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Changes to the proteome and targeted metabolites of xylem sap in *Brassica oleracea* in response to salt stress

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ABSTRACT

Root-to-shoot signalling via xylem sap is an important mechanism by which plants respond to stress. This signalling could be mediated by alteration in the concentrations of inorganic and/or organic molecules.

The effect of salt stress on the contents of xylem sap in *Brassica olarecea* has been analysed by mass spectrometry in order to quantify these changes. Subcellular location of arabinogalactan proteins (AGPs) by immunogold labelling and peroxidase isozymes was also analysed by isoelectrofocusing.

The xylem sap metabolome analysis demonstrated the presence of many organic compounds such as sugars, organic acids and amino acids. Of these, amino acid concentrations, particularly that of glutamine, the major amino acid in the sap, were substantially reduced by salt stress. The xylem sap proteome analysis demonstrated the accumulation of enzymes involved in xylem differentiation and lignification, such as cystein proteinases, acid peroxidases, and a putative hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase under salt stress. The peroxidase isozyme pattern showed that salt stress induced a high accumulation of an acid isoform.

These results suggest that xylem differentiation and lignification is induced by salt stress. The combination of different methods to analyse the xylem sap composition provides new insights into mechanisms in plant development and signalling under salt stress.

Key-words: arabinogalactan proteins; cystein proteinases; lignification; peroxidases; programmed cell death; phi cells; xylem differentiation.

INTRODUCTION

The movement of solutes from roots to the aerial parts of the plant is accomplished by the tracheary elements of the xylem, which was traditionally considered as the main conduit for water and minerals. Structurally the xylem is a complex tissue containing at least tracheary elements and parenchyma cells and other cell-types that function as supporting cells (Evert 2007). On the other hand, sieve tubes of phloem are the main pathway to transport the photosynthetic assimilates from leaves to fruits, roots and buds.

Probably for these reasons, xylem sap analysis has focused mainly on the mineral contents. However, xylem sap contains also organic solutes, including carbohydrates, amino acids, organic acids, hormones and proteins (Satoh 2006). Transport via the xylem could supply signal molecules other than water and nutrient from the root system and that modification in the concentrations of these signals under abiotic stress can play important roles in plant adaptation to stress (Dodd 2005). Differences in the concentrations of organic compounds in xylem sap from stressed and unstressed plants have been found for a number of species (Gollan, Schurr & Schulze 1992; Patonnier, Peltier & Marigo 1999; Chen *et al*. 2001; De Sousa & Sodek 2003; Albacete *et al*. 2009). Xylem sap analysis under salt stress focused mainly on the role of hormone signalling mediated by abscisic acid (ABA) (Wolf, Jeschke & Hartung 1990; Zhao, Munns & King 1991; Gomez-Cadenas *et al*. 1998; Chen *et al*. 2001; Albacete *et al*. 2009). However, detailed studies of other organic compounds such as sugars, organic acids, amino acids or proteins in xylem sap and the quantification of changes under salt stress are scarce in the literature.

In the last 5 years, there have been considerable advances in xylem sap analysis using proteomic approaches. The xylem sap proteomes of *Zea mays*, *Brassica napus*, *Glycine max*, *Vitis vinifera* and *Populus* have been studied using separation in 2D gel electrophoresis and mass spectrometric identification (Kehr, Buhtz & Giavalisco 2005; Alvarez *et al*. 2006; Djordjevic *et al*. 2007; Agüero *et al*. 2008; Aki *et al*. 2008; Dafoe & Constabel 2009). Xylem sap proteome analysis shows similar groups of proteins present in the different species investigated (Buhtz *et al*. 2004; Dafoe & Constabel 2009). These groups of proteins were classified as proteins involved in cell wall metabolism and remodelling (glycosyl hydrolases, arabino-furanosidases,

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polygalacturonases, peroxidases, lignifiying enzymes, etc.), defence (chitinases, 1-3-β-glucanases, heat shock proteins, etc.), programmed cell death (aspartyl–proteases, cystein–proteases, serine–proteases, etc.), redox regulation (superoxide dismutase, glutathione reductase, ascorbate peroxidase, thioredoxin) and metabolism.

Recently, the xylem sap proteome has also been analysed in relation to plant-microbe interactions in different species infected by fungi, such as *Fusarium oxysporum*-infected tomato plants (Houterman *et al*. 2007), *Phytostophthora sojae*-infected soybean plants (Subramanian *et al*. 2009) and *Verticillium longisporum*-infected *B. napus* plants (Floerl *et al*. 2008) or infected by bacteria, as in Pierce's disease, which is caused by the xylem-confined *Xylella fastidiosa* (Basha, Mazhar & Vasanthaiah 2010). In these studies, the accumulation of several defence proteins and peroxidases that inhibit xylem colonization by the pathogens was observed (Floerl *et al*. 2008; Basha *et al*. 2010). To our knowledge, the proteome analysis of xylem sap of plants under abiotic stress is restricted to a single study, in which the proteomic changes in the xylem sap of maize under drought stress was analysed (Alvarez *et al*. 2008), and no one applied advanced differential proteome analysis platforms such as 2D differential in gel electrophoresis (2D-DIGE).

Xylem differentiation in higher plants is a highly regulated process that is induced in the tracheary elements of the vascular tissues and occurs in several steps (Turner, Gallois & Brown 2007). The third and final step of xylem differentiation, which includes secondary cell wall formation and programmed cell death, takes places after mesophyll cells multidifferentiation and procambial cells differentiation to tracheary elements precursors (Demura *et al*. 2002). After secondary cell wall deposition, programmed cell is induced and cell content is degraded by a macro-autolysis process (Turner *et al*. 2007; Avci *et al*. 2008). Therefore, the proteomic analysis of xylem sap could be representative of the last stages of xylem differentiation when lignification and cell death are induced. Xylem lignification has been observed in several species under salt stress (Cachorro *et al*. 1993; Jbir *et al*. 2001; Sanchez-Aguayo *et al*. 2004; Fernandez-Garcia *et al*. 2009). Class III peroxidases are thought to be involved in the cross-linking of monolignols present in xylem cell walls, thereby inducing the lignification of xylem elements (Passardi, Penel & Dunand 2004; Marjamaa, Kukkola & Fagerstedt 2009). The presence of peroxidases in xylem sap has been widely demonstrated in all species analysed by proteomic approaches (Buhtz *et al*. 2004; Kehr *et al*. 2005; Djordjevic *et al*. 2007; Agüero *et al*. 2008; Aki *et al*. 2008; Dafoe & Constabel 2009). Therefore, the aim of the present study is to identify the molecular mechanism underlying the respond of xylem sap to salt stress. We have determined changes at the level of proteins, metabolites and ions that may be involved in the process of root-to-shoot signalling and that may be selected as biomarkers to study xylem differentiation and lignification under salt stress.

MATERIALS AND METHODS

Plant material and growth conditions

Broccoli (*B. oleracea* var. italica cv. Marathon) is the market standard variety. This variety is moderately sensitive to salinity. Broccoli seeds (*B. oleracea* cv. Marathon) were imbibed and aerated, de-ionised in water for 12 h and germinated in vermiculite, at 28 °C in an incubator, for 2 d. They were then transferred to a controlled-environment chamber with a 16-h light-8-h dark cycle and air temperatures of 25 and 20 °C, respectively. After 5 d, the seedlings were placed in 15 L containers with continuously-aerated modified Hoagland nutrient solution (Hernandez *et al*. 2010). Plants were treated with 0 and 80 mM NaCl, corresponding to electrical conductivities of 2 and 10 dS cm^{-1}. The experimental groups were:

1 Control group (designated control): plants were grown in control nutrient solution for 33 d.
2 Experimental group 1 (designated 24 h): grown in control nutrient solution for 32 days followed by 24 h of salinization (80 mM NaCl).
3 Experimental group 2 (designated 7 d): grown in control nutrient solution for 26 d followed by 7 d of salinization (80 mM NaCl).

To eliminate differences between plants from the different experimental groups due to plant maturity, all plants were sampled out at the same age, i.e. 33 d, corresponding to the end of the experimental period.

Xylem sap collection

To ensure that the composition of xylem sap collected from detopped plants accurately reflected what was flowing in the intact plants, pressure was applied using a Scholander chamber to induce sap flow at a positive value equal to the negative of leaf water potential, previously calculated on each plant (Goodger *et al*. 2005). Xylem sap was collected for up to 1 h after cutting the stem 0.5 cm above the base of the stem. To avoid modifications induced for the circadian cycle, xylem sap was collected after 4 h from the beginning of the light period. The cut end was washed with distilled water, blotted with filter paper and the first 200 μL of sap discarded. The sap was collected with a silicon tube fitted over the stem section and continuously transferred to a tube kept on ice. At intervals of less than 5 min, samples were frozen in liquid nitrogen. The samples were stored frozen (–80 °C) until analysis. Each treatment involved four replicates.

Ion analysis

For the anion analysis, xylem sap was diluted and injected into a Dionex-D-100 ion chromatograph as described by Hernandez *et al*. (2010).

Analyses of amino acids, sugars and organic acids by high-performance liquid chromatography/mass spectrometry

The analyses were carried out on a high-performance liquid chromatography/mass spectrometry (HPLC/MS) system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a thermostatted μ-wellplate autosampler and a capillary pump, and connected to an Agilent Ion Trap XCT Plus Mass Spectrometer (Agilent Technologies) using an electrospray (ESI) interface.

Standards with known concentrations of each amino acid (10, 25, 50, 75 and 100 μM) and xylem samples were prepared in the mobile phase A, consisting of water/acetonitrile/formic acid (89.9:10:0.1), and passed through 0.22 μm filters. Then, 5 μL of each standard or sample were injected into a Zorbax SB-C18 HPLC column (5 μm, 150 × 0.5 mm, Agilent Technologies, Santa Clara, CA, USA). The UV chromatogram was recorded at 210 nm with the DAD module (Agilent Technologies, Santa Clara, CA, USA).

The chromatogram of each amino acid, sugar and organic acid ion from either standards or samples was extracted and the peak area was quantified using the DataAnalysis program for LC/MSD Trap Version 3.2 (Bruker Daltonik GmbH, Bremen, Germany). The peak area data of the standards were used for the calculation of the calibration curve, from which the concentrations of each amino acid in the samples were obtained.

Quantification of hydrogen peroxide

The hydrogen peroxide (H_2O_2) content in xylem sap of broccoli was determined by the methodology described by Cheeseman (2006). To avoid degradation of H_2O_2, samples were directly measured from the extracted xylem sap (50 μL).

Glutathione determinations

1 mL of frozen samples of xylem sap was lyophilized and resuspended in 100 μL of 5% meta-phosphoric acid and centrifuged at 10 000 *g* for 10 min. The supernatants were used to determine reduced glutathione (GSH) and oxidized glutathione (GSSG).The methods for analysis of GSSG and GSH was as described by Anderson, Chevone & Hess (1992).

Quantification and immunogold labelling of Arabinogalactan proteins

The concentration of arabinogalactan proteins (AGPs) was determined spectrophotometrically with Yariv reagent as described by Lamport, Kieliszewski & Showalter (2006), purchased from Biosupplies Australia (Parkville, Australia).

