

RESEARCH PAPER

# The roles of the cation transporters CHX21 and CHX23 in the development of *Arabidopsis thaliana*

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## Abstract

The *Arabidopsis thaliana* genome encodes a family of 28 proteins whose members have been associated with the transport of monovalent cations across membranes. Experiments have been performed to elucidate the biochemical function and the role in plant development of two closely related members of this CHX family. A genotype carrying a knockout of the *AtCHX23* gene (*At1g05580*) showed no phenotype when grown in glasshouse conditions. In particular, it did not exhibit the reduced root growth phenotype observed for a knockout of its homologue *AtCHX21* when exposed to elevated sodium concentration. However, it was not possible to produce plants that were homozygous knockout for both *AtCHX21* and *AtCHX23*. Reverse transcription-PCR (RT-PCR) experiments revealed that both genes are highly expressed in flower buds, flowers, and pollen. However, examination of pollen grain viability and pollen tube growth through excised styles did not reveal a phenotypic difference between the *chx21<sup>-</sup>chx23<sup>-</sup>* condition and other haplotypes. Crosses between selected mutants and wild-type plants in which the *chx21<sup>-</sup>chx23<sup>-</sup>* haplotype was produced by either the male or female parent demonstrated unequivocally that the *chx21<sup>-</sup>chx23<sup>-</sup>* haplotype could not pass through the female line. This suggests that the genes share a critical function in the development and/or function of the female gametophyte and that this function cannot be provided by other members of the *AtCHX* gene family. Experiments were carried out using the heterologous expression of *AtCHX23* in *Saccharomyces cerevisiae* genotypes carrying combinations of deletions of genes involved in the transport of sodium or potassium across membranes. The results show that *CHX23* would only complement the poor colony growth phenotype associated with the deletion of the yeast gene *kha1*. The conclusion is that both *AtCHX21* and *AtCHX23* act in potassium homeostasis within the female gametophyte and this is discussed in terms of the diversification of gene sequence and function within the *CHX* gene family.

**Key words:** Cation transport, CHX, female gametophyte, gene knockout, yeast.

## Introduction

The sequencing of the *Arabidopsis* genome (*Arabidopsis* Genome Initiative, 2000) allowed the identification of large numbers of genes encoding putative membrane-bound transporters (e.g. Mäser *et al.*, 2001). These include members of the CPA (cation proton antiporter) superfamily (Chang *et al.*, 2004) which are considered to be involved in monovalent cation exchange in plants, animals, fungi, and bacteria. In *Arabidopsis*, the CPA1 group of transporters includes eight NHX (sodium/hydrogen exchanger) proteins. The two most highly characterized are *AtNHX1*, which is

localized in the vacuolar membrane and enables sodium compartmentalization in the vacuole (Apse *et al.*, 1999) and *AtNHX7* (*SOS1*) which is localized in the plasma membrane and is thought to be involved in sodium efflux (Wu *et al.*, 1996; Shi *et al.*, 2000). The CPA2 family consists of two cation transporter families: the KEA (potassium exchange antiporters) and CHX (cation hydrogen exchangers) families. There are six members of the KEA family in *Arabidopsis* which are thought to function as  $K^+/H^+$  antiporters, given their homology to proteins with this

function in bacteria. The *Arabidopsis* CHX family comprises 28 members and these are thought to be involved in cation/H<sup>+</sup> transport (Sze *et al.*, 2004).

The CHX group represents an interesting model for the study of the diversification of protein sequence and function. Many of the CHX proteins exist as pairs of close homologues, such as AtCHX13 and AtCHX14, and AtCHX21 and AtCHX23 (Sze *et al.*, 2004). Such relationships clearly suggest that members of each pair possessed a relatively recent common ancestor. *AtCHX21* and *AtCHX13* lie close together on chromosome 2. Their nearest homologues are *AtCHX23* and *AtCHX14*, respectively and these two genes lie close together on chromosome 1 (Supplementary Fig. S1 available at *JXB* online), suggesting that the current situation is a result of the duplication of part of an ancestral chromosome. This proposition is strengthened by the observation that *AtCHX8* and *AtCHX7* lie next to each other further along chromosome 2 and the closest homologues to these genes are *AtCHX6a* and *AtCHX5* which again lie next to one another on chromosome 1.

To date, only a few of the AtCHX transporters have been characterized in yeast and/or *in planta*. AtCHX17 is an endomembrane transporter which was shown to transport potassium in yeast and is therefore thought to have a role in potassium homeostasis (Cellier *et al.*, 2004; Maresova and Sychrova, 2006). Similarly, CHX20 has been shown to transport potassium in yeast, but only under alkaline conditions. This transporter is located in the guard cells and is thought to function in osmoregulation (Padmanaban *et al.*, 2007). *AtCHX13* restored the growth of yeast lines mutant at both *trk1* and *trk2* (Zhao *et al.*, 2008). The conclusion of the authors was that AtCHX13 has a role in K<sup>+</sup> acquisition and that it is involved in high-affinity potassium uptake. The gene is expressed in roots under conditions of potassium deficiency and, using <sup>86</sup>Rb as a tracer, the protein has been shown to mediate high-affinity K<sup>+</sup> uptake in both *Arabidopsis* and yeast. Green fluorescent protein (GFP)-tagged versions of the protein were localized within the plasma membrane in both yeast and plant.

Cellier *et al.* (2004) reported that expression of *AtCHX17* could be detected, using northern analysis, in anthers and also in roots that had been subjected to Na<sup>+</sup> stress, K<sup>+</sup> starvation, abscisic acid treatment, or low external pH. Using *GUS* constructs and *in situ* reverse transcription-PCR (RT-PCR), they showed that the gene is expressed in the epidermal and cortical cells of mature roots in response to these stresses. Gene knockouts in *Arabidopsis* accumulated less K<sup>+</sup> in their roots compared with the wild type in response to Na<sup>+</sup> stress and K<sup>+</sup> starvation. Their conclusion was that AtCHX17 is involved in K<sup>+</sup> acquisition and homeostasis. Maresova and Sychrova (2006) were subsequently able to demonstrate that *AtCHX17* cDNA complements a growth defect at increased pH phenotype of *Akhal* in yeast. They showed that a GFP-tagged AtCHX17 protein had a punctuate distribution resembling that of the Golgi apparatus. They speculated that AtCHX17 and

Skh1p may both function in pH and K<sup>+</sup> homeostasis in Golgi cisternae.

