# Inventory of the Superfamily of P-Type Ion Pumps in Arabidopsis[1]

**Kristian B. Axelsen and Michael G. Palmgren***

SwissProt Group, Swiss Institute of Bioinformatics, 1 rue Michel Servet, CH–1211 Geneva 4, Switzerland (K.B.A.); and Plant Physiology and Anatomy Laboratory, Department of Plant Biology, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK–1871 Frederiksberg C, Copenhagen, Denmark (M.G.P.)

A total of 45 genes encoding for P-type ATPases have been identified in the complete genome sequence of Arabidopsis. Thus, this plant harbors a primary transport capability not seen in any other eukaryotic organism sequenced so far. The sequences group in all five subfamilies of P-type ATPases. The most prominent subfamilies are $P_{1B}$ ATPases (heavy metal pumps; seven members), $P_{2A}$ and $P_{2B}$ ATPases ($Ca^{2+}$ pumps; 14 in total), $P_{3A}$ ATPases (plasma membrane $H^+$ pumps; 12 members including a truncated pump, which might represent a pseudogene or an ATPase-like protein with an alternative function), and $P_4$ ATPases (12 members). $P_4$ ATPases have been implicated in aminophosholipid flipping but it is not known whether this is a direct or an indirect effect of pump activity. Despite this apparent plethora of pumps, Arabidopsis appears to be lacking $Na^+$ pumps and secretory pathway (PMR1-like) $Ca^{2+}$-ATPases. A cluster of Arabidopsis heavy metal pumps resembles bacterial $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ transporters. Two members of the cluster have extended C termini containing putative heavy metal binding motifs. The complete inventory of P-type ATPases in Arabidopsis is an important starting point for reverse genetic and physiological approaches aiming at elucidating the biological significance of these pumps.

The P-type superfamily of ion pumps includes primary transporters energized by hydrolysis of ATP with a wide range of specificities for small cations and perhaps also phospholipids (Møller et al., 1996; Palmgren and Harper, 1999). P-type ATPases are characterized by forming a phosphorylated intermediate (hence the name P-type), by being inhibited by vanadate, and by having a number of sequence motifs in common (Serrano, 1989; Axelsen and Palmgren, 1998). Plant P-type ATPases are characterized structurally by having a single subunit, eight to 12 transmembrane (TM) segments, N and C termini exposed to the cytoplasm, and a large central cytoplasmic domain including the phosphorylation and ATP binding sites (Fig. 1).

The P-type ATPase family can be divided into five major evolutionarily related subfamilies, which group according to the ions they transport (Axelsen and Palmgren, 1998; Fig. 1). The P-type ATPases are involved in a wide range of fundamental cellular processes such as making and maintaining the electrochemical gradient used as the driving force for the secondary transporters ($H^+$-ATPases in plants and fungi and $Na^+/K^+$-ATPases in animals), cellular signaling ($Ca^{2+}$-ATPases), the transport of essential micronutrients ($Zn^{2+}$- and $Cu^{2+}$-ATPases), and extrusion of the same ions if they accumulate in amounts that are too high. P-type ATPases may also be involved in the generation of membrane lipid asymmetry (Tang et al., 1996; Gomes et al., 2000).

Although P-type ATPases can be completely absent from certain bacterial and archaean genomes (e.g. *Borrelia burgdorferi* and *Pyrococcus horikoshii*), they constitute a large and indispensable family in eukaryotes as demonstrated by the completely sequenced genomes of the organisms *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster*. With the completion of the Arabidopsis genome, it is possible for the first time to study the primary transport capabilities of a plant and to compare plant transport with that of animals, insects, and fungi.

## THE P-TYPE ATPase SUPERFAMILY IN ARABIDOPSIS

The first remarkable fact is the number of P-type ATPases found in Arabidopsis. A total of 45 P-type ATPases was identified in the genome of Arabidopsis (Table I). This is the highest number of P-type ATPases found so far in a single organism. Arabidopsis harbors more than double as many P-type ATPases than *C. elegans* (21), *D. melanogaster* (13; the number is increased by gene splicing), and the yeast *S. cerevisiae* (16). Even in the human genome, which is not completed yet, fewer genes are found. Table II gives an overview of how many P-type ATPases can be found in the different sequenced genomes and of the distribution among the different subfamilies. Complete sequences can be found at The P-type ATPase database Web site (http://www.Patbase.kvl.dk).

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* Corresponding author; e-mail palmgren@biobase.dk; fax 45–3528–3365.

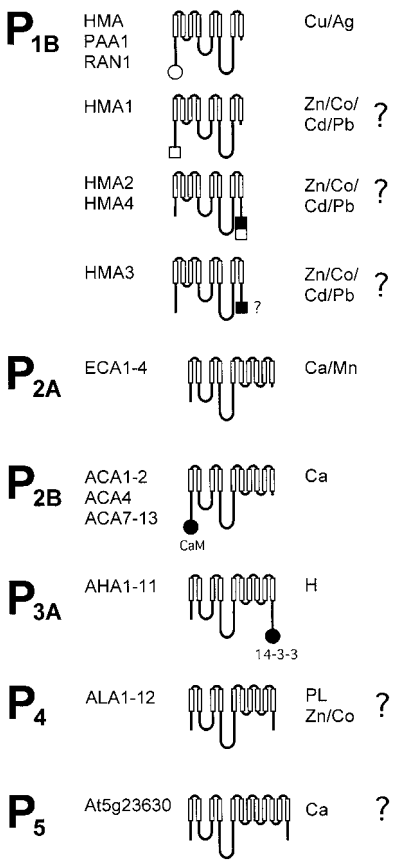


**Figure 1.** Overview of the Arabidopsis P-type ATPase superfamily. Families are designated by numerals on the left followed by gene names. The putative transported ions are indicated on the right. Boxes indicate transmembrane segments; black circles, regulatory domains containing autoinhibitory sequences; white circles, HMA domains; black boxes: CC dipeptide domains; white boxes, poly-His domains. HMA, CC dipeptide, and poly-His domains are putatively involved in heavy metal binding and sensing. Abbreviations are: 14-3-3, 14-3-3 protein binding region; CaM, calmodulin binding region; and PL, phospholipids.

