L  V  E  V  F  C  K  G      351
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 C  H  C  K  E  D  L  R  R  K  V  H  S  I  I  D  K  Y  A  E
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 R  G  L  R  S  L  A  V  A  R  Q  E  V  P  E  K  N  K  E  S      471
TCCGAGGGGGGCCATGGCAGTTCGTTGGTCTGTTGCGGGTGTTTGATCCGCCTAGGCATGA
 P  G  G  P  W  Q  F  V  G  L  L  R  V  F  D  P  P  R  H  D
CAGCGCTGAGACAATTCGCAAGGCTCTTGTTCTTGGTGTTAACGTCAAGATGATCACGGG    1740
 S  A  E  T  I  R  K  A  L  V  L  G  V  N  V  K  M  I  T  Q      511
TGATCAGCTTGCTATTGGTAAGGAGACTGGAAAGGAGGCTTGGCATGGGTACCAACATGTA
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CGTGAAGAGACTGCAGGAGAAGAAGCACATCGTTGGTATGACTGGAGATGGTGTCAACGA    1980
 V  K  R  L  Q  E  K  K  H  I  V  G  M  T  G  D  G  V  N  D      591
TGCCCCGGCTCTTAAGAAAGGCTGACATCGGTATTGCTGTTGCGGATGCTACTGACGCTGC
 A  P  A  L  K  K  A  D  I  G  I  A  V  A  D  A  T  D  A  A
GAGGAGTGCTTCCGACATTGTCCTTACTGAGCCAGGTCTTAGCGTAATTATCAGCGCCGT    2100
 R  S  A  S  D  I  V  L  T  E  P  G  L  S  V  I  I  S  A  V      631
CCTCACCAGCAGAGCAATCTTCCAGAGGATGAAGAACTACACCATCTATGCAGTTTCCAT
 L  T  S  R  A  I  F  Q  R  M  K  N  Y  T  I  Y  A  V  S  I
CACAATCCGTATAGTTCTCGGATTTATGCTCATTGCGTTGATCTGGCAATACGATTTCTC    2220
 T  I  R  I  V  L  G  F  M  L  I  A  L  I  W  Q  Y  D  F  S      671
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 G  I  V  L  Q  G  Y  L  A  L  M  T  V  I  F  F  W  A  M  H
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 K  T  D  F  F  S  D  K  F  G  V  R  S  I  R  D  S  E  H  E      751
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CCTCGACACGCGAATTGCATTCACACGGAAAAAAAGACCTTCGGAAAGGAGAGAGGGAGAC    2820
 L  D  T  R  I  A  F  T  R  K  K  D  L  R  K  G  E  R  E  A      871
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 F  N  D  K  S  S  Y  R  E  L  S  E  I  A  E  Q  A  K  R  R      911
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 K  L  K  G  L  D  I  D  T  I  Q  Q  N  Y  T  V  *              951
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TTTTCAATAGTGGAGTGTGCACGTGTTGTAATCGTTTTTACCTGCGTTTCCTCTAACTA    3300
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AAAAAAAAA
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**Figure 2.** Nucleotide and Deduced Amino Acid Sequences of *MHA2*.

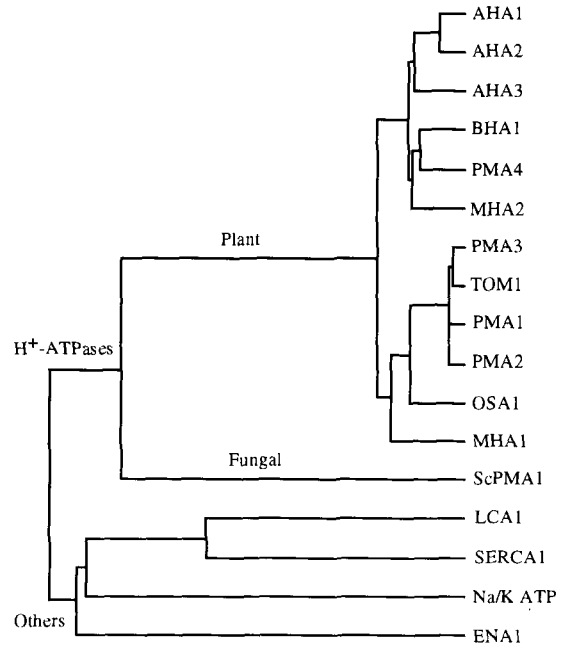


**Figure 3.** Dendrogram of the Comparison between *MHA2* and Different P-Type ATPases.

Amino acid sequences of several ATPases were compared with the program PILEUP (Genetics Computer Group, Madison, WI). Notation is as follows: *AHA1*, *AHA2*, and *AHA3* are PM H⁺-ATPase isoforms from Arabidopsis (Harper et al., 1989, 1990; Pardo and Serrano, 1989); *PMA1*, *PMA2*, *PMA3*, and *PMA4* are PM H⁺-ATPase isoforms from *Nicotiana plumbaginifolia* (Boutry et al., 1989; Perez et al., 1992; Moriau et al., 1993); *BHA1* (F. Campos, J.R. Perez-Castiñeira, and R. Serrano, unpublished data; EMBL accession number X85804) is a PM H⁺-ATPase from bean; *MHA1* (Jin and Bennetzen, 1994) is a PM H⁺-ATPase from maize; *TOM1*, *OSA1*, and *ScPMA1* are PM H⁺-ATPases from tomato (Ewing et al., 1990), rice (Wada et al., 1992), and yeast (Serrano et al., 1986), respectively; *LCA1* is a putative $Ca^{2+}$-ATPase from tomato (Wimmers et al., 1992); *SERCA1* is sarcoplasmic reticulum $Ca^{2+}$-ATPase isoform 1 from frog skeletal muscle (EMBL accession number X63009); *Na/K ATP* is the $Na^+/K^+$-ATPase from rat brain (Hara et al., 1987); *ENA1* is the putative $Na^+$-ATPase isoform 1 from yeast (Haro et al., 1991).