AtCHX21 has been shown to be localized on the plasma membrane of root endodermal cells and has been shown to play a role in the transport of sodium into the stele. Genotypes homozygous for a knockout allele of the gene exhibited lower root growth rates in the presence of elevated sodium or potassium ions. When exposed to elevated sodium concentrations around their roots, these genotypes were shown to contain lower levels of sodium within their transpiration stream and lower amounts of sodium accumulated in their leaves (Hall *et al.*, 2006).

Song *et al.* (2004) reported *AtCHX23* expression to be widespread around the plant following experiments employing RT-PCR and a *CHX23* promoter-*GUS* fusion. Work with a GFP fusion protein showed that AtCHX23 is located in the chloroplast envelope. They employed two types of mutant to affect the expression of this gene: an RNA interference (RNAi) line and a mutant produced using ethylmethane sulphonate (EMS) that resulted in a product with a point mutation (Phe761 to lysine). Both mutants displayed a striking phenotype in homozygous lines. Under 'ideal conditions' they exhibited a reduced growth rate and had yellower leaves (possessing less chlorophyll) than the wild type, and possessed smaller siliques with fewer seeds. The chloroplasts in the mutants lacked granal stacks and must have been severely compromised with regard to their photosynthetic capacity. Growth of seedlings on MS medium supplemented with 30 mM KCl increased the growth rate of the mutants and led to higher chlorophyll accumulation. On the basis of this observation, the authors suggested that CHX23 might function in K transport. However, they also reported that *chx23* mutants displayed a high sensitivity to NaCl, with seed germination being reduced in elevated salt conditions.

In contrast to the results of Song *et al.* (2004), Sze *et al.* (2004) reported that *AtCHX23* is preferentially expressed in pollen. This conclusion was based on the use of microarray data, RT-PCR experimentation, and the examination of transgenic plants bearing a *AtCHX23* promoter-*GUS* fusion. They were unable to explain the discrepancy between their results and those of Song *et al.* (2004). Sze *et al.* (2004) concluded that 18 of the *AtCHX* genes are specifically or preferentially expressed in the male gametophyte and speculated that some CHX proteins may play a role in osmotic adjustment and K<sup>+</sup> homeostasis as mature pollen desiccates and then rehydrates.

In this study, the role of *AtCHX23* in root growth was assessed to determine whether knocking out this gene results in a phenotype similar to that of plants with a mutant allele of *AtCHX21* (its closest homologue). Next, crosses were carried out between genotypes bearing mutant alleles of *AtCHX21* and *AtCHX23* in an attempt to determine the phenotypic characteristics of a double mutant. Since the results indicate that the genes have an overlapping function in reproduction, experiments were carried out to discover which part of this process is blocked when the genes are mutant. Finally, heterologous expression experiments were

performed in yeast to determine the ionic specificity of AtCHX23. The experimental approaches used here exploit the gene knockout resources available for two model organisms: *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. The results provide information about the function of AtCHX23 but also afford an opportunity to consider the processes by which two similar genes (AtCHX21 and AtCHX23) have diverged from one another in recent evolutionary history.

## Materials and methods

### Genetic resources and plant growth

A seed stock from the SAIL collection (Code 899 G8), carrying a putative T-DNA insert in gene AtCHX23 (At1g05580), was obtained from the Nottingham Arabidopsis Stock Centre. Genotypes homozygous for a knockout mutant allele of AtCHX21 (SLAT 01\_19\_03b) had already been produced (see Hall *et al.*, 2006). Seedling growth, DNA extraction, general PCR methodology, and DNA sequencing were as described in Hall *et al.* (2006) using the gene- and T-DNA specific primers shown in Supplementary Fig. S2 at JXB online.

### RT-PCR

RNA was initially extracted using a Trizol™-based method from pollen and flowers of the SAIL line (see above) and a (negative segregant) wild type in order to test whether the putative mutant produced AtCHX23 transcript. The RNA was DNase treated and used to produce cDNA by reverse transcription; primers 05580Fseq (TAATCACCACCGCAATCTTCG) and 05580R3 (ATCGTTTAAACCGGCAGTC ACCGG) were used to amplify AtCHX23 cDNA using PCR, and the products of the reaction were examined using agarose gel electrophoresis.

In order to determine the tissues in which AtCHX23 is expressed, RNA was then extracted from 100 mg of root, stem, leaf, bud, flower, and silique tissue (using Trizol extraction except for root and silique where the Qiagen RNeasy minikit was employed). RNA was then DNase treated, reverse transcribed, and the same primers were used to amplify AtCHX23 cDNA.

To test AtCHX23 expression further, a sample of mature pollen cDNA was kindly provided by Berger's group (Pina *et al.*, 2005). As the average length of the transcript was 600 bp, primers were designed in this region at the 3' end of the gene and using 2.5/5 ng of pollen cDNA.

### Complementation assays in yeast

A full length cDNA clone of AtCHX23 (clone RAFL09-91-J02) was obtained from the Riken collection. This clone was transformed into *Escherichia coli* and the insert amplified using primers containing NotI restriction sites, with the forward primer also containing a Kozak translation initiation sequence (Kozak, 1991). The PCR product was cloned into pGEM-T, transformed into *E. coli*, and the construct sequenced. The construct was then excised using NotI and cloned into pYES2 which is a yeast/*E. coli* shuttle vector with a galactose-inducible promoter upstream of the insert position. This recombinant plasmid was introduced into *E. coli* to facilitate further checking of the sequence. Along with the empty vector (pYES2) it was then transformed into the yeast strain, W303a, which is W303-1A MATa leu2-3/112 ura3-1 trp1<sup>-1</sup> his3-11/15 ade2-1 can1-100 GAL SUC2 mal10 (Wallis *et al.*, 1989). Derivatives that were subsequently used were: AB11c, which is  $\Delta$ ena1-4  $\Delta$ nha1  $\Delta$ nhx1; KTA40-2, which is  $\Delta$ ena1-4  $\Delta$ nha1  $\Delta$ nhx1  $\Delta$ kha1; LMM03, which is  $\Delta$ trk1  $\Delta$ trk2; and LMM04, which is  $\Delta$ trk1  $\Delta$ trk2  $\Delta$ tok1 (Maresova and Sychrova, 2005).

