Shoot chloride exclusion and salt tolerance in grapevine is associated with differential ion transporter expression in roots

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Abstract

Background: Salt tolerance in grapevine is associated with chloride (Cl\(^-\)) exclusion from shoots; the rate-limiting step being the passage of Cl\(^-\) between the root symplast and xylem apoplast. Despite an understanding of the physiological mechanism of Cl\(^-\) exclusion in grapevine, the molecular identity of membrane proteins that control this process have remained elusive. To elucidate candidate genes likely to control Cl\(^-\) exclusion, we compared the root transcriptomes of three Vitis spp. with contrasting shoot Cl\(^-\) exclusion capacities using a custom microarray.

Results: When challenged with 50 mM Cl\(^-\), transcriptional changes of genotypes 140 Ruggeri (shoot Cl\(^-\) excluding rootstock), K51-40 (shoot Cl\(^-\) including rootstock) and Cabernet Sauvignon (intermediate shoot Cl\(^-\) excluder) differed. The magnitude of salt-induced transcriptional changes in roots correlated with the amount of Cl\(^-\) accumulated in shoots. Abiotic-stress responsive transcripts (e.g. heat shock proteins) were induced in 140 Ruggeri, respiratory transcripts were repressed in Cabernet Sauvignon, and the expression of hypersensitive response and ROS scavenging transcripts was altered in K51-40. Despite these differences, no obvious Cl\(^-\) transporters were identified. However, under control conditions where differences in shoot Cl\(^-\) exclusion between rootstocks were still significant, genes encoding putative ion channels SLAH3, ALMT1 and putative kinases SnRK2.6 and CPKs were differentially expressed between rootstocks, as were members of the NRT1 (NAXT1 and NRT1.4), and CLC families.

Conclusions: These results suggest that transcriptional events contributing to the Cl\(^-\) exclusion mechanism in grapevine are not stress-inducible, but constitutively different between contrasting varieties. We have identified individual genes from large families known to have members with roles in anion transport in other plants, as likely candidates for controlling anion homeostasis and Cl\(^-\) exclusion in Vitis species. We propose these genes as priority candidates for functional characterisation to determine their role in chloride transport in grapevine and other plants.

Keywords: ABA signalling, ACA, CAX, mRNA, Salt overly sensitive (SOS), Woody perennial

Background

Grapevine (Vitis vinifera L.), used for wine, table grape and dried grape production, is an economically important crop plant that is moderately sensitive to salinity [1]. Grapevine salt stress symptoms include reduced stomatal conductance, reduced photosynthesis [2,3] and leaf burn [4], which are generally associated with increases in shoot chloride (Cl\(^-\)) rather than sodium (Na\(^+\)) concentrations [3]. Reduced vigour [5] and reduced yield [6] are further effects of salt stress, with a strong positive correlation between the two [5]. Certain non-vinifera Vitis spp. rootstocks are used commercially to constrain shoot Cl\(^-\) accumulation and confer improved salt tolerance to grafted V. vinifera scions [7,8]. Despite a detailed understanding of the physiology of shoot Cl\(^-\) accumulation in grapevine and other plants, the genes responsible for this process across the plant kingdom are not known [9]. This is in contrast to the control of long-distance Na\(^+\) transport in plants where numerous reports have targeted known genes in order to improve the salt tolerance of plants, particularly cereals e.g. [10-13]. Due to extensive natural variation in the shoot Cl\(^-\) exclusion capacity of Vitis spp.
[14,15] grapevine represents an ideal model to identify candidate genes involved in controlling shoot Cl\(^-\) exclusion.

Solute movement between the roots and shoot is essential for plant growth. Physiological studies using radioisotopes and fluorescent dyes in grapevine have indicated that the transfer of solutes to the xylem apoplast involves a symplastic step, and that rootstocks confer Cl\(^-\) exclusion to a grafted scion by reducing net xylem loading of Cl\(^-\) [15,16]. Patch clamp studies of xylem parenchyma protoplasts identified the passive quickly activating anion conductance (X-QUAC) as capable of catalysing the majority of Cl\(^-\) flux to the xylem of barley roots [17]. Cl\(^-\) entry to the root xylem is down-regulated by abscisic acid (ABA), as demonstrated by \(^{36}\)Cl\(^-\) fluxes in excised roots and whole seedlings of barley [18], and reduces X-QUAC of maize xylem parenchyma cells [19]. Given that ABA rises in concentration in plant roots exposed to salt stress [20], anion transporters expressed in cells that surround the root xylem, especially those that change activity when plants are salt treated, are likely to be good targets to explore for improving our understanding of how shoot Cl\(^-\) exclusion is conferred.

There have been a limited number of studies that have provided insights to the genetic elements that control long-distance transport of Cl\(^-\). Like grapevine, Citrus spp. are moderately salt-sensitive woody perennial crops frequently grown on salt-excluding rootstocks. Brumos et al. [21] compared the partial leaf transcriptomes of Citrus rootstocks Cleopatra mandarin (a good shoot Cl\(^-\) excluder) and Carrizo citrange (a poor shoot Cl\(^-\) excluder) after exposure to NaCl and KCl stress using a cDNA microarray covering 6,875 putative unigenes. They concluded that a nitrate (NO\(_3^-\)) transporter with homology to GmNRT1-2 from soybean was differentially expressed between rootstocks and therefore was deemed a candidate gene for influencing Cl\(^-\) movement. Using the same germplasm, Brumos et al. [22] used quantitative PCR to measure root expression of three candidate genes for the control of long-distance Cl\(^-\) transport derived from the literature. Candidates included a homolog of a cation chloride co-transporter (CcCCC1), CcICln1 (a putative regulator of chloride channel conductance) and CcSLAH1, a homolog of the plant guard cell slow anion channels (SLAC) [22]. Of these genes SLAH1 was more highly expressed in the chloride accumulating rootstock under 90 mM NaCl stress. In guard cells, SLAC chloride channels mediate ABA induced passive Cl\(^-\) efflux causing stomatal closure [23,24]. SLAC homologs (SLAH) in plant roots are therefore particularly interesting candidates for xylem loading of Cl\(^-\), but their role in roots remains uncharacterised. CCC was proposed to regulate retrieval of Na\(^+\), K\(^+\) and Cl\(^-\) from the Arabidopsis root xylem but was not regulated transcriptionally by salt [22,25], Furthermore, questions remain as to how CCC can act directly in xylem loading on the plasma membrane due to unfavourable electrochemical gradients [9]. ICln1 homologs from rat and Xenopus laevis elicit Cl\(^-\) currents in voltage clamp experiments [26]. In Citrus, ICln1 exhibited strong repression in the Cl\(^-\) excluder after application of 4.5 mM Cl\(^-\) [22]. However, ICln proteins from plants remain uncharacterised. Whilst these genes are good candidates for regulating Cl\(^-\) transport in Citrus, analyses of entire root transcriptomes is likely to provide a more complete list of factors that mediate long-distance transport of Cl\(^-\).