Three different antibodies were used – JIM13, LM2 and JIM4 – for subcellular location of AGPs. However, only JIM13 showed a positive labelling of the samples. Small pieces (1 × 1 mm) of leaves from control and salt treated roots were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), for 2 h at 4 °C, rinsed in the same buffer and dehydrated in an ethanol series. Samples were embedded in LR White. Ultra-thin sections (60–80 nm) were obtained with a Leica EM UC6 ultramicrotome (Leica Mikrosysteme, Hernalser Hauptstraße, Vienna, Austria) and collected on formvar-coated nickel grids and immunogold labelling was as described by Fernandez-Garcia, Piqueras & Olmos (2008). Samples were observed using a Philips Tecnai 12 electron microscope (Philips, Eindhoven, The Netherlands). At least 30 samples were analysed for statistical analysis.

Lignin staining

Fresh cut sections from the stem 0.5 cm above the base of the stem (150–200 μm thick) were obtained using a hand microtome. Lignin was detected using the method described by Fernandez-Garcia, Carvajal & Olmos (2004).

ABA quantification

ABA was quantified in triplicates by GC–MS, as described for gibberellins (GA) analysis (Rieu *et al*. 2008), but with modifications. Freeze-dried xylem sap samples were dissolved in 5 mL 80% (v/v) methanol/water in after which 25 ng [3-methyl-$^2H_3$]ABA was added as internal standard. Samples were purified as described previously except that the pooled ethyl acetate phases after elution from the Varian Bond Elut NH2 cartridge (100 mg; Kinesis, St. Neots, UK) were evaporated to dryness in vacuo and dissolved in ethyl acetate (20 μL), of which 2 μL was injected into a TR-1 capillary column (30 m × 0.25 mm × 0.25 mm film thickness; ThermoFisher ScientiWc, Madison, WI, USA) at 50 °C. The amount of ABA was determined from the peak areas for the ions *m*/*z* 190 and 193 by reference to a calibration curve.

Peroxidases quantification and IEF-gel electrophoresis

Proteins in the xylem sap were concentrated about 10-fold on Amicon 2 mL Ultra Centrifugal Filter Devices with a 10 kD MWCO (Millipore, Bedford, MA, USA). Protein concentrations in samples were determined by using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

Peroxidase activity in xylem sap was determined in assays containing 50 mM Tris-acetate buffer (pH 5.0) and 0.5 mM H_2O_2, using 50 μM syringaldazineor 1.0 mM 4-m4thoxy-*á*-naphtol. The reaction was initiated by the addition of enzyme. Peroxidase isozymes were separated by isoelectric focusing, using a MiniProtean III system (Bio-Rad) on 8.5% (w/v) polyacrilamide gels containing 1% glycerol and 5% ampholytes of Pharmalites (Pharmacia), pH 3–10 as described by (Hernandez *et al*. 2010).

Statistical analysis

The data was statistically analysed using parametric tests at a stringency of $P < 0.05$. The significance of variation in mean values was analysed using analysis of variance (ANOVA) and Tukey HSD tests (Statistix 8 program). Significant differences ($P < 0.05$) are indicated in the graphics by different letters (a, b or c), according to Tukey's test.

Proteomic analysis

2D DIGE

Protein labelling was performed using the CyDyes DIGE Fluors (Mackintosh *et al*. 2003) developed for fluorescence 2D DIGE technology (GE Healthcare, Amersham Place, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's recommendations. Fifty μg of each protein sample was covalently labelled with either Cy3 or Cy5 dye, and a mixture of 25 μg of each protein sample, with Cy2 dye which is used as technical replicate and internal standard. For statistical analysis, four biological replicates per group under comparison were used, and an experimental design for maximal randomization of groups and dye labels was applied in order to minimize biasing from the dyes (Alban *et al*. 2003).

2D Image analysis

The DIGE gels were scanned using a Typhoon 9410 variable mode scanner (Amersham Biosciences) set at appropriate wavelengths for each of the cyanine dyes. Image alignment, spot detection, matching and grouping were performed using SameSpots v3.0 with DIGE and Stats modules (Nonlinear Dynamics, Newcastle, UK). One of the Cy2-labeled pooled sample images was used as the reference gel. Statistical analysis of protein abundance changes between the sample groups was performed with a one-way ANOVA test and average ratio calculated for each matched spot as a measure of the differential protein expression. Matched spots with $P < 0.05$ and fold change above 1.5 were marked up as differentially expressed polypeptides.

Tryptic in-gel digestion

The selected spots from a Coomasie stained gel were excised from the gel and *in-gel* digested with trypsin endoprotease. Briefly, gel plugs were extensively washed with 25 mM ammonium bicarbonate to remove dye and SDS impurities, *in-gel*-reduced with 60 mM dithiothreitol (DTT) and S-alkylated with excess iodoacetamide followed by digestion with porcine trypsin (Promega) at 37 °C for 6 h. Peptides were extracted in 25 mM ammonium bicarbonate, then in 70% acetonitrile and finally in 1% formic acid. Extracted peptides were dried down in a speed-vac benchtop centrifuge and resuspended in 5 μL of 0.1% formic acid and 3% acetonitrile, suitable for HPLC-MS/MS analysis.

Mass spectrometry and de novo sequencing

Tryptic peptides were concentrated and desalted on a Zorbax 300SB-C18 cartridge (5 × 0.3 mm and 5 μm particle size, Agilent Technologies, Germany) and further separated on an analytical Zorbax 300SB RP C18 column (75 μm × 150 mm and 3.5 μm particle size, Agilent Technologies) using an Agilent 1200 HPLC system. Peptides were eluted at 300 nL min^{-1} flow rate by using a 60 min linear gradient from 5% to 40% solvent B (solvent A: 0.1% formic acid in water and solvent B: 0.1% formic acid in acetonitrile). The peptides were scanned and fragmented with an LTQ Orbitrap mass spectrometer (ThermoFisher Scientific) equipped with a Proxeon nano-ESI source (Proxeon). The three most intense precursor ions, ranging from 400 to 2000 *m/z*, were scanned and measured in the Orbitrap at a 60 000 resolution at *m/z* 400, and the corresponding fragment ions generated were measured in the Orbitrap at 7500 after higher-energy C-trap dissociation (HCD) fragmentation. Normalized collision energies used for HCD fragmentation was 40%.

Database searches were performed against NCBInr (non-redundant) database (ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz) containing 7873120 entries and using both SEQUEST and MASCOT search engines implemented in Proteome Discoverer software (Thermo Fisher Scientific). The following parameters were used for the searches: trypsin as the proteolytic enzyme, allowing for one missed cleavage; carboxyamidomethyl cisteine as fixed modifications; oxidation of methionine as variable modification. Precursor ion tolerances of 5 ppm and fragment ion tolerances of ±0.005 D were allowed. The resulting peptides were filtered to show the list of proteins identified with less than 1% false discovery rate. Positive identifications were considered only when two or more peptides where matched, and their score was >20 for MASCOT and >2.5 for SEQUEST.

RESULTS

Nutrient analysis

Salt-treated plants showed a rapid increase in Na^+ ions in xylem sap 24 h after the start of treatment, although the concentration was lower after 7 d (Fig. 1a). Similarly, an increase of K^+ ions in xylem sap was observed in salt-treated plants (Fig. 1a). The Ca^{2+} concentration in xylem sap was strongly reduced at 24 h of salt treatment. However, long-term treatments (7 d) induce a significantly higher concentration of calcium (Fig. 1b). Sulphur (S) concentrations were significantly reduced in xylem sap at 24 h of salt treatments but after 7 d of salt treatment the concentration was similar to control (Fig. 1b). Phosphorus (P) concentrations in xylem sap were not significantly affected by the salt treatments (Fig. 1b).

Chloride anions showed a much higher concentrations in xylem sap of salt-treated plants (Fig. 2) in parallel with Na^+ accumulation. Nitrate concentrations were highly increased in xylem sap by salt treatments, while phosphate concentrations were unaltered (Fig. 2), showing similar results to that for phosphorus. Sulphate concentrations were significantly reduced in the xylem sap after 24 h of salt treatment (from

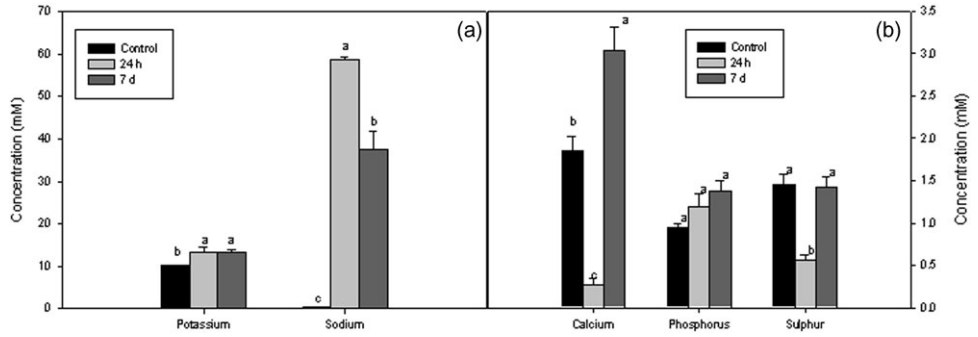

Figure 1. Contents of (a) potassium and sodium and (b) calcium, sulphur and phosphorus in xylem sap of *B. oleracea*, comparing control and plants treated with NaCl for 24 h and 7 d. Values represent the mean ± SD of five different samples. Significant differences ($P < 0.05$) are indicated by different letters, according to Tukey's test.

1.3 ± 0.38 mM in the control group to 0.66 ± 0.16 mM in the 24 h treated group, $P < 0.05$), although after 7 d of salt treatment, the concentration was again similar to that of control (Fig. 2), showing a similar trend to that of sulphur concentrations.

Organic solutes

We have identified more than 40 different organic solutes by mass spectrometry but we were able to quantify only 18 of the most abundant. Total amino acids were the main compounds of the xylem sap in control conditions, followed by sugars and organic acids (Table 1). However, xylem from salt-treated plants showed much higher concentrations of sugars than of amino acids.

The most abundant amino acids in control xylem sap were glutamine (Gln) and alanine (Ala), but their concentrations were greatly reduced after salt treatment, being 8- and 10-fold less, respectively, after 7 d, when the most abundant amino acids were Gln and leucine/isoleucine (Leu/Ile) (Table 1). Interestingly, Leu/Ile concentrations were unaltered by salt treatments (Table 1). The concentration of most of the amino acids was reduced by salt treatments, except for cysteine (Cys), which accumulated significantly after 7 d of treatment (Table 1).

Sugar concentrations were strongly increased in xylem sap after 7 d of salt treatments (Table 1). Sucrose was the major sugar present in xylem sap of control and salt treated plants.

Organic acid concentrations were little affected by salt treatments, except for citric acid, the concentration of which was significantly reduced after 7 d of salt treatment (Table 1).