For complementation testing using the conventional colony growth assay, yeast strains were grown in liquid culture to 1.0 A<sub>600</sub>, 10-fold serially diluted to 1/1000, and then 3  $\mu$ l aliquots were plated onto selective medium; 10-fold serial dilutions of these initial samples were then applied. Arginine phosphate (SDAP) selective medium (Rodriguez Navarro and Ramos, 1984) was used because of its low potassium level (Padmanaban *et al.*, 2007). Plates were incubated at 30 °C for assessment of colony growth. Each complementation assay was repeated on five different occasions using independent colonies, and the results shown were consistently obtained in all five tests.

## Results

### Molecular characterization of Atchx21 and Atchx23 knockout alleles

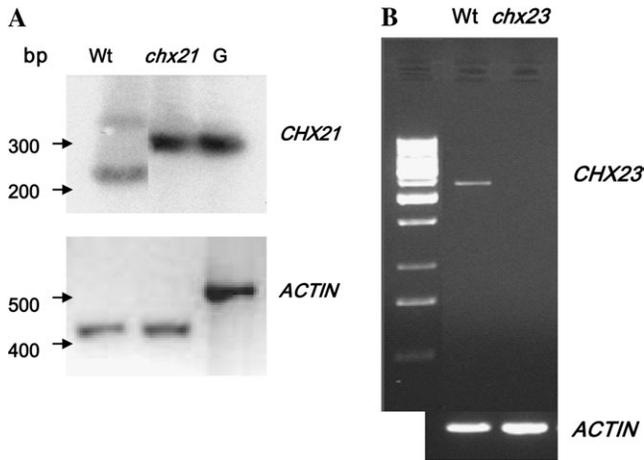
DNA was extracted from individual plants carrying a putative T-DNA insertion in gene AtCHX23 (At1g05580). Primers were designed to regions of the gene and the T-DNA insert and PCR products were sequenced. These demonstrated that the T-DNA was inserted in the predicted exon 5 of the gene (Supplementary Fig. S2 at JXB online). Since the seed stock received contained heterozygous lines with respect to the Atchx23 allele, it was also possible, by genetic segregation, to obtain lines homozygous for either the wild type or the mutant chx23 allele for use in comparisons in subsequent phenotypic tests.

RT-PCR was carried out to ensure that no transcript was produced in the Atchx23 insertional mutant. As AtCHX23 had been reported to be preferentially expressed in pollen (Sze *et al.*, 2004), whole flower cDNA was used to test for AtCHX23 expression from wild-type and insertional mutant lines. Figure 1 shows that the putative mutant form of Atchx23 is a knockout since the 1.5 kb cDNA amplified from wild-type flowers is absent. Additional characterization was also carried out on the Atchx21 insertional mutant previously described by Hall *et al.* (2006). Figure 1 also shows that the cDNA produced using template RNA from root tissue of wild-type plants was absent when RNA from the homozygous Atchx21 insertional mutant was used.

The copy numbers of the T-DNA insertions in the genotypes bearing the knockout Atchx21 and Atchx23 alleles were assessed. The use of a T-DNA-specific probe revealed a single T-DNA insert in the Atchx21 knockout line using Southern analysis (Supplementary Fig. S3 at JXB online). In addition, the use of an appropriate restriction enzyme showed that there is only one T-DNA insertion within the AtCHX21 locus (rather than a tandem insertion). Copy number in the Atchx23 mutant line was assessed using fluorescent *in situ* hybridization (FISH). Chromosomal spreads from flower buds from both wild-type and Atchx23 knockout plants were probed with a T-DNA sequence. No hybridization was detected in wild-type cells but hybridization was evident at a single locus in Atchx23 knockout cells (Fig. 2).

### Phenotypic analysis of plants containing combinations of knockout alleles

During detailed analyses of the growth of sets of Atchx23 knockout and wild-type plants grown under ideal conditions

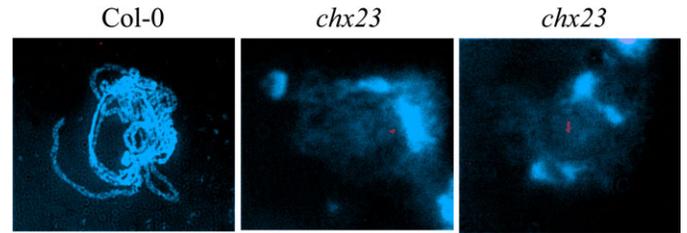


**Fig. 1.** RT-PCR analysis of *chx21* and *chx23* insertional mutants showing the absence of transcripts (compared with the wild type). Col(0) (wild type) root cDNA, *chx21* mutant root cDNA, and genomic DNA (G) were used as substrates in PCR amplifications using the *AtCHX21*-specific primers NaT<sub>1</sub>F<sub>5</sub> and NaT<sub>1</sub>R<sub>5</sub>, and then re-amplified using nested primers NaT<sub>1</sub>F<sub>6</sub> and NaT<sub>1</sub>R<sub>6</sub> (A). Actin was amplified using primers ActF and ActR. Flower cDNA samples from Col(0) (wild type) and the *chx23* mutant were used as substrates in PCR amplifications using primers 05580Fseq and 05580R3 (B).

in a glasshouse, no observations of differential chlorophyll content of the two genotypes were made (Evans, 2008). While roots of seedlings carrying a knockout of *Atchx21* had been shown to grow more slowly in the presence of 50 mM and 100 mM NaCl (Hall *et al.*, 2006), roots homozygous for the *Atchx23* knockout grew at the same rate as the wild type (not shown).