riboprobe showed the intrinsic contrast of collenchyma and sclerenchyma tissues (Figure 5G). In seedling roots, high expression was observed at the surface (Figures 6B and 6D) and in phloem tissue (Figures 6D and 6F). Control hybridizations

The sequence was obtained for both strands, as described in Methods. The deduced amino acid sequence is shown below the corresponding nucleotides. The predicted ATG start codon is at position 206, and the stop codon is at position 3050. The six amino acid motifs most conserved in ATPases with phosphorylated intermediate (Serrano, 1989) are underlined. The aspartate forming the phosphorylated intermediate is marked with an asterisk. The nucleotide sequence has EMBL accession number X85805.

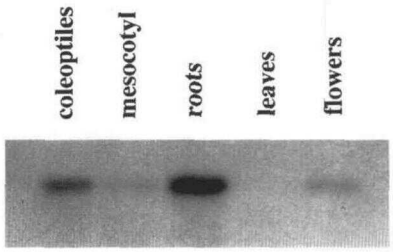


**Figure 4.** RNA Gel Blot Analysis of *MHA2* mRNA in Different Organs.

Each lane was loaded with 15 μg of total RNA from the indicated maize organs. After blotting, the filter was hybridized with the specific *MHA2* probe, as indicated in Methods. Autoradiography of the region containing the 3.6-kb ATPase band is shown.

with a sense riboprobe showed the intrinsic contrast of the root surface (Figures 6A and 6C), which was much less intense than the true hybridization signal at the root epidermis (Figures 6B and 6D).

### Regulation by Auxin in Coleoptile Segments

First experiments on regulation of *MHA2* by auxin were made with segments from the elongation zone of coleoptiles (Figure 7A). The results from numerous RNA gel blot analyses indicated a modest increase (30 to 40% after quantification by densitometry) of *MHA2* mRNA after auxin treatment. This change was insufficient to account for auxin-triggered acidification (Hager et al., 1971; Rayle and Cleland, 1977).

Given the enrichment of PM $H^+$-ATPase protein (Villalba et al., 1991) and mRNA (Figure 5D) in the vascular bundles of the coleoptiles, we speculated that this vascular PM $H^+$-ATPase might not be auxin regulated and that because of its abundance, it could mask regulatory changes in the PM $H^+$-ATPase of nonvascular tissue. Coleoptile segments can be split into vascular and nonvascular parts (see Methods), and after this fragmentation, auxin produced a clear increase in the level of *MHA2* mRNA in nonvascular parts (Figure 7B). Statistical quantitation was made by densitometry, and corrections for uneven loading of the lanes were made using both ethidium bromide–staining of rRNAs and the hybridization signal of tubulin mRNA. The results indicated an average induction factor of 3.0, with a standard deviation of 0.2 (values from three repetitions of the experiment).

The induction of *MHA2* mRNA correlated with the induction of PM $H^+$-ATPase protein levels. Vascular tissue had a higher PM $H^+$-ATPase protein content than did nonvascular tissue, but induction by auxin was very low in the former tissue. On the other hand, nonvascular tissue exhibited a clear induction of PM $H^+$-ATPase protein by auxin (Figure 8). The magnitude of the induction at the protein level (approximately a factor of 2), however, was lower than in the case of *MHA2* mRNA. A plausible explanation is that protein synthesis is more affected than mRNA synthesis by the wounding associated with the preparation of vascular and nonvascular parts.

To determine whether this localized PM $H^+$-ATPase induction correlated with auxin-triggered acidification, we split coleoptile segments into vascular and nonvascular parts and quantified the extrusion of protons in the absence and presence of auxin. The results indicate that the nonvascular part of a coleoptile segment responded most intensely to auxin with increased acidification. The vascular part was less active: the maximum acidification rate (Figure 9A) was much less than that of the nonvascular part, and total net acidification (Figure 9B) was negligible. Normalized to protein content, the acidification rates for vascular and nonvascular parts were 0.5 and 2.5 nmol of $H^+$ extruded per min per mg, respectively, a five-fold difference. In terms of fresh weight, the values were 2.5 and 9.3 nmol of $H^+$ extruded per min per g, a 3.7-fold difference. Because the vascular parts of coleoptile segments contain nonvascular tissue surrounding the bundles (see Figure 10), the actual differences between vascular and nonvascular tissues must be greater. A rough estimate, assuming that half of the weight of vascular parts corresponds to nonvascular tissue, indicates a difference of eight- to 10-fold. As indicated in Table 1, the growth response of vascular and nonvascular parts to acid buffer (pH 4) was very similar, but auxin-induced growth was greater in nonvascular parts.

## DISCUSSION

The *MHA2* ATPase gene characterized in this work corresponds to a major expressed isoform of the maize proton pump. *MHA2* mRNA can be easily detected by RNA gel blot analysis of total RNA preparations from maize tissues, and *MHA2* clones are well represented (with a probability of $\sim 10^{-4}$) in cDNA libraries from maize seedlings. Although several isoforms of this crucial enzyme are likely to be encoded in the genome of all plant species (Sussman, 1994), we have not been able to isolate PM $H^+$-ATPase cDNAs different from *MHA2* in maize seedlings. Screening conditions involved antibody detection in expression libraries and cross-hybridization with *MHA2* at reduced stringency. Also, polymerase chain reaction–based strategies utilizing cDNA from coleoptile tissue as a template and degenerate primers corresponding to conserved amino acid motifs again produced *MHA2* fragments (six analyzed) and no other isoforms (M.T. Caldeira and R. Serrano, unpublished observations). Therefore, other maize PM $H^+$-ATPase genes must have lower expression levels than does *MHA2* or must be expressed under special conditions or in specialized organs (see, for example, Parets-Soler et al., 1993; Houlné and Boutry, 1994). These other PM $H^+$-ATPase genes with low expression in maize seedlings include *MHA1*, a gene partially captured by the maize retroelement *Bs1* (Jin and Bennetzen, 1994) and very different from *MHA2* (Figure 3).

