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Special Issue

Ascorbate deficiency influences the leaf cell wall glycoproteome in *Arabidopsis thaliana*

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ABSTRACT

The cell wall forms the first line of interaction between the plant and the external environment. Based on the observation that ascorbate-deficient *vtc* mutants of *Arabidopsis thaliana* have increased cell wall peroxidase activity, the cell wall glycoproteome of *vtc2-2* was investigated. Glycoproteins were purified from fully expanded leaves by Concanavalin A affinity chromatography and analysed by liquid chromatography quadrupole time-of-flight mass spectrometry. This procedure identified 63 proteins with predicted glycosylation sites and cell wall localization. Of these, 11 proteins were differentially expressed between *vtc2-2* and wild type. In particular, PRX33/34 were identified as contributing to increased peroxidase activity in response to ascorbate deficiency. This is the same peroxidase previously shown to contribute to hydrogen peroxide generation and pathogen resistance. Three fasciclin-like arabinogalactan proteins (FLA1, 2 and 8) had lower abundance in *vtc2-2*. Inspection of published microarray data shows that these also have lower gene expression in *vtc1* and *vtc2-1* and are decreased in expression by pathogen challenge and oxidative stresses. Ascorbate deficiency therefore impacts expression of cell wall proteins involved in pathogen responses and these presumably contribute to the increased resistance of *vtc* mutants to biotrophic pathogens.

Key-words: Arabinogalactan; cell wall proteome; glycoproteins; hydrogen peroxide; pathogen response; peroxidase; reactive oxygen; vitamin C; *vtc* mutants.

INTRODUCTION

Ascorbate is an antioxidant and photoprotectant in plants (Müller-Moulé *et al*. 2004; Foyer & Noctor 2011; Smirnoff 2011; Page *et al*. 2012). Ascorbate-deficient *vtc* mutants of *Arabidopsis thaliana* that are affected in biosynthesis and related processes (Conklin *et al*. 1997, 2000, 2006, 2013; Dowdle *et al*. 2007) are more sensitive to environmental stresses including high light, salt, UV-B and temperature extremes (Smirnoff 2011). Similarly, mutants of ascorbate peroxidase and enzymes that recycle the oxidized forms of ascorbate (monodehydroascorbate reductase and glutathione-dependent dehydroascorbate reductase) are also more sensitive to abiotic stresses (Smirnoff 2011). In contrast, *vtc* mutants are more resistant to biotrophic pathogens such as *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis* (Barth *et al*. 2004; Pavet *et al*. 2005; Mukherjee *et al*. 2010), while being more sensitive to *Alternaria brassicicola*, a necrotrophic fungal pathogen (Botanga *et al*. 2012). In the case of *P. syringae*, increased resistance is dependent on salicylic acid (SA) and NPR1 (Pavet *et al*. 2005; Mukherjee *et al*. 2010). *vtc* mutants exhibit higher constitutive expression of SA and PAMP-inducible genes such as *PR1* (Pastori *et al*. 2003; Barth *et al*. 2004; Colville & Smirnoff 2008; Mukherjee *et al*. 2010; Kerchev *et al*. 2011). Therefore basal pathogen defences are primed by ascorbate deficiency in an SA-dependent manner, possibly also depending on accumulation of hydrogen peroxide.

A number of previous studies have found increased peroxidase activity in Arabidopsis with decreased ascorbate due to antisense suppression of the ascorbate biosynthesis enzyme L-galactose dehydrogenase (Gatzek *et al*. 2002) and in the ascorbate-deficient *vtc* mutants (Veljovic-Jovanovic *et al*. 2001; Colville & Smirnoff 2008). Since increased peroxidase activity was found in a range of different mutants, it is likely that it is caused by ascorbate deficiency and not by loss of function of any specific gene. The biggest increase in peroxidase activity in *vtc* mutants was found in a protein fraction that could be eluted from a crude cell wall preparation by $CaCl_2$ (Colville & Smirnoff 2008). The functions of the 73 class III peroxidases in *A. thaliana* have not been fully defined. They are mostly targeted to the apoplast or vacuole. Proposed roles include peroxidative cross linking of lignin monomers, cross linking of other wall polymers and hydrogen peroxide scavenging, particularly in the vacuole (Tognolli *et al*. 2002; Valério *et al*. 2004; Zipor & Oren-Shamir 2013). These biochemical functions therefore define diverse physiological roles in development and biotic/abiotic stress responses. As well as using hydrogen peroxide as a substrate, peroxidases can, in the presence of suitable co-substrates, generate hydrogen peroxide (Bolwell & Wojtaszek 1997; Frahry & Schopfer 1998) or hydroxyl radicals (Liszkay *et al*. 2004). The potential for hydrogen peroxide production raises the possibility that apoplastic peroxidases could generate hydrogen peroxide in response to pathogens along with the well-characterized contribution from plasma membrane