In order to determine whether the close structural homologues *AtCHX23* and *AtCHX21* possessed overlapping functions, attempts were made to produce homozygous double mutant lines for study. Crosses were made between lines homozygous for the knockout allele at either the *chx21* or *chx23* (unlinked) loci. The genotypes of the F<sub>1</sub> hybrids (*chx21*<sup>+/-</sup>*chx23*<sup>+/-</sup>) were confirmed using PCR with four pairs of allele-specific primers. The plants were allowed to self-pollinate and the F<sub>2</sub> population was screened. Of the 76 individuals examined, 1/16 would be expected to be homozygous knockout for both *chx21* and *chx23*; however, no such double mutants were observed. In a further test, 40 progeny of the F<sub>2</sub>s with the genotype *chx21*<sup>+/-</sup>*chx23*<sup>+/-</sup> and 40 with the genotype *chx21*<sup>-/-</sup>*chx23*<sup>+/-</sup> were allowed to self-fertilize and their progeny characterized using PCR. In both cases, one would expect 1/4 of the progeny to have the genotype *chx21*<sup>-/-</sup>*chx23*<sup>-/-</sup>. However, once again all other possible genotypes were observed except for the homozygous double mutant. The probability that a double knockout did not exist in the sample is >99.999%.

Since 100% germination was obtained in these experiments (data not shown), the reason for the absence of double mutants from the progeny populations appeared to lie at an earlier stage of development. To help establish the stage at which lethality is expressed, the expression of the



**Fig. 2.** Fluorescent *in situ* hybridization (FISH) to identify the number of T-DNA insertions in the mutant *Atchx23*. Cells undergoing prophase I were isolated from Col(0) and *chx23* flower buds. FISH was carried out using a digoxigenin (DIG) nick-translated pBluescript T-DNA-specific probe followed by anti-DIG-rhodamine (red label) and viewed using a fluorescence microscope. No hybridization to chromosomes was apparent in Col(0) chromosomes. Hybridization to a single locus is apparent for *chx23* chromosomes, and for some cells it is clear that the *chx23* genotype is homozygous for the T-DNA insertion since there is hybridization to the same region of sister chromosomes.

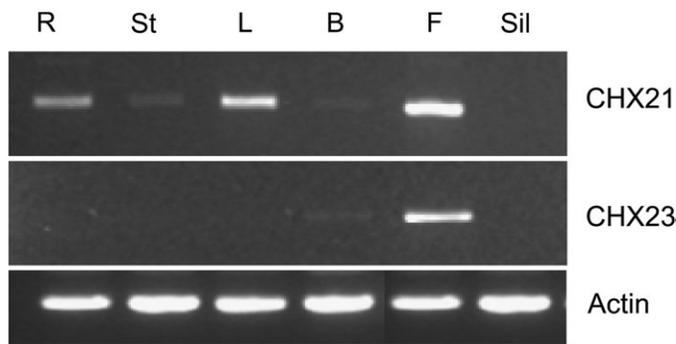
genes was assessed in different tissues. RNA was extracted from root, stem, leaf, flower bud, flower, and silique tissue, and locus-specific primers were designed to amplify the *CHX21* and *CHX23* transcripts using RT-PCR (Fig. 3). *AtCHX21* transcript was present in the root, leaf, and flower, and very low levels were present in the stem and flower bud. *AtCHX23* transcript was detected only in flower buds and flowers. Hence, *AtCHX21* has wider expression within the plant but both genes are expressed in flower buds and flowers. RT-PCR was next carried out on pollen cDNA. Using a cDNA library produced from the RNA of mature pollen, it was shown that both genes are expressed (Fig. 4). Hence, it appeared possible that the lethality observed in the absence of a functional copy of one of the genes may be due a lesion in pollen development.

Tests were carried out on pollen viability using the Alexander vital stain. Pollen was collected from plants that were wild type, were homozygous mutants for either *AtCHX21* or *AtCHX23*, or were homo-insufficient mutants recovered from the double knockout cross (*chx21*<sup>+/-</sup>*chx23*<sup>+/-</sup> or *chx21*<sup>-/-</sup>*chx23*<sup>+/-</sup>). Half of the pollen produced by the latter mutants will be *chx21*<sup>-</sup>*chx23*<sup>-</sup>. However, non-viability of a proportion of the pollen was not detected from any of the parental genotypes (Supplementary Fig. S4 at JXB online).

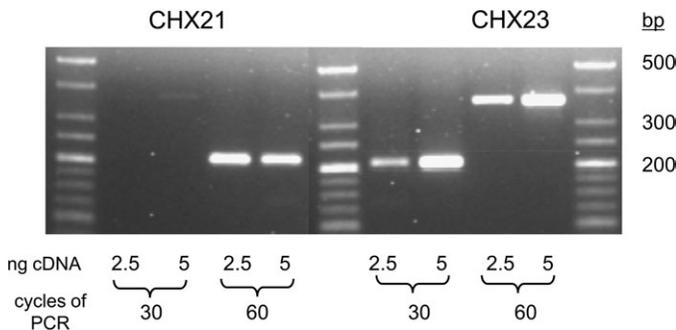
To assess pollen tube growth in a 'semi-*in vivo*' manner, wild-type pistils were removed from flower buds at anthesis, pollinated with wild-type or mutant pollen (homozygous knockout mutants or *chx21*<sup>+/-</sup>*chx23*<sup>+/-</sup> or *chx21*<sup>-/-</sup>*chx23*<sup>+/-</sup>), and examined microscopically over time. Wild-type pollen reached the base of the pistil 24 h after pollination. Indistinguishable results were obtained even though half of the pollen from some genotypes was *chx21*<sup>-</sup>*chx23*<sup>-</sup> (Fig. 5).

One method for testing for reduced transmission of genetic material through either gametophyte is to make reciprocal backcrosses of selected genotypes to the wild type. For example, half of the gametes produced by a *chx21*<sup>-/-</sup>*chx23*<sup>+/-</sup> parent will be *chx21*<sup>-</sup>*chx23*<sup>-</sup>. If this is

used as the male parent in a backcross to the wild type, and the *chx21chx23* function is required for pollen production, then one would observe a clear shift from the expected ratio



**Fig. 3.** RT-PCR analysis showing the expression of *AtCHX21*, *AtCHX23*, and an actin control in different tissues. Amplification was for 45 cycles for *AtCHX21* but 30 cycles for *AtCHX23* and the actin control. R, root; St, stem, L, leaf; B, bud; F, flower; Sil, silique.