The expression of *MHA2*, as detected by in situ hybridization with a specific probe, was highest in tissues previously

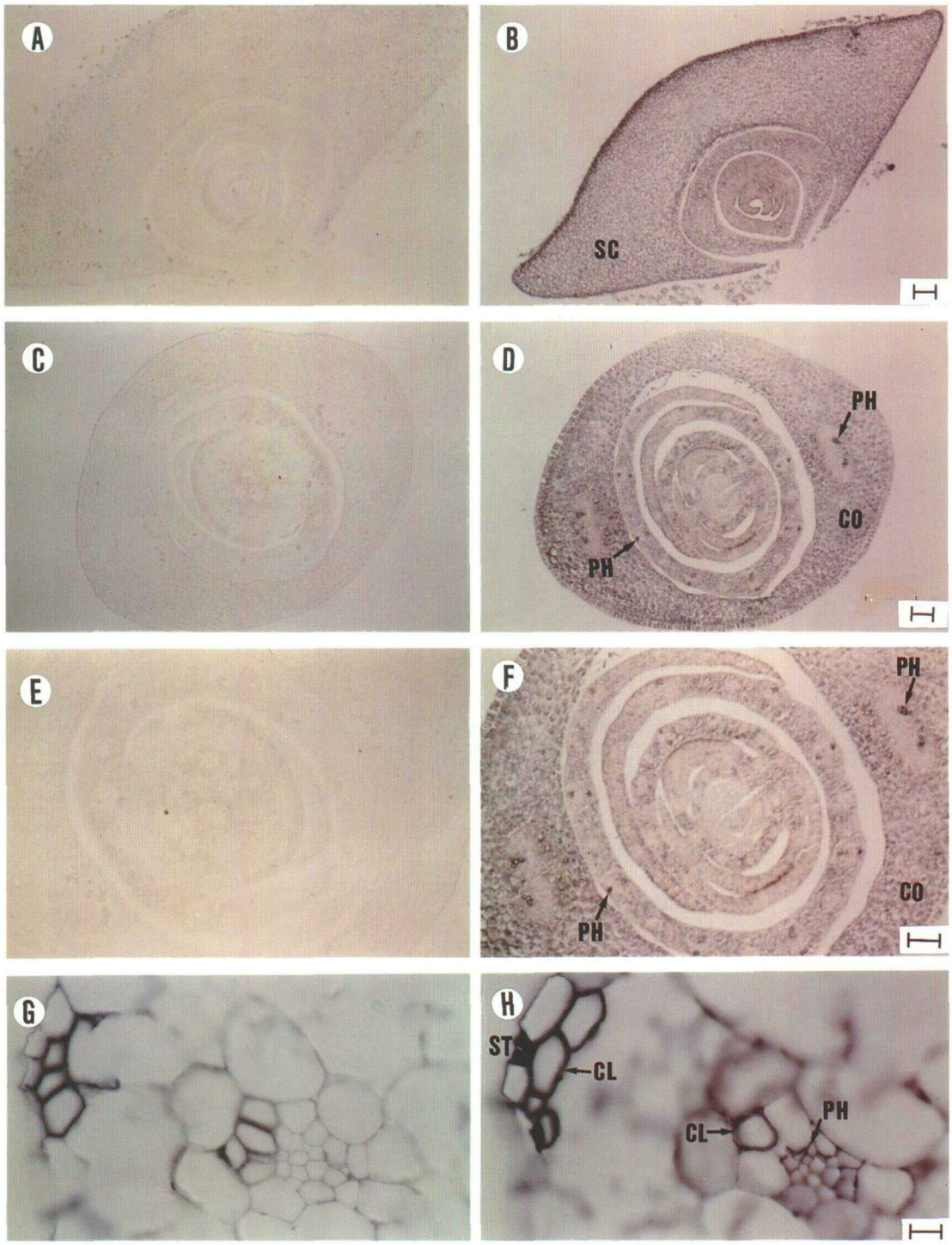


**Figure 5.** Localization of *MHA2* mRNA in Shoots of Maize Embryos and Seedlings.

**(A)** and **(B)** Transverse sections of plumules from embryos 30 days after pollination.
**(C)** and **(D)** Transverse sections of plumules from 4-day-old seedlings.
**(E)** and **(F)** Higher magnification of **(C)** and **(D)**.
**(G)** and **(H)** Detail of stomata and phloem. In **(G)**, artifactual labeling of collenchyma and sclerenchyma cells is apparent.
In situ hybridization was performed with either antisense (**[B]**, **[D]**, **[F]**, and **[H]**) or sense (**[A]**, **[C]**, **[E]**, and **[G]**) digoxigenin-labeled RNA probes from the 0.4-kb SacI-EcoRI fragment corresponding to the 3′ end of *MHA2* cDNA (Figure 1). Sections were visualized by bright-field microscopy, which gives a purple label to the hybrids. CL, collenchyma, sclerenchyma; CO, coleoptile; PH, protophloem; SC, scutellum; ST, stomata guard cells. Bars in **(B)**, **(D)**, and **(F)** = 100 μm; the bar in **(H)** = 10 μm.