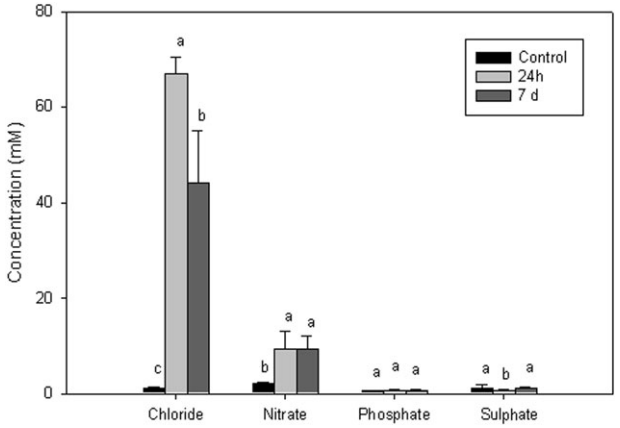

Figure 2. Chloride, nitrate, sulphate and phosphate content in xylem sap of *B. oleracea* comparing control and plants treated with NaCl for 24 h and 7 d. Values represent the mean ± SD of five different samples. Significant differences ($P < 0.05$) are indicated by different letters, according to Tukey's test.

Contents of hydrogen peroxide, reduced and oxidized glutathione and ABA

The concentration of H_2O_2 in xylem sap was reduced more than threefold after 24 h of salt treatments and remained at a lower concentration after7 d (Fig. 3).

The concentrations of reduced and oxidized glutathione were very low in the xylem sap from untreated plants (Fig. 4a), but there was a considerable increase in the concentration of both forms 24 h after initiating the salt treatment. However, after 7 d of salt treatment, the concentrations of reduced and oxidized glutathione were substantially decreased: in the case of GSSG, the level was similar to that of the control, while GSH concentration remained significantly higher in salt-treated plants, such that the GSH/GSSG ratio was highly increased by salt treatments (Fig. 4b).

ABA concentrations in xylem sap increased 5-fold within 24 h of the salt treatment, from which level it was substantially reduced after 7 d, although it was significantly higher than in sap from the untreated control (Fig. 5).

Arabinogalactan proteins quantification and immunolocation

Arabinogalactan proteins (AGPs) are glycoproteins that are probably involved in cell wall plasticity (Seifert &

Table 1. Amino acids, sugars and organic acids composition of the xylem sap of control, 24 h and 7 d after salt treatment (80 mM of NaCl) of *B. oleracea* plants

	Control μM	24 h μM	Fold change[a]	7 Days μM	Fold change[a]
Glutamine (Gln)	323.1 ± 55a	80.1 ± 24b	–4	43.5 ± 8.2b	–7.4
Alanine (Ala)	249.1 ± 37a	63.1 ± 17b	–3.9	25.3 ± 3.1c	–9.8
Leucine/Isoleucine(Leu/Ileu)	50.7 ± 15a	30.2 ± 10a	–1.7	45.8 ± 6.5a	–1.1
Glutamic (Glu)	47.4 ± 12a	21.6 ± 9b	–2.2	20.5 ± 6b	–2.3
Asparagine (Asn)	28.3 ± 7.2a	14.1 ± 5.4b	–2	22.7 ± 7.8a	–1.2
Valine (Val)	25.8 ± 6a	10.2 ± 2.2b	–2.5	10.5 ± 3.7b	–2.5
Threonine (Thr)	11.9 ± 3a	5.1 ± 2.8a	–2.3	9.2 ± 3.8a	–1.3
Cysteine (Cys)	10.8 ± 4b	5.6 ± 2.7b	–1.9	30.4 ± 7.5a	2.8
Phenylalanine (Phe)	8.5 ± 3.2a	1.5 ± 0.9b	–5.7	3.8 ± 1.2b	–2.2
Proline (Pro)	4.9 ± 2ab	2.8 ± 1.9b	–1.7	6.4 ± 2.9a	1.3
Glucose	93 ± 4b	79.1 ± 15b	–1.2	276.4 ± 42a	3
Fructose	25.4 ± 5.2c	54.1 ± 11b	2.1	135.5 ± 43a	5.3
Sucrose	234.5 ± 76b	301.3 ± 70b	1.3	760.8 ± 20a	3.2
Myo-Inositol	67.7 ± 21c	102.9 ± 9b	1.5	308.7 ± 4a	4.5
Succinic acid	53.3 ± 3a	62 ± 6a	1.2	58 ± 6a	1.1
Malic acid	2.4 ± 0.2a	2.8 ± 0.5a	1.2	2.5 ± 0.5a	1
Citric acid	16.6 ± 2.2a	16.2 ± 3.2a	1	11.9 ± 2.4b	–1.4
Fumaric acid	123.8 ± 30a	46.4 ± 10b	2.7	92.1 ± 27a	–1.3

Values represent the means ± SD of five different samples. Means within a range without a common letter are significantly different by Tukey test ($P < 0.05$). [a]Fold change compared to control.

Roberts 2007). We found a significant accumulation of AGPs in the xylem sap after 24 h of salt treatment (Fig. 6A). However, after 7 d of treatment the concentration of AGPs was similar to that of the control xylem sap (Fig. 6A). Immunogold labelling of AGPs using the antibody JIM13 (Fig. 6B) demonstrates that the main labelling was always in the cytoplasm of companion cells (CC) of xylem (Fig. 6B (b) (d)) and the secondary cell walls (SCW) of xylem elements (Fig. 6B (a) (c) and (d)). Quantification of gold labelling indicates that secondary cell walls of control xylem elements showed a higher density of labelling (10.4 ± 2.5, $n = 12$) (Fig. 6B (a) and (b)) than that of the salt-treated plants (4.9 ± 1.3, $n = 12$) (Fig. 6B (c) and (d)).

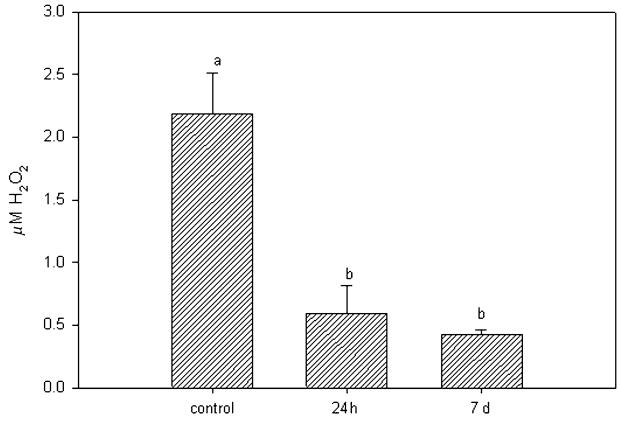

Figure 3. Hydrogen peroxide content in xylem sap of *B. oleracea* comparing control and plants treated with NaCl for 24 h and 7 d. Values represent the mean ± SD of five different samples. Significant differences ($P < 0.05$) are indicated by different letters, according to Tukey's test.

Peroxidase activity and isozymes pattern

The activity of peroxidases in the xylem sap was measured using different substrates: 4-methoxy-α-naphthol and syringaldazine (lignin analogue) as the electron donors (Fig. 7a & b). Our results demonstrate that salt treatments strongly increase the peroxidase activity independently of the substrate used in the assay.

Through isoelectrofocusing of the xylem sap samples, we have identified at least nine different peroxidase isozymes, four with a basic isoelectric point (B1 = 9.8; B2 = 9.1; B3 = 8.6 and B4 = 8.2, see Fig. 7c & d) and five with an acidic isoelectric point (A1 = 6.7; A2 = 4.1; A3 = 3.9; A4 = 3.8 and A5 = 3.4, see Fig. 7c & d).

The isoelectric pattern of xylem sap peroxidases were compared with the total isoelectric pattern of the whole root (Fig. 7d, marked as R). Only the isoform A5 was observed in both samples. This fact demonstrated that the majority of the peroxidases are specific for the xylem sap and the apoplast of companion cells.

We analysed the relative activity of each isozyme using a densitometric system (Fig. 7c). We observed that the isozyme A5 was the most abundant but it was unaltered by salt treatments. However, isozyme A1 was highly induced by salt treatments at 24 h and 7 d. Similarly, isozymes A2 and A4 were also induced by salt treatments. Basic isozymes seem to be slightly induced by salt treatments. Only B4 is significantly induced by salt treatments at 7 d.

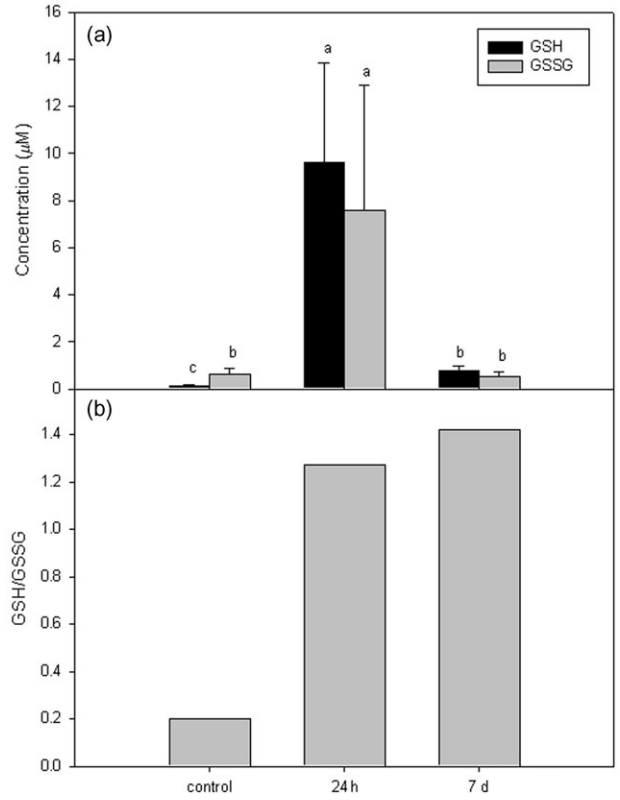

Figure 4. (a) Reduced glutathione and oxidized glutathione content and (b) ratio of reduced glutathione and oxidized glutathione in xylem sap of *B. oleracea* comparing control and plants treated with NaCl for 24 h and 7 d. (b) Values represent the mean ± SD of five different samples. Significant differences ($P < 0.05$) are indicated by different letters, according to Tukey's test.

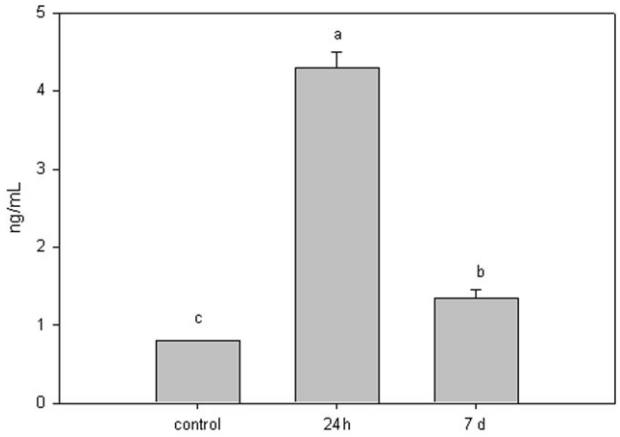

Figure 5. Abscisic acid content in xylem sap of *B. oleracea* comparing control and plants treated with NaCl for 24 h and 7 d. Values represent the mean ± SD of four different samples. Significant differences ($P < 0.05$) are indicated by different letters, according to Tukey's test.

Xylem lignification

Xylem lignification was analysed by phlorogucinol staining in control and salt-treated plants after 7 d (Fig. 8). The treatment increased lignification and reduced the size of xylem elements (Fig. 8).