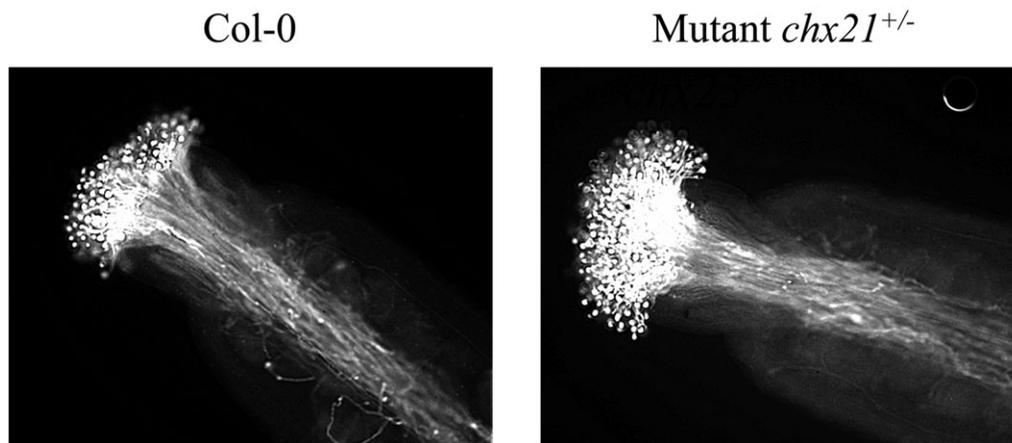
**Fig. 4.** RT-PCR analysis of *AtCHX21* and *AtCHX23* using mature pollen cDNA. *AtCHX21* cDNA was initially amplified for 30 cycles using NaT<sub>1</sub>F<sub>7</sub> and NaT<sub>1</sub>R<sub>7</sub>, and *AtCHX23* cDNA using 05580F6 and 05580R<sub>3</sub>. PCR products were then amplified for a further 30 cycles using the same primers.

of genotypes in the progeny produced; none of the progeny would carry the *chx21*<sup>-</sup> or *chx23*<sup>-</sup> allele. This test was used with four classes of backcrosses, each involving 25 pairs of plants. The results (Table 1) demonstrate that where the male parent was *chx21*<sup>+/-</sup>*chx23*<sup>-/-</sup> or *chx21*<sup>-/-</sup>*chx23*<sup>+/-</sup>, the ratio of genotypes in the progeny population was as expected, with normal transmission of the mutant through the male gamete. However, the data show that there was no transmission of the mutant alleles through the female line. This is clear evidence that the *chx21*<sup>-</sup>*chx23*<sup>-</sup> condition is lethal at some stage in the development or function of the female gametophyte.

If female gametophyte function is compromised where both *AtCHX21* and *AtCHX23* are absent, one would expect this to be reflected in the number of developing seeds in the siliques of specific genotypes. This prediction was confirmed when seed number was found to be lower in siliques of the self-pollinated genotypes *chx21*<sup>+/-</sup>*chx23*<sup>-/-</sup> and *chx21*<sup>-/-</sup>*chx23*<sup>+/-</sup> than in the wild type ( $P=0.001$ ) (Fig. 6). As a consequence, the former genotypes also have shorter siliques ( $P=0.001$ ). Examination of siliques revealed undeveloped ovules/aborted seeds (Fig. 7) which failed to mature along with the other seeds.

#### Heterologous expression of *AtCHX23* in yeast

The results obtained using gene knockouts *in planta* suggest that *AtCHX21* and *AtCHX23* have overlapping functions that are required for the development of female gametes when the plant is grown under 'ideal conditions'. Sequence homology strongly suggests that these genes encode mono-valent cation transporters and there is experimental evidence, using heterologous expression, that some members of the *AtCHX* family act to transport potassium. In order to test whether *AtCHX23* functions to move potassium across a membrane, a full-length cDNA derived from the *AtCHX23* gene was incorporated into the vector pYES2 and transformed into yeast cells. This vector has

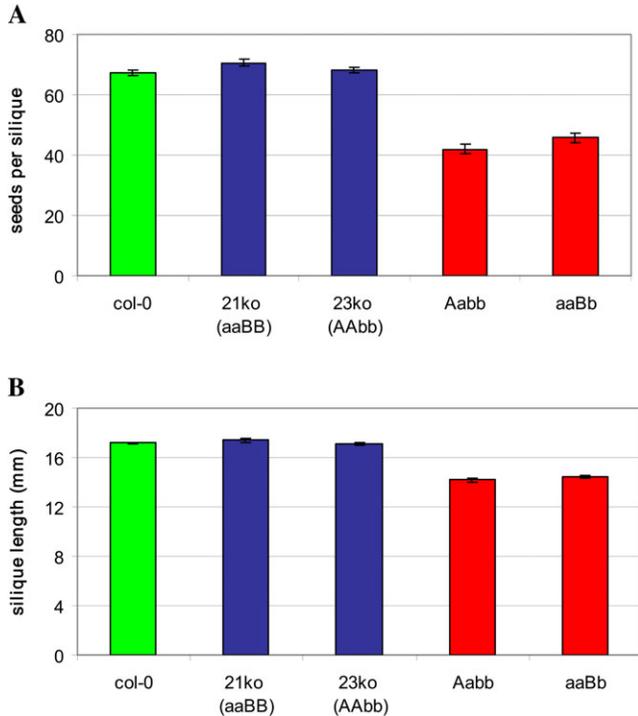


**Fig. 5.** 'Semi-*in vivo*' pollen tube growth. Emasculated buds from Col(0) (wild-type) plants were pollinated with pollen from Col(0) or a mutant (*chx21*<sup>+/-</sup>*chx23*<sup>-/-</sup>) genotype. Twenty-four hours later the stigmas were fixed, stained with aniline blue, and viewed under a UV microscope using a DAPI (4',6-diamidino-2-phenylindole) filter. No differences in pollen tube growth were observed for this mutant or when pollen from a *chx21*<sup>-/-</sup>*chx23*<sup>+/-</sup> mutant was used (not shown).

**Table 1.** The expected and actual ratios of genotypes obtained following reciprocal crosses between the wild type (Col-0) and  $chx21^{+/-}chx23^{-/-}$  or  $chx21^{-/-}chx23^{+/-}$

For each cross, the male parent is given first.