A phylogenetic analysis of the conserved regions common to all P-type ATPases (Axelsen and Palmgren, 1998) reveal that Arabidopsis harbors ATPases belonging to all the five major subfamilies (Fig. 2). Several of the subfamilies such as the $P_{2B}$ ATPases (the calmodulin-regulated $Ca^{2+}$-ATPases) and the $P_{3A}$ ATPases (plasma membrane $H^+$-ATPases) form closely related clusters, whereas other subfamilies are more distantly related, most notably the $P_{1B}$ ATPases (heavy metal-transporting ATPases).

### $P_{1B}$ ATPases

Arabidopsis is equipped with seven $P_{1B}$ ATPases. Plant $P_{1B}$ ATPases have recently been reviewed (Williams et al., 2000) and are expected to be involved in the transport of heavy metals. In other organisms, pumps belonging to this subfamily typically exhibit two substrate specificities: either $Cu^{2+}/Ag^{2+}$ or $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ (Solioz and Odermatt, 1995; Beard et al., 1997; Rensing et al., 1997, 1998; Thelwell et al., 1998). Multiple alignments between all $P_{1B}$ ATPases identified so far in bacteria, archaea, and eukaryotes indicate that Arabidopsis HMA1, HMA2, HMA3, and HMA4 are likely to be $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ ATPases, whereas RAN1, PAA1, and HMA5 are candidate $Cu^{2+}/Ag^{2+}$ ATPases. HMA2, HMA3, and HMA4 form a subcluster in the phylogenetic analysis of Arabidopsis ATPases (Fig. 2), supporting the notion that they might have a similar function.

The cDNAs of four of the $P_{1B}$ ATPase genes (*RAN1*, *PAA1*, *HMA1*, and *HMA4*) have been cloned. Only RAN1, which is 50% similar to the human Menkes and Wilson $Cu^{2+}$-ATPases, has been characterized in some detail. RAN1 has been shown to be important for the delivery of copper ions to receptors for the plant hormone ethylene (Hirayama et al., 1999; Woeste and Kieber, 2000) and perhaps also to additional cuproenzymes (Woeste and Kieber, 2000). Closely related proteins found in yeast and humans have equivalent roles, as exemplified by the yeast $Cu^{2+}$-ATPase Ccc2p, which delivers copper ions to the iron oxidase Ftr3p involved in iron uptake (Yuan et al., 1995), and the Menkes disease symptoms, which can be explained by the general deficiency of copper for copper-requiring enzymes (Danks et al., 1972).

Eukaryotes apart from plants appear to have a limited number of $P_{1B}$ ATPases (one or two in each genome) and so far those that have been identified belong to the $Cu^{2+}/Ag^{2+}$ cluster. $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ ATPases are common in bacteria and have not been observed in animals and fungi (Table II). Therefore, it is noteworthy that four Arabidopsis $P_{1B}$ ATPase genes encode pumps belonging to the $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$-transporting cluster. Biochemical or genetic evidence is needed to confirm the actual transport specificity of these pumps.

In Arabidopsis, a large number of carrier proteins are involved in the transport of $Zn^{2+}$, $Co^{2+}$, and $Cd^{2+}$ (Mäser et al., 2001). These heavy metal transporters belong to two families: the Nramp family, which contains at least seven members, five of which have been characterized (Curie et al., 2000; Thomine et al., 2000); and the ZIP family of approximately 13 members, four of which have been characterized (Eide et al., 1996; Grotz et al., 1998; Korshunova et al., 1999; Guerinot, 2000). The Nramp family of proteins codes both high- and low-affinity transporters, which mainly transport $Fe^{2+}$ into the plants, but have a broad substrate specificity. It has been demonstrated that they also transport $Mn^{2+}$ and $Cd^{2+}$ (Curie et al., 2000; Thomine et al., 2000). The ZIP transporters mainly transport $Zn^{2+}$ and $Fe^{2+}$, but they also have a broad substrate range.

Why would Arabidopsis need additional primary transporters for the transport of $Zn^{2+}$, $Co^{2+}$, and $Cd^{2+}$?