Proteomic analysis

The xylem sap samples were analysed following 2D DIGE. More than 300 protein spots can be observed in the gels (Fig. 9a). Gel image analysis identified 76 proteins that were differently expressed in xylem sap of control and salt treated plants (Fig. 9b). These spots were excised from 2D gels, digested *in situ* with the protease trypsin followed by peptide sequencing after HPLC-MS/MS analysis and databases searches. This led to the identification of 40 spots matching proteins included in NCBI database (Table 2).

The identified proteins whose abundance changed fell into four major biological categories (see Fig. 10): cell wall

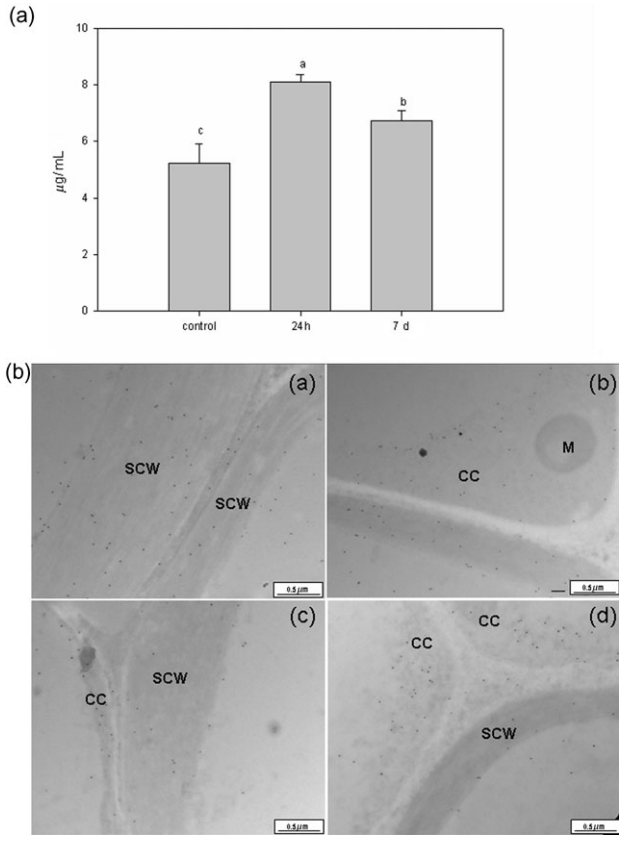

Figure 6. (A) Arabinogalactan proteins content in xylem sap of *B. oleracea* comparing control and plants treated with NaCl for 24 h and 7 d. Values represent the mean ± SD of five different samples. Significant differences ($P < 0.05$) are indicated by different letters, according to Tukey's test. (B) Immunlocalization of AGPs in xylem and companion cells of the xylem elements using the antibody JIM13 in control (a and b) and after 7 d of salt treatment (c and d). CC = companion cells; M = Mitochondria; SCW = Secondary cell wall.

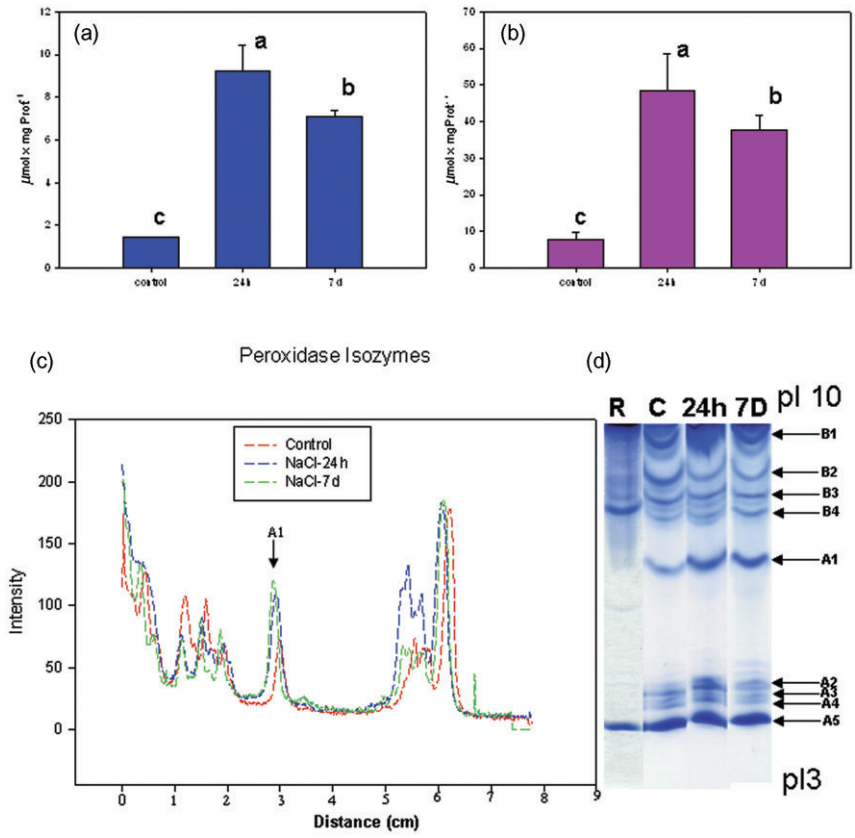

Figure 7. Total peroxidase activity using different substrates as 4-methoxy-á-naphtol (a) and syringaldazine (b). Peroxidase isoforms detected by isoelectrofocusing gel electrophoresis (pH 3-10) and densitrometic scan of the samples (c and d). The protein concentration loaded in each well was the same. R = Total root proteins.

metabolism, programmed cell death, plant defence metabolism and plant metabolism (mainly glycolysis metabolism).

Cell wall metabolism

Fourteen different spots were observed in this category. The majority of them can be classified as enzymes involved in cell wall remodelling and included glycosyl–hydrolases, polygalacturonases and α-L-arabinofuranosidases. Some of these protein spots led to the identification of the same protein in two or more different protein spots of similar molecular weight, but with a variable isoelectric point. The occurrence of similar proteins in different spots is a common feature observed in 2D gel electrophoretic analysis and may be caused by post-transcriptional modifications of distinct amino acids of single gene products, protein degradation or different protein isoforms. The expression of these isoforms was decreased by short (24 h) and long (7 d) salt treatments (Table 2, Fig. 10). We also identified a protein (spot 113, gi 15239747) with close homology with an hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase (HCT) from *Arabidopsis thaliana* that may be involved in cell wall lignification. This protein was significantly induced by salt treatments (see Table 2, Fig. 10).

Finally, a protein with homology to a peroxidase of *B. napus* (spot 29, gi 67772580) was induced by salt treatment (Fig. 10). Peroxidases are highly represented in plant genomes and many of them participate in the lignifications process of the cell walls, but they have also been shown to participate in plant defence mechanism.

Programmed cell death

Xylem sap of *B. oleracea* contained two different families of proteases that are decreased (3 aspartyl-proteases, spots 54, 64 and 114) or increased (2 Cys-proteases, spots 1 and 31) by salt treatment. Of these, a Cys-protease (spot 1, XCP1, *A. thaliana* gi 18418684) showed the largest change under salt stress after 24 h and 7 d (over 6-fold). A trypsin inhibitor (spot 6, *B. oleracea* var. botritys, gi183988816) that accumulated over 3.5-fold after 7 d of salt treatment was also identified (Table 2, Fig. 10).

Plant defence metabolism

This group includes proteins that are induced by pathogen infection (disease resistance proteins, β-glucanases), heat shock proteins and antioxidant enzymes [superoxide dismutases (SOD) and germin-like proteins]. A β-glucanase (spot 10 homology with *B. rapa*, gi 62361691) enzyme was decreased by salt stress. Two proteins (22 and 39) with homology to disease resistance-responsive family proteins of *A. thaliana* (gi 5222633) accumulated at 24 h of salt treatment but were significantly reduced at 7 d. Two heat shock proteins (spot 12, *A. thaliana*, gi123593; spot 17, *Petunia* × *hybrida*, gi20559) also accumulated in the xylem

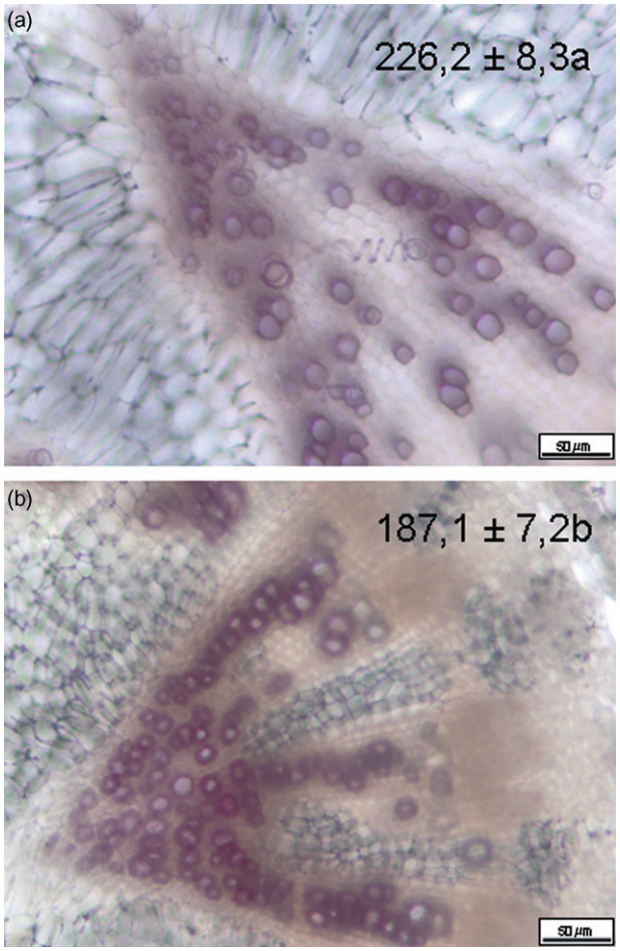

Figure 8. Fresh hand-microtome cross-sections of control (a) and 7 d after salt treatment (b) of *B. oleracea* stem bases showing the xylem stained with phloroglucinol. Xylem area is shown in the top right of each figure. Data were determined as described in material and methods. Values represent the mean ± SD of 100 different xylem elements. Significant differences ($P < 0.05$) are indicated by different letters, according to Tukey's test.

sap of salt-treated plants (Table 2, Fig. 10). Other proteins that accumulated after salt treatment were a Mn-SOD (spot 103, MSD1, *A. thaliana*, gi79313181) and a putative germin protein, represented by two spots (spot 4 and 24; *A. thaliana*, gi26449711), which increased > threefold.

Plant metabolism (glycolysis)

The majority of these proteins were involved in the last steps of glycolysis (Table 2, Fig. 10). Two different spots were identified as putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (spot 33, *A. thaliana*, gi18391066; spot 162, *A. thaliana*, gi21537260), which catalyses the interconversion of 3-phosphoglycerate and 2-phosphoglycerate. Both proteins were decreased under salt stress. Another protein was identified as enolase (spot 74, *B. rapa,* gi90194338), which catalyses the reversible dehydration of 2-phosphoglycerate to phosphoenolpyruvate (PEP). This protein accumulated in salt-treated plants, as did two fructokinases (spots 60, *A. thaliana*, gi14423528; spot 93, *Z. mays*, gi162460362).

