

An *Arabidopsis* quiescin-sulfhydryl oxidase regulates cation homeostasis at the root symplast–xylem interface

Santiago Alejandro¹, Pedro L Rodríguez¹,
Jose M Bellés¹, Lynne Yenush¹,
María J García-Sánchez², José A Fernández²
and Ramón Serrano^{1,*}

¹Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, Camino de Vera s/n, Valencia, Spain and

²Departamento de Biología Vegetal, Facultad de Ciencias, Universidad de Málaga, Campus de Teatinos, Málaga, Spain

A genetic screen of *Arabidopsis* ‘activation-tagging’ mutant collection based on tolerance to norspermidine resulted in a dominant mutant (*par1-1D*) with increased expression of the *QSO2* gene (*At1g15020*), encoding a member of the quiescin-sulfhydryl oxidase (QSO) family. The *par1-1D* mutant and transgenic plants overexpressing *QSO2* cDNA grow better than wild-type *Arabidopsis* in media with toxic cations (polyamines, Li⁺ and Na⁺) or reduced K⁺ concentrations. This correlates with a decrease in the accumulation of toxic cations and an increase in the accumulation of K⁺ in xylem sap and shoots. Conversely, three independent loss-of-function mutants of *QSO2* exhibit phenotypes opposite to those of *par1-1D*. *QSO2* is mostly expressed in roots and is upregulated by K⁺ starvation. A *QSO2::GFP* fusion ectopically expressed in leaf epidermis localized at the cell wall. The recombinant *QSO2* protein, produced in yeast in secreted form, exhibits disulfhydryl oxidase activity. A plausible mechanism of *QSO2* action consists on the activation of root systems loading K⁺ into xylem, but different from the *SKOR* channel, which is not required for *QSO2* action. These results uncover QSOs as novel regulators of ion homeostasis.

The EMBO Journal (2007) 26, 3203–3215. doi:10.1038/sj.emboj.7601757; Published online 14 June 2007

Subject Categories: membranes & transport; plant biology

Keywords: activation-tagging; K⁺ transport; salinity; red-ox regulation

Introduction

The homeostasis of monovalent cations is a fundamental activity of living cells, with both permissive and regulatory roles in many cellular functions. The transport of K⁺, Na⁺ and H⁺ determines membrane potential, turgor, intracellular cation concentrations and pH, and these basic parameters are

crucial for nutrient uptake, metabolism, cellular integrity, cell death, growth and differentiation (Hoffman, 1964; Harold, 1986; Hager *et al*, 1991; Yenush *et al*, 2002, 2005; Adams *et al*, 2006). Although the basic mechanisms of monovalent cation transport have been identified, our knowledge of their regulatory systems is rather fragmentary (Sanders and Bethke, 2000; Serrano and Rodríguez-Navarro, 2001; Pollard and Earnshaw, 2002).

Fungi and plants share general transport mechanisms at the plasma membrane based on an H⁺ chemiosmotic circuit. The primary pump is the electrogenic H⁺-pumping P-ATPase that generates an electrochemical H⁺ gradient (Serrano, 1989; Sussman and Harper, 1989; Morsomme and Boutry, 2000). This H⁺ gradient drives the secondary transport of K⁺ mediated by channels and carriers (Very and Sentenac, 2002; Rodríguez-Navarro and Rubio, 2006), as well as the uptake of nutrients by H⁺-symporters and the efflux of Na⁺ by H⁺-antiporters (Sanders and Bethke, 2000).

In plants, the regulation of H⁺-ATPases and K⁺ channels and carriers involves changes in both expression and activity of these systems. Auxin increases the levels of H⁺-ATPase (Hager *et al*, 1991; Frías *et al*, 1996; Rober-Kleber *et al*, 2003) and inward K⁺ channels (Philippart *et al*, 1999) in elongating tissues and blue light activates the H⁺-ATPase of guard cells by inducing its phosphorylation (Kinoshita and Shimazaki, 1999). The fungal toxin fusaric acid activates the H⁺-ATPase by binding to its phosphorylated C-terminus and recruiting 14-3-3 proteins (Würtele *et al*, 2003).

Abscisic acid inhibits the activity of the inward K⁺ channels of *Arabidopsis* guard cells (KAT1 and KAT2) through a signal transduction pathway involving reactive oxygen species and Ca²⁺ (Kwak *et al*, 2003). This hormone also represses the expression of the outward K⁺ channel (SKOR) at the root pericycle and xylem parenchyma of *Arabidopsis* (Gaymard *et al*, 1998). K⁺ starvation induces the expression of high-affinity K⁺ carriers such as Hak5 (Maathuis *et al*, 2003; Shin and Schachtman, 2004; Gierth *et al*, 2005) by a mechanism including hydrogen peroxide production (Shin and Schachtman, 2004). Again, the signal transduction pathways of these regulations remain largely unknown.

Na⁺ efflux in *Arabidopsis* is mediated by the SOS1 H⁺-antiporter, a transporter activated by the SOS2/CIP24-SOS3/CBL4 protein kinase–calcium sensor complex (Qiu *et al*, 2002; Quintero *et al*, 2002). Another protein kinase–calcium sensor complex, namely CIPK23-CBL1 (or CBL9), has recently been described to activate the AKT1 K⁺ uptake channel of *Arabidopsis* roots (Xu *et al*, 2006). These two protein kinases are the only regulatory components of plant cation homeostasis characterized at the molecular level.

One approach to identify novel regulators of cation transport consists of the identification of genes that upon gain of function improve tolerance to toxic cations (Serrano *et al*,

*Corresponding author. Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, Camino de Vera s/n, Valencia 46022, Spain. Tel.: +34 96 387 7883; Fax: +34 96 387 7859; E-mail: rserrano@ibmcp.upv.es

Received: 22 January 2007; accepted: 18 May 2007; published online: 14 June 2007

1999). Gain-of-function mutants obviate genetic redundancy and can identify bottlenecks in biological pathways. We have screened the collection of ‘activation-tagging’ *Arabidopsis* mutants (Weigel *et al*, 2000) for tolerance to the toxic cation norspermidine (Hamana *et al*, 1989), and report here that the *QSO2* gene (*At1g15020*), encoding a quiescin-sulphydryl oxidase (QSO), is a novel regulator of monovalent cation transport at the root symplast–xylem interface. QSOs are animal and plant enzymes proposed to participate in oxidative folding of disulfide-containing secreted proteins. Quiescins have been implicated in the regulation of growth and the elaboration of extracellular matrix of animal cells (Coppock *et al*, 1998; Thorpe *et al*, 2002). Our work suggests that these eukaryotic proteins may also regulate ion homeostasis.

Results

A screening based on tolerance to norspermidine resulted in the *par1-1D* mutant

We have screened the ‘activation-tagging’ mutant collection of the plant *Arabidopsis thaliana* (Weigel *et al*, 2000) for tolerance to toxic cations. Previous screenings for tolerance to Na^+ in *Arabidopsis* resulted in mutants defective in either abscisic acid biosynthesis or perception. The high concentrations of NaCl required for toxicity during the germination assay (greater than 0.1 M) have significant osmotic effects, which trigger the biosynthesis of abscisic acid and this

hormone inhibits germination and early growth (González-Guzmán *et al*, 2002). Alternatively, polyamines are toxic at millimolar concentrations, which pose no osmotic stress, and the presence of several positive charges per molecule makes their uptake very sensitive to membrane potential, negative inside. Norspermidine is a non-metabolizable polyamine (Hamana *et al*, 1989), and this type of toxic cation has been successfully used to demonstrate changes in membrane potential in yeast mutants affected in K^+ transport (Forment *et al*, 2002).

We have screened 16 398 lines selecting for tolerance to 3.2 mM norspermidine, a concentration that completely inhibits growth of wild-type seeds. The most resistant mutant was named *par1-1D*, from ‘polyamine resistant’, gene 1, allele 1, Dominant (Meinke and Koornneef, 1997).

As indicated in Figure 1A and B, *par1-1D* seeds germinate and grow better than wild-type plants on plates containing norspermidine, spermidine, Li^+ or Na^+ . Experiments in soil with adult plants also demonstrate tolerance to Li^+ and Na^+ (Figure 2). This tolerance is reflected both in the total size (Figure 2A) and in the discoloration of the leaves (Figure 2B and C) of stressed plants. Therefore *par1-1D* seems to have a pleiotropic phenotype of tolerance to toxic cations.

The mutation was monogenic and dominant, because F1 plants exhibited tolerance to toxic cations and there was a 3:1 segregation of the phenotype in the F2 generation. Actual data were 156:44 ($c^2 = 0.96$).

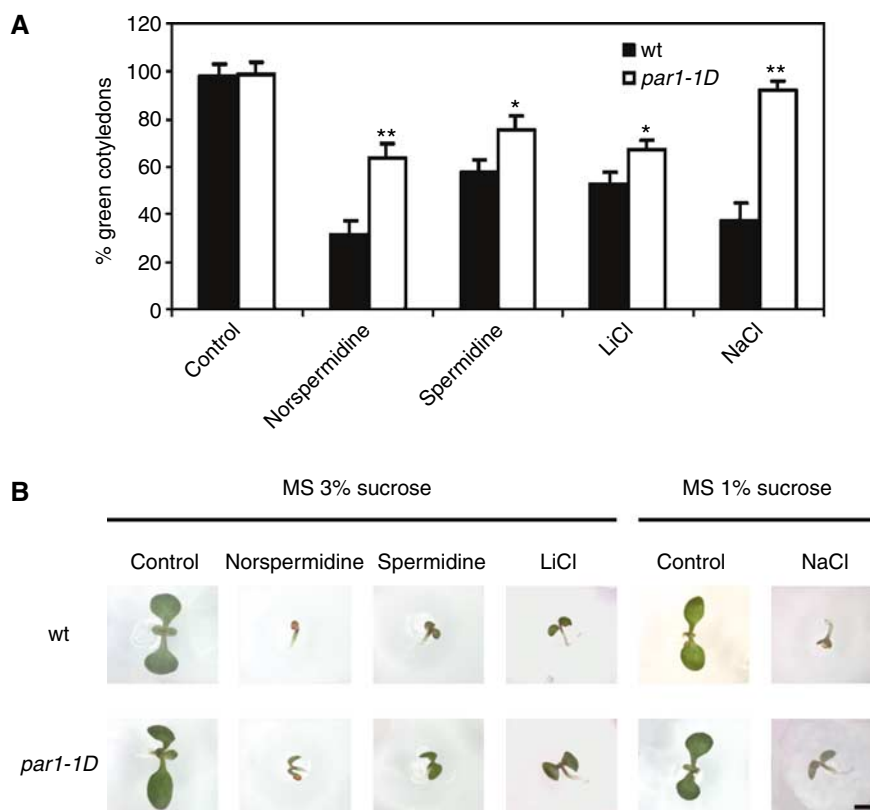


Figure 1 Seedlings of the *par1-1D* mutant are more tolerant to toxic cations than wild type (wt). (A) Percentage of seeds that germinated and developed green cotyledons 6 days after sowing in MS medium supplemented with the indicated toxic cation. Error bars represent the s.d. of three independent experiments, with three replicates each ($n = 9$). * $P < 0.05$ and ** $P < 0.01$ by Student’s test. (B) Representative seedlings from the experiment in panel A. The concentrations of toxic cations were as follows: none (control), norspermidine (2.8 mM), spermidine (7 mM), lithium (20 mM LiCl) and sodium (140 mM NaCl). Sucrose concentration was 3%, except in the case of NaCl, where 1% was utilized. Controls were sown in media with the two sucrose concentrations. Scale bar corresponds to 1 mm.