**Table I.** *P-type ATPases in Arabidopsis*

| SWISS-PROT Accession No. | Chrromosome | Specificity | Protein Name | Arabidopsis Genome Initiative Name | Genomic Locus | Expressed Sequence Tags (ESTs) | Length |
|---|---|---|---|---|---|---|---|
| $P_{1B}$ ATPases | | | | | | | |
| Q9M3H5 | 4 | – | HMA1 | At4g37270 | C7A10.90 | 3 | 819 |
| Q9SZW4 | 4 | – | HMA2 | At4g30110 | F6G3.140 | 1 | 951 |
| Q9SZW5 | 4 | – | HMA3 | At4g30120 | F6G3.150 | 0 | 760[a] |
| O64474 | 2 | – | HMA4 | At2g19110 | T20K24.12 | 13 | 1,172 |
| Q9S7J8 | 5 | $Cu^{2+}$ | RAN1 | At5g44790 | K23L20.14/T19K24.18 | 11 | 1,001 |
| Q9SZC9 | 4 | – | PAA1 | At4g33520 | F17M5.280/T16L1.10 | 3 | 949 |
| Q9SH30 | 1 | – | HMA5 | At1g63440 | F2K11.18 | 0 | 995[b] |
| $P_{2A}$ ATPases | | | | | | | |
| P92939 | 1 | $Ca^{2+}$ | ECA1/ACA3 | At1g07810 | F24B9.9 | 5 | 1,061 |
| O23087 | 4 | – | ECA2/ACA5 | At4g00900 | A_TM018A10.4 | 0 | 1,054 |
| Q9SY55 | 1 | – | ECA3/ACA6 | At1g10130 | T27I1.16/F14N23.1 | 6 | 998 |
| Q9XES1 | 1 | – | ECA4 | At1g07670 | F24B9.24 | 5 | 1,061 |
| $P_{2B}$ ATPases | | | | | | | |
| Q37145 | 1 | – | ACA1/PEA1 | At1g27770 | T22C5.23/F28L5.1 | 3 | 1,020 |
| O81108 | 4 | $Ca^{2+}$ | ACA2 | At4g37640 | F19F18.130 | 15 | 1,014 |
| O22218 | 2 | $Ca^{2+}$ | ACA4 | At2g41560 | T32G6.8 | 15 | 1,030 |
| O64806 | 2 | – | ACA7 | At2g22950 | T20K9.16 | 0 | 1,015 |
| Q9LF79 | 5 | $Ca^{2+}$ | ACA8 | At5g57110 | MUL3.5 | 6 | 1,074 |
| Q9LU41 | 3 | – | ACA9 | At3g21180 | MXL8.3 | 0 | 1,073 |
| Q9SZR1 | 4 | – | ACA10 | At4g29900 | F27B13.140 | 7 | 1,069 |
| Q9M2L4 | 3 | – | ACA11 | At3g57330 | F28O9.180 | 3 | 1,025 |
| Q9LY77 | 3 | – | ACA12 | At3g63380 | MAA21_10 | 3 | 1,033 |
| Q9LIK7 | 3 | – | ACA13 | At3g22910 | F5N5.8 | 0 | 1,017 |
| $P_{3A}$ ATPases | | | | | | | |
| P20649 | 2 | $H^+$ | AHA1 | At2g18960 | F19F24.16 | 120 | 949 |
| P19456 | 4 | $H^+$ | AHA2 | At4g30190 | F9N11.40 | 84 | 948 |
| P20431 | 5 | $H^+$ | AHA3 | At5g57350 | MJB24.16 | 9 | 949 |
| Q9SU58 | 3 | – | AHA4 | At3g47950 | T17F15.180 | 9 | 960 |
| Q9SJB3 | 2 | – | AHA5 | At2g24520 | F25P17.18 | 0 | 949[c] |
| Q9SH76 | 2 | – | AHA6 | At2g07560 | F9A16.7 | 1 | 949 |
| Q9LY32 | 3 | – | AHA7 | At3g60330 | F27H5_120 | 0 | 961 |
| Q9M2A0 | 3 | – | AHA8 | At3g42640 | T12K4_90 | 2 | 948 |
| Q42556 | 1 | – | AHA9 | At1g80660 | F23A5.1/T21F11.1 | 4 | 954 |
| Q43128 | 1 | – | AHA10 | At1g17260 | F20D23.4 | 0 | 947[d] |
| Q9LV11 | 5 | – | AHA11 | At5g62670 | MRG21.9 | 14 | 956 |
| $P_4$ ATPases | | | | | | | |
| P98204 | 5 | – | ALA1 | At5g04930 | MUG13.22 | 3 | 1,158 |
| P98205 | 5 | – | ALA2 | At5g44240 | MLN1.17 | 10 | 1,107[e] |
| Q9XIE6 | 1 | – | ALA3 | At1g59820 | F23H11.14 | 4 | 1,123 |
| Q9LNQ4 | 1 | – | ALA4 | At1g17500 | F1L3.21 | 2 | 1,216[f] |
| Q9SGG3 | 1 | – | ALA5 | At1g72700 | F28P22.11 | 3 | 1,228 |
| Q9SLK6 | 1 | – | ALA6 | At1g54280 | F20D21.10 | 1 | 1,244 |
| Q9LVK9 | 3 | – | ALA7 | At3g13900 | MDC16.2 | 0 | 1,247[g] |
| Q9LK90 | 3 | – | ALA8 | At3g27870 | K16N12.9 | 1 | 1,189 |
| Q9SX33 | 1 | – | ALA9 | At1g68710 | F24J5.6 | 0 | 1,200 |
| Q9LI83 | 3 | – | ALA10 | At3g25610 | T5M7.5 | 0 | 1,202 |
| Q9SAF5 | 1 | – | ALA11 | At1g13210 | F3F19.24 | 6 | 1,203 |
| P57792 | 1 | – | ALA12 | At1g26130 | F28B23.19 | 0 | 1,184 |
| $P_5$ ATPases | | | | | | | |
| Q9LT02 | 5 | – | – | At5g23630 | MQM1.11 | 6 | 1,179 |
| P-type ATPase-like proteins | | | | | | | |
| Q9T0E0 | 4 | – | AHA12 | At4g11730 | T5C23.160 | 0 | 813[h] |
| Q9LVV1 | 5 | – | – | At5g53010 | MNB8.7 | 0 | 1,095[i] |
| O22180 | 2 | – | – | At2g23280 | T20D16.9 | 0 | 215[j] |

[a] The sequenced genome has a 1-bp deletion at position 70,787 (positions from AL161576). This leads to a truncation in the universally conserved domain close to the ATP-binding site. We have corrected for this error by inserting 1 bp to obtain a full-length protein. Thus, inserting an A at this position leads to a better gene prediction by moving the exon donor site at position 70,781 to position 70,927. The SWISS-PROT entries indicated contain the corrected sequences. [b] The predicted C-terminal domain is a repeated domain found in more than 30 copies in the genome. The new C-terminal domain is found by removing the last two exons (deleting 186 amino acids) and prolonging the third last exon to the nearest stop codon (inserting four amino acids; change exon acceptor site at position 65,737 to stop codon at position 65,722;

(*Legend continues on facing page.*)