Gene expression studies of V. vinifera have been greatly aided by the draft genome sequence of Pinot Noir inbred line PN40024 [27,28]. These studies have concentrated on berry development [29,30], leaf responses to heat stress [31] and to UV radiation [32]. The most comprehensive grapevine expression study to date compared the transcriptome of 54 samples representing different vegetative and reproductive organs at various developmental stages [33]. Although abiotic stress was not analysed in this study, grapevine roots were found to express more organ-specific transcripts than leaves [33]. This is consistent with findings from Tillett et al. [34] who compared large-scale EST libraries from roots and shoots of Cabernet Sauvignon and identified 135 root enriched transcripts. These findings indicate that shoot expression analyses of grapevine, while useful, might not give a complete picture of root gene expression patterns, and therefore studies into root responses to abiotic stresses are required. Two microarray studies have examined the effect of salinity stress on transcript levels of Cabernet Sauvignon shoot tips [35,36]. Increased levels of a transcript encoding a putative NRT were observed, while decreased expression of a chloride channel (CLC) with sequence similarity to Arabidopsis AtCLC-d was detected by two probe sets, but this was not statistically significant [36].

We performed a comparative microarray of mRNAs derived from roots of salt stressed and control Cabernet Sauvignon, 140 Ruggeri and K51-40 rooted leaves as an unbiased method to identify candidates for long-distance transport of Cl\(^-\). We aimed to test the hypothesis that the differences in Cl\(^-\) exclusion between rootstocks 140 Ruggeri and K51-40 could be due to expression differences in genes that encode membrane transport proteins which facilitate root-to-shoot Cl\(^-\) translocation. The identification of genes that prevent excessive shoot Cl\(^-\) accumulation in grapevine will facilitate continued rootstock development by providing genetic markers for rootstock breeding programs. Furthermore, this study will aid a greater understanding of plant Cl\(^-\) homeostasis by using grapevine as a model species to elucidate genes that underpin the Cl\(^-\) exclusion trait in plants in general.
Methods
Preparation of rooted-leaves
Grapevine, being a woody perennial crop, is challenging to use in controlled conditions experiments, especially where large amounts of material and multiple replicates are required. We therefore used the method of Schachtman and Thomas [37] where leaves are excised from a parent plant and grown as rooted-leaves. This is consistent with previous studies of Cl− accumulation in vines, where it was demonstrated that root and leaf phenotypes acquired with this system are similar to field observations [15,16]. Rooted leaves were established from pot-grown grapevines of K51-40 (Vitis champinii × Vitis riparia), 140 Ruggeri (Vitis berlandieri × Vitis rupestris) and Cabernet Sauvignon (Vitis vinifera) established from cuttings and maintained in a glasshouse as described previously [15]. After approximately 3 weeks, rooted-leaves were transferred to aerated hydroponic tanks containing modified Hoagland Solution with the following nutrients (in mM) for a two-week pre-treatment period: KNO3, 1.0; Ca(NO3)2·4H2O, 1.0; MgSO4·7H2O, 0.4; KH2PO4, 0.2; H3BO3, 4.6 × 10−2; MnCl2·4H2O, 9.1 × 10−3; ZnSO4·7H2O, 7.6 × 10−3; CuSO4·5H2O, 3.2 × 10−3; Na2MoO4·2H2O, 2.4 × 10−3; EDTA-Fe·Na, 7.1 × 10−2 (pH 6.5) [15].

Response of intact rooted-leaves to short term salinity
Rooted-leaves of K51-40, 140 Ruggeri and Cabernet Sauvignon were subjected to nutrient solution only (control) or to 50 mM Cl− (Na+:Ca2+:Mg2+ = 6:1:1) in nutrient solution for 4 days. At harvest, the rooted-leaves of each genotype were washed in de-ionised water, blotted dry with paper towel, weighed, then separated into lamina, petiole and roots. Fresh weights of all plant parts were also obtained. Samples were divided equally for RNA extraction and ion composition analysis. Samples for RNA extraction were snap frozen in liquid nitrogen and stored at minus 80°C. Root, petiole and lamina samples for ion analysis were weighed before being dried in an oven at 60°C and retained for Cl− analysis.

For stele and cortex expression studies roots were salt-treated and harvested as described above, lateral roots were removed from main roots and then cortex was stripped from stele of the main root using fine tweezers. Three biological replicates were harvested, each consisting of dissected tissue from three rooted-leaves. Tissue samples were immediately frozen in liquid nitrogen and stored at minus 80°C for RNA extraction.

Ion analyses
Laminae, petiole and root samples were dried at 60°C for at least 72 h and ground to a fine powder using a mortar and pestle. Cl− concentration was measured by silver ion titration with a chloridometer (Model 442–5150, Buchler Instruments, Lenexa, Kansas, USA) from extracts prepared by digesting 20–100 mg dry samples in 4 mL of acid solution containing 10% (v/v) acetic acid and 0.1 M nitric acid overnight before analysis.

RNA extraction
Frozen root tissues were grounded to a fine powder in liquid nitrogen using a mortar and pestle. RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma, St. Louis, Missouri, USA) following the manufacturer’s protocol. RNA was DNase I treated with Turbo DNA-free (Life Technologies, Carlsbad, California, USA) for 1 hour at 37°C to remove contaminating genomic DNA. RNA was precipitated at minus 80°C overnight in 5 volumes of 100% ethanol (v/v) and 1/10 volumes of 3 M NaOAC. After ethanol precipitation, RNA was resuspended in nuclease free water and analysed on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Only RNA samples with 260/280 and 260/230 absorbance ratios greater than 1.8 were used. RNA integrity was screened on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA) and only RNA samples with an RNA integrity number (RIN) above 8.5 were used.

Microarray chip design, labelling and hybridisation
Custom 8x60K gene expression microarrays were designed using eArray (Release 7.6) (Agilent Technologies). Oligonucleotide probes (60-mers) were designed against 26,346 annotated V. vinifera transcripts from the 12x Genoscope build available from http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/. The Agilent 60-mer probe format is considered more tolerant to sequence mismatches than 25-mers, and more suitable for analysis of polymorphic DNA sequences [38]. Also, the use of a custom Agilent expression array enabled us to print a subset of probes for 90 putative anion transporters multiple times on the array (Additional file 1). This multi-probe approach increases the robustness of the expression values obtained when the probes for these genes are averaged. Probes that detect differential gene expression many times show a greater probability of genuine differential expression when the B-statistic probability (log-odds) of differential gene expression is calculated. The higher the B-statistic, the greater the chance that the gene is differentially expressed (B-statistic = 0 represents 50:50 chance of differential gene expression).