DISCUSSION

Nutrient and cation movement through xylem sap is affected by salt stress

In a previous paper, we have observed that long salt stress treatments induced thickening (phi thickening) in the radial cell walls of the innermost layers of cortical cells (phi cells), in direct contact with the endodermis (Fernandez-Garcia *et al*. 2009). Similarly, we have also observed that phi thickening was induced after seven days of salinization (data not shown). One of the mechanisms that contributes to plant salinity tolerance is reduction of Na^+ loading into the xylem (Munns & Tester 2008). Along with other mechanisms, this can be accomplished by minimizing entry of sodium from the root apoplast (Plett & Moller 2010). Therefore, our observation that the concentration of Na^+ and Cl^- in the xylem sap is significantly reduced after seven days of salt treatment

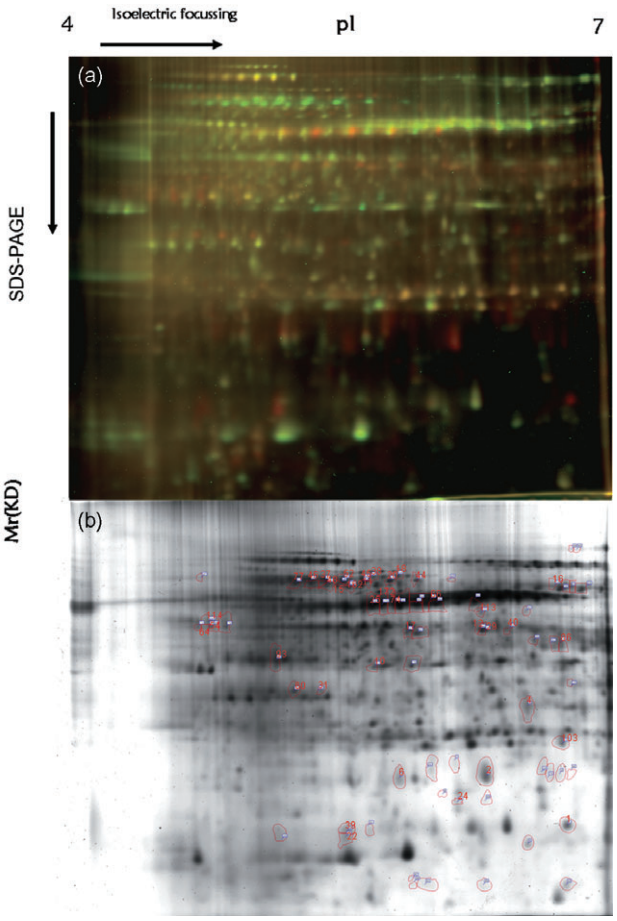

Figure 9. 2DIGE of *B. oleracea* xylem sap proteins. (a) Example of fluorescence gel image acquired using a Typhoon 9410. (b) Selected spots from stained gel. Numbers correspond to spots that were identified and listed in Table 2.

Table 2. Protein name and fold change of the proteins identified as being significantly regulated under salt stress classified according to their biological function

Spot N°	Fold Change[a] 24 h	Fold Change[a] 7 d	ANOVA (p)	Protein Similarity	pI/MW exp	pI/MW theo	GI accession	Organism matched	Scores (S/M)[b]	N° of Peptides matched	Functional Classification[c]
1	4.88	6.43	1.93E-05	XCP1 (Xylem cysteine peptidase 1)	6.7/16.9	5.6/39.6	gi18418684	*A. thaliana*	21.4(S)	10	PCD
2	6.39	4.99	8.85E-05	Disease resistance-responsive family protein	6.3/22.3	8.4/21.4	gi15236570	*A. thaliana*	15.2(S)	7	PDM
4	2.02	3.58	0.00017	Putative germin	6.5/32.8	8.4/23.5	gi26449711	*A. thaliana*	4.1(S)	2	PDM
6	3.38	3.04	0.00043	Trypsin inhibitor (TPI)	5.8/22.1	7.8/17.5	gi183988816	*B. oleracea*	3.5(S)	4	PCD
16	1.63	2.93	0.00146	EDA28/MEE23	6.6/63.7	6.0/59.6	gi15226830	*A. thaliana*	1.8(S)	2	PM
24	1.63	2.71	0.02744	Putative germin	6.1/19.4	8.4/23.5	gi26449711	*A. thaliana*	9.6(S)	5	PDM
25	1.97	2.69	0.00554	Curculin-like(mannose-binding) lectin family protein	5.6/57.6	7.8/49	gi15219200	*A. thaliana*	7.7(S)	4	CWM
31	2.62	2.64	0.00566	XCP1 (Xylem cysteine peptidase 1)	5.4/35.8	5.6/39.6	gi18418684	*A. thaliana*	8.7(S)	4	PCD
12	3.10	2.51	0.00514	Heat shock 70 kDa protein	6.2/50.1	5.2/70.5	gi123593	*Z. mays*	19.4(S)	11	PDM
40	2.40	2.48	0.00127	EDA28/MEE23	6.4/50.3	6.0/59.6	gi15226830	*A. thaliana*	126.8(M)	2	PM
29	2.66	2.45	0.00173	Peroxidase	6.3/49.7	5.8/38.9	gi67772580	*B. napus*	284.0(M)	4	CWM
17	2.92	2.35	0.00172	Heat shock protein (AA6-651)	5.8/49.6	5.1/70.8	gi20559	*Petunia x hybrida*	16.5(S)	7	PDM
60	2.10	2.15	0.00352	Putative frucktokinase	5.2/35.7	5.3/35.2	gi14423528	*A. thaliana*	15.3(S)	6	PM
66	1.25	2.08	0.00366	ALDH6B2 (Aldehyde dehydrogenase)	6.0/59.1	9.0/64.7	gi145328284	*A. thaliana*	155.0(M)	2	PM
74	1.30	1.97	0.00819	Los (enolase)	5.8/58.1	5.5/47.5	gi90194338	*B. rapa subsp. chinensis*	12.4(S)	6	PM
86	1.49	1.89	0.00076	EDA28/MEE23	6.7/46.5	6.0/59.6	gi15226830	*A. thaliana*	162.4(S)	2	PM
103	1.81	1.79	0.00128	MSD1(Manganese superoxide dismutase1)	6.7/26.6	8.5/25.3	gi79313181	*A. thaliana*	7.2(S)	2	PDM
113	1.64	1.77	0.00076	Transferase family	6.2/54.9	6.2/48	gi15239747	*A. thaliana*	168.0(M)	2	CWM
93	1.86	1.76	0.00342	Fructokinase 1	5.1/42.5	4.9/34.7	gi162460362	*Z. mays*	4.1(S)	2	PM
172	1.11	1.54	0.00924	Putative leucine aminopeptidase	5.7/57.6	5.1/22.2	gi62321351	*A. thaliana*	9.5(S)	3	PDM
22	2.76	1.33	0.00079	Disease resistance-responsive family protein	5.5/16	8.4/20.7	gi15222633	*A. thaliana*	7.2(S)	4	PDM
39	2.48	1.31	0.00021	Disease resistance-responsive family protein	5.5/16.5	8.4/20.7	gi15222633	*A. thaliana*	5.5(S)	2	PDM
162	–1.18	–1.60	0.02837	Putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	5.5/63.5	5.5/60.7	gi21537260	*A. thaliana*	12.7(S)	4	PM
114	–1.77	–1.60	0.00038	Putative aspartyl protease	4.8/52.6	8.1/52.4	gi21595063	*A. thaliana*	7.8(S)	3	PCD
77	–1.92	–1.95	0.00304	Glycosidase hydrolase family 28/polygalacturonase	5.2/65	5.5/54.7	gi15236514	*A. thaliana*	24.1(S)	8	CWM
64	–1.91	–2.10	0.00010	Putative aspartyl protease	4.8/51.2	8.1/52.4	gi21595063	*A. thaliana*	4.6(S)	2	PCD
54	–2.21	–2.17	2.88E-05	Putative aspartyl protease	4.8/51.3	8.1/52.4	gi21595063	*A. thaliana*	4.7(S)	3	PCD
52	–1.83	–2.29	0.01594	Glycosidase hydrolase family 28/polygalacturonase	5.5/66	5.5/54.7	gi15236514	*A. thaliana*	24.3(S)	10	CWM
48	–1.74	–2.34	0.00156	Pectinesterase family protein	5.4/64.7	5.9/60.4	gi15242495	*A. thaliana*	249.0(M)	2	CWM
46	–2.07	–2.38	0.00106	Alpha-L-arabinofuranoside	5.3/65.6	8.1/83.8	gi74355968	*R. sativus*	19.7(S)	5	CWM
45	–1.87	–2.39	0.01670	Alpha-L-arabinofuranoside	5.6/66	8.1/83.8	gi74355968	*R. sativus*	21.2(S)	10	CWM
44	–1.97	–2.39	0.01027	Alpha-L-arabinofuranoside	5.9/65.7	8.1/83.8	gi74355968	*R. sativus*	29.9(S)	15	CWM
10	–3.17	–2.47	5.73E-05	Beta-1,3-glucanase	5.7/40.9	9.3/40.7	gi62361691	*B. rapa subsp. chinensis*	9.4(S)	5	PDM
38	–2.00	–2.50	0.02129	Glycosidase hydrolase family 28/polygalacturonase	5.6/66.9	5.5/54.7	gi15236514	*A. thaliana*	16.7(S)	7	CWM
37	–2.11	–2.50	0.02131	Glycosidase hydrolase family 28/polygalacturonase	5.4/65.7	5.5/54.7	gi15236514	*A. thaliana*	47.1(S)	21	CWM
33	–2.03	–2.6	0.00144	Putative 2,3-biphosphoglycerate-independent phosphoglycerate mutase	5.5/63.9	5.3/60.6	gi18391066	*A. thaliana*	10.8(S)	3	PM
20	–2.36	–2.88	0.00101	Alpha-L-arabinofuranoside	5.7/65.6	8.1/83.8	gi74355968	*R. sativus*	16.9(S)	5	CWM
18	–2.29	–2.90	0.01383	Alpha-L-arabinofuranoside	5.8/67.4	8.1/83.8	gi74355968	*R. sativus*	12.6(S)	6	CWM
15	–2.58	–2.99	0.00438	Glycosidase hydrolase family 28/polygalacturonase	5.5/65	5.5/54.7	gi15236514	*A. thaliana*	21.3(S)	11	CWM
11	–2.50	–3.10	0.0029	Glycosidase hydrolase family 28/polygalacturonase	5.6/65.1	5.5/54.7	gi15236514	*A. thaliana*	20.2(S)	8	CWM

[a]Fold change: Salt stress vs. control.
[b]S = Search by SEQUEST; M = Search by MASCOT.
[c]CWM, Cell wall metabolism; PCD, Programmed cell death; PDM, Plant defence metabolism; PM, Plant metabolism (Others).