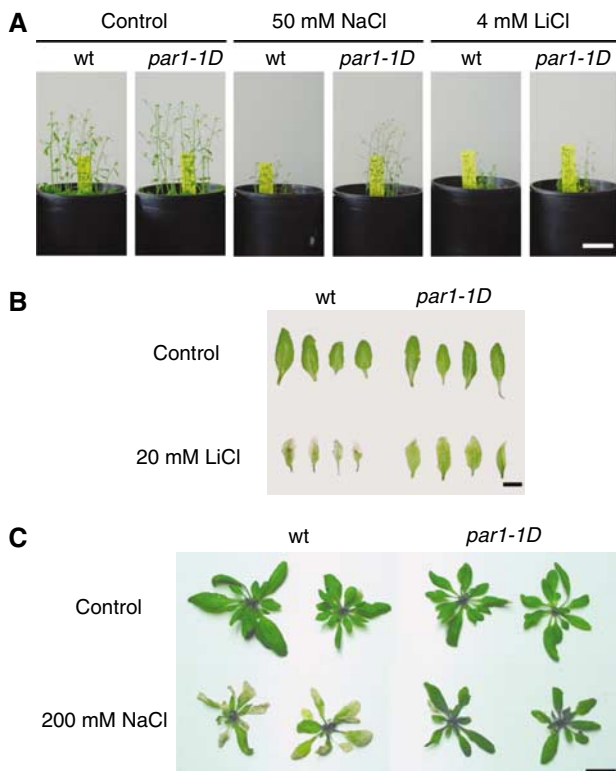


Figure 2 Plants of the *par1-1D* mutant are more tolerant to lithium and sodium than wild type (wt). (A) Plants sown in soil and irrigated with either normal solution (control) or with irrigation solution supplemented with 50 mM NaCl or 4 mM LiCl, twice a week for 25 days. Scale bar corresponds to 5 cm. (B) Leaves 6 days after treatment with 2 mM LiCl. (C) Rosettes 11 days after treatment with 200 mM NaCl. Plants in panels B and C were sown in soil and the treatment started when the rosette was fully developed (about 20 days). Scale bar corresponds to 1 and 2 cm, respectively.

The *par1-1D* mutation is caused by overexpression of *At1g15020* (QSO2), a limiting factor for toxic cation tolerance

Homozygous *par1-1D* plants were crossed to Columbia wild-type plants. From the segregating F2 generation, norspermidine-tolerant plants were selected and scored for the presence of the T-DNA by Southern blot and PCR analyses. As a result, the *par1-1D* mutation was shown to be linked to the T-DNA because of 60 F2 norspermidine-tolerant plants analyzed, all of them contained T-DNA (data not shown). Plasmid rescue of the T-DNA and sequencing indicated that the insertion was in the genomic region depicted in Figure 3A, with the transcriptional enhancer close to the *At1g15020* gene. Northern analysis confirmed that this gene was overexpressed about three-fold in the mutant (Figure 3B, left panel). As expected, the expression of the adjacent *At1g15030* gene, the promoter of which was interrupted by the insertion, was greatly decreased (Figure 3B, right panel).

In order to test if overexpression of *At1g15020* was responsible for the *par1-1D* phenotype, transgenic plants were generated, which overexpressed *At1g15020* cDNA from the 35S promoter. Most (7 out of 11) of the transgenic lines overexpressed the *At1g15020* gene (Figure 4A). Two overexpressing lines (numbers 2 and 10) were further analyzed and found to be more tolerant to toxic cations than controls

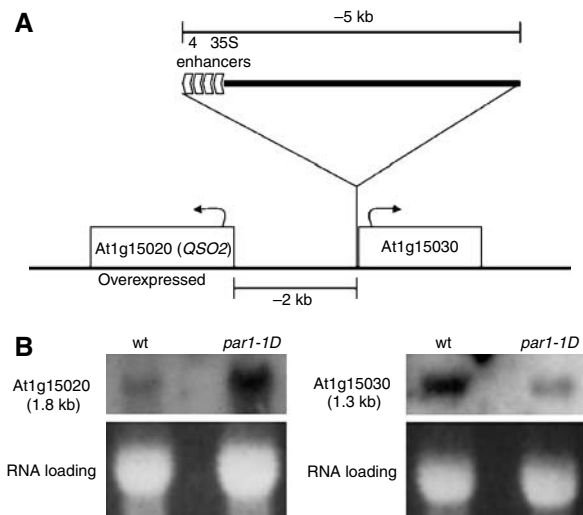


Figure 3 Position of T-DNA insertion in the *par1-1D* mutant and expression analysis of adjacent genes. (A) Location of T-DNA in the 5' UTR region of *At1g15030* gene, as determined by plasmid rescue. (B) Upper panels correspond to the Northern blot analysis of *At1g15020* (left) and *At1g15030* (right) mRNAs from wild type (wt) and *par1-1D* mutant; lower panels correspond to ethidium bromide staining of major ribosomal RNAs as loading controls.

(Figure 4B and C). This suggests that the *par1-1D* mutation was caused by overexpression of *At1g15020*.

At1g15020 encodes a sulfhydryl oxidase of the quiescin family and it has been previously named *AtQSOX2*, because there is a homologous gene, *At2g01270*, named *AtQSOX1* (Thorpe *et al*, 2002). A recent review of the family (Houston *et al*, 2005) has reversed the numbers of the two genes. We propose the three-letter, italic name *QSO2* to conform community standards for *Arabidopsis* genetics (Meinke and Koornneef, 1997), and maintain the numbers of the first publication (Thorpe *et al*, 2002). QSOs are flavoproteins containing a thioredoxin domain and which oxidize disulfhydryl groups in proteins to disulfides, with reduction of oxygen to hydrogen peroxide. They are secreted proteins located at the endoplasmic reticulum, Golgi and outside the cell. The domain structure of QSO2 is shown in Supplementary Figure 1S. QSOs are found only in multicellular organisms, plants and animals, while yeast cells contain enzymes lacking the thioredoxin domain but containing the sulfhydryl oxidase flavoprotein or ERV domain (Thorpe *et al*, 2002).

Three T-DNA insertion mutants of *At1g15020* were identified in the TAIR collection (www.arabidopsis.org) and the homozygous lines named *par1-2* (SALK_066130), *par1-3* (SALK_025237) and *par1-4* (SALK_072829). A scheme of the T-DNA insertions is presented in Figure 5A. Quantitative RT-PCR analysis indicated that QSO2 expression levels in the mutants were 13 (*par1-2*), 18 (*par1-3*) and less than 1% (*par1-4*) of wild type. The three mutant lines displayed more sensitivity to toxic cations than control plants, both as seedlings grown 'in vitro' (Figure 5B; details of seedlings shown for *par1-2* in Figure 5C), and as plants grown in soil (details of plants shown for *par1-2* in Figure 5D). Taken together with the results of the overexpression approach, these experiments demonstrate that the QSO2 sulfhydryl oxidase is a limiting factor for tolerance to toxic

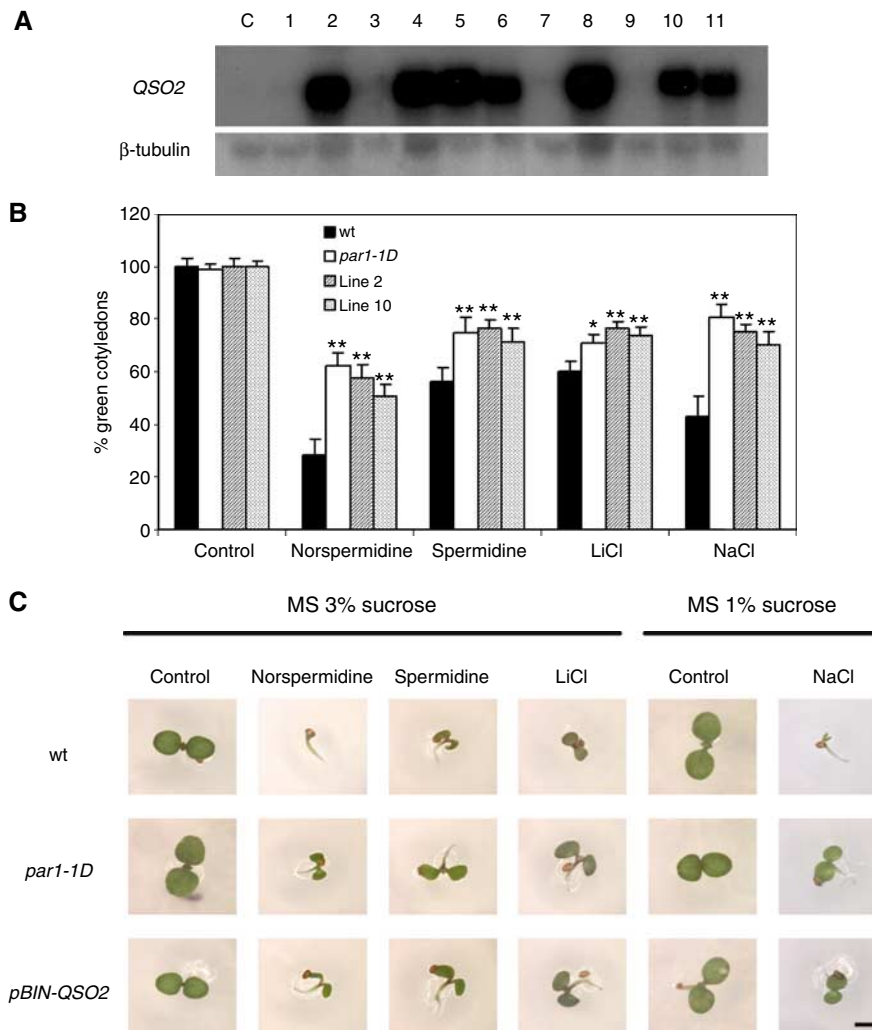


Figure 4 Transgenic plants overexpressing At1g15020 (*QSO2*) cDNA are more tolerant to toxic cations. (A) Northern blot analysis of 11 independent transgenic lines. (B) Percentage of seeds that germinated and developed green cotyledons. Lines 2 and 10 are two transgenic lines from panel A. (C) Representative seedlings from the experiment in panel B. Experiments were performed as in Figure 1, and pictures of representative seedlings were taken 6 days after sowing. Scale bar corresponds to 1 mm. Errors bars represent s.d. of three independent experiments, with three replicates each one ($n = 9$). * $P < 0.05$ and ** $P < 0.01$ by Student's test.

cations. On the other hand, a knockout mutant of the homologous gene *At2g01270* has no detectable phenotype (data not shown).