Twenty-two microarrays were used which consisted of 4 biological replicates for Cabernet Sauvignon (±50 mM Cl−), 4 biological replicates of K51-40 (±50 mM Cl−) and 3 biological replicates of 140 Ruggeri (±50 mM Cl−). Each biological replicate consisted of roots from 4 rooted-leaves pooled together. Single colour labelling, hybridisations and image analysis were performed at the Ramaciotti...
Centre for Gene Function Analysis (University of New South Wales, Australia).

Functional annotation of genes
Gene functional annotation, which included InterPro descriptions, Gene Ontology terms and Arabidopsis orthologs, was obtained from BioMart at EnsemblPlants (plants.ensembl.org/biomart/martview/). Additional functional annotation was gathered from Grimplet et al. [39], and this annotation was used for the tables and figures presented in this manuscript.

Microarray data analysis
Scanned images were analysed with Feature Extraction Software 10.7.3 (Agilent Technologies, Santa Clara, California, USA) and the Cy3 median signal intensities for each spot on the arrays were imported into R for further processing. The data was log(2) transformed and quantile normalized. Since the microarray hybridizations were performed at different dates we observed batch effects that we corrected for with the ComBat package [40]. The quality of the microarray hybridisation and reproducibility amongst biological replicates was validated using array-QualityMetrics version 3.12.0 [41]. Differentially expressed genes were identified using the Linear Model for Micro-array Data (LIMMA) package [42], and the Benjamini and Hochberg correction method was applied to account for multiple testing [43]. To filter the probes, the probe sequences were blasted against the predicted cDNAs of the 12xV1 genome sequence at EnsemblPlants. Probes with an e-value $\geq 1 \times 10^{-10}$ and probes that showed no blast hit were excluded from the initial analyses. Gene expression changes were considered significant when a threshold fold change of greater than or equal to 1.41 was reached (log(2) FC $\pm 0.5$) and a false discovery rate (FDR) corrected probability of $P \leq 0.05$. The raw data for the microarray experiments are available at the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE57770.

Hierarchical clustering and co-expression analysis was performed using Genesis 1.7.6 [44] using tab delimited text files of the log(2) fold change values of gene expression of averaged probes. Transcripts and experiments were clustered using the average linkage method. Singular enrichment analysis was performed using Agrigo [45]. At the time of writing, the Agrigo server is incompatible with 12xV1 V. vinifera gene IDs. Therefore transcripts that were differentially expressed (identified after filtering) were entered into the Agrigo server using the 12xV0 transcript ID’s (Genoscope). The hypergeometric method with Hochberg (FDR) multi-test adjustment was used to identify statistically significant GO terms ($P < 0.05$).

Phylogenetic analyses
V. vinifera protein sequences of interest were obtained from EnsemblPlants using the 12xV1 gene IDs. V. vinifera amino acid sequences were used as a query in a protein-protein BLAST (blastp) at the National Centre for Biotechnology Information (NCBI) against non-redundant protein sequences limited to Arabidopsis thaliana (taxid: 3702). Arabidopsis sequences with the best total score were reciprocally blasted at EnsemblPlants against the Vitis vinifera peptide database. Arabidopsis and grapevine sequences that were obtained using this approach were aligned using Clustal W2 [46]. Phylogenetic trees were generated with Geneious 6.1.2 (Biomatters) using the neighbour-joining method and the Jukes-Cantor genetic distance model. A consensus tree was generated by resampling 1000 times using the bootstrap method. Branch lengths are proportional to the amount of divergence between nodes in units of substitutions per site. Gene identifiers for the protein sequences used are shown in Additional file 2, while the multiple sequence alignment is shown in Additional file 3.

Quantitative real-time PCR (qRT-PCR)
One microgram of total RNA was reverse transcribed in a 20 μL reaction using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA). The procedure was modified from the manufacturer’s to include an initial RNA denaturation step of 65°C for 5 minutes then incubation on ice for 1 minute, and cDNA synthesis step of 42°C for 1 hour. cDNA was diluted 1 in 5. Two microliters of cDNA was used as a template for PCR and qRT-PCR reactions. PCR targets were first amplified from cDNA using KAPA taq (KAPA Biosystems, Woburn, Massachusetts, USA) following manufacturer’s procedures. Fragments of the correct size and target were confirmed by agarose gel and sequencing. PCR fragments, or linearised plasmid containing the PCR fragment, were serially diluted and used as a template for qRT-PCR in duplicate. Standard curves were generated using iCycler iQ optical system software version 3.1 (Bio-Rad), which also calculates the reaction efficiency of each primer pair using the formula $E = 10^{1/slope}$. qRT-PCR was performed on a Bio-Rad iCycler. Reactions consisted of 250 nM forward and reverse primer, 1x KAPA SYBR FAST qPCR Master Mix (KAPA Biosystems), and 2 μL of diluted cDNA. Reactions were performed in triplicate following a three-step protocol consisting of 40 cycles of the following: 95°C 15 sec, 56°C 20 sec, 72°C 10 sec (plus data acquisition). Melt curve analysis was performed by heating PCR products from 52°C to 92°C for 20 seconds increasing at 0.5°C per cycle with continuous fluorescence detection. Relative expression ratios were calculated using the primer pair efficiency and the formula described by Pfaffl [47], with the geometric mean of VvActin1, VvUbiquitin-L40 and
**Results**

Salt treatment, grapevine growth and ion accumulation

Following 4-days of 50 mM Cl\(^-\) treatment, roots of 140 Ruggeri retained significantly more Cl\(^-\) compared to those of Cabernet Sauvignon and K51-40 (Figure 1A). Conversely, Cabernet Sauvignon and K51-40 petioles and laminae accumulated significantly more Cl\(^-\) compared to 140 Ruggeri (Figure 1B and C). K51-40 accumulated the highest amount of Cl\(^-\) in aerial tissues under salt stress (Figure 1B and C). Under control conditions, 140 Ruggeri also accumulated significantly less petiole and laminae Cl\(^-\) than K51-40, indicating that the Cl\(^-\) exclusion mechanism may be active in low Cl\(^-\) conditions (Figure 1B and C). Overall, the shoot Cl\(^-\) accumulation of varieties can be expressed as 140 Ruggeri < Cabernet Sauvignon < K51-40.