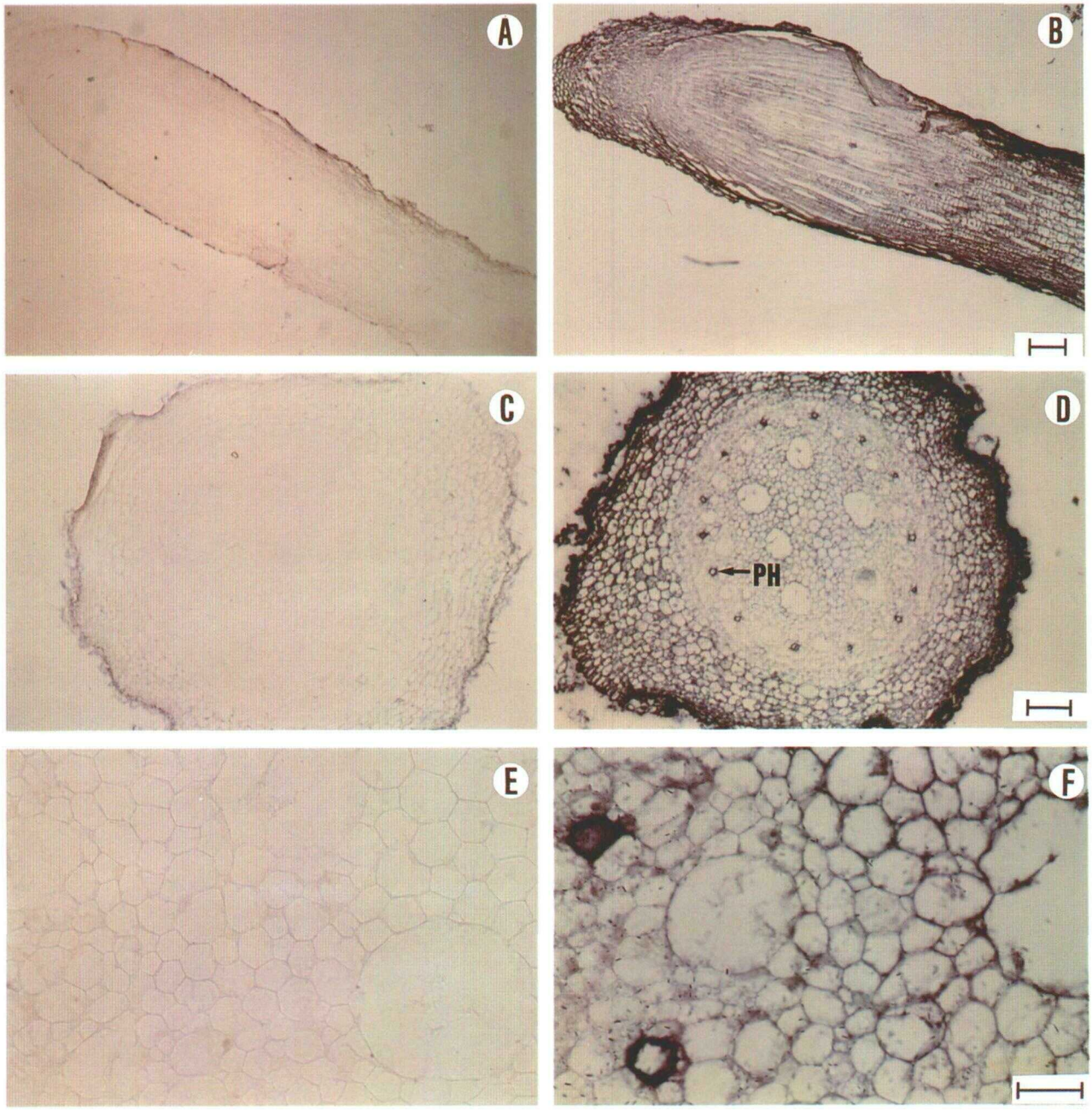


**Figure 6.** Localization of *MHA2* mRNA in Roots of Maize Seedlings.

**(A)** and **(B)** Longitudinal sections of roots from 4-day-old seedlings.
**(C)** and **(D)** Transverse sections of roots from 4-day-old seedlings.
**(E)** and **(F)** Higher magnification of **(C)** and **(D)**.
In situ hybridization was performed with either antisense (**[B]**, **[D]**, and **[F]**) or sense (**[A]**, **[C]**, and **[E]**) digoxigenin-labeled RNA probes from the 0.4-kb SacI-EcoRI fragment corresponding to the 3′ end of *MHA2* cDNA (Figure 1). Sections were visualized by bright-field microscopy, which gives a purple label to the hybrids. The arrow in **(D)** indicates a protophloem cell (PH), which is shown at a higher magnification in **(F)**. The bar in **(B)** = 100 μm; the bar in **(D)** = 40 μm; the bar in **(F)** = 10 μm.

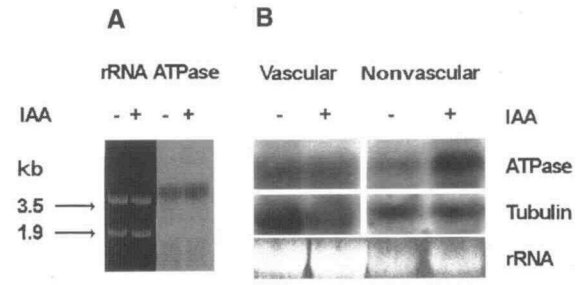


**Figure 7.** Effect of Indole-3-Acetic Acid on the Level of *MHA2* mRNA in Coleoptile Segments and Fragments.

**(A)** RNA gel blot analysis of total RNA (15 μg) from whole coleoptile segments incubated without (−) or with (+) auxin. rRNA lanes are ethidium bromide–stained gels to show the two rRNA bands. ATPase lanes show hybridization with the 0.4-kb SacI-EcoRI fragment corresponding to the 3′ end of *MHA2* cDNA (Figure 1) after autoradiography. **(B)** As shown in **(A)**, but the coleoptile segments were divided into vascular and nonvascular fragments before incubation without (−) or with (+) auxin and only specific bands are shown. ATPase indicates the 3.6-kb hybridization band detected with the 0.4-kb SacI-EcoRI fragment corresponding to the 3′ end of *MHA2* cDNA (Figure 1). Tubulin indicates the 1.9-kb hybridization band detected with the α3-tubulin gene of Arabidopsis (Ludwig et al., 1987). rRNA indicates the 3.5-kb ethidium bromide–stained band of rRNA.