***QSO2* inversely regulates the accumulation of K^+ and toxic cations**

One mechanism of tolerance to toxic cations is based on decreasing cation accumulation. We have measured the initial rates of uptake (Figure 6A) and the final accumulation levels (Figure 6B) of norspermidine and Na^+ in *Arabidopsis* plants (wild type, *par1-1D* and *par1-2*) incubated in liquid culture. While the initial rate of uptake (less than 20 min) was not significantly affected by either gain- or loss-of-function of *QSO2*, both the uptake at longer times (1 h) and the accumulation after 2 days of both toxic cations correlated inversely with *QSO2* function. We have also measured the level of K^+ (Figure 7A) and the initial rate of Rb^+ uptake (as indication of K^+ transport; Figure 7B). Again, the initial rate of uptake was not affected by *QSO2* but, opposite to the results with

toxic cations, both the uptake at long times and the accumulation of K^+ were directly correlated with *QSO2* function.

***QSO2* modulates the loading of cations into the xylem**

One determinant of cation accumulation, which could be affected by *QSO2* is the plasma membrane electrical potential (Mulet *et al*, 1999). However, we have measured this parameter in root epidermal cells of wild type and plants with gain and loss of function of *QSO2*, and found no significant differences (Supplementary Table 1S). Another determinant of cation accumulation is the plasma membrane H^+ -ATPase, the primary pump that energizes all secondary transporters. However, neither the amount nor the activity of the enzyme was affected by *QSO2* mutations (Supplementary Figure 2S). Also, the protein level of the root K^+ uptake channel AKT1 (Hirsch *et al*, 1998) was not affected by *QSO2* mutations (Supplementary Figure 3S A) and overexpression of *QSO2* still confers tolerance to toxic cations in the *akt1-1* mutant (Supplementary Figure 3S B).

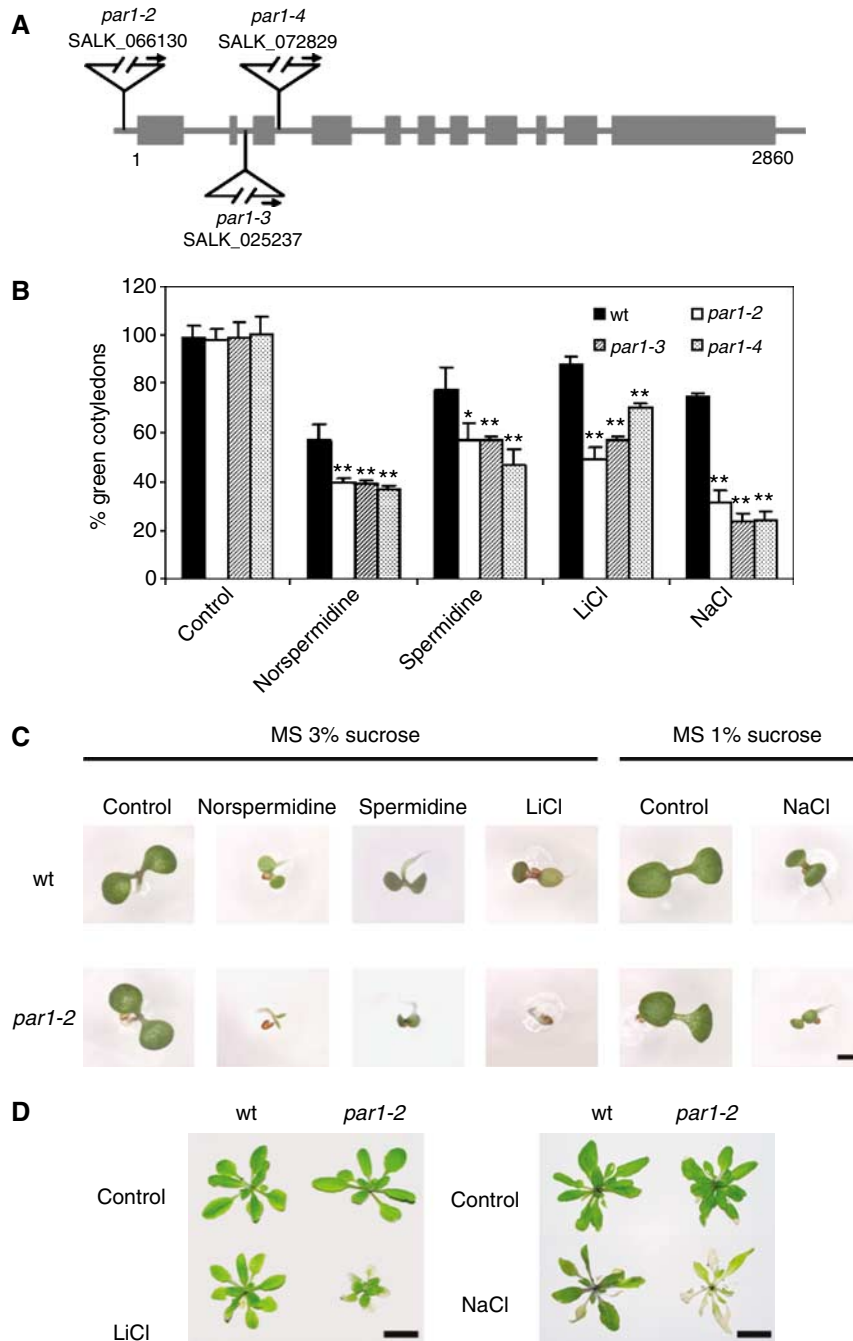


Figure 5 The loss-of-function mutants of *QSO2* are sensitive to toxic cations. **(A)** Scheme of the *At1g15020* gene and localization of the T-DNA insertions in the *par1-2*, *par1-3* and *par1-4* mutants. Blocks indicate exons. Nucleotide numbering begins at the ATG translation start codon. The T-DNA left border primer (pROKLBb1) that was used to localize the T-DNA insertion is indicated by an arrow. **(B)** Percentage of seeds that germinated and developed green cotyledons 6 days after sowing in MS medium supplemented with the indicated toxic cation. Error bars represent the s.d. of three independent experiments, with three replicates each ($n=9$). * $P<0.05$ and ** $P<0.01$ by Student's test. **(C)** Representative *par1-2* seedlings from the experiment in panel A. Scale bar corresponds to 1 mm. The concentrations of toxic cations were as follows: none (control), norspermidine (2.4 mM), spermidine (6 mM), lithium (18 mM LiCl) and sodium (120 mM NaCl). Sucrose concentration was 3%, except in the case of NaCl, where 1% was utilized. Controls were sown in media with the two sucrose concentrations. **(D)** Soil-grown plants of the *par1-2* mutant are more sensitive to lithium and sodium than wild type (wt). Representative rosettes of plants irrigated with either normal solution (control) or with irrigation solution supplemented with either 2.5 mM LiCl (left panel) or 150 mM NaCl (right panel), twice a week for 14 days. Plants were sown in soil and the treatment started when the rosette was fully developed (about 20 days). Scale bars correspond to 2.5 cm.

As *QSO2* mutations affect cation accumulation at long times but not the initial rate of uptake, we hypothesized that this QSO regulates cation loading at the root xylem and subsequent cation accumulation at the shoot. The results of

Figure 8A and B indicate that, effectively, *QSO2* positively regulates shoot accumulation of K^+ , while inhibiting shoot accumulation of toxic cations. Figure 8C and D show the same effect of *QSO2* in the case of xylem sap concentrations.

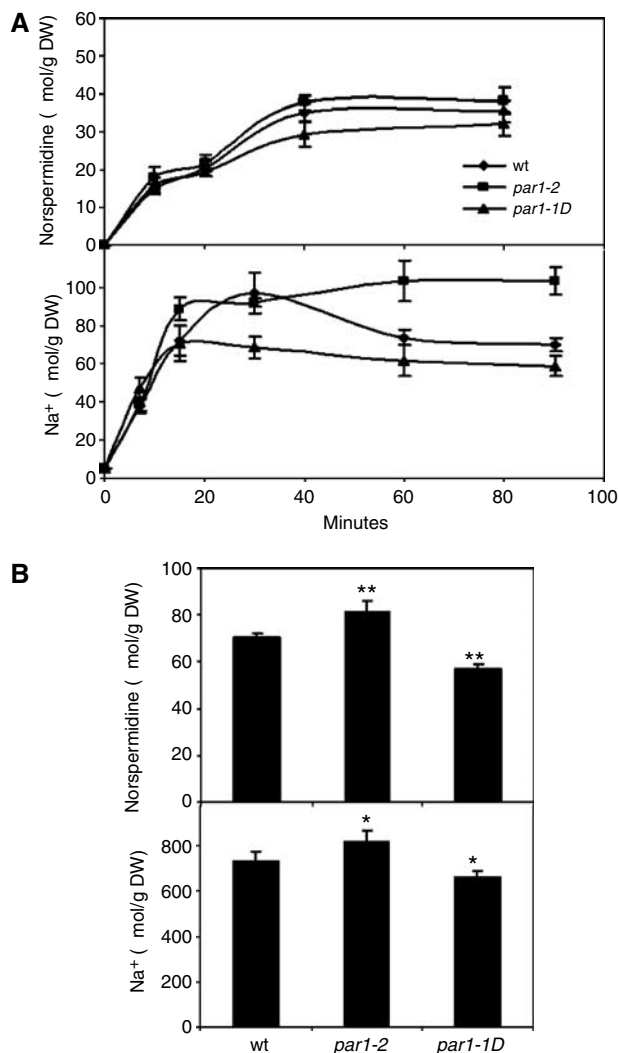


Figure 6 Homeostasis of toxic cations is altered in QSO2 mutants. (A) Initial rate of uptake of norspermidine and Na⁺ in wild type (wt) and mutants with gain (*par1-1D*) and loss (*par1-2*) of function of QSO2. Ten-day-old plants grown in liquid culture were transferred to fresh medium supplemented with either 2.8 mM norspermidine or 120 mM NaCl. Error bars correspond to s.d.s of the mean ($n=6$). (B) Steady-state accumulation of norspermidine and Na⁺ in wild type (wt) and mutants with gain (*par1-1D*) and loss (*par1-2*) of function of QSO2. Ten-day-old plants grown in liquid culture were transferred to fresh medium that was supplemented with either 2.8 mM norspermidine or 120 mM NaCl and further incubated for 2 days. The average of three experiments, with three replicates each is shown ($n=9$). Errors bars represent s.d. * $P<0.05$ and ** $P<0.01$ by Student's test. DW, dry weight.