Validation of microarray data using real-time quantitative PCR (qRT-PCR)

To validate the microarray expression data and further quantify mRNA expression levels, we measured the expression of 12 genes by qRT-PCR and compared the datasets. Expression ratios of genes from control and 50 mM Cl\(^-\) treated samples were analysed by linear regression and an R\(^2\) value of 0.88 was observed, indicating good correlation (Additional file 5a). Similarly, qRT-PCR and microarray ratios for 12 genes were compared between varieties under control conditions, which provided an R\(^2\) value of 0.89, also demonstrating good correlation (Additional file 5b).

Differentially expressed genes due to chloride stress

Following Cl\(^-\) stress 1361 unique genes were differentially expressed in at least one grapevine variety (Figure 2). The number of differentially expressed genes due to Cl\(^-\) treatment was positively correlated with Cl\(^-\) accumulation in shoot tissues. The Cl\(^-\) accumulator K51-40 had the highest number of Cl\(^-\) responsive transcripts (817), followed by the intermediate accumulator Cabernet Sauvignon (511), while the Cl\(^-\) excluder 140 Ruggeri had the least number of Cl\(^-\) responsive transcripts (252) (Figure 2). This correlation is consistent with findings in *Citrus* leaves when salt tolerant and sensitive rootstocks were compared after salt stress [21].

Cluster analysis

The transcript profiles of Cabernet Sauvignon, 140 Ruggeri and K51-40 roots exposed to high Cl\(^-\) were grouped by hierarchical clustering (Figure 3). 140 Ruggeri and Cabernet Sauvignon had the most similar transcriptional response to Cl\(^-\) in roots, while the Cl\(^-\) includer K51-40 had a unique response (Figure 3, top dendrogram). Gene clusters were examined by singular enrichment analysis (SEA) of gene ontology (GO) terms. Three clusters of interest showed enrichment of GO biological processes (Figure 3). Other gene clusters showed no significant enrichment of GO terms.

In 140 Ruggeri, Cl\(^-\) treatment induced the expression of transcripts involved in abiotic stress tolerance (Figure 3, Cluster A), including glutathione-S-transferases (GST).
and heat shock proteins (HSP) (Additional file 7). Overexpression of GSTs in tobacco enhances growth under salt stress [50], while HSPs act as molecular chaperones that help maintain correct protein conformation under abiotic stress [51]. These unique transcriptional changes might enable 140 Ruggeri to perform better under salt stress relative to other grapevine genotypes.

In K51-40, Cl\textsuperscript{-} treatment repressed genes involved in the hypersensitive response and flavonoid biosynthesis (Figure 3, Cluster B; Additional file 8). Flavonoids have diverse roles in plants including scavenging of reactive oxygen species (ROS) and pathogen defence [52]. Under salt stress, leakage of photosynthetic and respiratory electrons may react with oxygen, leading to ROS production and subsequent oxidative stress [53]. Therefore the transcriptional regulation of flavonoid biosynthesis in K51-40 might prevent damage from excessive ROS production. In Cabernet Sauvignon, Cl\textsuperscript{-} treatment repressed mitochondrial specific transcripts, such as NADH dehydrogenases, c-type cytochromes and pentatricopeptide repeat (PPR) domain proteins (Figure 3, Cluster C; Additional file 9). Transcriptional repression of respiratory transcripts in Cabernet Sauvignon probably functions to reduce ROS production.

The stress-inducible phytohormone ABA restricts anion entry to the root xylem [18] and inward anion currents (anion efflux) from xylem parenchyma protoplasts from barley [17] and maize [19]. We therefore investigated whether high Cl\textsuperscript{-} treatment reduces the expression of genes likely to facilitate Cl\textsuperscript{-} transport to aerial tissues of 140 Ruggeri. Only four membrane transporters were repressed in 140 Ruggeri upon Cl\textsuperscript{-} treatment and none were predicted to facilitate anion movement across membranes (Additional file 10).

**Transcriptional differences between grapevine varieties under control conditions**

Given that Cl\textsuperscript{-} accumulation in shoot tissues was significantly different between grapevine varieties in the absence of salt stress (Figure 1B and C), we hypothesised that there might be a difference in gene expression of anion transporters under control conditions. Under these conditions, 4527 genes were differentially expressed between 140 Ruggeri and K51-40 with approximately half (2310 genes) being lower in 140 Ruggeri (Additional file 11). Genes encoding 214 membrane integral proteins were expressed differently between roots of K51-40 and 140 Ruggeri (Additional file 12). Multigene families have been proposed as regulators of salt tolerance and anion homeostasis in plants, including NRT, ALMT, SLAH and CLC [9,54]. Members from these and other gene families encoding membrane proteins, as well as possible regulatory proteins, that were expressed differently between rootstocks, are summarised (Table 1) and described below. As an alternative analysis, genes with a high B-statistic (log-odds) for differential expression between rootstocks are listed in Table 2.

**NRT/POT**

The NRT or proton dependent oligopeptide (POT) gene family is involved in the acquisition and whole plant homeostasis of nitrogen; different family members transport NO\textsubscript{3}\textsuperscript{-}, amino acids and various peptides [55]. In our study, 8 NRT1 genes were expressed differently between rootstocks (Table 1). Grapevine NRT1 gene family members were poorly annotated in functional databases. To assign putative functions, we produced a phylogeny of the grapevine NRTs uncovered in our microarray screen using Arabidopsis NRT1s. Homologs of AtNRT1.4, AtNRT1.11, nitrate excretion transporter 1 (AtNAXT1), AtNAXT2 and glucosinolate transporter 1 (AtGTR1) were identified, as well as three other Vitis NRTs with uncharacterised Arabidopsis homologs (Figure 4). Two grapevine NRTs homologous to Arabidopsis AtNRT2.5 and AtNRT2.7, as well as a homolog of Arabidopsis oligopeptide transporter 4 (OPT4) were more abundantly expressed in 140 Ruggeri (Table 1). Differential expression of VvNAXT1, VvNAXT2, VvNRT1.11 (all higher in 140 Ruggeri) and VvNRT1.4 (higher in K51-40) was also highly significant (Table 2).