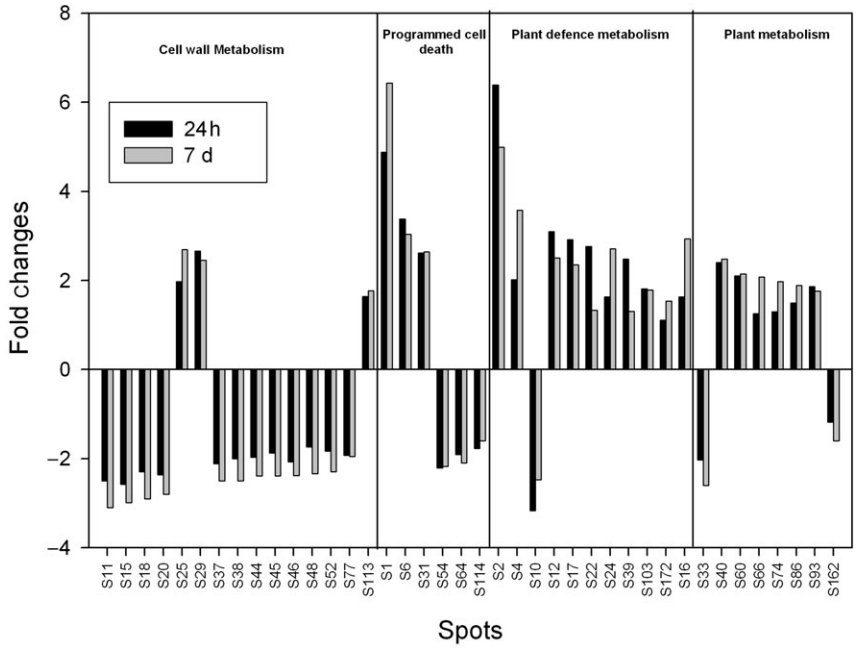

Figure 10. General classification and fold changes related to control of xylem proteins after 24 h and 7 d of NaCl treatment in *B. oleracea*.

relative to 24 h could be explained by phi thickening producing a partial apoplastic barrier to cation movement from the cortex to the stele (Fernandez-Garcia *et al*. 2009).

B. oleracea plants have been grown hydroponically, where the only source of nitrogen was nitrate. Roots take up the nitrate and convert a portion into nitrite (via nitrate reductase) that is transformed into ammonia (via nitrite reductase) and then assimilated in amino acids, mainly glutamine and/or asparagine. These amino acids can be stored in the roots or transported by the xylem to the aerial part where they are chemically reduced. Salinity reduces nitrate reductase and nitrite reductase activities and therefore reduces nitrate conversion into amino acids (Carrillo *et al*. 2005; Surabhi *et al*. 2008). Our results demonstrated that xylem sap of salt-stressed plants accumulated nitrate (about 5-fold relative to non-stressed plants), while glutamine concentration was highly reduced. This could be explained by the study of Shelp (1987), in which *B. oleracea* plants grown in high nitrate concentrations in the absence of ammonium accumulated nitrate in the xylem sap while glutamine concentration was reduced (from 75 to 42% of total amino acids concentration). Hence, these results suggest that salinity may induce nitrate loading into the root xylem and inhibit nitrate assimilation into amino acids, then transporting nitrate via xylem sap to the aerial part where is chemically reduced (Shelp 1987). Recently, Wilkinson, Bacon & Davies (2007) have proposed that an increase in the concentration of nitrate transported by xylem sap can act as a signal for drought stress, closing stomata and reducing leaf growth. These authors have proposed a mechanism that is mediated by a pH-based ABA redistribution and nitrate accumulation in the xylem sap. Therefore, xylem sap of *B. oleracea* after short salt treatments (24 h) could be transporting higher nitrate and ABA to the leaves, inducing stomatal closure, so reducing stomatal conductance and leaf growth, as we have previously observed in this species (Fernandez-Garcia *et al*. 2009). Moreover, glycophyte species decrease the rate of leaf and shoot growth after long salt treatments. A hormonal control of plant growth during salt adaptation mediated by the interaction of ABA and gibberellins has been proposed. ABA can inhibit leaf and shoot elongation by lowering the content of active GAs (Munns & Tester 2008).

Glutathione and Cys are considered to be the main thiols present in xylem sap of trees and herbaceous plants (Kostner *et al*. 1998) and their presence has been demonstrated in several species, including *B. napus* (Kostner *et al*. 1998; Mendoza-Cozati *et al*. 2008). Glutathione and Cys transport through xylem elements from the root to shoot may be an important mechanism of sulphur transport in the form of thiols compounds. Glutathione was highly accumulated in xylem sap of *B. olereacea* after 24 h of salt treatment and reduced after 7 d. Interestingly, *B. oleracea* roots also showed a high increase of glutathione content after 24 h of salt treatment (Fernandez-Garcia *et al*. 2009). It is possible that salt stress induces glutathione synthesis in the roots under short-term stress via sulphate assimilation in Cys (both compounds decreased their concentrations after 24 h of salt treatments) and is then loaded into xylem to be transported to the aerial part. Moreover, the GSH/GSSG ratio was highly increased by salt stress, which could be due to a higher GR activity in the xylem sap. Recently, Wang *et al*. (2008) have described the presence of GR in xylem sap of poplar that is induced under salt stress in the salt sensitive genotypes.

Xylem differentiation and lignification is induced by salt stress

The role of xylem lignification and the formation of new tracheary elements under different stresses, mainly salt

stress, seems to be important in water and nutrient movement (Sanchez-Aguayo *et al*. 2004; Fernandez-Garcia *et al*. 2009). Our results demonstrate that under salt stress two Cys proteases (homologues to *Arabidopsis* XCP1) were highly accumulated in xylem sap. In *A. thaliana*, it has been demonstrated by immunolabelling that XCP1 and XCP2 are specifically located and accumulated in the last steps of tracheary element differentiation (Avci *et al*. 2008). These Cys proteases are considered to carry out micro-autolysis within the intact central vacuole, where they may be degrading proteins as part of nutrient recycling and probably participating in the last steps of the macro-autolysis, initiated by vacuolar implosion. Similarly, when xylem differentiation was induced in cell cultures of *Zinnia elegans*, ZCP4 (a homolog of XCP1) accumulated in the last stage of xylem differentiation (Demura *et al*. 2002). Furthermore, three putative aspartic peptidases were found, of which only one increased significantly in the last stage of xylem differentiation. However, in *B. oleracea* the three putative aspartic peptidases were decreased by salt stress. Interestingly, it has also been observed that salt stress induced the accumulation of a trypsin inhibitor in xylem sap of *B. oleracea*. The presence of proteinase inhibitors could be a safety mechanism in the apoplast involved in the control of proteases that acts during tracheary element formation (Endo, Demura & Fukuda 2001). These facts probably indicate that xylem differentiation was induced under salt stress. It seems to be a finely controlled mechanism, in which proteinases, mainly Cys proteases, are involved.

We have previously observed that roots of *B. oleracea* were lignified under long salt-treatments (Fernandez-Garcia *et al*. 2009). Herein, the analysis of the stem bases demonstrated that after 7 d of salinization the xylem was also lignified. Previous studies of the xylem sap proteome of *B. oleracea* and *B. napus* have demonstrated the abundance of acid and cationic peroxidases (Buhtz *et al*. 2004; Kehr *et al*. 2005). Our proteomic results demonstrate a significant accumulation of one putative peroxidase isozyme under salt stress. In 2D electrophoresis, we used an isolectric pH range from 4 to 7, so basic peroxidases were not resolved in these gels. Surprisingly, the studies of peroxidase activity in xylem sap have received scarce study in the literature. Biles & Abeles (1991) analysed the peroxidase isozymes of the xylem sap of several species, observing that the majority were acid isozymes. However, the isozyme pattern of *B. oleracea* showed both acid and basic isozymes. The analysis of the total peroxidase activity demonstrated much higher peroxidase activity in *B. oleracea* xylem sap under salt stress. This increase was principally due to the activity of acid isozymes, mainly an isozyme with a pI of 6.7, showing a similar pI to that observed with 2D analysis. Quiroga *et al*. (2001) have described an increase in the amount of a cationic peroxidase (pI 9.6) in tomato roots under salt stress. However, it is not clear whether acidic or basic peroxidases are specifically related to lignification under different stresses (Passardi *et al*. 2004; Marjamaa *et al*. 2009). Strikingly, hydrogen peroxide concentration was highly reduced in xylem sap under salt stress. In our opinion, this might be explained by the higher peroxidase activity present in xylem sap of salt-treated plants that consumes hydrogen peroxide in the polymerization of monolignols during lignification (Marjamaa *et al*. 2009).

In addition, the hypothesis that lignification is induced is also suggested by higher amounts of a putative hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase (HCT)-like transferase (spot 113, gi 5239747) after salt treatments. This enzyme has been implicated in the monolignol pathway, catalysing the reactions both immediately preceding and following the insertion of the 3-hydroxyl group into monolignol precursors (Hoffmann *et al*. 2004). Transgenic alfalfa (*Medicago sativa* L.) plants with a 50% reduction in HCT activity induced by gene silencing through antisense expression showed a reduced lignin content (Shadle *et al*. 2007). Therefore, the higher levels of this protein observed in *B. oleracea* under salt stress might be related to higher lignification observed in the xylem elements.

The presence of AGPs in the xylem sap has been described in *Cucurbita maxima* where it was the main polysaccharide component of the xylem sap (Iwai *et al*. 2003). *B. oleracea* xylem sap contains a similar concentration of total AGPs to *C. maxima* (in *C. maxima* 3.9 μg/mL and *B. oleracea* 5.2 μg/mL). The role of these proteins is not totally understood but they have been implicated in many biological processes such as cell proliferation and survival, cell–cell interaction, pattern formation and growth, plant microbe interaction, etc. (Seifert & Roberts 2007). Motose, Sugiyama & Fukuda (2004) have demonstrated that an AGP protein, previously referred to as xylogen (Motose, Sugiyama & Fukuda 2001), accumulates in the cell walls of differentiating tracheary elements. This xylogen induced xylem differentiation in *Z. elegans* cells cultured *in vitro* (Motose *et al*. 2001). These authors also demonstrated that a double mutant of *A. thaliana* that encoded xylogen proteins showed defects in vascular development (Motose *et al*. 2004). However, the role of xylogen in the response to abiotic stress has been scarcely studied in the literature. Different cell cultures (tobacco BY-2, tomato, acacia and *Arabidopsis*) adapted to high salinity showed an accumulation of AGPs in the cell culture media (Lamport *et al*. 2006). These authors have speculated that AGPs can act under salt stress as cell wall plasticizers or 'xylem conditioners' affecting hydraulic conductance and probably contributing to xylem sap desalination (Zimmermann *et al*. 2002; Zwieniecki, Thompson & Holbrook 2002; Lamport *et al*. 2006). Interestingly, our results confirm a significant increase in AGP content after 24 h of salt treatments. Immunolocation of AGPs in *B. oleracea* demonstrated their presence in the secondary cell walls of xylem elements. A similar location of JIM13 (AGP epitope) has been observed in the secondary cell walls of xylem in maize coleoptiles and *Z. elegans* (Schindler, Bergfeld & Schopfer 1995; Stacey *et al*. 1995; Zhang *et al*. 2003). However, the JIM13 was present a lower levels in the secondary cell walls of xylem elements of salt treated plants of *B. oleracea*. AGPs are actively excreted by the companion cells of xylem elements and accumulated in

the xylem sap but their presence is reduced in the secondary cell wall, so reducing its plasticity. A reduction in the levels of enzymes, such as pectinesterases, arabinofuranosidases and glycosyl hydrolases, which are implicated in cell wall degradation suggests that a reduction of cell wall stiffening is also induced by salt stress.