Table I. (*Legend continued from facing page.*)
positions from AC008047). Furthermore, the exon acceptor site at position 66,992 has been moved to position 67,028, deleting 12 amino acids. [c] The predicted N-terminal domain is wrong. An exon should be added at positions 3,244 through 3,284 (positions from AC006954), and an acceptor site should be inserted at position 3,505, four amino acids upstream of the chosen ATG. It has not been possible to detect the first A of the ATG, which is situated on an exon apart. [d] One change has been performed in the predicted part of the amino acid sequence (Harper et al., 1994). A donor site has been moved from position 4,682 to position 4,685 (positions from S74033) inserting one amino acid. [e] The predicted protein contains several errors. According to a cDNA sequence (M.K. Jakobsen, personal communication), the ATG should be moved from position 76,277 to position 76,191 (positions from AB005239), and the acceptor site of exon 2 should be moved from position 76,455 to position 76,412, resulting in the replacement of 13 amino acids with 56. The acceptor site at position 78,739 should be moved to position 78,781, deleting 14 amino acids. An unconventional donor site chosen at position 81,455 (TA) should be GT found at position 81,454. The chosen donor site leads to the prediction of a wrong C-terminal domain because the remainder of protein is translated from a wrong reading frame. The last 82 amino acids have been exchanged with 164. [f] The predicted protein contains multiple prediction errors. The first five exons of the predicted protein should be deleted and the ATG placed at position 72,499 (replacing 187 amino acids with 22; positions from AC022492). The donor sites at positions 74,522, 75,514, and 75,705 should be moved to positions 74,468, 75,475, and 75,696, respectively (deletes 18, 134, and three amino acids, respectively). [g] The predicted protein contains errors. The acceptor sites at positions 13,373 and 14,282 (positions from AB019229) should be moved to positions 13,361 and 14,309, respectively (inserts four and deletes nine amino acids, respectively). [h] Amino acids one through 460 and 480 through 768 of the predicted sequence is 73% identical to amino acids one through 461 and 567 through 856 from PMA3, respectively. The PMA3 amino acids 462 through 566 missing from At4g11730p are situated in the large cytoplasmic loop and includes the entire conserved segment f (Axelsen and Palmgren, 1999), including the motif VKMIT**GD**Q (amino acids conserved in all P-type ATPases are indicated in bold). [i] Probable pseudogene. Amino acids 128 through 1,035 of the predicted sequence are 43% identical to amino acids 102 through 1,004 of ACA8. The motif DKTGTLT is completely missing and the universally conserved motifs T**GE**S (in ACA8; TASD in At5g53010p), **P**EGL (PVGL), VRMVT**GD**N (VCMVTDND), **P**ND**K** (PNDN), and VAVT**GDG**T**ND**APAL (VAATGMGI-HDPKTL) are mutated in several of the amino acids crucial for function (amino acids conserved in all P-type ATPases are indicated in bold). [j] Probable pseudogene. Amino acids one through 120 of the predicted sequence are 65% identical to amino acids 487 through 611 situated in the large cytoplasmic loop of HMA1.

Important roles for primary heavy metal transporters could be the accumulation of these or other heavy metals in subcellular compartments and/or ensuring that the levels inside the cells do not reach toxic levels. Furthermore, the members of the $P_{1B}$ ATPases could be involved in delivering heavy metal ions to specific proteins, like is the case for RAN1 (Hirayama et al., 1999; Woeste and Kieber, 2000). The elucidation of the tissue distribution and subcellular locations of plant $P_{1B}$ ATPases would help determine which (if not all) of these possibilities are correct.

$P_{1B}$ ATPases typically have short C-terminal domains and very large N-terminal domains containing heavy metal-associated (HMA) domains of 31 amino acids with the signature GMTCxxC (Bull and Cox, 1994). The HMA domains bind heavy metals and might serve a role as heavy metal sensors. Analysis of the $P_{1B}$ ATPase sequences in Arabidopsis shows a number of interesting features. HMA domains are found in the N-terminal regions of the candidate $Cu^{2+}/Ag^{2+}$ ATPases RAN1 (one domain), PAA1 (two domains), and HMA5 (two domains). However, the Arabidopsis subgroup of putative $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ ATPases (HMA1-4) contain no HMA domains in their sequences. Instead, several CC dipeptides and His-rich domains can be found in HMA2 and HMA4. The His-rich domain is most evident in HMA4. It has been proposed that CC dipeptides together with His-rich domains could be part of non-HMA domains that are also involved in heavy metal binding (Solioz and Vulpe, 1996; Williams et al., 2000). Furthermore, HMA2 and HMA4 are the only $P_{1B}$ ATPases identified so far, which are predicted to have a long C-terminal domain. It is interesting that the CC dipeptides and the His-rich domains are found in the prolonged C termini of HMA2 and HMA4 and not in the N-terminal domain where HMA domains are always found (Fig. 1). The N-terminal end of HMA1 also harbors a poly-His domain (Fig. 1).

Because a cDNA representing *HMA2* has not been cloned, the predicted primary sequence of the encoded protein with an extended C-terminal domain might be wrong. However, the prolonged C-terminal end of HMA2 is encoded by a long exon including the two last transmembrane segments and part of the universally conserved ATP binding domain, and the far C terminus shows similarity to the far C terminus of HMA4. Therefore, the predicted gene model is likely to be correct.

The locus *HMA5* is also predicted to have a prolonged C-terminal domain, but contrary to *HMA2* this domain is found on an exon of its own and it is similar to a repeated domain found in more than 30 copies dispersed in the genome. Furthermore, it does not contain any putative heavy metal binding domains, whereas two HMA domains can be found in the N terminus of the protein. Therefore, we believe the C-terminal domain is not part of locus *HMA5* and have instead identified the C terminus of the protein by removal of the two last exons and prolongation of the third last exon to the first stop codon and found four amino acids downstream of the proposed splice site. This yields a C terminal similar to that normally seen in $P_{1B}$ ATPases. No expressed sequence tags (ESTs) corresponding to this protein have yet been found.