Therefore, a plausible mechanism of QSO2 action is the activation of K⁺ efflux at the root symplast–xylem interface while inhibiting the efflux of toxic cations at this location.

The *skor-1* mutant is sensitive to toxic cations but overexpression of QSO2 still confers tolerance in this mutant

Although QSO2 could act on several transporters, the possibility exist that its primary target is K⁺ efflux at the symplast–xylem interface, and that activation of this system indirectly inhibits the efflux of toxic cations. The rationale is that activation of K⁺ efflux would hyperpolarize the

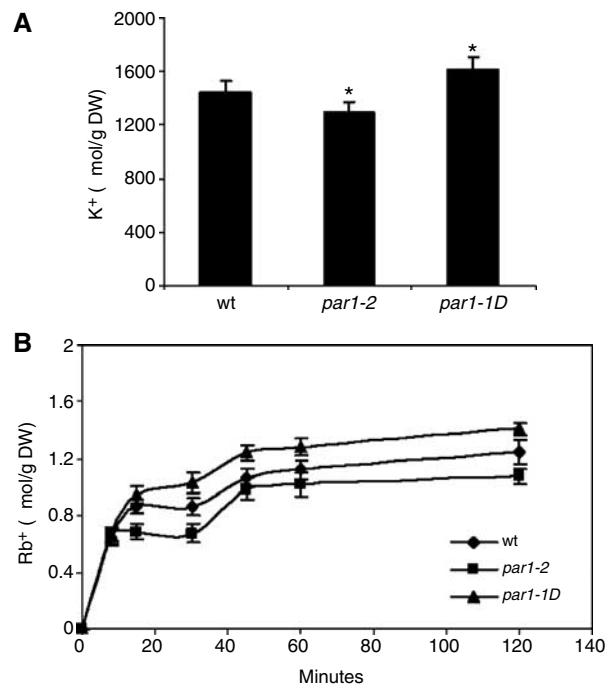


Figure 7 K⁺ homeostasis is altered in QSO2 mutants. (A) Intracellular K⁺ level in wild type (wt) and mutants with gain (*par1-1D*) and loss (*par1-2/qso2*) of function of QSO2. Five-day-old seedlings were grown in liquid culture for 10 days and intracellular K⁺ measured as indicated in Materials and methods. The average of four experiments, with three replicates each is shown ($n=12$). Error bars represent s.d.s. * $P<0.05$ by Student's test. (B) K⁺ transport activity was measured by the initial rate of Rb⁺ uptake. Ten-day-old plants grown in liquid culture were transferred to K⁺-free medium supplemented with 1 mM RbCl and samples taken at the indicated times for intracellular Rb⁺ measurement as indicated in Materials and methods. Errors bars represent s.d.s ($n=6$). DW, dry weight.

potential difference across the symplast/xylem boundary (xylem positive), and this elevated potential would inhibit the efflux of toxic cations mediated by nonspecific channels.

One way to test our hypothesis was to compare the phenotypes of the *par1-2*- and *skor-1*-null mutants. SKOR is an outward K⁺ channel mediating K⁺ release into the xylem sap (Gaynard *et al*, 1998). As predicted by our model, the *skor-1* mutant is sensitive to toxic cations in addition to K⁺ depletion (Figure 9A and B).

Although this result can be considered as a 'proof of concept' for the mechanism of QSO2 suggested above, SKOR is not the only system loading K⁺ into xylem. Actually, overexpression of QSO2 still confers tolerance to toxic cations and to K⁺ depletion in the *skor-1* mutant (Figure 9A and B). Therefore, other unknown systems involved in xylem loading of K⁺ must be affected by QSO2.

Characterization of QSO2 expression and encoded protein

Quantitative RT-PCR analysis of *Arabidopsis* tissues indicates that QSO2 is mostly expressed in roots (Figure 10A). Transcription in roots is induced about two-fold by K⁺ depletion (Figure 10B), and this regulation is in agreement with the proposed activation by QSO2 of systems loading K⁺ into the xylem.

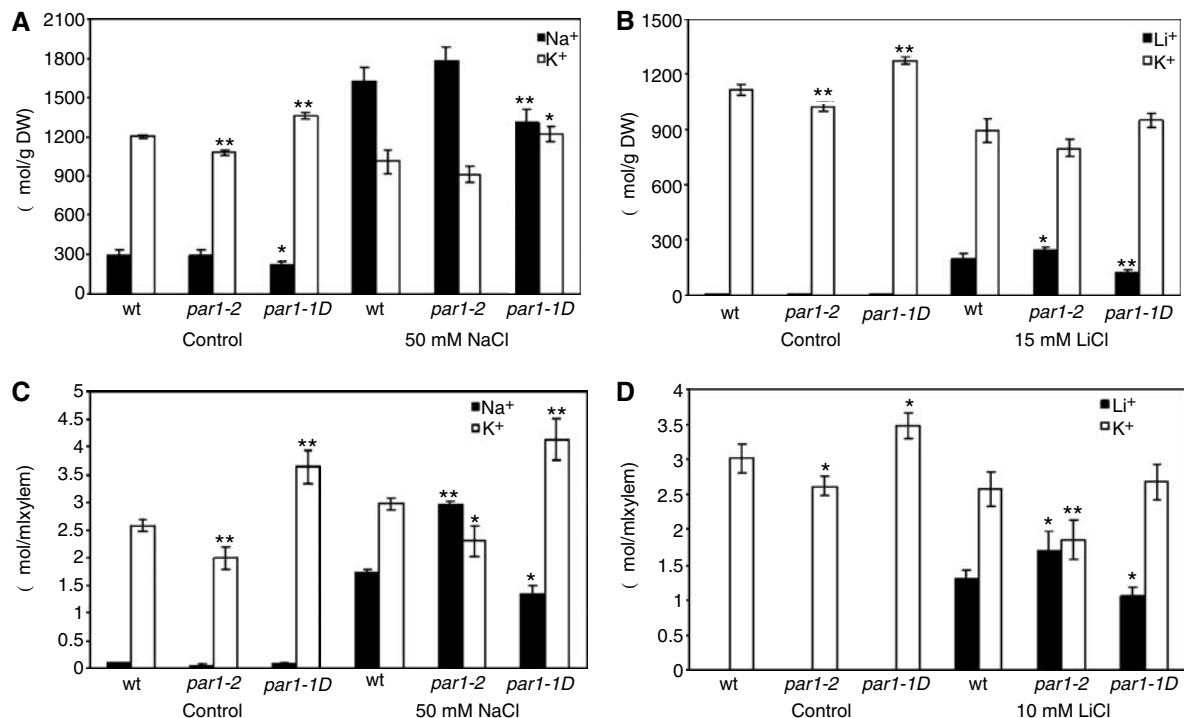


Figure 8 *QSO2* positively regulates shoot and xylem accumulation of K^+ while inhibiting accumulation of toxic cations. (A, B) Shoot cation content in wild type (wt) and mutants with gain (*par1-1D*) and loss (*par1-2*) of function of *QSO2*. Na^+/K^+ and Li^+/K^+ contents in the shoot were measured under irrigation with 50 mM NaCl or 15 mM LiCl during 5 or 3 days, respectively. Control plants were irrigated with normal solutions (control). (C, D) Cation content in xylem sap in wild type (wt) and mutants with gain (*par1-1D*) and loss (*par1-2/qso2*) of function of *QSO2*. Na^+/K^+ and Li^+/K^+ content in the xylem were measured under irrigation with 50 mM NaCl or 10 mM LiCl, during 3 or 2 days, respectively. The average of two experiments, with three replicates each is shown ($n = 6$). Errors bars represent \pm s.d. * $P < 0.05$ ** $P < 0.01$ by Student's test.

The localization of expression of more than 22 000 genes in the *Arabidopsis* root has been described by Birnbaum *et al* (2003). *QSO2* (*At1g15020*) behaves as a housekeeping gene, because it is expressed at similar levels (within a factor of 2) in different root tissues (stele, endodermis, cortex and epidermis) and zones (meristematic, elongation and mature zone with root hairs).

A *QSO2::GFP* fusion transiently expressed in *Nicotiana benthamiana* epidermal cells is present at the cell surface (Figure 11A), as expected from its N-terminal signal peptide. Plasmolysis of cells indicates that the fusion protein is associated with the cell wall and not the plasma membrane (Figure 11B). After expression in yeast about 30% of the recombinant *QSO2* protein is solubilized by digestion of the cell wall in the process of making yeast protoplasts (Supplementary Figure 4S B). This fraction corresponds to the extracellularly secreted form of the enzyme.

We have purified *QSO2* after expression in yeast (Supplementary Figure 4S B). Assay of sulfhydryl oxidase activity demonstrated specificity for dithiol compounds such as dithioerythryl and dithiothreitol, and insignificant activity (less than 1%) with monothiols such as mercaptoethanol and glutathione. The turnover number measured with simple dithiols (14 min^{-1}) is within the range of values detected for sulfhydryl oxidases (Levitan *et al*, 2004). The optimum pH for activity is 7.5, but it has considerable activity (40–60% of optimum) at the pH values reported to prevail at the apoplast (from 5.5 to 6.5; Gao *et al*, 2004).