In Arabidopsis roots, AtNRT1.8 is induced and AtNRT1.5 repressed by salt and cadmium stress [56]. AtNRT1.5 is the only NRT1 isoform with a confirmed role in root xylem loading of NO\textsubscript{3} [57], and mutants of atnrt1.5 grow...
better under NaCl stress than wildtype [58]. Conversely, AtNAXT1 effluxes NO₃⁻ under acid load, and is regulated at the post-transcriptional level [59]. We further investigated expression patterns of Vitis orthologs of these genes. VvNRT1.8 and VvNRT1.5 were identified phylogenetically (Figure 4). They were oppositely regulated by salt stress in Cabernet Sauvignon and 140 Ruggeri, but not K51-40, although the expression changes were small (Figure 5A and B). VvNAXT1 was unresponsive to salt in all three genotypes (Figure 5C), which is consistent with the response of its homolog in Arabidopsis [59]. Interestingly, VvNRT1.4 was strongly repressed (3 fold) by salt stress in K51-40 (Figure 5D). In spite of these differences in salt response, the largest transcriptional differences in grapevine NRT1 mRNAs were observed between genotypes under control conditions, especially between the contrasting rootstocks 140 Ruggeri and K51-40 (Figure 5E – H). This suggests of a role of some of these genes in Cl⁻ exclusion in the absence of stress (Figure 6). Arabidopsis AtNRT1.5 is considered important for plant salt tolerance [58], possibly due a role in anion loading to the xylem [57]. In grapevine, VvNRT1.5 was not preferentially expressed in the root stele under salt stress (Additional file 13), which contrasts with AtNRT1.5 [57]. Furthermore, VvNRT1.5 was more abundant in 140 Ruggeri than K51-40 (Figure 5F; Figure 6B). These data reduce the likelihood of VvNRT1.5 having a role in xylem loading of Cl⁻ in grapevine. Based on transcriptional data, we suggest that VvNRT1.4 is the best NRT1 candidate for xylem loading of Cl⁻ due to a much greater abundance in K51-40 roots under control conditions (Figure 5D; Figure 6C).

ALMT
Chelation of toxic aluminium in the rhizosphere by the efflux of organic acids from roots is facilitated by plasma membrane aluminium-activated malate transporters
| Probe ID | 12xV1 blast hit | 12xV0 gene ID | Arabidopsis homolog | Log(2) FC | p-value | Functional annotation |
|----------|----------------|---------------|---------------------|-----------|---------|----------------------|
| CUST_15333_17284 | VIT_02s0012g01270 | GSVIVT01013161001 | AT4G17870 | 1.41 | 1.1E-09 | Abscisic acid receptor PYL1, RCAR11 |
| NG2_36172_20391 | VIT_06s0008g00170 | GSVIVT01036162001 | AT1G04400 | -0.69 | 3.02E-04 | Aluminum activated malate transporter 1 |
| CUST_44694_7793 | VIT_06s0009g00450 | GSVIVT01037570001 | AT1G04400 | 0.78 | 1.90E-03 | Aluminum activated malate transporter 1 |
| CUST_46237_21897 | VIT_08s0105g00250 | GSVIVT01111480001 | AT3G11680 | 1.30 | 2.51E-04 | Aluminum activated malate transporter 1 |
| CUST_8680_62299 | VIT_11s0052g00320 | GSVIVT01029900001 | AT4G29900 | -0.70 | 3.31E-04 | Calcium ATPase 10 (ACA10), plasma membrane |
| NG2_12175_47390 | VIT_07s0129g00110 | GSVIVT01000116001 | AT4G37640 | 0.58 | 8.86E-03 | Calcium ATPase 2 (ACA2), plasma membrane |
| CUST_16133_33172 | VIT_07s0129g00110 | GSVIVT01000116001 | AT4G37640 | -0.69 | 3.02E-04 | Aluminum activated malate transporter 1 |
| CUST_46046_19308 | VIT_01s0010g02150 | GSVIVT01010291001 | AT1G12580 | 0.70 | 2.43E-03 | Calcium ATPase 13 (ACA13) |
| NG2_35892_10569 | VIT_06s0004g06570 | GSVIVT01024741001 | AT3G51860 | 1.44 | 5.00E-12 | Calcium ATPase 10 (ACA10), plasma membrane |
| CUST_50946_56104 | VIT_02s0025g04520 | GSVIVT01019868001 | AT1G12580 | 0.70 | 2.43E-03 | Calcium ATPase 13 (ACA13) |
| NG11_49713_18843 | VIT_08s0068g02190 | GSVIVT01022542001 | AT1G17870 | 0.52 | 2.67E-02 | Calcium-dependent protein kinase 1 |
| NG11_40688_11883 | VIT_09s0015g01840 | GSVIVT01016118001 | AT3G33200 | 0.99 | 1.10E-05 | Cation exchanger (CAX2) |
| NG11_57150_10097 | VIT_06s0004g03520 | GSVIVT01025107001 | AT3G45650 | 1.27 | 7.0E-19 | Calcium ATPase 12 (ACA12) |
| NG11_44542_25973 | VIT_06s0004g03530 | GSVIVT01025106001 | AT3G45650 | 1.61 | 1.24E-32 | Nitrate transporter 1.11 |
| NG11_46422_21127 | VIT_06s0004g01570 | GSVIVT01015552001 | AT1G64410 | 0.87 | 1.65E-06 | Oligopeptide transporter OPT4 |
| CUST_37073_22417 | VIT_06s0004g03530 | GSVIVT01015552001 | AT1G64410 | 0.87 | 1.65E-06 | Oligopeptide transporter OPT4 |
| CUST_42271_1540 | VIT_14s0066g00850 | GSVIVT01032430001 | AT5G14940 | 0.64 | 3.31E-07 | Proton-dependent oligopeptide transport (POT) family protein |
| CUST_27252_1533 | VIT_16s0004g00650 | GSVIVT01035643001 | AT1G72140 | 0.89 | 7.91E-14 | Proton-dependent oligopeptide transport (POT) family protein |
| NG11_25530_14040 | VIT_17s0000g03580 | GSVIVT01008072001 | AT3G47960 | 0.54 | 1.88E-04 | Glucosinolate transporter 1 (GTR1) |
| NG11_7897_10153 | VIT_14s0066g02020 | GSVIVT01032550001 | AT5G14940 | 0.64 | 3.31E-07 | Proton-dependent oligopeptide transport (POT) family protein |
| NG11_35177_1429 | VIT_18s0014g00670 | GSVIVT01026058001 | AT1G72140 | 0.89 | 7.91E-14 | Proton-dependent oligopeptide transport (POT) family protein |
| NG11_31776_20297 | VIT_16s0005g01860 | GSVIVT01028789001 | AT5G24030 | 0.54 | 4.61E-05 | SLAH3 (SLAC1 Homologue 3) |
| CUST_21950_56777 | VIT_07s0191g00070 | GSVIVT01034190001 | AT4G40010 | -1.01 | 1.28E-04 | SNF1-related protein kinase 2.5 (SnRK2.5) |
| CUST_41758_42394 | VIT_06s0105g00220 | GSVIVT01032389001 | AT4G33950 | -0.56 | 1.54E-02 | SNF1-related protein kinase 2.6 (SnRK2.6) |
| CUST_27252_1533 | VIT_16s0001g06550 | GSVIVT01011573001 | AT2G01980 | -2.30 | 1.53E-05 | SOS1 (Na+/H + antiporter) |
| CUST_15165_41173 | VIT_06s0004g07830 | GSVIVT01024587001 | AT5G8380 | -0.73 | 1.72E-06 | SOS2 (salt overly sensitive 2) |
| CUST_27642_7432 | VIT_16s0009g01870 | GSVIVT01038549001 | AT5G24270 | -0.67 | 8.65E-03 | SOS3 (salt overly sensitive 3) |
ALMT homologs were differentially expressed between rootstocks (Table 1). Whether these proteins mediate Cl fluxes, and the directionality of such fluxes, remains unresolved, but Cl exclusion could arise through Cl transport across the tonoplast (AtALMT9) [62]. Root ALMTs might therefore have a role in Cl exclusion.