### $P_{2A}$ ATPases

The $P_{2A}$ ATPases encompass $Ca^{2+}$ ATPases similar to the animal $Ca^{2+}$-ATPases of the sarco- and endo-

**Table II.** *Distribution of P-type ATPases in entire genomes according to phylogenetic relationship*[a]

| Organism | ATPase Type | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1A K | 1B HM | 2A Ca | 2B Ca | 2C Na/K, H/K | 2D Na | 3A H | 3B Mg | 4 PL?, Zn? | 5? | Others[b] | Total |
| Bacteria | | | | | | | | | | | | |
| *Escherichia coli* | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 4 |
| *Mycobacterium tuberculosis* | 1 | 7 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 12 |
| *Synechocystis* PCC6803 | 1 | 4 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 9 |
| Archaea | | | | | | | | | | | | |
| *Methanobacterium thermoautotrophicum* | 0 | 3 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| *Methanococcus jannaschii* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| Eukarya | | | | | | | | | | | | |
| Fungi | | | | | | | | | | | | |
| *S. cerevisiae* | 0 | 2 | 1 | 1 | 0 | 5 | 2 | 0 | 5 | 2 | 0 | 16 |
| Plants | | | | | | | | | | | | |
| Arabidopsis | 0 | 7 | 4 | 10 | 0 | 0 | 11 | 0 | 12 | 1 | 0 | 45 |
| Animals | | | | | | | | | | | | |
| *C. elegans* | 0 | 1 | 2 | 3 | 5 | 0 | 0 | 0 | 6 | 4 | 0 | 21 |
| *D. melanogaster* | 0 | 1 | 2 | 1 | 2 | 0 | 0 | 0 | 6 | 1 | 0 | 13 |
| Human | 0 | 2 | 5 | 4 | 6 | 0 | 0 | 0 | 20 | 3 | 0 | 40 |

[a] The categories in the table correspond to the families of P-type ATPases defined in earlier work (Axelsen and Palmgren, 1999). HM, Heavy metals; PL, phospholipids. [b] This group contains bacterial ATPases, for which the ion specificity is not known and which do not group into any of the major families defined in Axelsen and Palmgren (1999). [c] P-type ATPases found in the draft genome which covers ~94% of the entire genome.

plasmatic reticulum (SERCA pumps) and the fungal and animal secretory pathway ATPases (ATP2C1 in animals; PMR1 in yeast). These ATPases are also involved in $Mn^{2+}$ transport (Lapinskas et al., 1995; Liang et al., 1997; Dürr et al., 1998). The $Ca^{2+}$ ATPases of Arabidopsis have recently been reviewed (Evans and Williams, 1998; Geisler et al., 2000a; Sze et al., 2000). Four $P_{2A}$ ATPases can be found in the Arabidopsis genome. All four of the genes have been cloned and *ECA1-3* have been characterized (Liang et al., 1997; Pittman et al., 1999). ECA1 and ECA4 are the two most closely related of the $P_{2A}$ ATPases (97% identical) and the corresponding genes are found within 50 kb of each other on chromosome 1, indicating a recent duplication event. ECA1, ECA2, and ECA4 form a closely related cluster with ECA3 being more distant (Fig. 2). There is only evidence for the subcellular location of ECA1, which has been localized to endoplasmic reticulum membranes.

The yeast *S. cerevisiae* is equipped with a well-characterized $Ca^{2+}$-ATPase, PMR1, which is situated in the secretory pathway. Here it is involved in the correct processing of proteins exported from the cells. Very similar ATPases have been identified in mammals (e.g. human ATP2C1 and KIAA0703). These pumps form a distinct cluster of ATPases in the $P_{2A}$ subfamily (Axelsen and Palmgren, 1998). In Arabidopsis, however, none of the four $P_{2A}$ ATPases identified resemble the secretory pathway $Ca^{2+}$-ATPases. Thus, all four pumps are much more similar to animal SERCA $Ca^{2+}$-ATPases (approximately 50% identity) than to the secretory pathway pumps ATP2C1 or PMR1 (approximately 32% identity).

The absence of PMR1-like pumps in Arabidopsis does not exclude the possibility that this plant can have a $Ca^{2+}$-ATPase situated in the secretory pathway: A number of the 14 Arabidopsis $P_{2A}$ and $P_{2B}$ ATPases have been demonstrated in different endomembranes. Thus, it remains a possibility that one or more of these are positioned in the secretory pathway.

### $P_{2B}$ ATPases

The $P_{2B}$ ATPases also transport $Ca^{2+}$. They are most similar to the mammalian plasma membrane $Ca^{2+}$-ATPases (PMCA pumps) and the PCA1 ATPase from yeast. The $P_{2B}$ ATPases have a total of 10 members in Arabidopsis and form three clusters in the Arabidopsis P-type ATPase tree (Fig. 2). The overall sequence identity is rather high, ranging from 45% to 92% between the different members; but even so, the genomic organization of the different genes differs fundamentally. Where *ACA12* and *ACA13* are coded in only one exon each, the *ACA8* and *ACA10* genes consist of 34 exons.

Four of the 10 proteins (ACA1, ACA2, ACA4, and ACA8) have been cloned and characterized in some detail (Huang et al., 1993; Harper et al., 1998; Bonza et al., 2000; Geisler et al., 2000b). Each protein seems to be present in a specific membrane such as the plasma membrane (ACA8; Bonza et al., 2000), the membrane of small vacuoles (ACA4; Geisler et al., 2000b), and perhaps the chloroplast envelope (ACA1; Huang et al., 1993). An N-terminal calmodulin-binding domain was first identified in the cauli-

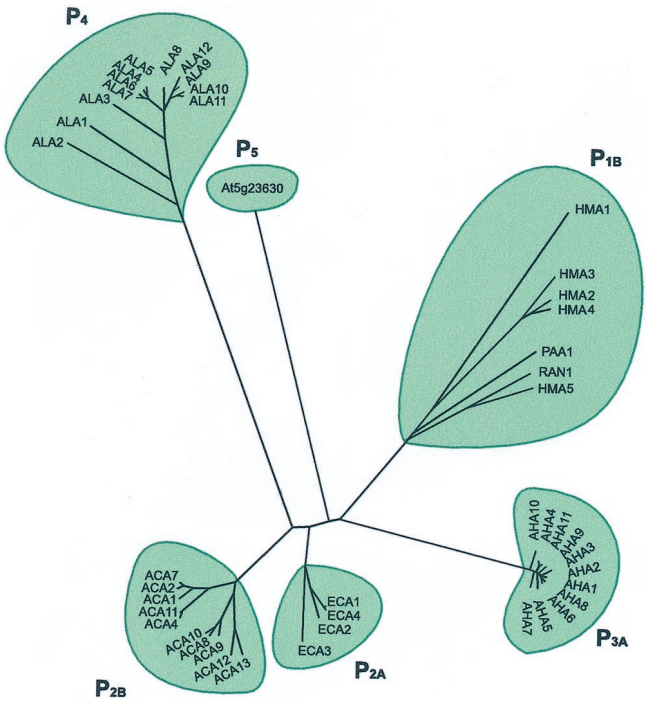


**Figure 2.** Phylogenetic tree of Arabidopsis P-type ATPases. Conserved segments present in all P-type ATPases were extracted from the sequences and were aligned using T-COFFEE (Notredame et al., 2000). The alignment was used to perform a phylogenetic analysis using the Protdist and Fitch program from the Phylip package (Felsenstein, 1989). The resulting phylogenetic tree reveals five major branches, which are named according to Axelsen and Palmgren (1998).