Discussion

QSOs are animal and plant enzymes proposed to participate in oxidative folding of disulfide-containing secreted proteins. The induction of human *QSO1* when fibroblasts enter quiescence has suggested the name of the family and it has been proposed that the formation of the extracellular matrix may regulate growth (Thorpe *et al*, 2002). However, the lack of mutants in animal QSOs has prevented demonstration of the physiological roles of these enzymes. One report with neuroblastoma cells with gain and loss of function of a QSO has shown a role in the sensitization of these cells to apoptosis induced by interferon gamma (Wittke *et al*, 2003).

Our results with mutants of *Arabidopsis QSO2* indicate that this QSO regulates cation homeostasis. *Arabidopsis QSO2* is a positive regulator, directly or indirectly, of root stellar K^+ efflux systems involved in xylem loading, and this physiological role is supported by the induction of *QSO2* expression under conditions of K^+ starvation.

Although the K^+ efflux channel *SKOR* (Gaynard *et al*, 1998) and efflux systems for toxic cations (Li^+ , Na^+ , polyamines) could also be regulated, the simplest mechanism of *QSO2* action is the activation of a K^+ efflux system different from *SKOR* (because *QSO2* overexpression still occurs in the *skor-1* mutant). The activation of this system probably results from *QSO2*-mediated oxidation of sulfhydryl groups of the transporter exposed to the external side of the plasma

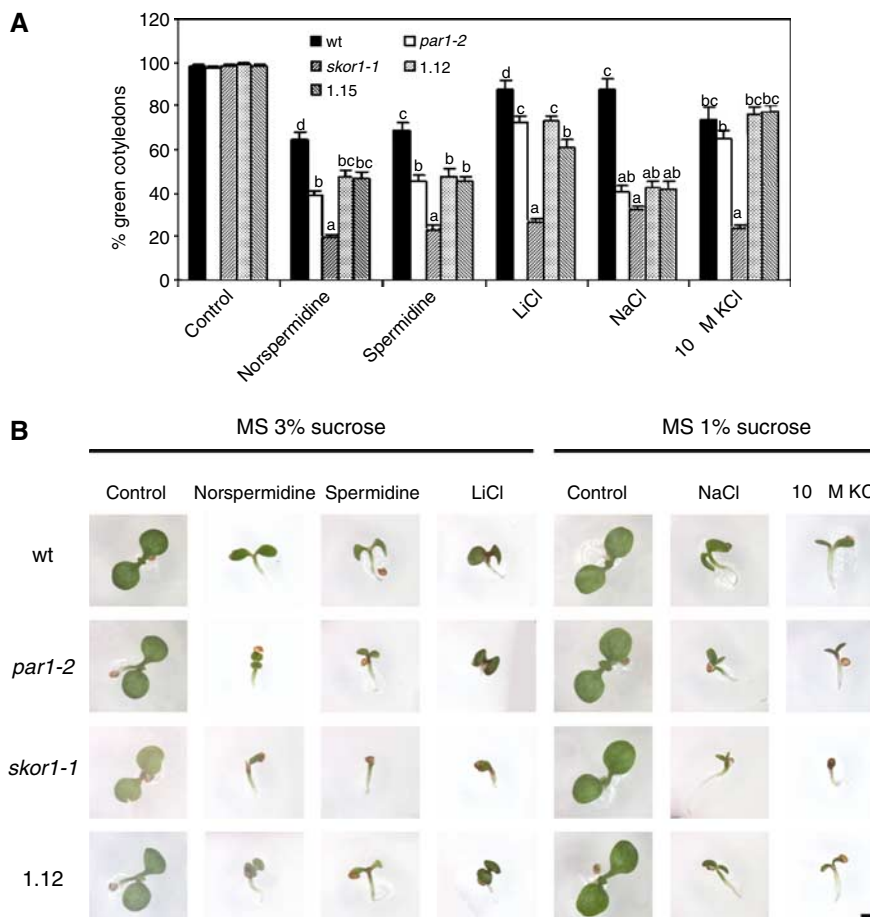


Figure 9 The *skor-1* mutant is sensitive to toxic cations and overexpression of *QSO2* still confers tolerance in this mutant. **(A)** Percentage of seeds that germinated and developed green cotyledons, 5 days after sowing in MS medium supplemented with the indicated toxic cation. Errors bars represent s.d. of three independent experiments, with three replicates each ($n=9$). Columns marked with different letters represent significantly different means according to statistical analysis ($P<0.05$, ANOVA, Tukey's test). **(B)** Representative seedlings from the experiment in panel A. Scale bar corresponds to 1 mm. The concentrations of toxic cations were as follows: none (control), norspermidine (2.4 mM), spermidine (6 mM), lithium (18 mM LiCl), sodium (120 mM NaCl) and a K^+ -free medium supplemented with 10 μ M KCl. Sucrose concentration was 3%, except in the case of NaCl and K^+ -free medium, where 1% was utilized. Controls were sown in media with the two sucrose concentrations. Wt, wild-type Columbia control; 1.12 and 1.15, transgenic lines derived from the *skor1-1* mutant overexpressing *QSO2*.

membrane. The opposite effects of *QSO2* mutations on the transport of K^+ and toxic cations can be explained by changes in membrane potential. The activation of K^+ efflux through this unknown system would hyperpolarize the symplast/xylem boundary, and this membrane potential, positive outside, would inhibit the efflux of toxic cations mediated by other channels. Unfortunately, the membrane potential of xylem parenchyma cells is not easily measured, because of the uncertainty of electrode location after penetrating the epidermal layer. The potential difference across the symplast/xylem boundary can be estimated from the trans-root electrical potential and the root epidermis potential (De Boer and Volkov, 2003), but such measurements have never been made in *Arabidopsis*. The measured membrane potentials at the symplast/xylem boundary are relatively low, about 80 mV, while the potential of epidermal and cortical cells are much higher, 150–170 mV (De Boer and Volkov, 2003). Therefore it is very likely that the membrane potential of xylem parenchyma cells is low because it is dominated by the 'leak' (channels or electrogenic cotransporters). Although SKOR does not seem to be target of *QSO2*, we have shown that

the *skor-1* mutant is sensitive to toxic cations, in addition to K^+ depletion. This result was predicted by our model and can be considered as a 'proof of concept' for the mechanism of *QSO2* suggested above.

We are investigating the nature of the *Arabidopsis* K^+ transport system regulated by *QSO2*, by testing possible epistatic relationships between loss-of-function mutations of different monovalent cation transporters and the overexpression of *QSO2*. The antiporter SOS1 and many channels of the CNGC family have sulfhydryl groups susceptible of oxidation from the external side of the membrane and are therefore the first candidates.

Although we detect *QSO2::GFP* at the cell wall during transient expression, the regulation of activity or stability of this unknown K^+ transporter by formation of disulfide bridges (or by possible disulfide isomerase activity of *QSO2*; see Houston *et al*, 2005) could also occur during its movement to the plasma membrane. The identification of the K^+ transporter regulated by *QSO2* and electrophysiological studies of the symplast-xylem interface in *QSO2* mutants may be crucial for the understanding of monovalent cation homeostasis in plants.

Finally, the observation that a plant QSO regulates ion homeostasis may be of general significance and deserves investigation in the case of animal QSOs (Thorpe *et al*, 2002).

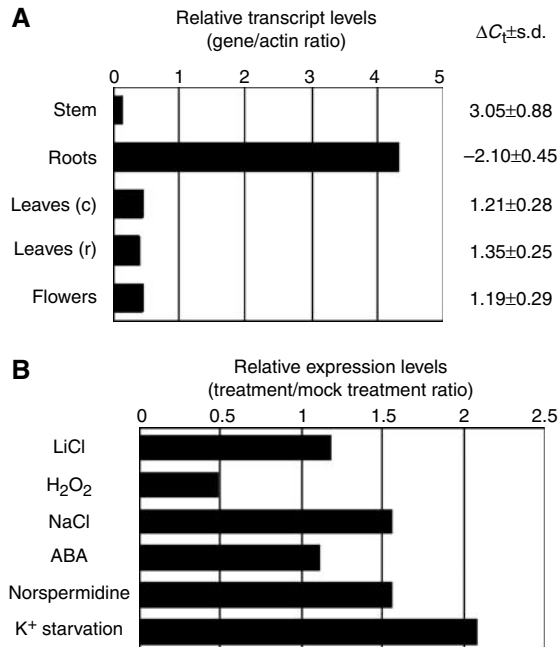


Figure 10 Expression analysis of *QSO2*. **(A)** Quantitative RT-PCR analysis of the *QSO2* gene expression pattern in different organs. Values are mean $\Delta C_t \pm s.d.$ (right) and relative transcript levels (left) were calculated as $2^{-\Delta C_t}$. **(B)** Induction of *QSO2* by K⁺ starvation. Seedlings of wild-type *Arabidopsis* (Columbia) were treated with either 2.8 mM norspermidine, 25 mM LiCl, 120 mM NaCl, 10 mM H₂O₂, 10 μ M ABA or K⁺-free medium for 16 h, as indicated. Quantitative RT-PCR analyses were conducted in three independent biological experiments. Expression values are relative to those of non-stressed control seedlings (taken as 1).

Materials and methods

Plant material and growth conditions

A. thaliana plants (ecotype Columbia) were grown under greenhouse conditions (16 h light/8 h dark, at $23 \pm 2^\circ\text{C}$ and $70 \pm 5\%$ relative humidity) in pots containing a 1:2 vermiculite:soil mixture. For *in vitro* culture, seeds were surface sterilized by soaking in 70% ethanol containing 0.1% Triton X-100 for 15 min, followed by commercial bleach (2.5%) containing 0.05% Triton X-100 for 10 min and rinsing three times with sterile water. Stratification of the seeds was conducted during 3 days at 4°C .

The agar medium contained Murashige and Skoog (1962) salts (MS) with 3 or 1% (w/v) sucrose, 10 mM morpholino ethane-sulfonic acid and 0.8% (w/v) agar, pH adjusted to 5.7 with Tris base. Different concentrations of norspermidine, spermine, NaCl and LiCl were prepared by adding appropriate amounts of reagents to the basal medium after autoclaving. For liquid culture, the MS medium was prepared without agar, and using 250-ml flasks or suspension-culture-6-well plates (Greiner). Plates were sealed and incubated in a controlled environment growth room at 23°C under long-day conditions (16 h light/8 h dark) at $80\text{--}100 \mu\text{mol/m}^2/\text{s}$. When appropriate, seedlings (5–7 days old) were transferred to vermiculite:soil mixture and grown to maturity. Plants were irrigated twice a week with nutrient solution during 3 weeks (Naranjo *et al*, 2003). For stress experiments, NaCl and LiCl were added to the nutrient solution.