Three ALMTI homologs were differentially expressed between rootstocks (Table 1). Whether these proteins mediate Cl fluxes, and the directionality of such fluxes, remains unresolved, but Cl exclusion could arise through efflux of Cl to the rhizosphere (higher expression in 140 Ruggeri).
or reduced Cl\(^{-}\) entry in the cortex and restricted xylem loading of Cl\(^{-}\) (lower expression in 140 Ruggeri, VIT_06s0080g00170) (Table 1; Figure 6D and G).

**Calcium transporters (CAX and ACA)**

Calcium exchangers (CAX) mediate Ca\(^{2+}\)/cation antiport activity across the tonoplast. Roles of CAXs include cell specific storage of Ca\(^{2+}\) by CAX1 [63], while Arabidopsis cax3 mutants are sensitive to NaCl, LiCl and acidic pH, suggesting a possible role in salt tolerance [64]. Three grapevine CAX transcripts were more abundant in roots of 140 Ruggeri compared to K51-40 (Table 1). In addition to CAX, the plant plasma and vacuolar membranes harbour auto-inhibited Ca\(^{2+}\)-ATPases (ACA), of which Arabidopsis ACA4 can improve salt tolerance of yeast [65]. Six ACAs were differentially expressed between 140 Ruggeri and K51-40. These data indicate that genes regulating cytosolic free calcium ([Ca\(^{2+}\)]\(_{cyt}\)) in roots could be important for grapevine Cl\(^{-}\) exclusion.

**CLC**

Two CLCs showed differential expression between rootstocks under control conditions. A gene homologous to Arabidopsis AtCLCb (VIT_14s0068g02190) was less abundant in 140 Ruggeri (Table 1). Another CLC with homology to AtCLCf (VIT_19s0015g01850) was less abundant in K51-40 (Table 1). Differential expression of VvCLCf was also identified as highly statistically significant (Table 2).

**SLAH3 and ABA signalling**

Homologs of the Arabidopsis SLAC1 anion channel (AtSLAH1 and AtSLAH3) are plasma membrane localized, expressed in the root vasculature, and functionally complement guard cell anion efflux in the slac1 mutant [23]. This indicates that SLAHs might be involved in anion homeostasis [23] and loading to the xylem sap [54]. VvSLAH3 was more abundant in the Cl\(^{-}\) excluder 140 Ruggeri compared to K51-40 under control conditions (Table 1; Figure 6E). This contrasts with *Citrus*, where CcSLAH1 was up-regulated by 90 mM salt stress.
in a Cl\(^-\) accumulating rootstock [22]. Reconstitution in X. laevis oocytes has demonstrated that plant SLAC/SLAH activity is tightly regulated by kinase/phosphatase activity following an ABA signal [66]. Homologs of the Arabidopsis ABA signalling machinery were differentially expressed between rootstocks. The ABA receptor VvPYL1/RCAR11 was more highly expressed in 140 Ruggeri (Table 1). Two SNF1-related protein kinase 2
Figure 6 Relative transcript abundances of membrane proteins in roots of grapevine genotypes under control conditions measured by qRT-PCR, and a model indicating possible molecular mechanisms for reduced net xylem loading of Cl\(^{-}\) in 140 Ruggeri. (A – B) relative expression levels of VvNAXT1 (A) and VvNRT1.5 (B) measured by qRT-PCR, which represent possible avenues for cortical or epidermal efflux of Cl\(^{-}\) out of roots. (C – E) relative expression levels of VvNRT1.4 (C), VvALMT1 (D) and VvSLAH3 (E) measured by qRT-PCR, which represent possible avenues for xylem loading of Cl\(^{-}\). Bars represent the mean of four biological replicates ± SEM. Transcript abundance is relative to the Cabernet Sauvignon biological replicate with the lowest cycle threshold (Ct) value, which was set to 1. Statistical differences were determined using one way ANOVA with Holm-Sidak’s multiple comparisons test to compare the means. (F - G) proposed model for reduced net xylem loading of Cl\(^{-}\) in 140 Ruggeri relative to K51-40. (F) In 140 Ruggeri, anion efflux from cortical or epidermal root cells could be mediated through putative anion channels VvNRT1.5 and VvNAXT1 which are transcriptionally more abundant in the Cl\(^{-}\) excluder. Xylem loading of Cl\(^{-}\) could be restricted through reduced VvNRT1.4 abundance, or inhibition of VvSLAH3 and VvALMT by higher [Ca\(^{2+}\)]\(_{cyt}\) mediated by VvCAX3 (directly, or in partnership with Ca\(^{2+}\) dependent protein kinases). (G) In K51-40, anion efflux to the xylem apoplast could be enhanced through increased abundance of VvALMT1 and VvNRT1.4, and activation of VvALMT1 and VvSLAH3 by SnRK2 kinases.
(SnRK2) family members including the Vitis ortholog of SnRK2.6 (open stomata 1 (OST1)) were repressed in 140 Ruggeri, and multiple calcium dependent protein kinases (CPK) were differentially expressed between rootstocks (Table 1). Homologs of these genes in other plants have proven roles in ABA induced activation of SLAC1 in guard cells [67] and might be involved in SLAH3 regulation in Vitis roots.

Other candidates
Two ABC transporters were significantly differentially expressed between rootstocks; a C-type (ABCC12) (VIT_09s0002g02430) (higher in 140 Ruggeri) and multidrug resistance 12-type (VIT_02s0025g00930) (higher in K51-40) (Table 2). A role in Cl\(^{-}\) transport has not been identified for ABC transporters in plants, although reports suggest roles in arsenic tolerance [68] and salt tolerance [69]. A C3HC4-type ring finger protein was more abundant in the tolerant variety 140 Ruggeri (Table 2). A C3HC4 protein was potentially crucial for abiotic stress tolerance in rice roots [70]. A phospholipase A2 precursor (VIT_11s0016g02570) was also expressed alternatively between rootstocks (Table 2). The product of a phospholipase A2 activates a tonoplast H\(^{+}\)/Na\(^{+}\) antiporter in cultured cells of California poppy [71].