Crosses (male×female)	Expected ratio	Observed ratio
Col-0 × $chx21^{+/-}chx23^{-/-}$	1:1 ( $chx21^{+/-}:chx21^{+/-}$ )	25:0
Col-0 × $chx21^{-/-}chx23^{+/-}$	1:1 ( $chx23^{+/-}:chx23^{+/-}$ )	25:0
$chx21^{-/-}chx23^{+/-}$ × Col-0	1:1 ( $chx23^{+/-}:chx23^{+/-}$ )	13:12



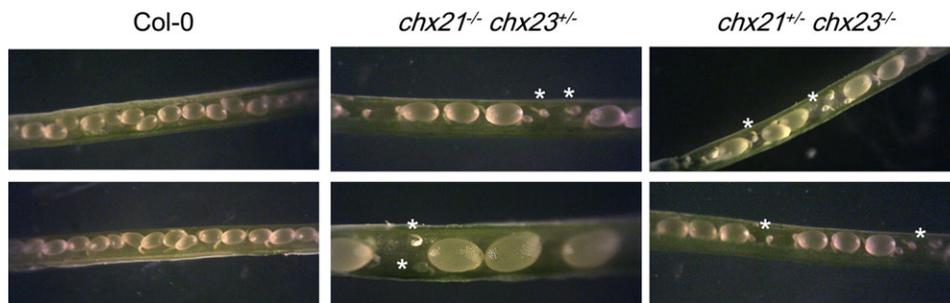
**Fig. 6.** Testing for transmission of double mutant haplotypes through male and female gametophytes. Self-pollination was allowed for a range of genotypes, and the effect on seed number and siliqua length was assessed. (A) Mean seed number per siliqua. (B) Mean siliqua length. Col(0), wild type; 21ko,  $chx21^{-/-}chx23^{+/-}$ ; 23ko,  $chx21^{+/-}chx23^{-/-}$ ; AaBB,  $chx21^{+/-}chx23^{+/-}$ ; aaBb,  $chx21^{-/-}chx23^{+/-}$ .

a galactose-inducible promoter upstream of the introduced sequence so that expression can be induced as desired. Initially, the transgenes were expressed in yeast strain W303a; the cells were homogenized and protein samples subjected to western analysis. The results (Fig. 8) showed that the transgene was expressed following induction using galactose.

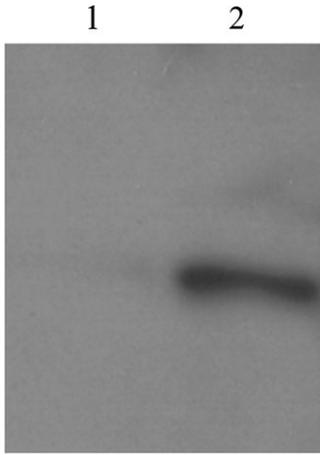
Colony growth tests were then carried out using strains of yeast that are deficient in several monovalent cation transporter functions. Since *AtCHX21* has been shown to export sodium *in planta* (Hall *et al.*, 2006) a test for sodium tolerance in yeast was performed using the triple mutant AB11c; this strain lacks both the sodium-extruding ATPase genes (*ena1-4*) and sodium/proton antiporters (*nhal* and *nhx1*). The expression of *AtCHX23* within AB11c did not rescue colony growth under sodium stress (not shown).

There is evidence to suggest that the *AtCHX* gene family may have a role in potassium homeostasis (Sze *et al.*, 2004). To test a potential role for *AtCHX23* in high affinity potassium uptake, the gene was transferred to LMM03, a yeast strain lacking the high affinity potassium transporters *trk1* and *trk2*. Mutants lacking these two genes were unable to sustain colony growth below 10 mM KCl at pH 5.7; the addition of *AtCHX23* failed to restore growth under these conditions. As the yeast gene *tok1* also contributes to some potassium uptake when this cation is at low concentrations, the assays were repeated using strain LMM04 which is  $\Delta trk1$ ,  $\Delta trk2$ , and  $\Delta tok1$ . However, no restoration of colony growth below 10 mM KCl was observed (not shown).

*kha1p* is the closest yeast protein homologue to the plant *AtCHX* family and is also a member of the CPA2 superfamily. A phenotype of  $\Delta kha1$  is reduced yeast colony growth at alkaline pH which disappears with increasing potassium concentration. Strain KTA 40-2 is  $\Delta ena1-4$ ,  $\Delta nhx1$ ,  $\Delta nhx1$ , and  $\Delta kha1$ , and colonies of this genotype did not grow well on medium adjusted to pH 7.5 containing 1, 5, or 25 mM KCl. However, the addition of *AtCHX23* complemented this mutation at 1 mM and 5 mM KCl (Fig. 9). Also, at pH 4.5 and 5.5, strains containing *AtCHX23* were more tolerant to 800 mM KCl compared with those bearing the empty vector (Fig. 9). To confirm that the results were due to the expression of *AtCHX23* under the control of the galactose-inducible promoter, the



**Fig. 7.** Seed development was monitored in self-pollinated Col(0) (wild type) and  $chx21^{-/-}chx23^{+/-}$  and  $chx21^{+/-}chx23^{-/-}$  plants. Unfertilized ovules/aborted seeds were observed in the mutants compared with normal seed development in Col(0) siliques.



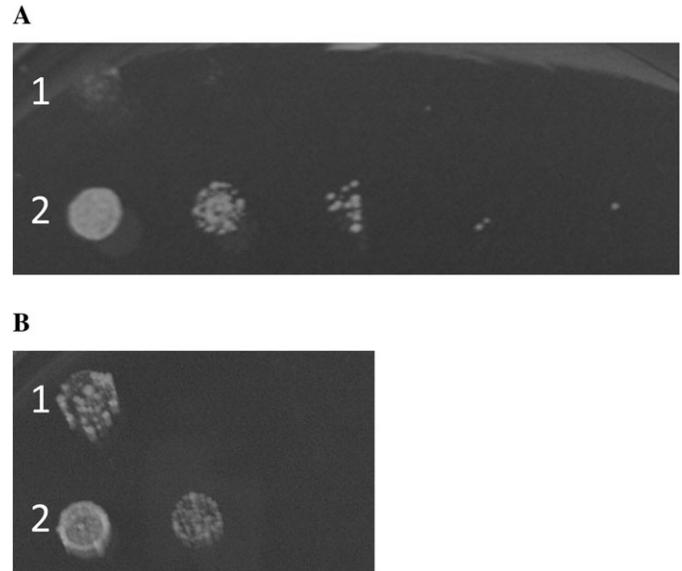
**Fig. 8.** Demonstration that a transgenic yeast line produces AtCHX23. Yeast transformants containing the *AtCHX23* construct in pYES2 within yeast strain W303a were grown in liquid YNB in glucose followed by (inductive) galactose until stationary phase. P100 protein fractions were extracted, resolved using 8% SDS-PAGE, and subjected to western analysis. The filter was probed with anti-CHX23 (diluted 1/1000), followed by goat anti-rat secondary antibody conjugated to horseradish peroxidase. AtCHX23 is absent from the lane containing protein extracted from transformed yeast cells growing in glucose (lane 1) but occurs in cells transferred to galactose (lane 2: galactose induces transgene expression in the construct used). Further details of the primary antiserum production are available in the Supplementary data at JXB online.