shown to be enriched in PM H+-ATPase by immunolocalization: root epidermis (Parets-Soler et al., 1990; Samuels et al., 1992), stomatal guard cells (Villalba et al., 1991), and phloem (Parets-Soler et al., 1990; Villalba et al., 1991; Samuels et al., 1992). Immunolocalization has been performed with antibodies that are unlikely to differentiate between isoforms (Roldán et al., 1991; Bouché-Pillon et al., 1994); therefore, the results represent the additive contribution of all of the PM H+-ATPase isozymes. The fact that *MHA2* mRNA exhibits tissue distribution similar to total PM H+-ATPase antigen is in agreement with our finding that *MHA2* is a major expressed isoform of maize PM H+-ATPase (see above), probably involved in plasma membrane energization. In this respect, the high *MHA2* ATPase expression at the scutellum epidermis facing the endosperm (Figure 5B) is in accordance with the important role of this cell layer in the nutrition of the embryo (Raven et al., 1986). A translational fusion of the Arabidopsis *AHA3* isoform to the β-glucuronidase reporter gene exhibited expression in phloem but not in root epidermis or stomata (DeWitt et al., 1991). The expression of this Arabidopsis gene seems more restricted than that of *MHA2*, but no in situ hybridization studies were performed. In situ hybridization indicated that the *ZHA1* gene (for Zostera H+-ATPase gene 1) of the seagrass *Zostera marina* was expressed specifically in leaf epidermal cells (Fukuhara et al., 1996). This preferential localization is not observed with PM H+-ATPases of terrestrial plants and represents an adaptation to sea water.

The most significant regulatory feature of *MHA2* identified so far is a threefold increase in steady state mRNA level induced by auxin in the nonvascular tissue of coleoptiles (Figure 7B). These results may provide a molecular mechanism for the "acid-growth" theory of auxin action. By increasing the expression of the proton pump, auxin would reduce apoplastic pH and activate wall-loosening processes resulting in elongation growth (Hager et al., 1971; Rayle and Cleland, 1977, 1992). The induction of coleoptile PM H+-ATPase mRNA by auxin detected in this work is in agreement with the results of Hager et al. (1991), who showed a cycloheximide- and cordycepin-sensitive increase in the PM H+-ATPase antigen when coleoptile segments were treated with auxin.

No significant regulation of the *MHA2* mRNA (Figure 7B) and PM H+-ATPase protein (Figure 8) by auxin was observed in the vascular part of coleoptile segments, which is enriched in PM H+-ATPase. The amounts of *MHA2* mRNA (normalized to total RNA) and PM H+-ATPase protein (normalized to total

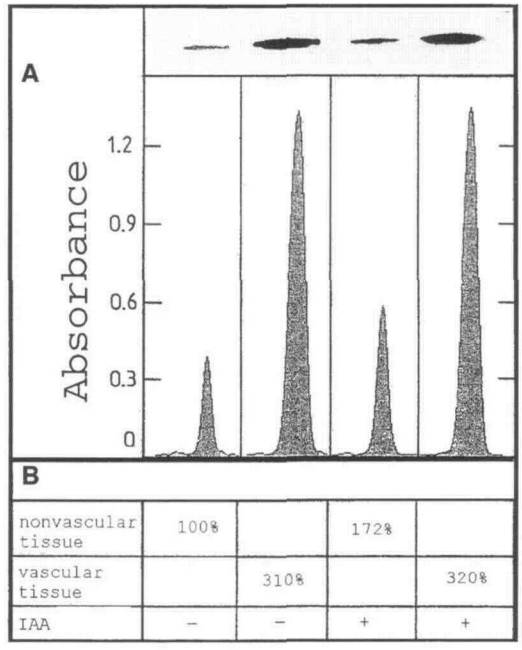


**Figure 8.** Effect of Indole-3-Acetic Acid on the Level of PM H+-ATPase Antigen in Vascular and Nonvascular Parts of Coleoptile Segments.

**(A)** Protein gel blot analysis of membrane proteins (20 μg) from coleoptile segments split into vascular and nonvascular parts and incubated for 40 min with either 20 μM indole-3-acetic acid (IAA +) or 20 μM benzoic acid as control (IAA −). The PM H+-ATPase was visualized by using specific antibody and a chemiluminescence detection system (bands at the upper part). Quantification was performed by densitometry (scans at the lower part).

**(B)** PM H+-ATPase quantification. Average values of two experiments such as the one indicated in **(A)** are shown. The content of nonvascular tissue without auxin treatment was taken as 100%, and values from the two averaged experiments differed by <10%.