Discussion
Shoot chloride exclusion is one of several traits that underpins salt tolerance. However, the root-localised anion transport proteins (or their regulators) thought to be crucial for salt tolerance remain unidentified [9,15,16,22]. We therefore analysed the genome wide transcriptional response of grapevine roots to Cl\(^{-}\) stress. Cabernet Sauvignon repressed transcripts encoding respiratory proteins, probably to reduce ROS production under salt stress. Although ROS may act as signalling molecules in eukaryotes [72,73], it has previously been reported that ROS production in grapevine cells that contrast in salt-tolerance represents a manifestation of cellular damage rather than an adaptive response [74]. We therefore propose a hierarchy exists in the magnitude of transcriptional responses to Cl\(^{-}\) stress (K5140> > Cabernet Sauvignon> > 140 Ruggeri) that correlates with the amount of damage in the laminae. However, these differences in varietal responses to stress do not explain differential Cl\(^{-}\) exclusion, which was statistically significant before salt stress (Figure 1).

Studies indicate that there is natural variation in the ability to tolerate salt stress in various plant species including Citrus [22], rice [70], barley [75,76], and Arabidopsis [77]. Our data support the hypothesis that Cl\(^{-}\) exclusion in grapevine is mediated by anion transporters or channels that are differentially expressed between non-stressed 140 Ruggeri and K51-40. To this end, we have proposed a testable model for Cl\(^{-}\) exclusion based on the expression levels of candidate genes identified in our study (Figure 6). These candidate genes for Cl\(^{-}\) exclusion are subsequently discussed in the context of this model and existing literature.

In plant roots, anion movement across the plasma membrane of xylem parenchyma cells for loading to xylem vessels occurs through unidentified anion channels with fast and slow activation kinetics (X-QUAC and X-SLAC respectively) [17]. Anion conductances in Arabidopsis guard cells with homologous activation kinetics have been characterised, and the channels eliciting these currents identified. The slowly activating anion conductance in guard cells has been attributed to AtSLAC1 and AtSLAH3 channels [24,78], while guard cell QUAC is mediated by AtALMT12 [79]. It is therefore feasible that X-SLAC and X-QUAC arise from SLAH and ALMT channels in root cells. We identified VvSLAH3 and three VvALMT1 transcripts that were expressed differently between rootstocks. Thermodynamics predicts that the loading of Cl\(^{-}\) into the xylem under low apoplastic [Cl\(^{-}\)] occurs by passive transport [80]. Therefore, transcripts that encode putative anion transport proteins with a high abundance in K51-40 are good candidates for controlling xylem loading of Cl\(^{-}\). Our results suggest that VvALMT1 may be involved in xylem loading of Cl\(^{-}\) (Figure 6D and G). VvSLAH3 transcript was more abundant in 140 Ruggeri (Table 1; Figure 6E). For it to be involved in xylem loading of Cl\(^{-}\) there are two alternatives. Arabidopsis SLAH3 has been shown to be much more permeable for NO\(_3\) than Cl\(^{-}\) [78]. If this is the case in grapevine, the pathway for anion transport in 140 Ruggeri could be more NO\(_3\) selective than in K51-40, thus resulting in greater discrimination against Cl\(^{-}\) loading of the xylem in 140 Ruggeri. Alternatively, SLAH3 could be permeable to Cl\(^{-}\) but the extent of post-translational control differs between varieties, as elaborated below.

Cellular anion conductance must be tightly regulated to avoid uncontrolled electrolyte efflux [54], and for this reason complex signalling networks exist in plants. Upon an ABA induced rise in [Ca\(^{2+}\)\(_{\text{cyt}}\)], guard cell SLAC may be activated by calcium dependent protein kinases CPK23 and CPK21 [67]. Alternatively, the Ca\(^{2+}\) independent kinase SnRK2.6 (OST1) can activate both guard cell SLAC and QUAC in response to ABA [81]. In contrast, opposite regulation by ABA and [Ca\(^{2+}\)\(_{\text{cyt}}\)] occurs in root cells, with X-QUAC being inhibited by ABA and by high [Ca\(^{2+}\)\(_{\text{cyt}}\)] [17,19]; whether kinases are involved in this regulation have not yet been determined. A transcript encoding the VvPYL1/RCAR11 ABA receptor was significantly more abundant in roots of 140 Ruggeri compared to K51-40. The Cl\(^{-}\) excluder might therefore be more sensitive to ABA, or may be primed for any
slight increase in ABA concentration. Differential expression of vacuolar CAXs and ACAs between rootstocks might function to maintain \([\text{Ca}^{2+}]_{\text{cyt}}\) signals in root cells of 140 Ruggeri, thus participating in the \(\text{Ca}^{2+}\) dependent down-regulation of X-QUAC and X-SLAC (Figure 6F). AtCPK20 interacts with AtSLAH3 in Arabidopsis pollen tubes [82]. Differential expression of \(VvCPK20\), among other CPKs, between rootstocks might indicate an involvement of these kinases in \(VvSLAH3\) regulation. In addition, differential expression of \(VvSnRK2.6\) and \(VvSnRK2.7\) between rootstocks implicates both the \(\text{Ca}^{2+}\) dependent and independent ABA signalling machinery in grapevine roots as possible mediators of \(\text{Cl}^-\) exclusion (Figure 6G). The shear number of genes potentially involved in X-QUAC and X-SLAC mediated pathways, possible kinase redundancy or multiple kinase targets, could explain the observations that \(\text{Cl}^-\) exclusion in grapevine is polygenic [15,83].