Salt stress induces accumulation of defence proteins in the xylem sap

The presence of the Halliwell-Asada-Foyer cycle in the apoplast and xylem sap in plants remains to be demonstrated. However, the enzymatic activity of some of these enzymes, e.g. SOD, APX, DHAR, GR in apoplast and xylem sap has been described in the literature (Vanacker, Carver & Foyer 1998; Hernandez *et al*. 2001; Wang *et al*. 2008), and their presence has been demonstrated by proteomic analysis (Djordjevic *et al*. 2007; Aki *et al*. 2008; Dafoe & Constabel 2009). Extracellular forms of SOD have been found in Scots pine needles (Streller & Wingsle 1994), maize root apoplast (Kukavica, Vucinic & Vuletic 2005) and immunolocalized in the apoplast of spinach, *Pinus sylvestris* and *Z. elegans* (Ogawa, Kanematsu & Asada 1996; Karpinska *et al*. 2001; Karlsson *et al*. 2005). In agreement with these observations, *B. oleracea* xylem sap showed a significant accumulation of a putative Mn-SOD induced by salt stress. Similarly, Wang *et al*. (2008) recently reported SOD activity in xylem sap of poplar. The activity was induced under saline conditions, but only in salt-sensitive genotypes. These authors proposed that antioxidant enzyme activity may function to prevent ROS accumulation in xylem sap, but SOD activity may also be involved in hydrogen peroxide production for lignification (Kukavica *et al*. 2009). A NADPH oxidase present in the plasma membrane of the tracheary elements of xylem under development and/or parenchyma cells associated with xylem elements may be a source of radical superoxide production that is converted into hydrogen peroxide by apoplastic oxidereductases such as peroxidases (Barcelo 2005) and/or superoxide dismutase (Ogawa, Kanematsu & Asada 1997; Karlsson *et al*. 2005). We consider that this role of SOD could have its main function in the xylem cell walls and xylem sap. Interestingly, two putative germins were also induced by salt treatments. These proteins have been described as having oxalate oxidase and superoxide dismutase activities generating hydrogen peroxide in the apoplast (Dunwell *et al*. 2008). Therefore, different roles have been proposed for germins and germin-like proteins, including plant development, embryogenesis, lignification, senescence, biotic and abiotic responses (Dunwell *et al*. 2008). Germins have been mainly implicated in germination and stress response (Bernier & Berna 2001). Dani *et al*. (2005) have observed an enhancement accumulation of a germin-like protein in the apoplast of *Nicotiana tabacum* under salt stress.

Finally, we have also observed the accumulation of three proteins related disease resistance. The accumulation of these proteins in the xylem sap has been described for several species infected with bacteria or fungi (Houterman *et al*. 2007; Floerl *et al*. 2008; Subramanian *et al*. 2009; Basha *et al*. 2010). Similarly, we have also observed the accumulation of two heat-shock proteins in the xylem sap of salt stressed plants of *B. oleracea*. These proteins participate in maintaining proteins in their functional conformations, acting as chaperonins. They can also act by preventing aggregation of non-native proteins, promoting refolding of denatured proteins to regain their functional conformation and removal of non-functional but potentially harmful polypeptides. The accumulation of heat shock proteins under different abiotic stresses seems to be a general adaptation mechanism to protect cells against the stress conditions (Timperio, Egidi & Zolla 2008).

In this work, we have investigated the composition of the xylem sap under salt stress conditions. We have analysed ionic content, 18 different organic solutes by HPLC MS/MS and proteins by DIGE technique. We have found differences in the content of small molecules and proteins in the xylem sap of *B. oleracea* under salt stress conditions. These differences may be related to the response mechanisms to salinity of the plant to avoid the negative effects of salt in growth and development. Based on our results, we hypothesise that xylem differentiation and lignification might be involved in adaptation to salt stress conditions, probably by affecting the root-to-shoot water and nutrient transport. We have also observed the accumulation of defence proteins, which may be considered a general mechanism of response to stress conditions such as drought or pathogen infection.

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REFERENCES

Agüero C.B., Thorne E.T., Ibanez A.M., Gubler W.D. & Dandekar A.M. (2008) Xylem sap proteins from *Vitis vinifera* L. Chardonnay. *American Journal Enology Viticulture* **59,** 306–311.

Aki T., Shigyo M., Nakano R., Yoneyama T. & Yanagisawa S. (2008) Nano scale proteomics revealed the presence of regulatory proteins including three FT-like proteins in phloem and xylem saps from rice. *Plant and Cell Physiology* **49,** 767–790.

Albacete A., Martinez-Andujar C., Ghanem M.E., Acosta M., Sanchez-Bravo J., Asins M.J., Cuartero J., Lutis S., Dodd I.C. & Perez-Alfocea F. (2009) Rootstock-mediated changes in xylem ionic and hormonal status are correlated with delayed leaf senescente, and increased leaf area and crop productivity in salinized tomato. *Plant, Cell & Environment* **32,** 928–938.

Alban A., David S.O., Bjorkesten L., Andersson C., Sloge E., Lewis S. & Currie I. (2003) A novel experimental design for comparative two dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics* **3,** 36–44.

Alvarez S., Goodger J.Q.D., Marsh E.L., Chen M.S., Asirvatham V.S. & Schachtman D.P. (2006) Characterization of the maize xylem sap proteome. *Journal of Proteome Research* **5,** 963–972.

Alvarez S., Marsh E.L., Schroeder S.G. & Schachtman D.P. (2008) Metabolomic and proteomic changes in the xylem sap of maize under drought. *Plant, Cell & Environment* **31,** 325–340.

Anderson J.V., Chevone B.I. & Hess J.L. (1992) Seasonal-variation in the antioxidant system of eastern white-pine needles-Evidence for thermal dependence. *Plant Physiology* **98,** 501–508.

Avci U., Petzold H.E., Ismail I.O., Beers E.P. & Haigler C.H. (2008) Cysteine proteases XCP1 and XCP2 aid micro-autolysis within the intact central vacuole during xylogenesis in Arabidopsis roots. *The Plant Journal* **56,** 303–315.

Barcelo A.R. (2005) Xylem parenchyma cells deliver the H_2O_2 necessary for lignification in differentiating xylem vessels. *Planta* **220,** 747–756.

Basha S.M., Mazhar H. & Vasanthaiah H.K.N. (2010) Proteomics approach to identify unique xylem sap proteins in Pierce's disease-tolerant *Vitis* species. *Applied Biochemistry Biotechnology* **160,** 932–944.

Bernier F. & Berna A. (2001) Germins and germin-like proteins: plant do-all proteins. But what do they do exactly? *Plant Physiology and Biochemistry* **39,** 545–554.

Biles C.L. & Abeles F.B. (1991) Xylem sap proteins. *Plant Physiology* **96,** 597–601.

Buhtz A., Kolasa A., Arlt K., Walz C. & Kehr J. (2004) Xylem sap protein composition is conserved among different plant species. *Planta* **219,** 610–618.

Cachorro P., Ortiz A., Barcelo A.R. & Cerda A. (1993) Lignin deposition in vascular tisúes in *Phaseolus-vulgaris* roots in response to salt stress and Ca^{+2} ions. *Phyton-Annales Rei Botanicae* **33,** 33–40.

Carrillo P., Mastrolonardo G., Nacca F. & Fuggi A. (2005) Nitrate reductase in durum wheat seedling as affected by nitrate nutrition and salinity. *Functional Plant Biology* **32,** 209–219.

Cheeseman J.M. (2006) Hydrogen peroxide concentrations in leaves under natural conditions. *Journal of Experimental Botany* **57,** 2435–2444.

Chen S., Li J., Wang S., Huttermann A. & Altman A. (2001) Salt, nutrient uptake and transport, and ABA of *Populus euphratica*; a hybrid in response to increasing soil NaCl. *Trees* **15,** 186–194.

Dafoe N.J. & Constabel C.P. (2009) Proteomic analysis of hybrid poplar xylem sap. *Phytochemistry* **70,** 856–863.

Dani V., Simon W.J., Duranti M. & Croy R.D.R. (2005) Changes in the tobacco leaf apoplast proteome in response to salt stress. *Proteomics* **5,** 737–745.

De Sousa C.A.F. & Sodek L. (2003) Alanine metabolism and alanine aminotransferase activity in soybean (*Glycine max*) during hypoxia of the root system and subsequent return to normoxia. *Environmental and Experimental Botany* **50,** 1–8.

Demura T., Tashiro G., Horiguchi G., *et al.* (2002) Visualization by comprehensive microarray analysis of gene expression programs during transdifferentiation of mesophyll cells into xylem cells. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 15794–15799.

Djordjevic M.A., Oakes M., Li D.X., Hwang C.H., Hocart C.H. & Gresshoff P.M. (2007) The *Glycine max* xylem sap and apoplast proteome. *Journal of Proteome Research* **6,** 3771–3779.

Dodd I.C. (2005) Root-to-shoot signaling: assessing the roles of 'up' in the up and down world of long-distance signaling in plant. *Plant and Soil* **274,** 251–270.

Dunwell J.M., Gibbings J.G., Mahmood T. & Naqvi S.M.S. (2008) Germin and germin-like proteins: evolution, structure, and function. *Critical Reviews in Plant Science* **27,** 342–375.

Endo S., Demura T. & Fukuda H. (2001) Inhibition of proteosome activity by the TED4 protein in extracellular space: a novel mechanism for protection of living cells from injury caused by dying cells. *Plant and Cell Physiology* **42,** 9–19.

Evert R.F. (2007) Xylem cell types and developmental aspects. In *Esau's Plant Anatomy. Meristems, Cells, and Tissues of the Plant Body – Their Structure, Function, and Development* (ed. R.F. Evert) 3rd edn, pp. 255–290. Wiley-Interscience, Hoboken, NJ, USA.

Fernández-García N., Carvajal M. & Olmos E. (2004) Graf union formation in tomato plants: peroxidase and catalase involvement. *Annals of Botany* **93,** 53–60.

Fernandez-Garcia N., Piqueras A. & Olmos E. (2008) Sub-cellular location of H_2O_2 peroxidases and pectin epitopes in control and hyperhydric shorts of carnation. *Environmental and Experimental Botany* **62,** 168–175.

Fernandez-Garcia N., Lopez-Perez L., Hernandez M. & Olmos E. (2009) Role of phi cells and the endodermis under salt stress in *Brassica oleracea*. *New Phytologist* **181,** 347–360.

Floerl S., Druebert C., Majcherczyk A., Karlovsky P., Kües U. & Polle A. (2008) Defence reactions in the apoplástica proteome of oilseed rape (*Brassica napus var. napus*) attenuate *Verticillium longisporum* growth but not disease symptoms. *BMC Plant Biology* **8,** 129.

Gollan T., Schurr U. & Schulze E.D. (1992) Stomatal response to drying soil in relation to changes in the xylem sap composition of *Helianthus annuus*. I. The concentration of cations, anions, amino acids in, and pH of, the xylem sap. *Plant, Cell & Environment* **15,** 551–559.

Gomez-Cadenas A., Tadeo F.R., Primo-Millo E. & Talon M. (1998) Involvement of abcisic acid and ethylene in the responses of citrus seedling to salt shock. *Physiologia Plantarum* **103,** 475–484.