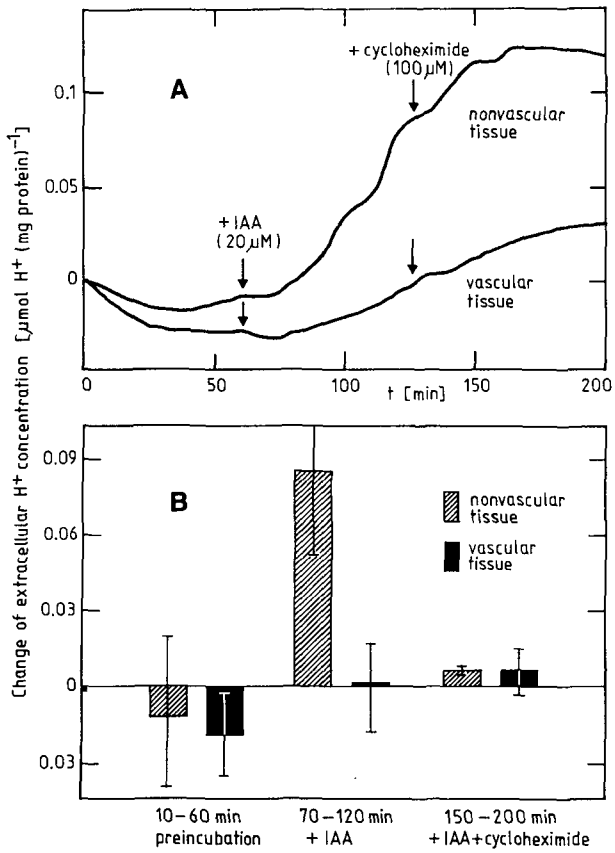


**Figure 9.** Effect of Indole-3-Acetic Acid on the Proton Extrusion of Vascular and Nonvascular Parts of Coleoptile Segments.

**(A)** Time course of extracellular acidification. IAA and cycloheximide indicate the addition of 20 μM indole-3-acetic acid and 100 μM cycloheximide, respectively.

**(B)** Total acidification. Average values of three experiments such as the one indicated in **(A)** are shown. Three successive periods of 50 min were computed: before indole-3-acetic acid (preincubation), during indole-3-acetic acid action, and after the addition of cycloheximide. Bars correspond to standard deviation.

protein) in the vascular part of coleoptile segments were 2.6- and 3.1-fold greater, respectively, than those of the nonvascular part (average of three determinations ±0.4 SD). The contribution of this unregulated PM H+-ATPase in preparations from whole coleoptile segments explains the small effects of auxin observed with this material (a 30 to 40% increase in *MHA2* mRNA). These small changes are difficult to reproduce, which may explain the difficulties experienced by other laboratories in detecting auxin induction of the PM H+-ATPase (Ewing and Bennett, 1994; Cho and Hong, 1995).

An important correlation between molecular and physiological results is that auxin-induced external acidification occurs mostly in the nonvascular part of coleoptile segments, the same tissue that exhibits an increase in PM H+-ATPase expression in response to auxin (Figure 9). Therefore, the PM H+-ATPase of vascular bundles is not regulated by auxin and does not contribute significantly to acidification. Despite the difficulties with the elongation measurements of Table 1, due to the large and variable wound areas of the tissue strips and their curling tendency, it seems that both parts of the coleoptile segments have similar growth responses to acidification but exhibit different capabilities for auxin-induced acidification and consequent growth. Nonvascular parts have a more dramatic response to auxin than do vascular parts in terms of acidification, elongation growth, and induction of *MHA2* mRNA, and this correlation supports a role for the *MHA2*-encoded proton pump in auxin action.

The exclusive induction of the PM H+-ATPase in nonvascular coleoptile tissue resembles the behavior of other genes induced by auxin known as small auxin upregulated RNAs. These small auxin upregulated RNAs are induced primarily in epidermal and cortical cells of elongating hypocotyl sections, with little induction in vascular tissue (Franco et al., 1990). Apparently, vascular bundle cells lack the signal transduction machinery required for auxin responses. Alternatively, it is possible that the vascular tissue of cut coleoptile sections is inactivated by a wound response (Bowles, 1991). The special features of vascular cells may make them more sensitive to wounding than are nonvascular cells. The lack of participation of vascular PM H+-ATPase in auxin-induced acidification may be due to a wound-induced inactive state of either the enzyme or other systems required for acidification. Sustained proton efflux catalyzed by the PM H+-ATPase requires, in addition to ATP synthesis, metabolic conversion of sugars into acids (to prevent intracellular pH increase during H+ efflux) and concomitant K+ uptake (for electrical balance during H+ efflux) (Serrano, 1989). Any of these systems may have little activity in the vascular tissue of coleoptile segments.

The increased expression of the proton pump detected in a previous report (Hager et al., 1991) and in this work may explain the need for protein synthesis in auxin-induced acidification and coleoptile elongation (Hager et al., 1991; Rayle and Cleland, 1992). Other inducers of coleoptile acidification

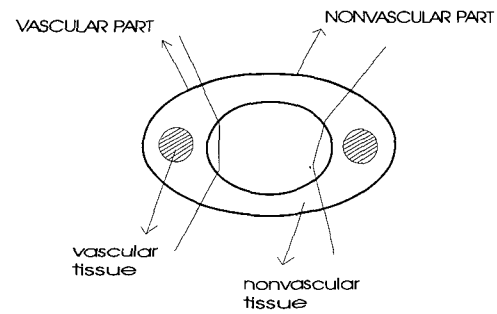


**Figure 10.** Scheme of a Cross-Section of a Coleoptile Segment to Illustrate the Fragmentation into Vascular and Nonvascular Parts.

As indicated, vascular parts contain nonvascular tissue.