flower $P_{2B}$ ATPase BCA1 (Malmström et al., 1997). On the basis of sequence analyses, calmodulin-binding domains could be identified in the first 50 amino acids of all Arabidopsis $P_{2B}$ ATPases (not shown). There is experimental evidence for the presence of calmodulin-binding sequences in the N-terminal domains of ACA2 (Harper et al., 1998), ACA4 (Geisler et al., 2000b), and ACA8 (Bonza et al., 2000). In these pumps, the N terminus is likely to form an autoinhibitory regulatory domain (Geisler et al., 2000b; Hwang et al., 2000a, 2000b).

### $P_{3A}$ ATPases

$P_{3A}$ ATPases encompass a subfamily of plasma membrane $H^+$-ATPases only found in plants and fungi. This group consists of 11 very closely related members being at least 66% identical and a truncated gene, which might be a pseudogene or have alternative functions (AHA12; see below). The $P_{3A}$ ATPases have recently been reviewed (Palmgren, 2001). They all have the same structure with a prolonged C-terminal regulatory domain comprising a 14-3-3 binding site and a phosphorylation site at the penultimate Thr residue. The two most expressed P-type ATPases in Arabidopsis can be found in this family. Thus, AHA1 and AHA2 are five to eight times more abundant than any of the other proteins as based on number of ESTs identified for each (Table I).

### $P_4$ ATPases

Twelve proteins from Arabidopsis are members of the large subfamily of $P_4$ ATPases, which includes a substantial number of the P-type ATPases found in other eukaryotic organisms (Table II). A bioinformatic study of the $P_4$ ATPases in Arabidopsis has recently been published (Gomes et al., 2000). They form two closely related clusters (pumps being >75% identical in each cluster) consisting of ALA4 through ALA7 and ALA9 through ALA12, with ALA8 placed in between the clusters and ALA1, ALA2, and ALA3 more distant from the other subfamily members.

Two of the Arabidopsis $P_4$ ATPases have been cloned (*ALA1*, Gomes et al., 2000; *ALA2*, M.K. Jakobsen, personal communication), but only *ALA1* has been characterized, and it was demonstrated that this gene is involved in cold tolerance of Arabidopsis plants (Gomes et al., 2000). ALA1 (Gomes et al., 2000), bovine ATPase II (Tang et al., 1996), and yeast DRS2 (Tang et al., 1996; Gomes et al., 2000), all belonging to the $P_4$ ATPase cluster, have been implicated in flipping of aminophospholipids. Studies with four recombinant isoforms of bovine ATPase II, all $P_4$ pumps, revealed that the potential substrate phosphatidyl-Ser is essential for the dephosphorylation of the phosphorylated reaction cycle intermediate and for continuation of its catalytic cycle (Ding et al., 2000). Dephosphorylation of P-type ATPases is normally triggered by the transported species and results in the conformational change that is associated with transport (Møller et al., 1996). The human $P_4$ ATPase FIC1 (Bull et al., 1998) is involved in the transport of conjugated bile acids. Overexpression of yeast NEO1 (Prezant et al., 1996), which resembles DRS2, results in resistance to the aminoglucoside neomycin by a mechanism that is not understood.

From the above it appears that $P_4$ ATPases are involved in the transport of relatively large amphipathic compounds. However, it is not known whether this is a direct or indirect effect of $P_4$ ATPases. Thus, the transport capabilities of none of these pumps have been characterized following their purification and reconstitution in an artificial membrane. It should be noted that a phenotype of *drs2* cells is sensitivity toward $Zn^{2+}$ and $Co^{2+}$ (Siegmund et al., 1998). Whether this reflects a role for this and other $P_4$ ATPases in the transport of transition metals is not known. Disruption of *DRS2* also results in a defect of ribosome assembly, a process known to be dependent upon $Zn^{2+}$ (Tal, 1969).

### $P_5$ ATPases

There is only a single member of $P_5$ ATPases in Arabidopsis. The substrate specificity of this subfam-

ily, found only in eukaryotes, is unknown because none of its members have been characterized biochemically.

Deletion of the two $P_5$ ATPases in the yeast *S. cerevisiae* are nonlethal, but deletion of *SPF1* leads to glycosylation defects (Suzuki and Shimma, 1999) and deficient ubiquitin-dependent degradation of an enzyme in the mevalonate biosynthetic pathway (Cronin et al., 2000). The latter phenotype could be partially reversed by adding high $Ca^{2+}$ to the medium. Therefore, it was suggested that SPF1 could be a $Ca^{2+}$-ATPase important for maintaining $Ca^{2+}$ homeostasis in a membrane system in the secretory pathway (Cronin et al., 2000). The *spf1* phenotype partially mimics the phenotype of a yeast strain deleted for the secretory pathway $Ca^{2+}/Mn^{2+}$ ATPase PMR1 because the degradation of a protein, misfolded caboxypeptidase protein Y, was affected in the *pmr1* strain (Dürr et al., 1998). However, ubiquitin-dependent degradation is not affected in this strain. If SPF1 is a $Ca^{2+}$ ATPase, a novel calcium-binding site must be found in the $P_5$ ATPases because only one of the residues important for coordination of $Ca^{2+}$ ions in SERCA1a, a $P_{2A}$ $Ca^{2+}$-ATPase of which the structure has been solved at 2.6-Å resolution (Toyoshima et al., 2000), are conserved in $P_5$ ATPases (Fig. 3).