same tests were performed on cultures grown in glucose rather than galactose. No complementation was observed in strain KTA 40-2 under these conditions.

## Discussion

The examination of the growth and development of a *Atchx23* knockout mutant has not revealed a phenotype. The plants grew and flowered at the same rate as the wild type in a glasshouse. As in the study of *Sze et al.* (2004), the striking phenotype with reduced growth and less chlorophyll reported by *Song et al.* (2004) was not observed. These authors were working with two genotypes exhibiting lowered expression of *AtCHX23*. It was not possible to explain this difference. The lack of phenotype of the *Atchx23* knockout in the present study extends to the absence of an effect on root growth rate in the presence of elevated sodium concentrations which had previously been reported for a knockout of the close homologue *AtCHX21* (Hall *et al.*, 2006). This result for *AtCHX21* was accompanied by the demonstration of its role in the endodermis loading sodium ions into the transpiration stream when that cation is present in high concentrations around the roots.

In spite of the lack of a mutant phenotype for the *Atchx23* knockout, it was demonstrated that it is not possible to produce progeny plants that are homozygous



**Fig. 9.** Colony growth test, in SDAP medium, of yeast strain KTA 40-2 (*Δena1-4 Δnha1 Δnhx1 Δkha1*) containing: 1, pYES2 ('empty vector'); 2, pYES2-*CHX23* (A) in the presence of 800 mM KCl at pH 4.5 and (B) in the presence of 1 mM KCl at pH 7.5. The inoculated samples were serially diluted (10-fold) from left to right.

knockout for both *Atchx23* and *Atchx21*. This must mean that there is functional redundancy and that both genes can play a role that is required at some point in the reproductive process. This function appears to be absolutely required since when no functional copy of either gene is present, progeny plants are not produced. Hence, the sequence homology of these recently diverged genes is matched by the conservation of a critical shared function. It also means that the function is required when monovalent cation levels are not elevated since the attempts to produce the double mutants were carried out under ideal glasshouse conditions. Further, it means that the essential function performed by *AtCHX23* and *AtCHX21* is not provided by any of the many other *AtCHX* genes within the *Arabidopsis* genome.

Following this discovery, the effect of the removal of this function on pollen development was tested. Although both genes are expressed in pollen [according to the present RT-PCR results and the reports of both *Song et al.* (2004) and *Sze et al.* (2004)], it was not possible to detect an altered phenotype in either pollen grains or pollen tube growth within excised styles. Indeed, a critical experiment designed to determine the location of the lesion in reproductive development using mutant genotypes as either male or female parents in crosses with wild-type plants revealed unequivocally that homozygous double knockouts cannot be obtained because the *chx21<sup>-</sup>chx23<sup>-</sup>* genotype is not transmitted through the female line. This finding is consistent with the observation of undeveloped ovules/aborted seeds in the shorter siliques that have arisen from the self-pollination of plants that will produce some *chx21<sup>-</sup>chx23<sup>-</sup>* gametes. These results mean that experimentation should now be carried out to determine the precise female

gametophyte tissue in which *AtCHX21* and *AtCHX23* share their essential function; an interesting question is whether the genes act before or after fertilization.

*Sze et al.* (2004) concluded that 18 of the *AtCHX* genes are specifically or preferentially expressed in the male gametophyte and speculated that some CHX proteins may play a role in osmotic adjustment and K<sup>+</sup> homeostasis as mature pollen desiccates and then rehydrates. The present results do not preclude a key role for *AtCHX23* and/or *AtCHX21* in pollen development and function. The removal of their function might pass unobserved if there is sufficient functional redundancy and their specific function is shared by other *CHX* genes. If these genes do play a role in pollen biology then one would assume that the molecular function, cation specificity, and membrane location would be the same in both male and female gametophytes.

*Song et al.* (2004) reported that *AtCHX23* is located in the chloroplast envelope following an experiment with a CHX23–GFP fusion protein. Furthermore, they reported that the chloroplasts in the mutants lacked granal stacks. As in most plants, plastids are maternally inherited and it is interesting to speculate that a lesion in a plastid function is responsible for the breakdown of the female gametophyte in these mutants. As *Song et al.* (2004) point out, conditions that impair various metabolic functions of the plastid (which include, for example, the synthesis of amino acid-specific lipids, carotenoids, purines, and pyrimidines) will inevitably lead to wider disturbances in the biochemistry and physiology of the plant.

The experiments involving the heterologous expression of *AtCHX23* made use of a range of yeast genotypes bearing combinations of gene deletions. The results of the complementation assays suggest that *AtCHX21* and *AtCHX23* are involved in potassium tolerance. Tolerance levels varied with pH, with complementation occurring at high (800 mM) potassium concentrations at pH 4.5 and low levels (1–25 mM) at pH 7.5. As 800 mM potassium is a much higher concentration than naturally occurring levels, the most probable function of these CHXs appears to be in potassium homeostasis. The ability to rescue yeast colony growth at alkaline pH at low potassium concentrations has similarities to the *Δkha1* phenotype. *Maresova and Sychrova* (2005) showed that the reduced growth rate of the *Δkha1* strain at alkaline pH could be overcome by the addition of potassium. *kha1* is the closest yeast homologue to the CHXs, being a member of the CPA2 gene family. The present experiments demonstrating complementation of the yeast *kha1* gene by *AtCHX23* follow those of *Maresova et al.* (2006) and *Padmanaban et al.* (2007) who reported complementation of this yeast gene by *AtCHX17* and *AtCHX20*, respectively. In contrast, *Zhao et al.* (2008) showed that *AtCHX13* complements mutations at the *trk1* and *trk2* loci that are involved in high-affinity potassium uptake.