**Table 1.** Elongation of Vascular and Nonvascular Parts of Coleoptile Segments in Response to Indole-3-Acetic Acid (20 μM) and to Citrate-Phosphate Buffer (75 mM, pH 4.0)[a]

| Fragment | Acid-Induced Growth | Auxin-Induced Growth |
|---|---|---|
| Nonvascular part | 670 | 90 |
| Vascular part | 620 | 55 |

[a] Results are expressed as the percentage of increase of the elongation rate over the basal rate at pH 5.8 without auxin. Absolute values of basal elongation rates of tissue strips from vascular and nonvascular parts were 3.2 and 1.2 μm $min^{-1}$, respectively. Values are the average of two determinations differing by <20%. The elongation rate of the tissue strips was measured by stretching them at a constant load (2 g) for a period of 30 min in an extensiometer equipped with a linear displacement transducer.

and growth, such as fusicoccin (Hager et al., 1991), lactic acid (endogenously produced by a short period of anaerobiosis; Hager, 1980), or acetate esters (which generate acetic acid within the cytosol upon hydrolysis; Vesper and Evans, 1979; Hager and Moser, 1985), do not require protein synthesis and must directly activate the proton pump. Because the H+-ATPase has an acidic pH optimum (Serrano, 1989), it may be activated by a decrease in intracellular pH (Hager and Moser, 1985). Fusicoccin probably acts by displacement of the inhibitory domain at the C terminus of the PM H+-ATPase through interaction with the fusicoccin–receptor complex (Johansson et al., 1993).

It cannot be excluded that auxin, in addition to increasing the amount of proton pump, also activates the enzyme by some other mechanisms. A relatively direct activation of the PM H+-ATPase (not requiring protein synthesis) could be involved in the rapid (less than 5 min) effects of auxin on membrane potential detected by the patch-clamp technique in a whole-cell configuration (Lohse and Hedrich, 1992; Rück et al., 1993) in the change of affinity for ATP induced by auxin in several experimental systems (Gabathuler and Cleland, 1985; Santoni et al., 1991), and in the decrease in molecular activity triggered by suboptimal auxin in tobacco calli (Altabella et al., 1990).

There is another experimental system in which a correlation has been made between an increased acidification and increased expression of PM H+-ATPase: the "washing" or "adaptative aging" of plant tissue sections. This classic system in transport studies is obtained by sectioning plant tissues into slices and incubating them for a few hours in water. This treatment results in a cycloheximide-sensitive stimulation of transport of inorganic and organic substrates, membrane potential, and proton efflux; all of these effects can be inhibited by cycloheximide (Lüttge and Higinbotham, 1979). A recent report with sections from sugar beet leaves demonstrates a twofold increase in the amount of PM H+-ATPase protein and mRNA triggered by the washing treatment (Noubhani et al., 1996). Therefore, plants may regulate proton efflux in response to different stimuli by modulating the expression level of the PM H+-ATPase.

Future work on the induction of *MHA2* by auxin should explore the presence of auxin-responsive elements (Li et al., 1994; Liu et al., 1994; Ballas et al., 1995; Takahashi et al., 1995) in the promoter of this PM H+-ATPase gene. There are also two regulatory features in the *MHA2* cDNA that merit future investigation. The 5′ leader region contains a short upstream reading frame starting with an ATG 62 nucleotides before the start codon of the PM H+-ATPase reading frame (Figure 2). As suggested for tobacco *PMA1* (Michelet et al., 1994), this finding may indicate translational regulation by reinitiation. On the other hand, the 3′ untranslated region contains the sequence $GGAX_{11}ACAGATX_9TTX_2GTA$, 46 nucleotides after the stop codon (X corresponds to unspecified nucleotides; see Figure 2). This sequence fits the DST consensus element conferring rapid decay to many auxin-inducible mRNAs (Newman et al., 1993).

## METHODS

### Plant Material and Incubation Conditions

*Zea mays* cv Lixis (Nungesser GmbH, Darmstad, Germany) was grown on moistened cellulose for 4 days, as described by Hager et al. (1991). Coleoptiles varying in length between 3 and 4 cm were selected for experiments. Segments of 2 cm were cut 3 mm below the tip, and the enclosed leaves were pulled out. For some experiments, coleoptile segments were sliced longitudinally with a razor blade, resulting in two strings each of vascular and nonvascular parts or fragments (see Figure 9). Vascular parts contained nonvascular tissue surrounding the bundles. During preparation, coleoptile segments and fragments were stored in ice-cold water. The fresh weights of vascular and nonvascular parts for a typical experiment were 2.4 and 2.5 g, respectively (average of three determinations ±0.4 SD). Protein in homogenates was determined according to Lowry et al. (1951) with a Bio-Rad detergent-compatible protein assay kit. The protein contents of vascular and nonvascular parts (in milligrams per gram fresh weight) were 5.0 and 3.7, respectively (average of three determinations ±1.3 SD).

For testing the induction of *MHA2* mRNA by auxin, we preincubated coleoptile segments and fragments (vascular and nonvascular parts) for 30 min at 30°C in 5 mM Mes-KOH buffer, pH 5.8, in a shaking water bath, followed by the addition of 20 μM indole-3-acetic acid–treated samples or benzoic acid (control samples) and incubation for an additional 40 min. Treatment was stopped by placing the treated samples in liquid nitrogen.

The time course of elongation growth in fragments of coleoptile segments was measured by stretching the tissue at a constant load (2 g) in an extensometer equipped with a linear-displacement transducer (Hager and Moser, 1985). Incubation media were as described above for *MHA2* mRNA measurements. Buffer that was adjusted to pH 4.0 was used for acid-induced growth (Hager et al., 1991).

For the determination of proton extrusion, coleoptile fragments (2.4 to 2.5 g fresh weight) were incubated in 60 mL of a solution containing 10 mM KCl and 1 mM each of $Na_2SO_4$, $NaNO_3$, $CaCl_2$, and $Mg(NO_3)_2$. Carbon dioxide–free air was continuously bubbled through the solution (20 mL $min^{-1}$), and the pH was adjusted to 6.0 by a bidirectional

pH controller (pH-Stat; Radiometer, Copenhagen, Denmark). The consumption of titrant (1 mM HCl or 1 mM NaOH) was monitored and converted into changes in proton extrusion.