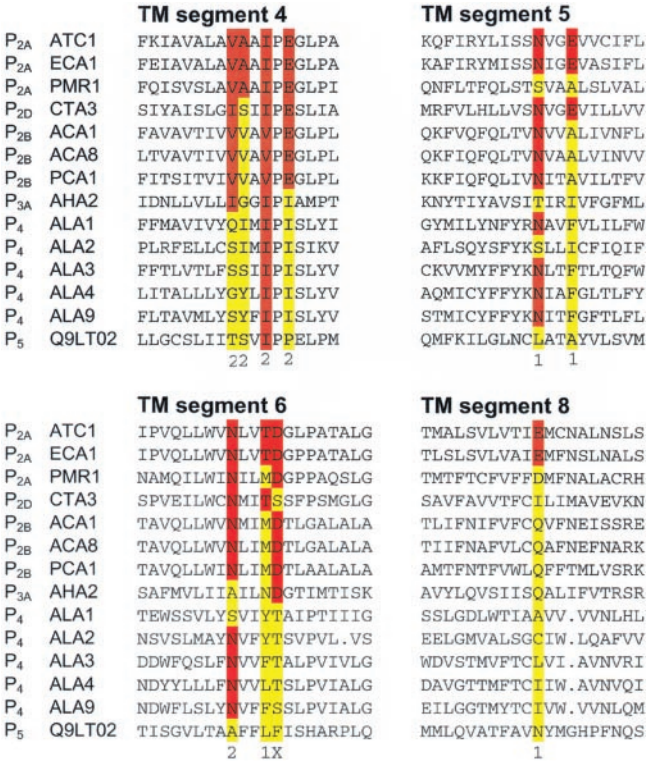


**Figure 3.** Alignment of $Ca^{2+}$ binding residues in TM segments from different P-type ATPases. An alignment of the full length Arabidopsis ATPases was performed using T-COFFEE (Notredame et al., 2000). The TM segments 4–6 and 8 were extracted from the alignment. The overall alignment was reliable for TM segments 4–6, whereas the alignment of TM 8 is more dubious as the similarity between the $P_{2A}$, $P_4$ and $P_5$ ATPases is low in this region. The alignment includes the $P_{2A}$ ATPase SERCA1a from rabbit (ATC1) the $Ca^{2+}$ ATPases PMR1 ($P_{2A}$) and PCA1 ($P_{2B}$) from *S. cerevisiae*, the $P_{2D}$ $Ca^{2+}$ ATPase CTA3 from *Schizosaccharomyces pombe* as well as a selection of ATPases from Arabidopsis. Residues involved in coordination of $Ca^{2+}$ in the two $Ca^{2+}$ binding sites (Site I and Site II) found in the 2.6 Å crystal structure of SERCA1a (Toyoshima et al., 2000) are marked with 1 and 2, respectively. The position marked X is a residue involved in coordination of both $Ca^{2+}$ ions. Residues with hydrophobic side chains in TM 4 contribute with backbone carbonyl oxygens.

## SODIUM TRANSPORT IN ARABIDOPSIS

$Na^+$ is excluded from the cytoplasm of most living cells because it generates osmotic stress and has specific toxic effects (Bohnert et al., 1995). Animals and fungi have $Na^+$-/$K^+$-ATPases ($P_{2C}$ ATPases) and $Na^+$-ATPases ($P_{2D}$ ATPases), respectively, that carry out this task (Axelsen and Palmgren, 1998). No Arabidopsis P-type ATPase group in a phylogenetic tree together with $P_{2C}$ or $P_{2D}$ ATPases. This would suggest that Arabidopsis is not equipped with pumps that can transport $Na^+$.

Plant cells are more $Na^+$ sensitive than animal and fungal cells, except in some species adapted to $Na^+$-rich environments. Exclusion of $Na^+$ from the cytoplasm of Arabidopsis is probably mediated by $Na^+/H^+$ antiporters in two membrane systems: the plasma membrane and the vacuolar membrane. The gene *SOS1* in Arabidopsis may encode a plasma membrane antiporter (Shi et al., 2000), whereas *NHX1* may encode a vacuolar antiporter (Apse et al., 1999; Gaxiola et al., 1999). Arabidopsis harbors at least three evident $Na^+/H^+$ antiporters similar to SOS1, and four similar to vacuolar antiporters.

## P-TYPE ATPASES WITH ALTERNATIVE ROLES

The analysis of the Arabidopsis genome resulted in the identification of a $P_{3A}$ ATPase (AHA12; At4g-11730p) lacking a conserved domain involved in ATP binding. In addition, the complete C-terminal autoinhibitory domain is missing and is replaced by 50 amino acid residues having no similarity to other $P_{3A}$ ATPases (see Table I). Besides these peculiar features, the protein closely resembles other Arabidopsis $P_{3A}$ ATPases (72% identity to AHA3) and the transmembrane regions are intact, suggesting that $H^+$ binding can occur. This raises the question of whether *AHA12* is a pseudogene (it is not represented by an EST) or whether it is an example of a gene coding for an ATPase like protein with an alternative function.

P-type ATPase-like proteins lacking parts of the universally conserved domains and/or TM segments have been characterized in animal systems. One example is a $P_4$ ATPase like protein from rabbit lacking TM segment 4, which is universally conserved in the P-type ATPase family. The protein is situated in the inner nuclear membrane and was identified because of its ability to bind RING finger domains of a RUSH

transcription factor (Mansharamani et al., 2001). Another example is the splice variants of the human $P_{1B}$ ATPases Menkes and Wilson disease proteins, which lack several universally conserved domains as well as either two or eight of the transmembrane segments, resulting in the splice variants being cytosolic (Yang et al., 1997; Reddy and Harris, 1998; Reddy et al., 2000). The shortest splice variant of the Menkes protein only codes for a 103-amino acid protein, which contains a nuclear targeting signal, a single HMA domain, and a short C-terminal domain. A final example is the protein identified in the genome of the archaea *M. jannaschii* resembling the large cytoplasmic domain of P-type ATPases (Ogawa et al., 2000). It has been demonstrated that this soluble ATPase shows ATPase activity, autophosphorylation, and inhibition by vanadate (Ogawa et al., 2000).