Goodger J.Q.D., Sharp R.E., Marsh E. & Schachtman D.P. (2005) Relationships between xylem sap constituents and leaf conductance of well-watered and water-stressed maize across three xylem sap sampling techniques. *Journal of Experimental Botany* **53,** 2389–2400.

Hernandez J.A., Jimenez A., Ferrer M.A., Jimenez A., Barcelo A.R. & Sevilla F. (2001) Antioxidant systems and $O^{-2}(.-)/H_2O_2$ production in the apoplast of pea leaves. Its relation with salt-induced necrotic lesions in minor veins. *Plant Physiology* **127,** 817–831.

Hernandez M., Fernandez-Garcia N., Diaz-Vivancos P. & Olmos E. (2010) A different role of hydrogen peroxide and the antioxidative system under short and long salt stress in *Brassica oleracea* roots. *Journal of Experimental Botany* **61,** 521–535.

Hoffmann L., Besseau S., Geoffroy P., Ritzenthaler C., Meyer D., Lapierre C., Pollet B. & Legrand M. (2004) Silencing of hydroxyl-cinnamoyl-coenzyme A shikimate/quinate hydroxycinnamoyltransferase affects phenylpropanoid biosynthesis. *The Plant Cell* **16,** 1446–1465.

Houterman P.M., Speijer D., Dekker H.L., Koster C.G., Cornelissen B.J.C. & Rep M. (2007) The mixed xylem sap proteome of *Fusarium oxysporum*-infected tomato plants. *Molecular Plant Pathology* **8,** 215–221.

Iwai H., Usui M., Hoshino H., Kamada H., Matsunaga T., Kakegawa K., Ishii T. & Satoh S. (2003) Analysis of sugars in squash xylem sap. *Plant and Cell Physiology* **44,** 582–587.

Jbir N., Chaibi W., Ammar S., Jemmali A. & Ayadi A. (2001) Root growth and lignification of two wheat species differing in their sensitivity to NaCl, in response to salt stress. *Comptes Rendus de l'Academie des Sciences. Serie III-Sciences de la Vie-Life Science* **324,** 863–868.

Karlsson M., Melzer M., Prokhorenko I., Johansson T. & Wingsle G. (2005) Hydrogen peroxide and expression of hipI-superoxide dismutase are associated with the development of secondary cell walls in *Zinnia elegans*. *Journal of Experimental Botany* **56,** 2085–2093.

Karpinska B., Karlsson M., Schingel H., Streller S., Suss K.H., Melzer M. & Wingsle G. (2001) A novel superoxide dismutase with a high isoelectric point in higher plants. Expression, regulation, and protein localization. *Plant Physiology* **126,** 1668–1677.

Kehr J., Buhtz A. & Giavalisco P. (2005) Analysis of xylem sap proteins from *Brassica napus*. *BMC Plant Biology* **5,** 11.

Kostner B., Schupp R., Schulze E.D. & Rennenberg H. (1998) Organic and inorganic sulfur transport in the xylem sap and the sulfur budget of *Picea abies* trees. *Tree Physiology* **18,** 1–9.

Kukavica B., Vucinic Z. & Vuletic M. (2005) Superoxide dismutase, peroxidase, and germin-like protein activity in plasma membranes and apoplast of maize roots. *Protoplasma* **226,** 191–197.

Kukavica B., Mojovic M., Vucinic Z., Maksimovic V., Takahama U. & Jovanovic S.V. (2009) Generation of hydroxyl radical in isolated pea root cell wall, and the role of cell wall-bound peroxidase, Mn-SOD and phenolics in their production. *Plant and Cell Physiology* **50,** 304–317.

Lamport D.T.A., Kieliszewski M.J. & Showalter A.M. (2006) Salt stress upregulates periplasmic arabinogalactan proteins: using salt stress to analyse AGP function. *New Phytologist* **169,** 479–492.

Mackintosh J.A., Choi H.Y., Bae S.H., Veal D.A., Bell P.J., Ferrari B.C., Van Dyck D.D., Verrills N.M., Paik Y.K. & Caruso P. (2003) A fluorescent natural product for ultra sensitive detection of proteins in one-dimensional and two-dimensional gel electrophoresis. *Proteomics* **3,** 2273–2288.

Marjamaa K., Kukkola E.M. & Fagerstedt K.V. (2009) The role of xylem class III peroxidases in lignification. *Journal of Experimental Botany* **60,** 367–376.

Mendoza-Cozati D.G., Butko E., Springer F., Torpey J.W., Komives E.A., Kehr J. & Schroeder J.I. (2008) Identification of high levels of phytochelatins, glutathione and cadmium in the phloe sap of *Brassica napus*. A role for thio-peptides in the long-distance transport of cadmium and the effect of cadmium on iron translocation. *The Plant Journal* **54,** 249–259.

Motose H., Sugiyama M. & Fukuda H. (2001) An arabinogalactan protein(s) is a key component of a fraction that mediates local intercellular communication involved in tracheary element differentiation of *Zinni*a mesophyll cells. *Plant and Cell Physiology* **42,** 129–137.

Motose H., Sugiyama M. & Fukuda H. (2004) A proteoglycan mediates inductive interaction during plant vascular development. *Nature* **429,** 873–878.

Munns R. & Tester M. (2008) Mechanisms of salinity tolerance. *Annual Review of Plant Biology* **59,** 651–681.

Ogawa K., Kanematsu S. & Asada K. (1996) Intra- and extracellular localization of 'citosolic' CuZn-superoxide dismutase in spinach leaf and hypocotyls. *Plant and Cell Physiology* **37,** 790–799.

Ogawa K., Kanematsu S. & Asada K. (1997) Generation of superoxide anion and localization of CuZn-superoxide dismutase in the vascular tissue of spinach hypocotyls: their association with lignification. *Plant and Cell Physiology* **38,** 1118–1126.

Passardi F., Penel C. & Dunand C. (2004) Performing the paradoxical: how plant peroxidases modify the cell wall. *Trends in Plant Science* **9,** 534–540.

Patonnier M.P., Peltier J.P. & Marigo G. (1999) Drought-induced increase in xylem malate and mannitol concentrations and closure of *Fraxinus excelsior* L. stomata. *Journal of Experimental Botany* **50,** 1223–1229.

Plett D.C. & Moller I.S. (2010) Na^+ transport in glycophytic plants: what we know and would like to know. *Plant, Cell & Environment* **33,** 612–626.

Quiroga M., deForchetti S.M., Taleisnik E. & Tigier H.A. (2001) Tomato root peroxidase isozymes: kinetic studies of the coniferyl alcohol peroxidase activity, immunological properties and role in response to salt stress. *Journal of Plant Physiology* **158,** 1007–1013.

Rieu I., Ruiz-Rivero O., Fernandez-Garcia N., *et al*. (2008) The gibberellin biosynthetic genes AtGA20ox1 and AtGA20ox2 act, partially redundantly, to promote growth and development throughout the Arabidopsis life cycle. *The Plant Journal* **53,** 488–504.

Sanchez-Aguayo I., Rodriguez-Galan J.M., Garcia R., Torreblanca J. & Pardo J.M. (2004) Salt stress enhance xylem development and expression of S-adenosyl-L-methionine synthase in lignifying tissues of tomato plants. *Planta* **220,** 278–285.

Satoh S. (2006) Organic substances in xylem sap delivered to above-ground organs by the roots. *Journal of Plant Research* **119,** 179–187.

Schindler T., Bergfeld R. & Schopfer P. (1995) Arabinogalactan proteins in maize coleoptiles: developmental relationship to cell death during xylem differentiation but not to extention growth. *The Plant Journal* **7,** 25–36.

Seifert G.J. & Roberts K. (2007) The biology of arabinogalactan proteins. *Annual Review of Plant Biology* **58,** 137–161.

Shadle G., Chen F., Reddy M.S.S., Jackson L., Nakashima J. & Dixon R.A. (2007) Down-regulation of hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase in transgenic alfalfa affects lignification, development and forage quality. *Phytochemistry* **68,** 1521–1529.

Shelp B.J. (1987) The composition of Phloem exudates and xylem sap from broccoli (*Brassica oleracea var. italica*) supplied with NH_4^+, NO_3^- or NH_4NO_3. *Journal of Experimental Botany* **38,** 1619–1636.

Stacey N.J., Roberts K., Carpita N.C., Wells B. & McCann M.C. (1995) Dynamic changes in cell surface molecules are very early events in the differentiation of mesophyll cells from *Zinnia elegans* into tracheary elements. *The Plant Journal* **8,** 891–906.

Streller S. & Wingsle G. (1994) *Pinus sylvestris* L. needles contain extracellular CuZn superoxide dismutase. *Planta* **192,** 195–201.

Subramanian S., Cho U.H., Keyes C. & Yu O. (2009) Distinct changes in soybean xylem sap proteome in response to pathogenic and symbiotic microbe interactions. *BMC Plant Biology* **9,** 119.

Surabhi G.K., Reddy A.M., Kumari G.J. & Sudhakar C. (2008) Modulations in key enzymes of nitrogen metabolism in two high yielding genotypes of mulberry (*Morus alba* L.) with differential sensitivity to salt stress. *Environmental and Experimental Botany* **64,** 171–179.

Timperio A.M., Egidi M.G. & Zolla L. (2008) Proteomics applied on plant abiotic stresses: role of heat shock proteins (HSP). *Journal of Proteomics* **71,** 391–411.

Turner S., Gallois P. & Brown D. (2007) Tracheary element differentiation. *Annual Review of Plant Biology* **58,** 407–433.

Vanacker H., Carver T.L.W. & Foyer C.H. (1998) Pathogen-induced changes in the antioxidant status of the apoplast of barley leaves. *Plant Physiology* **117,** 1103–1114.

Wang R., Chen S., Zhou X., *et al*. (2008) Ionic homeostasis and reactive oxygen species control in leaves and xylem sap of two poplars subjected to NaCl stress. *Tree Physiology* **28,** 947–957.

Wilkinson S., Bacon M.A. & Davies W.J. (2007) Nitrate signaling to stomata and growing leaves: interactions with soil drying, ABA, and xylem sap pH in maize. *Journal of Experimental Botany* **58,** 1705–1716.

Wolf O., Jeschke W.D. & Hartung W. (1990) Long distance transport of abscisic acid in NaCl-treated plants of *Lupinus albus*. *Journal of Experimental Botany* **41,** 593–600.

Zhang Y., Brown G., Whetten R., Loopstra C.A., Neale D., Kieliszewski M.J. & Sederoff R.R. (2003) An arabinogalactan

protein associated with secondary cell wall formation in differentiating xylem of loblolly pine. *Plant Molecular Biology* **52,** 91–102.

Zhao K., Munns R. & King R.W. (1991) Abscisic acid synthesis in NaCl-treated barley, cotton and saltbush. *Australian Journal of Plant Physiology* **18,** 17–24.

Zimmermann U., Schneider H., Wegner L.H., Wagner H.J., Szimtenings M., Haase A. & Bentrup F.W. (2002) What are the driving forces for water lifting in the xylem conduit? *Physiologia Plantarum* **114,** 327–335.

Zwieniecki M.A., Thompson M.V. & Holbrook N.M. (2002) Understanding the hydraulics of porous pipes: tradeoffs between water uptake and root length utilization. *Journal of Plant Growth Regulation* **21,** 315–323.

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