Isolation of mutants and genetic analysis

T-DNA ‘activation tagging’ lines were constructed in the laboratory of D Weigel (Salk Institute, La Jolla, CA) using the pSKI15 vector (Weigel *et al*, 2000). A total of 16 398 lines (stock numbers N21995 and N21991) was provided by the European *Arabidopsis* Stock Centre (NASC). The distributed T-DNA pools consisted of T4 seeds. These were screened at high density (170 Petri plates of 9 cm diameter containing ~1000 seeds per plate) on MS medium (3% sucrose) with 3.2 mM norspermidine (Sigma). After 7 days, seedlings were considered norspermidine resistant only if they produce fully green expanded cotyledons. Two hundred and ten putative mutants were transferred to soil and grown to maturity. Aliquots of seeds (T5) from the putative mutants were screened again at low seed density (50–100 seeds per 9 cm diameter Petri plate) with 2.8 mM norspermidine. Of the 210 putative mutants, only eight exhibited normal phenotype on agar plates without

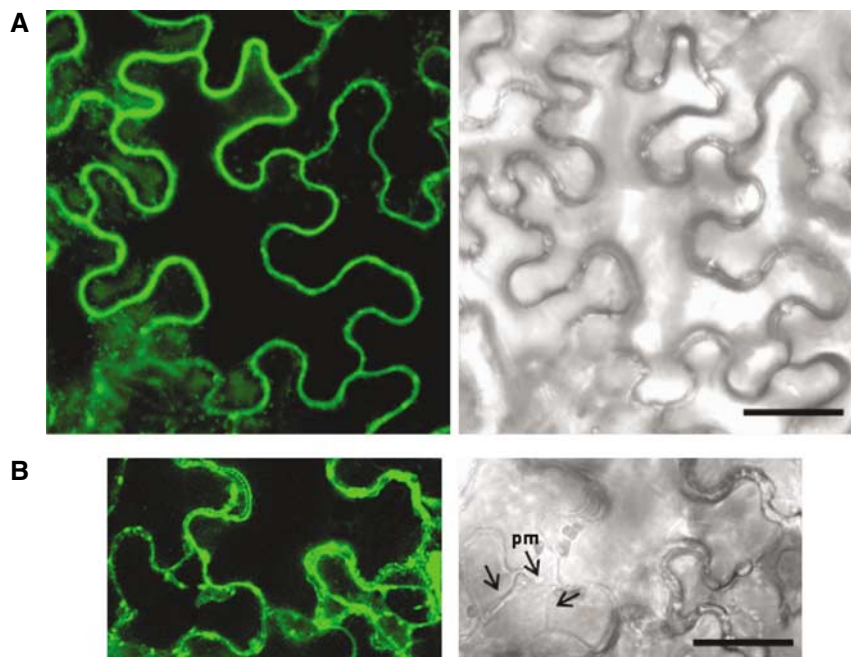


Figure 11 A *QSO2::GFP* fusion protein is secreted at the cell wall. **(A)** Laser scanning confocal microscopy image (LSCM) of *N. benthamiana* epidermal cells expressing a *QSO2::GFP* fusion, showing GFP fluorescence along the cell periphery. **(B)** LSCM of plasmolyzed epidermal cells expressing *QSO2::GFP* fusion, showing GFP fluorescence in the cell wall. Left, fluorescent image; right, bright-field image. Arrows indicate plasma membrane separated from cell wall. Scale bar corresponds to 20 μm .

norspermidine and fully green expanded cotyledons on plates with norspermidine. The best of them was called *par1-1D* for 'polyamine resistant' gene 1, allele 1, Dominant, following community standards for *Arabidopsis* genetics (Meinke and Koornneef, 1997).

The *par1-1D* mutant was crossed with the wild type (Columbia ecotype) by transferring pollen to the stigmas of emasculated flowers. F1 and F2 seeds were scored for germination in 3 mM norspermidine. From the segregating F2 generation, 60 resistant individuals were selected and DNA was extracted (see below) individually to check the correlation between T-DNA presence and resistance to norspermidine.

Plasmid rescue and sequencing

For plasmid rescue, 1 g of plant tissue was harvested from liquid culture and genomic DNA was extracted as described (Weigel and Glazebrook, 2002). DNA (2–3 µg) was digested with *EcoRI* (rescue left T-DNA border) and *BamHI* (rescue right T-DNA border) in a 40 µl reaction volume. After a phenol–chloroform extraction, samples were ligated overnight at 14°C in a total volume of 100 µl. Ligated DNA was precipitated with ethanol–sodium acetate and a one-third portion was transformed by electroporation into XL1-MRF *Escherichia coli* cells.

Plasmids rescued across the left T-DNA border were sequenced with the following primers: 5'LB (5'-AGA TTT CCG AAT TAG AAT AA-3') and T7 (5'-TAA TAC GAC TCA CTA TAG GG -3'); for those with right T-DNA border: 5' ECORI (5'-GAA ATG GAT AAA TAG CCT TGC-3').

Extraction and analysis of nucleic acids

Genomic DNA was extracted from seedlings and young leaves, as described (Weigel and Glazebrook, 2002). For Southern blotting, 3–4 µg DNA was digested with *BamHI*. CaMV 35S enhancer was used as a T-DNA-specific probe. RNA for Northern analysis was extracted from seedlings as described (Ecker and Davis, 1987). RNA (30 µg) was resolved by electrophoresis on formaldehyde–agarose gel. The products were transferred by capillary action onto nylon membranes (Amersham) and hybridized with random-priming ³²P-labeled probes (Feinberg and Vogelstein, 1983). Signals were quantified with a Fujifilm BAS-1500 phosphorimager. The *QSO2* and *TUB* (β-tubulin) probes were prepared by PCR amplification from genomic DNA of wild-type Columbia plants. Primers were *QSO2* (5'-TCC ATT TAA AAA GGC ACG TGA-3' and 5'-CAT CTC TCC TTT TCC CTT TCA-3') and *TUB* (5'-CCT GAT AAC TTC GTC TTT GG-3' and 5'-GTG AAC TCC ATC TCG TCC AT-3').

Overexpression of QSO2 in transgenic plants

QSO2 cDNA containing the complete open reading frame was obtained from Genomics Science Center, RIKEN, Japan. The cDNA was subcloned into the *NotI* site of pBS-SK+ (Stratagene, USA), yielding to pBS-*QSO2*. A fragment including the cDNA from *QSO2* was subcloned into pBIN121 (Jefferson *et al*, 1987) by replacing the *GUS* coding region between the *XbaI* and *SacI* sites. This results in a construct for overexpression of *QSO2* under the control of the CaMV 35S promoter. The plasmid was introduced into *Agrobacterium tumefaciens* strain C58C1 by electroporation. *A. thaliana* (Columbia ecotype) wild-type plants were transformed by flower infiltration (Bechtold *et al*, 1993). Transgenic plants with 35S:*QSO2* construction were screened on MS agar medium containing 50 mg/l kanamycin, and the expression of the transgene was further confirmed by Northern analysis, as described above.

Determination of K⁺, Na⁺ and norspermidine

Five-day-old seedlings were grown in liquid culture for 12 days and then transferred onto a second liquid culture supplemented with NaCl or norspermidine. For sampling at different times, seedlings were collected, rinsed twice briefly with cold 30 mM MgCl₂ and once with cold distilled water. For K⁺ and Na⁺ determination, samples were dried at 50°C for 3–4 days. After dry weight measurement, samples were extracted with 0.1 M nitric acid for 30 min at room temperature, cell debris were removed by filtration and monovalent cations were determined by atomic absorption spectrophotometry (Naranjo *et al*, 2003). In the case of norspermidine, samples were weighed and frozen in liquid nitrogen, and norspermidine was extracted and determined by HPLC, as described (Bellés *et al*, 1993).

Measurement of rubidium uptake

Potassium-free medium was prepared with the following ingredients: 2.5 mM Ca(NO₃)₂, 2 mM MgSO₄, 0.1 mM NaFeEDTA, 80 µM Ca(H₂PO₄)₂, 25 µM CaCl₂, 25 µM H₃BO₃, 2 µM ZnSO₄, 2 µM MnSO₄, 0.5 µM CuSO₄, 0.5 µM Na₂MoO₄, 0.01 µM CoCl₂, 1% sucrose and 2.5 mM MES. NH₄⁺ was added as NH₄H₂PO₄ at final concentration of 2 mM. The pH was adjusted to 5.7 with CaOH.

For measurement of potassium uptake, using Rb⁺ as a tracer, 5-day-old seedlings from MS agar plates were transferred to suspension-culture well containing 7 ml of liquid MS medium. After 10 days, the seedlings were collected, rinsed briefly with water and added to a 7 ml uptake solution containing potassium-free medium supplemented with 1 mM RbCl. The uptake was performed at 23°C under white fluorescent light. At the completion of uptake, the seedlings were rinsed twice with 7 ml of cold 30 mM MgCl₂ and once with water. Finally, the seedlings were treated similar to Na⁺ samples, and Rb⁺ content was determined by atomic absorption spectrophotometry.

Measurements of Li⁺, Na⁺ and K⁺ contents in shoots and xylem

Seeds were grown in MS medium for 5 days and transplanted to soil for 2–3 weeks. Plants were irrigated with NaCl or LiCl solutions as indicated, shoots collected, weighed and dried at 50°C for 3–4 days. Ions were extracted with 0.1 M nitric acid for 30 min at room temperature, cell debris were removed by filtration, ion content was determined by atomic absorption spectrophotometry.

Xylem sap was obtained from pressurized shoot of mature plants grown in MS medium for 5 days, and transplanted to soil for 3–4 weeks. Plants were then subjected to 50 mM NaCl or 10 mM LiCl by a single irrigation with nutritive solution for 3 or 2 days, respectively. The inflorescence stem was cut with a very sharp razor blade and placed in a pressure chamber, with the rest of the plant emerging out. Pneumatic pressure (10–30 bars) was applied. The first two drops emerging were discarded to prevent contamination of the xylem sap with contents from damaged cells or phloem sap. Ion content was determined by atomic absorption spectrophotometry.