The functions of the partially deleted isoforms found in animals are unknown, but it has been suggested (Reddy et al., 2000) that shortened $P_{1B}$ ATPases could be involved in regulation of $Cu^{2+}$-ATPase activity, $Cu^{2+}$ sensing, or in directing $Cu^{2+}$ to the nucleus. These findings, however, do not lead to an evident suggestion for the function of AHA12.

Two other genes in the Arabidopsis genome with homology to P-type ATPases (At2g23280 and At5g53010) are likely to represent pseudogenes. The first half of At2g23280 is very similar to 100 amino acids of the large cytoplasmic domain of $P_{1B}$ ATPases, whereas the second half has no resemblance to P-type ATPases. At5g53010 is similar to $P_{2A}$ ATPases, but is lacking the first and last 100 amino acids, and it is also lacking or has mutations in most of the universally conserved residues. It is most notable that in the large cytoplasmic domain encompassing the phosphorylation and ATP binding sites, the entire DKTGTLT motif is missing and the TGD and GDGND motifs have been distorted. The rudimentary structure, the lack of conserved regions, and the fact that neither of the two proteins in question are supported by ESTs are indicatives of a pseudogene nature of these genes.

## WHY ARE THERE SO MANY P-TYPE ATPASES IN ARABIDOPSIS?

Genetic redundancy in Arabidopsis is the result of (a) a polyploidization event in an ancestral plant around 150 million years ago, and (b) a number of local gene duplications resulting in the generation of tandem gene arrays (The Arabidopsis Genome Initiative, 2000). Such events might partly explain the high number of P-type ATPase genes in Arabidopsis. However, the various P-type ATPases subfamilies evolved well before the evolution of plants (Axelsen and Palmgren, 1998). Thus, the divergence of $P_1$ and $P_2$ ATPases occurred before the split between bacteria, archaea, and eukarya, and the evolution of $P_4$ and $P_5$ ATPases occurred before the separation of plants from fungi and animals.

Another reason for the large number of P-type ATPases in Arabidopsis could be the lack of alternative splicing events taking place in plants. Thus, protein diversity in animals can be obtained by alternative splicing of identical genes. A SWISS-PROT database search revealed that documented splice variants found in the human P-type ATPases produce at least 80 different P-type ATPase protein species (data not shown).

Plants are immobile and thus have to adapt to more varying conditions such as temperature and availability of water and nutrition, and furthermore distribute messages of the changes of these conditions. Perhaps one parameter to achieve this ability of adaptation is to have a large variety of isoforms within each protein family, in this way enabling the plant to quickly fine-tune its response to the conditions given at any time.

In different subfamilies of P-type ATPases, isoform divergency might serve different purposes. The high number of $P_3$ ATPases in Arabidopsis and in other plants might be a means to facilitate expression of a sufficient amount of $H^+$-ATPases in different cells and tissues at different stages of development (Oufattole et al., 2000; Palmgren, 2001). The evidence available so far supports the notion that all $P_3$ ATPases are expressed in the plasma membrane (DeWitt et al., 1996). However, different Arabidopsis $Ca^{2+}$-ATPases appear to be expressed in different cellular membranes. Here they serve a role pumping $Ca^{2+}$ into intracellular compartments in addition to extruding $Ca^{2+}$ from the cell (Geisler et al., 2000a). Some organelles, such as the endoplasmic reticulum, even harbor more than one $Ca^{2+}$-ATPase (Hong et al., 1999). By analogy, one might speculate that $P_{1B}$ ATPases, depending on their membrane location, could be involved in both extrusion and sequestration of heavy metals.

$Ca^{2+}$-ATPases and $Ca^{2+}/H^+$ antiporters in concert keep cytoplasmic calcium concentrations in the submicromolar range, which is a prerequisite for $Ca^{2+}$ signaling (Sanders et al., 1999; Sze et al., 2000). It has been shown recently that a mutation in *DET3*, which encodes a subunit of an Arabidopsis V-type $H^+$ pump that supplies the driving force for vacuolar membrane $Ca^{2+}/H^+$ antiporter(s), results in specific distortions in signal-induced $Ca^{2+}$ oscillations in Arabidopsis stomatal guard cells (Allen et al., 2000). Reverse genetic approaches might prove valuable to solve the question of whether individual $Ca^{2+}$-ATPase isoforms might play a role in encoding specificity in plant $Ca^{2+}$ signaling.

## CONCLUSIONS AND FUTURE PROSPECTS

The complete inventory of Arabidopsis P-type ATPases has revealed a surprising large number of transporters belonging to this family. At one level, the complexity of P-type pumps reflects the various

ion specificities these transporters are equipped with. At a second level, different isoforms are expressed in a tissue- and cell-type-specific manner. At a third level, isoforms belonging to the same subfamily are expressed in different membranes in the cell.

Some important directions for future research include the assignment of transport specificities to $P_4$ and $P_5$ ATPases, establishing the structural basis for regulation of P-type ATPases by terminal autoinhibitory domains, and assigning physiological roles to the various P-type pumps. We can expect rapid advances in our understanding of the function of P-type pumps with the combination of physiological and molecular genetic approaches in the coming years. Reporter gene analyses (Haseloff, 1999; Moriau et al., 1999) and DNA microarray technology (Schena et al., 1995) will be employed on a large scale to study gene expression as a function of space, time, and environmental conditions. In the different subfamilies, knockout mutants for all members will be isolated and multiple knockouts will be generated by crossing these lines (Young et al., 2001). The phenotypes of knockout lines will be studied carefully under all thinkable conditions. In this context, it will be of particular interest to learn whether additional roles for P-type pumps can be identified.

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