How do the current results relate to the previously reported role of *AtCHX21* in endodermal sodium transport (*Hall et al.*, 2006)? It has been confirmed that both *AtCHX21* and *AtCHX23* are expressed in root and flowers. It is proposed that the observed action of *AtCHX21* in loading Na into the

transpiration stream when the roots are exposed to high sodium levels is not of adaptive value to the plant. Most *Arabidopsis* genotypes (including Col-0, the genetic background of the genotypes used in these studies) are not adapted to saline conditions, and the phenotype observed appears to occur when the plants are exposed to high salinity conditions that they cannot survive in nature. The loading of sodium ions into the transpiration stream appears physiologically perverse and, in the light of the current results, it is proposed that the adaptive role played by the *AtCHX21* protein within the endodermal cells is actually the control of potassium levels in the stele. Because of the demonstration that *AtCHX21* (or *AtCHX23*) is required for the normal function of the female gametophyte, it is now apparent that *AtCHX21* can play a role in more than one tissue. It may also play a role in pollen development, although if so the present results suggest that this function can also be covered by the products of other CHX genes. This represents an interesting model for the study of the diversification of sequence and function within a gene family.

## Supplementary data

Supplementary data are available at *JXB* online.

**Figure S1.** The relative positions of nine members of the *AtCHX* gene family. The arrows show the closest homologues between pairs of genes; the numbers indicate the percentage identity at the amino acid level.

**Figure S2.** Diagram representing the knockout mutant of gene At1g05580, showing primer positions relative to base 1 of the At1g05580 coding region (arrows) and the regions they amplify.

**Figure S3.** Southern analysis.

**Figure S4.** Pollen viability testing.

## References

- Apse MP, Aharon GS, Snedden WA, Blumwald E.** 1999. Salt tolerance conferred by over-expression of a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter in *Arabidopsis*. *Science* **285**, 1256–1258.
- Arabidopsis Genome Initiative.** 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**, 796–815.
- Cellier F, Conejero G, Ricaud L, Luu DT, Lepetit M, Gosti F, Casse F.** 2004. Characterization of *AtCHX17*, a member of the cation/H<sup>+</sup> exchangers, CHX family, from *Arabidopsis thaliana* suggests a role in K<sup>+</sup> homeostasis. *The Plant Journal* **39**, 834–846.
- Chang AB, Lin RK, Studley W, Tran CV, Saier MH.** 2004. Phylogeny as a guide to structure and function of membrane transport proteins. *Molecular Membrane Biology* **21**, 171–181.
- Evans A.** 2008. Functional analysis of *Arabidopsis thaliana* cation transporters *in planta* and through expression in yeast (*Saccharomyces cerevisiae*). PhD thesis, University of Birmingham, UK.
- Hall D, Evans AR, Newbury HJ, Pritchard J.** 2006. Functional analysis of CHX21: a putative sodium transporter in *Arabidopsis*. *Journal of Experimental Botany* **57**, 1201–1210.

- Kozak M.** 1991. An analysis of vertebrate messenger RNA sequences—intimations of translational Control. *Journal of Cell Biology* **115**, 887–903.
- Maresova L, Sychrova H.** 2005. Physiological characterization of *Saccharomyces cerevisiae* kha1 deletion mutants. *Molecular Microbiology* **55**, 588–600.
- Maresova L, Sychrova H.** 2006. *Arabidopsis thaliana* CHX17 gene complements the kha1 deletion phenotypes in *Saccharomyces cerevisiae*. *Yeast* **23**, 1167–1171.
- Mäser P, Thomine S, Schroeder JI, et al.** 2001. Phylogenetic relationships within cation transporter families of *Arabidopsis*. *Plant Physiology* **126**, 1646–1667.
- Padmanaban S, Chanroj S, Kwak JM, Li XY, Ward JM, Sze H.** 2007. Participation of endomembrane cation/H<sup>+</sup> exchanger AtCHX20 in osmoregulation of guard cells. *Plant Physiology* **144**, 82–93.
- Pina C, Pinto F, Feijo JA, Becker JD.** 2005. Gene family analysis of the *Arabidopsis* pollen transcriptome reveals biological implications for cell growth, divisions control and gene expression regulation. *Plant Physiology* **138**, 744–756.
- Rodriguez Navarro A, Ramos J.** 1984. Dual system for potassium transport in *Saccharomyces cerevisiae*. *Journal of Bacteriology* **159**, 940–945.
- Shi HZ, Ishitani M, Kim CS, Zhu JK.** 2000. The *Arabidopsis thaliana* salt tolerance gene SOS1 encodes a putative Na<sup>+</sup>/H<sup>+</sup> antiporter. *Proceedings of the National Academy of Sciences, USA* **97**, 6896–6901.
- Song C-P, Guo Y, Qiu Q, Lambert G, Galbraith DW, Jagendorf A, Zhu J-K.** 2004. A probable Na<sup>+</sup> (K<sup>+</sup>)/H<sup>+</sup> exchanger on the chloroplast envelope functions in pH homeostasis and chloroplast development in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA*, **101**. 10211–10216.
- Sze H, Padmanaban S, Cellier F, et al.** 2004. Expression patterns of a novel AtCHX gene family highlight potential roles in osmotic adjustment and K<sup>+</sup> homeostasis in pollen development. *Plant Physiology* **136**, 2532–2547.
- Wallis JW, Chrebet G, Brodsky G, Rolfe M, Rothstein R.** 1989. Recombination mutation in *S. cerevisiae* identifies a novel eukaryotic topoisomerase. *Cell* **58**, 409–419.
- Wu SJ, Ding L, Zhu JK.** 1996. SOS1, a genetic locus essential for salt tolerance and potassium acquisition. *The Plant Cell* **8**, 617–627.
- Zhao J, Cheng N-H, Motes CM, Blancaflor EB, Moore M, Gonzales N, Padmanaban S, Sze H, Ward JM, Hirschi KD.** 2008. AtCHX13 is a plasma membrane K<sup>+</sup> transporter. *Plant Physiology* **148**, 796–807.