Selection of At1g15020 and At3g02850 knock-out Arabidopsis lines

Three *At1g15020* (*QSO2*) *Arabidopsis* independent knockout mutant lines (SALK_066130, SALK_025237 y SALK_072829) and one *At3g02850* (*SKOR*) *Arabidopsis* knock-out mutant line (SALK_132944) were obtained from the Salk Institute Genomic Analysis Laboratory (<<http://signal.salk.edu/cgi-bin/tdnaexpress>> <http://signal.salk.edu/cgi-bin/tdnaexpress>). The seeds were sown and grown on MS medium with 50 µg/ml of kanamycin for 6 days, resistant seedlings were transplanted to soil for 3 weeks, after which genomic DNA was extracted from the individual plants. Homozygous knockout mutant plants were selected by PCR using three primers. In all the cases, a T-DNA-specific primer pROK1bb1 5'-CGCTGGACCGCTTGCTGCAAC T-3' was used. For each line, two specific primers were used: SALK_066130 (*par1-2LP* 5'-TCAAGGCT CAGCAGACCCAAC-3' and *par1-2RP* 5'-TGCACATGATCGATACTTT TTGGTG-3') SALK_025237 (*par1-3LP* 5'-TTTGTCTCAACGGAAG GAG-3' and *par1-3RP* 5'-TTTAGGAGGAGCCAAAAGAG-3'), SALK_072829 (*par1-4LP* 5'-CTTGTGTTGGTCTGCTGAG-3' and *par1-4RP* 5'-TGCCTATCTGCTTGTGATCC-3') and SALK_132944 (*skorLP* 5'-TCAAGAATCTTTAGATCCGGACAGA-3' and *skorRP* 5'-ACAGGCGC CAATTTT AGGCAT-3').

Quantitative real-time RT-PCR analysis

Plant organs were harvested from four-week-old flowering plants grown in greenhouse. Organs were sampled as follows: opened flowers, all cauline leaves, a 1-cm section of the inflorescence stem and rosette leaves. Roots were sampled from 2-week-old seedlings grown vertically on MS. For the measurement of induced levels of *QSO2*, plants were grown on MS medium supplemented with 1% sucrose. After 5 days, 5–7 seedlings were transferred to liquid culture for 12 days, and then were either mock or treated with 2.8 mM spermidine, 25 mM LiCl, 120 mM NaCl, 10 mM H₂O₂, 10 µM ABA and K⁺-free medium for 16 h. All plant tissues were frozen in liquid nitrogen. Total RNA was isolated as described (Verwoerd *et al*, 1989). For reverse transcription, 2 µg of RNA previously treated with *DNase I* was incubated with Buffer RT ×1 (Fermentas), 1 mM dNTPs, 0.2 µg oligo(dT)₁₅ primer and 10 U

MuLV reverse transcriptase (Fermentas), to finally obtain a 40 µl cDNA solution. The sequence of the primers used for PCR amplifications were as follows: for *QSO2* (At1g15020) 5'-AGG ACC CAG CAG AAG AAC CGG-3' and 5'-CAT CTC TCC TTT TCC CTT TCA-3', and for *actin 8* (At1g49240) 5'-AGT GGT CGT ACA ACC GGT ATT GT-3' and 5'-GAG GAT AGC ATG TGG AAG TGA GAA-3'. The real-time PCR was performed in a total volume of 20 µl, including 0.5 µl of cDNA and gene-specific primers (500 nM final concentration). Amplification of PCR products was monitored via intercalation of Eva-green (Biotium), using an ABI PRISM 7000 sequence detection system (Perkin-Elmer Applied Biosystems). Amplification was performed for 40 cycles and the relative quantification of gene expression data in different treatments was carried using the comparative C_t method (Livak and Schmittgen, 2001). Expression levels were normalized using the C_t values obtained for the *actin 8* gene. The relative transcript level for *QSO2* was calculated by normalizing to *actin 8* as follows: $RTL = 2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_t(QSO2) - \Delta C_t(actin 8)$. The presence of a single PCR product was confirmed by dissociation analysis and agarose gel electrophoresis. Three independent replicate experiments were conducted.

Construction of *QSO2:GFP* fusion for expression in plants

The open reading frame of *QSO2* gene used in this fusion was obtained by PCR from pBS-*QSO2* construct (see above), using primers *QSO2*XbaI 5'-TGC TCT AGA GTA ATA AAC ACC AGA GAC CTT-3' and *QSO2*BamHI 5'-GCG GGA TCC TCT CTC CTT TTC CAC TTC ACT-3'. The amplified DNA was cloned into the *XbaI* and *BamHI* sites of a pBS-*GFP* vector (Chiu *et al*, 1996). The new construction pBS-*QSO2::GFP* harbored a translational fusion between the *QSO2* and *GFP* coding sequences. The *QSO2::GFP* coding sequence and the *NOS* terminator was subcloned into *XbaI-EcoRI* doubly digested pB1121. In this new construct (pB1121-*QSO2::GFP*), the expression of *QSO2:GFP* fusion was driven by 35S promoter. It was used to transform *A. tumefaciens* (strain C58C1).

Agrobacterium-mediated transient gene expression in *N. benthamiana*

Agrobacterium-based transient transformation was made as described in Wieland *et al* (2006) with some modifications. Briefly, *A. tumefaciens* culture was grown until saturation in LB medium (10 ml) at 28°C for 16 h. The culture was centrifuged and resuspended in the same volume of 10 mM MgCl₂, 10 mM MES pH 5.6 and 200 µM acetosyringone. The culture was kept at room temperature for 3 h, without shaking. A syringe (without needle) was used to inject the *Agrobacterium* culture into the leaf. Plants were grown for 3–4 days in greenhouse before GFP localization was performed. To plasmolyze cells, the tissue samples were soaked in 1 M mannitol on glass slides for 30 min at room temperature. Fluorescence of GFP was observed by a Leica TCS-SL confocal microscope and laser scanning confocal imaging system. A 488 nm excitation wavelength and a 510 nm emission wavelength were used.

Construction of His-tagged *QSO2* for expression in yeast

The coding region of the *QSO2* cDNA was amplified by PCR from the pBS-*QSO2* construct (see above), using primers *QSO2*-MssI5 5'-TTA TTA GTT TAA ACG TAA TAA ACA CCA GAG AC-3' and *QSO2*-MssI3 5'-TAT ATA GTT TAA ACT TTC TCT CCT TTT CCC-3'. The PCR product was subcloned into the *PmeI* site of pCM262 vector, yielding the pCM262-*QSO2* construct. pCM262 vector is derived from pCM190 (Gari *et al*, 1997), and it contains tetracycline-regulable promoter, three copies of the HA epitope and 6 × histidine tail fused to the C-terminus of the target gene. Once the pCM262-*QSO2* construct was verified by sequencing, it

was used to transform *Saccharomyces cerevisiae* strain W303 generating SA76 strain.

Purification of His-tagged *QSO2* from yeast

Yeast SA76 strain was grown in minimal medium (2% sucrose, 0.7% yeast nitrogen base and 50 mM succinic acid pH 5.5) to an absorbance at 660 nm of 0.4–0.5, and cells were harvested by centrifugation, suspended in homogenization buffer (50 mM Tris-HCl, pH 7.6, 0.1 M KCl, 10% sucrose and protease inhibitor cocktail) and lysed by vortexing with glass beads (0.5 mm). The lysate was centrifuged first at 2000 r.p.m. for 5 min at 4°C and further centrifuged at 13 000 r.p.m. (16 000 g) for 30 min at 4°C. The final supernatant was used for purification.

Ni²⁺ affinity resin (His-Bind Resin, Novagen) was packed onto a column and washed with 3 ml H₂O and 4 ml charge buffer (50 mM NiSO₄). After equilibration with 3 ml binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) the centrifuged yeast extract was loaded onto the column and washed with 8 ml of binding buffer and 4 ml washing buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). His-tagged *QSO2* was eluted with 10 ml of elution buffer (0.3 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The peak fractions (0.5 ml) were dialyzed against 50 mM Tris-HCl, pH 8 and used immediately in a assay of sulphydryl oxidase activity.

SDS-PAGE and protein immunoblotting

Protein content was measured with dye binding method (Bradford, 1976). BSA was employed as the protein standard. A 5 µg weight of total protein was resuspended with Laemmli (1970) buffer and loaded onto 8% (w/v) linear acrylamide minigels. After electrophoresis, the gels were prepared for immunoblotting. SDS-PAGE-separated proteins were electrophoretically transferred onto nitrocellulose membranes (Millipore). Protein content on the membrane was detected by Ponceau S staining. Following transfer, membrane was blocked with Tris-buffered saline (100 mM Tris, 150 mM NaCl) containing 0.1% (v/v) Tween 20 and 2% non-fat milk powder, for 30 min on a rocker at room temperature. Blocked membrane was incubated overnight at 4°C on a rocker with anti-HA (1/10 000), the membrane was washed five times with Tris-buffered saline (100 mM Tris, 150 mM NaCl) containing 0.1% (v/v) Tween 20 for 10 min each on a rocker at room temperature, followed by the addition of a 1:5000 dilution of anti-mouse secondary antibody. Anti-HA blots were visualized using the ECL detection system (Amersham).

Enzymatic assay of *QSO2* activity

Purified *QSO2* protein (corresponding to 20 pmol/reaction) was incubated in 0.1 ml of phosphate-buffered saline buffer (75 mM potassium phosphate buffer pH 7.5 and 3 mM EDTA), together with reduced substrates corresponding to 50 nmol of reduced thiol groups. All the reactions were carried out for 5 min at 25°C. *QSO2* activity was measured by determination of thiol content, as described (Levitan *et al*, 2004). Results are expressed as turnover numbers (per min).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

This work was supported by grants BFU2005-06388-C04-01 of the Spanish MEC (Madrid) and CPE03-006-C6-4 of the Spanish INIA (Madrid).