### Cloning of *MHA2* cDNAs

A λgt11 cDNA expression library from 8-day-old W22 maize seedlings (Clontech, Palo Alto, CA; random and oligo(dT) priming combined) was screened using monoclonal antibody 46E5/B11C10 raised against the maize plasma membrane (PM) $H^+$-ATPase (Villalba et al., 1991). One positive clone containing a 1.9-kb insert (λif) was isolated, phage DNA was purified (Miller, 1987), and the insert was subcloned into the pBluescript KS− plasmid (Stratagene, La Jolla, CA) and sequenced. The cDNA corresponded to a PM $H^+$-ATPase gene truncated at both ends, and it was used as hybridization probe to rescreen the library. The probe was labeled by the digoxigenin method of Boehringer Mannheim. Hybridization and washes were performed at 50°C, according to Church and Gilbert (1984). Positive clones were purified, phage DNA was isolated (Miller, 1987), and the inserts were subcloned into the pBluescript KS− plasmid (Stratagene) and sequenced. Another PM $H^+$-ATPase fragment of 1.9 kb (λmt) containing the 3′ end of the PM $H^+$-ATPase along with a 308-bp 3′ noncoding region was obtained in all cases (Figure 1). A third screening of the λgt11 library was performed using the 313-bp XhoI-XhoI fragment of clone λif (Figure 1) as a probe. In this case, the probe was labeled with α-$^{32}$P-dCTP. Hybridization and wash temperatures were 65°C. Several positive clones were isolated, and the 0.9-kb insert of one of them (λjr) was isolated and sequenced as described above. This fragment contained the 5′ end of the PM $H^+$-ATPase clone as well as a 205-bp-long 5′ noncoding region (Figure 1). Inserts of λif, λmt, and λjr were sequenced on both strands by the method of Sanger et al. (1977), using Sequenase (Amersham). DNA sequence analysis was performed with software from the Genetics Computer Group (Madison, WI).

### DNA and RNA Gel Blot Analyses

Genomic DNA was isolated from leaves of 10-day-old plants, according to Doyle and Doyle (information from Focus, Vol. 12, 1990, pp. 13–15, published by Bethesda Research Laboratories). Samples digested with restriction enzymes were run in 1% agarose gels, transferred to nylon membranes (Kempter et al., 1991), and hybridized (Church and Gilbert, 1984). Two different probes were used: the 1.9-kb insert of the λif clone (Figure 1), and the 0.4-kb SacI-EcoRI fragment of the λmt clone corresponding to the 3′ end of *MHA2* cDNA (Figure 1). Probes were labeled with α-$^{32}$P-dCTP by the method of Feinberg and Vogelstein (1983). High-stringency conditions corresponded to 65°C for hybridization and washes. For low-stringency conditions, the temperature was lowered to 50°C.

Total RNA was prepared as described by Logemann et al. (1987), separated by electrophoresis in formaldehyde gels, and blotted to nylon membranes (Kroczek and Siebert, 1990). For *MHA2* transcript analysis in different organs, roots and coleoptiles were obtained from plants grown for 4 days in the dark. Primary leaves were collected from 10-day-old plants and female flowers from the first stages of development. Hybridization and washes were at 65°C, and the probe was the 0.4-kb SacI-EcoRI fragment corresponding to the 3′ region of *MHA2* cDNA (Figure 1) random prime labeled with α-$^{32}$P-dCTP. To correct for uneven loading of the lanes, we made two different controls. Before blotting, ethidium bromide–stained gels were photographed, and the intensities of the rRNA bands were used as a reference. Filters already hybridized with the PM $H^+$-ATPase probe were washed at 95°C to remove radioactivity and rehybridized with a 1.9-EcoRI fragment comprising the $\alpha_3$-tubulin gene of Arabidopsis (Ludwig et al., 1987). Hybridization and wash temperatures were reduced to 50°C for this heterologous probe. Quantitative densitometry of photographs and autoradiographs was made with an electronic camera of 12 bits (Gelprinter; TDI, Madrid) and image analysis software from Advanced American Biotechnology (Fullerton, CA).

### Quantitative Determination of PM $H^+$-ATPase by Antibody

Vascular and nonvascular parts of coleptile segments (0.6 g) were incubated with either indole-3-acetic acid or benzoic acid as described above, and a membrane fraction was prepared by homogenization and centrifugation (Hager et al., 1991). Proteins were separated by SDS-PAGE and blotted to nitrocellulose, and the PM $H^+$-ATPase was visualized by incubation with affinity-purified antibody and enhanced chemiluminescence (ECL system; Amersham). Bands on the chemiluminescence-sensitive film were quantified by densitometry, as described by Hager et al. (1991).

### In Situ Hybridization

Digoxigenin-labeled RNA probes were prepared according to the manufacturer's instructions (Boehringer Mannheim). Hybridization was performed on paraffin-embedded sections of different maize tissues, as described by Jackson et al. (1991). Antibody binding and substrate reaction steps were performed as described by Coen et al. (1990). Sense and antisense RNA probes were transcribed from the 0.4-kb SacI-EcoRI fragment of the λmt clone corresponding to the 3′ end of *MHA2* cDNA (Figure 1) subcloned in the pBluescript KS− vector (Stratagene). For antisense probes, the plasmid was linearized with SacI and transcription was performed with T3 RNA polymerase. For control sense probes, the plasmid was linearized with HindIII, and transcription was performed with T7 RNA polymerase.

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