Arabidopsis has 53 \(NRT1\) genes and rice has 80 \(NRT1\) members. This has led to the question [55]: are there unidentified anionic substrates for NRTs beyond just nitrate or peptides to account for such large family genes? The large number of \(NRT1\) genes identified in our screen suggests they might play some key role in \(\text{Cl}^-\) homeostasis. Plasma membrane localisation of plant NRTs heightens the possibility for roles in cellular \(\text{Cl}^-\) fluxes. However, the anion selectivities of plant NRTs have been rarely examined [9]. \(AtNAXT1\) was shown not to transport \(\text{Cl}^-\) [59], but characterisation of the remaining 6 Arabidopsis NAXTs is yet to be reported. If permeable to \(\text{Cl}^-\), greater abundance of \(VvNAXT1\) and \(VvNAXT2\) in 140 Ruggeri compared to K51-40 (Table 1; Table 2; Figure 6A) could allow the \(\text{Cl}^-\) excluding rootstock to excrete \(\text{Cl}^-\) back to the external medium instead of transporting it to the shoot (Figure 6F). This function might be enhanced under salinity stress if cytosolic pH is reduced, as \(AtNAXT1\) actively excretes anions under acid load, possibly to balance proton extrusion by \(H^+\)-ATPases [59]. This cannot work as the sole mechanism of \(\text{Cl}^-\) exclusion, as 140 Ruggeri still retains more \(\text{Cl}^-\) in the roots compared to Cabernet Sauvignon and K51-40 (Figure 1A). Other stress responsive plant NRTs (\(VvNRT1.8, VvNRT1.5\)) showed similar expression profiles to orthologous genes in Arabidopsis. However, \(VvNRT1.5\) was less abundant in the root stele compared to the cortex (Additional file 13), indicating that this gene is more likely to be involved in cortical efflux of \(\text{Cl}^-\) rather than \(\text{Cl}^-\) loading to the xylem (Figure 6F and G). Excessive \(\text{Cl}^-\) in the root zone or cytoplasm could inhibit \(\text{NO}_3^-\) transport (both uptake and efflux) due to the well-documented antagonism between these anions [84,85]. Therefore, differences in \(NRT1\) expression in salt stressed grapevine roots also could be a compensatory mechanism to overcome this ionic antagonism.

Multiple studies have linked plant CLCs to salt tolerance [86-89]. \(VvCLCb\) and \(VvCLClf\) were expressed differently between rootstocks under control conditions. In Arabidopsis, \(AtCLCb\) is a vacuolar \(\text{NO}_3^-/\text{H}^+\) exchanger [90], as is \(AtCLCa\) [91]. A single missense mutation in \(AtCLCa\) changes the selectivity from \(\text{NO}_3^-\) to \(\text{Cl}^-\) [92,93]. It is therefore possible that \(VvCLCb\) in \(Vitis\) roots participates in \(\text{Cl}^-\) sequestration in cell vacuoles, although greater expression in K51-40 does not fully support this. \(AtCLClf\) is associated with the trans-Golgi network [94], so a role in salt tolerance is less likely. Further study into \(Vitis\) CLCs is therefore needed before concluding a role in grapevine salt tolerance.

Candidate genes for plant \(\text{Cl}^-\) exclusion identified by Brumos et al. [22] were not highlighted in our study. Our array design had two probes for the putative \(\text{Cl}^-\) conductance regulator \(VvICln\) (\(VIT_16s0022g01560\)) and neither were salt responsive, consistent with short-term stress response in \(Citrus\) but contrasting with the long-term results [22]. On the other hand, one probe showed statistically significant differential expression between varieties under control conditions but was greater in the \(\text{Cl}^-\) excluding rootstock (data not shown). This means \(VvICln\) contributes to \(\text{Cl}^-\) exclusion in 140 Ruggeri, it must act as a negative regulator of \(\text{Cl}^-\) conductance. This seems unlikely given data in animals and plants [22,26]. \(VvCCC\) and \(VvSLAH1\) were also not differentially expressed, and so if they are involved may be regulated at the post-translational level, which cannot be highlighted by microarray technology. Indeed the activity of many plant anion channels is modified by phosphorylation events such as \(AtNRT1.1\) [95], \(AtSLAC1\) [96] and \(AtCLCa\) [97]. Differences in expression of SLAC/SLAH regulators \(SnRK2.6\) and \(CPK\) between rootstocks ensures \(VvSLAH1\) remains a candidate for \(\text{Cl}^-\) homeostasis in \(Vitis\). In future studies, it would be valuable to identify interacting partners of the protein kinases identified as differentially expressed in this study, and any functional changes induced by such interactions.

Conclusions
Using a whole root transcriptome approach, a detailed analysis of root mRNA profiles of contrasting grapevine genotypes is presented. This provides a complement to earlier physiological studies of the same varieties that have demonstrated the mechanism of shoot \(\text{Cl}^-\) exclusion as the restriction of its net xylem loading at the root [15,16,98]. A valuable list of candidate genes likely to mediate shoot \(\text{Cl}^-\) exclusion has been identified. Future functional characterisation of these genes, including the elucidation of protein-protein interactions, may enable their use in grapevine rootstock breeding efforts. More broadly the further study of these genes and their
homologs in other species will aid our understanding of long distance Cl− transport in plants.

Availability of supporting data
Data supporting the results of this article are available in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE57770. An ArrayQualityMetrics report is available at http://dx.doi.org/10.6070/H4CZ354H.

Additional files

Additional file 1: List of 90 predicted V. vinifera genes encoding putative anion transporters, and their predicted functional annotation, that were spotted onto the custom Agilent microarray slides multiple times for B-statistic analysis.

Additional file 2: Gene identifiers and annotations of the amino acid sequences used in the phylogenetic analysis of interesting NRT1 members.

Additional file 3: Multiple sequence alignment of Arabidopsis and grapevine NRT members for the data presented in Figure 4. Shading is representative of the Blosum62 score matrix as follows: black (100% similar) dark grey (80% similar) light grey (60% similar) unshaded (<60% similar).

Additional file 4: List of primers used in this study.

Additional file 5: Correlation between expression ratios determined by Agilent custom gene expression array and qRT-PCR. (a) Gene expression ratios of control to salt treated samples were compared for 12 genes (VIT_01s0011g06550, VIT_02s0012g01160, VIT_06s0004g03520, VIT_06s0080g00170, VIT_08s0040g01890, VIT_08s0040g03220, VIT_11s0016g05170, VIT_13s0019g00330, VIT_14s0078g02090) in both grapevines and the 1.41 fold change. (b) Levels of VvNRT1.5 transcript in enriched fractions of the root cortex and stele of salt stressed (50 mM Cl−) grapevine determined by qRT-PCR. Bars are SEM of 3 biological replicates. Asterisk represents significant difference in the stele compared to the cortex (Student’s t-test, P < 0.05). (b) Levels of VvNRT1.5 transcript in enriched fractions of the root cortex and stele of salt stressed (50 mM Cl−) grapevine determined by qRT-PCR. Bars are SEM of 3 biological replicates. Asterisk represents significant difference in the stele compared to the cortex (Student’s t-test, P < 0.05). VvNRT1.5 is known to be expressed in the root stele [99], and is therefore used as a control for adequate enrichment of stele and cortex root fractions in (a).

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
SWH, ARW, RRW and MG contributed to project conception. SWH, ARW, RRW and DB harvested rooted leaves for expression studies. UB analysed the raw microarray data, performed microarray data normalization and performed the statistical analysis. DB propagated and maintained the plant material and performed ion content measurements. SWH performed all other analyses, drafted the initial manuscript and prepared the final version with MG. MG and RRW supervised the research. All authors read and approved the final manuscript.

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