References

- Adams DS, Robinson KR, Fukumoto T, Yuan TS, Albertson RC, Yelick P, Kuo L, McSweeney M, Levin M (2006) Early, H⁺-V-ATPase-dependent proton flux is necessary for consistent left-right patterning of non-mammalian vertebrates. *Development* **133**: 1657–1671
- Bechtold N, Ellis J, Pelletier G (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis* plants. *CR Acad Sci Paris/Life Sci* **316**: 1194–1199
- Bellés JM, Pérez-Amador MA, Carbonell J, Conejero V (1993) Correlation between ornithine decarboxylase and putrescine in tomato plants infected by citrus exocortis viroid or treated with ethephon. *Plant Physiol* **102**: 933–937
- Birnbaum K, Shasha DE, Wang JY, Jung JW, Lambert GM, Galbraith DW, Benfey PN (2003) A gene expression map of the *Arabidopsis* root. *Science* **302**: 1956–1960

- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254
- Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. *Curr Biol* **6**: 325–330
- Coppock DL, Cina-Poppe D, Gilleran S (1998) The quiescin Q6 gene (QSCN6) is a fusion of two ancient gene families: thioredoxin and ERV1. *Genomics* **54**: 460–468
- De Boer AH, Volkov V (2003) Logistics of water and salt transport through the plant: structure and functioning of the xylem. *Plant Cell Environ* **26**: 87–101
- Ecker JR, Davis RW (1987) Plant defense genes are regulated by ethylene. *Proc Natl Acad Sci USA* **84**: 5202–5206
- Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**: 6–13
- Forment J, Mulet JM, Vicente O, Serrano R (2002) The yeast SR protein kinase Skyp1p modulates salt tolerance, membrane potential and the Trk1, 2 potassium transporter. *Biochim Biophys Acta* **1565**: 36–40
- Frías I, Caldeira MT, Pérez-Castíñeira JR, Navarro-Aviñó JP, Culiñeiz-Maciá FA, Kuppinger O, Stransky H, Pagés M, Hager A, Serrano R (1996) A major isoform of the maize plasma membrane H⁺-ATPase: characterization and induction by auxin in coleoptiles. *Plant Cell* **8**: 1533–1544
- Gao D, Knight MR, Trewavas AJ, Sattelmacher B, Plieth C (2004) Self-reporting *Arabidopsis* expressing pH and [Ca²⁺] indicators unveil ion dynamics in the cytoplasm and in the apoplast under abiotic stress. *Plant Physiol* **134**: 898–908
- Gari E, Piedrafitá L, Aldea M, Herrero E (1997) A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in *Saccharomyces cerevisiae*. *Yeast* **13**: 837–848
- Gaymard F, Pilot G, Lacombe B, Bouchez D, Bruneau D, Boucherez J, Michaux-Ferrière N, Thibaud J-B, Sentennac H (1998) Identification and disruption of a plant shaker-like outward channel involved in K⁺ release into the xylem sap. *Cell* **94**: 647–655
- Gierth M, Mäser P, Schroeder JI (2005) The potassium transporter *ATHAK5* functions in K⁺ deprivation-induced high-affinity K⁺ uptake and *AKT1* K⁺ channel contribution to K⁺ uptake in *Arabidopsis* roots. *Plant Physiol* **137**: 1105–1114
- González-Guzmán M, Apostolova N, Bellés JM, Barrero JM, Piqueras P, Ponce MR, Micol JL, Serrano R, Rodríguez PL (2002) The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde. *Plant Cell* **14**: 1833–1846
- Hager A, Debus G, Edel H-G, Stransky H, Serrano R (1991) Auxin induces exocytosis and rapid synthesis of a high-turnover pool of plasma-membrane H⁺-ATPase. *Planta* **185**: 527–537
- Hamana K, Matsuzaki S, Hosaka K, Yamashita S (1989) Interconversion of polyamines in wild-type strains and mutants of yeast and the effects of polyamines on their growth. *FEMS Microbiol Lett* **61**: 231–236
- Harold FM (1986) *The Vital Force: a Study of Bioenergetics*. New York: WH Freeman
- Hirsch RE, Lewis BD, Spalding EP, Sussman MR (1998) A role for the AKT1 potassium channel in plant nutrition. *Science* **280**: 918–921
- Hoffman JF (1964) *The Cellular Functions of Membrane Transport*. Englewood Cliffs, NJ: Prentice-Hall
- Houston NL, Fan C, Xiang Q-Y, Schulze J-M, Jung R, Boston RS (2005) Phylogenetic analysis identifies 10 classes of the protein disulfide isomerase family in plants, including single domain protein-disulfide isomerase-related proteins. *Plant Physiol* **137**: 762–778
- Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* **6**: 3901–3907
- Kinoshita T, Shimazaki K (1999) Blue light activates the plasma membrane H⁺-ATPase by phosphorylation of the C-terminus in stomatal guard cells. *EMBO J* **18**: 5548–5558
- Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, Jones JD, Schroeder JI (2003) NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signalling in *Arabidopsis*. *EMBO J* **22**: 2623–2633
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685
- Levitan A, Danon A, Lisowsky T (2004) Unique features of plant mitochondrial sulphhydryl oxidase. *J Biol Chem* **279**: 20002–20008
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**: 402–408
- Maathuis FJM, Filatov V, Herzyk P, Krijger GC, Axelsen KB, Chen S, Green BJ, Li Y, Madagan KL, Sanchez-Fernandez R, Forde BG, Palgren MG, Rea PA, Williams LE, Sanders D, Amtmann A (2003) Transcriptome analysis of root transporters reveals participation of multiple gene families in the response to cation stress. *Plant J* **35**: 675–692
- Meinke D, Koornneef M (1997) Community standards for *Arabidopsis* genetics. *Plant J* **12**: 247–253
- Morsomme P, Boutry M (2000) The plant plasma membrane H⁺-ATPase: structure, function and regulation. *Biochim Biophys Acta* **1465**: 1–16
- Mulet JM, Leube MP, Kron SJ, Ríos G, Fink G, Serrano R (1999) A novel mechanism of ion homeostasis and salt tolerance in yeast: the Hal4 and Hal5 protein kinases modulate the Trk1-Trk2 potassium transporter. *Mol Cell Biol* **19**: 3328–3337
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* **15**: 473–497
- Naranjo MA, Romero C, Bellés JM, Montesinos C, Vicente O, Serrano R (2003) Lithium treatment induces a hypersensitive-like response in tobacco. *Planta* **217**: 417–424
- Philippart K, Fuchs I, Lüthen H, Hoth S, Bauer CS, Haga K, Thiel G, Ljung K, Sandberg G, Böttger M, Becker D, Hedrich R (1999) Auxin-induced K⁺-channel expression represents an essential step in coleoptile growth and gravitropism. *Proc Natl Acad Sci USA* **96**: 12186–12191
- Pollard TD, Earnshaw WC (2002) *Cell Biology*. Philadelphia: Saunders, pp 89–174
- Qiu QS, Guo Y, Dietrich MA, Schumaker KS, Zhu JK (2002) Regulation of SOS1, a plasma membrane Na⁺/H⁺ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. *Proc Natl Acad Sci USA* **99**: 8436–8441
- Quintero FJ, Ohta M, Shi H, Zhu JK, Pardo JM (2002) Reconstitution in yeast of the *Arabidopsis* SOS signaling pathway for Na⁺ homeostasis. *Proc Natl Acad Sci USA* **99**: 9061–9066
- Rober-Kleber N, Albrechtova JTP, Fleig S, Huck N, Michalke W, Wagner E, Speth V, Neuhaus G, Fischer-Iglesias C (2003) Plasma membrane H⁺-ATPase is involved in auxin-mediated cell elongation during wheat embryo development. *Plant Physiol* **131**: 1302–1312
- Rodríguez-Navarro A, Rubio F (2006) High-affinity potassium and sodium transport systems in plants. *J Exp Bot* **57**: 1149–1160
- Sanders D, Bethke P (2000) Membrane Transport. In *Biochemistry and Molecular Biology of Plants*, Buchanan BB, Gruissem W, Jones RL (eds), Chapter 3, pp 110–158. Rockville, MD: American Society of Plant Physiology
- Serrano R (1989) Structure and function of plasma membrane ATPase. *Annu Rev Plant Physiol Plant Mol Biol* **40**: 61–94
- Serrano R, Mulet JM, Ríos G, Marquez JA, de Larrinoa IF, Leube MP, Mendizabal I, Pascual-Ahuir A, Proft M, Ros R, Montesinos C (1999) A glimpse of the mechanisms of ion homeostasis during salt stress. *J Exp Bot* **50**: 1023–1036
- Serrano R, Rodríguez-Navarro A (2001) Ion homeostasis during salt stress in plants. *Curr Opin Cell Biol* **13**: 399–404
- Shin R, Schachtman DP (2004) Hydrogen peroxide mediates plant root cell response to nutrient deprivation. *Proc Natl Acad Sci USA* **101**: 8827–8832
- Sussman MR, Harper JF (1989) Molecular biology of the plasma membrane of higher plants. *Plant Cell* **1**: 953–960
- Thorpe C, Hooper KL, Raj S, Glynn NM, Burnside J, Turi GK, Coppock DL (2002) Sulphydryl oxidases: emerging catalysts of protein disulfide bond formation in eukaryotes. *Arch Biochem Biophys* **405**: 1–12
- Verwoerd TC, Dekker BM, Hoekema A (1989) A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Res* **17**: 2362
- Very AA, Sentenac H (2002) Cation channels in the *Arabidopsis* plasma membrane. *Trends Plant Sci* **7**: 168–175

- Weigel D, Ahn JH, Blazquez MA, Borevitz JO, Christensen SK, Fankhause C, Ferrandiz C, Kardailsky I, Malancharuvil EJ, Neff MM, Nguyen JT, Sato S, Wang ZY, Xia Y, Dixon RA, Harrison MJ, Lamb CJ, Yanofsky MF, Chory J (2000) Activation tagging in *Arabidopsis*. *Plant Physiol* **122**: 1003–1013
- Weigel D, Glazebrook J (2002) *Arabidopsis. A Laboratory Manual*, p 165 Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press
- Wieland WH, Lammers A, Schots A, Orzaez DV (2006) Plant expression of chicken secretory antibodies derived from combinatorial libraries. *J Biotechnol* **122**: 382–391
- Wittke I, Wiedemeyer R, Pillmann A, Savelyeva L, Westermann F, Schwab M (2003) Neuroblastoma-derived sulphhydryl oxidase, a new member of the sulphhydryl oxidase/quiescin6 family, regulates sensitization to interferon gamma-induced cell death in human neuroblastoma cells. *Cancer Res* **63**: 7742–7752
- Würtele M, Jelich-Ottmann C, Wittinghofer A, Oecking C (2003) Structural view of a fungal toxin acting on a 14-3-3 regulatory complex. *EMBO J* **22**: 987–994
- Xu J, Li H-D, Chen L-Q, Wang Y, Liu L-L, He L, Wu W-H (2006) A protein kinase, interacting with two calcineurin B-like proteins, regulates K⁺ transporter AKT1 in *Arabidopsis*. *Cell* **125**: 1347–1360
- Yenush L, Merchan S, Holmes J, Serrano R (2005) pH-responsive, posttranslational regulation of the Trk1 potassium transporter by the type1-related Ppz1 phosphatase. *Mol Cell Biol* **25**: 8683–8692
- Yenush L, Mulet JM, Ariño J, Serrano R (2002) The Ppz protein phosphatases are key regulators of K⁺ and pH homeostasis: implications for salt tolerance, cell wall integrity and cell cycle progression. *EMBO J* **21**: 920–929