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NADPH oxidases (Torres *et al*. 2002). Arabidopsis mutants with decreased activity of the peroxidase isoforms PRX33 and PRX34 produced about 50% of the apoplastic reactive oxygen species (ROS) associated with the transient microbial-associated molecular pattern (MAMP)-induced oxidative burst associated with basal resistance. These mutants had less cell wall callose deposition in response to the Flg22 and Elf26 peptides (derived from bacterial MAMPs) and were less resistant to *P. syringae* (Bindschedler *et al*. 2006; Daudi *et al*. 2012; O'Brien *et al*. 2012). Therefore, there is strong evidence that peroxidases contribute to basal defence by generating hydrogen peroxide and *vtc* mutants have both increased cell wall peroxidase activity and increased resistancee to biotrophic pathogens. The question arises as to which peroxidase isoforms are affected in *vtc* mutants. In addition, since the cell wall is the first point of contact between plants and invading pathogens, the influence of ascorbate deficiency on the cell wall proteome is of interest. Transcriptomic analysis of *vtc1* and *vtc2* shows that MAMP-responsive genes have altered expression compared with wild type and many of these encode cell wall localized proteins (Pastori *et al*. 2003; Colville & Smirnoff 2008; Kerchev *et al*. 2011). However, there is no information on the extent to which these differences in gene expression affect cell wall protein levels. This study aimed to identify components of the Arabidopsis leaf cell wall proteome since this has not been previously investigated. The focus was on ionically bound glycoproteins because this fraction includes peroxidases. This approach showed that ascorbate deficiency affected a number of cell wall proteins and PRX 33 and 34 as the peroxidases with increased activity in ascorbate-deficient plants. Furthermore, a group of fasciclin-like arabinogalactan proteins (FLAs), which have decreased gene expression in *vtc* mutants, had decreased protein levels.

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana vtc1-1, *vtc2-1*, *vtc3-1* and *vtc2-2* mutants were obtained from Patricia Conklin (State University of New York, Cortland) and all were in the Col-0 background (Conklin *et al*. 2000). Surface-sterilized seeds were cold treated for 3 d at 4 °C and sown onto medium containing four parts Levington F2 compost (DeJex, Doncaster, UK) to one part vermiculite. Plants were grown in controlled environment growth rooms under short day conditions (8 h light, 16 h dark) at a photosynthetic photon flux density (PPFD) of 100 μmol m^{-2} s^{-1}, 23 °C and 65% relative humidity for 7 weeks.

Extraction of cell wall proteins

Cell wall proteins were extracted using a modified version of a previously published method (Feiz *et al*. 2006). 1 g of leaf tissue was extracted with 5 mL 5 mM potassium acetate buffer pH 4.6 with 0.5 M sucrose + 1 mM ethylenediaminetetraacetic acid (EDTA) and one mini EDTA-free protease inhibitor cocktail tablet (Roche, Basel, Switzerland). The homogenate was centrifuged at 13 000 *g* for 10 min at 4 °C. The supernatant containing soluble protein was discarded and the pellet was washed and centrifuged (13 000 *g* for 10 min) six times with 5 mL of 5 mM potassium acetate buffer (pH 4.6) to remove sucrose and protease inhibitor. The supernatant was discarded and the cell wall proteins were extracted with 2.5 mL 0.2 M $CaCl_2$ followed by two extractions with 2.5 mL 2 M LiCl. Total protein concentration was measured by the Bradford assay (Bradford 1976) using a γ-globulin standard.

Purification of glycoproteins by lectin affinity chromatography

The cell wall proteins prepared as described above were purified by affinity chromatography using Concanavalin A (ConA) to enrich for glycoproteins (Minic *et al*. 2007). A 1 mL Supelclean™ LC-NH_2 SPE tube (SUPELCO, Bellefonte, PA, USA) was emptied of its original contents and then filled with 1 mL of ConA Sepharose slurry (Sigma Chemical Co., St Louis, MO, USA) and washed with 3 mL of washing buffer (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$). The ionically bound cell wall protein extracts (1 mL) were loaded onto the gel and then washed with 3 mL of washing buffer. The proteins were eluted with 3 mL of 0.2 M methyl-α-glucopyranoside in the same buffer. The elute was collected and concentrated by Spin-X UF 500 concentrators with a 10 kDa molecular weight cut-off (Corning, Amsterdam, the Netherlands).

Trypsin digestion

10 μL of concentrated sample was mixed with 4 μL of 10 mM dithiothreitol and 2 μL of RapiGest MS-compatible detergent (Waters Corporation, Milford, MA, USA), and incubated for 20 min at 50 °C. 4 μL of 50 mM iodoacetamide was then added to the same mixture followed by incubation at room temperature for 20 min in the dark. Proteins were digested with 0.1 μg trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, Madison, WI, USA) and incubated at 37 °C for 16 h. 1 μL of 1% trifluoroacetic acid was added to stop the reaction and the samples were stored at –20 °C until analysis.

Proteomics analysis

Peptides were separated and detected by LC-ESI-QToF MS/MS and label free quantitative analysis was carried out using Progenesis LC-MS software (Non Linear Dynamics, Newcastle, UK). The trypsinized samples were analysed with an Agilent 6520 (Agilent Technologies, Santa Clara, CA, USA) QToF MS coupled to an high-performance liquid chromatography (HPLC)-Chip interface system and the samples were loaded onto a large-capacity micro C_{18} reverse-phase analytical column (Agilent Protein Identification Chip, Agilent Technologies) comprising of a 160 nL enrichment column, and a 150 mm × 75 μm analytical column packed

with Zorbax 300SB-C_{18} 5 μm material. The enrichment column flow rate was 3 μL min^{-1} and the analytical column flow rate was 0.4 μL min^{-1}. Mobile phase A was 2% methanol with 0.1% formic acid in water and B was 95% methanol with 0.1% formic acid in water. Peptides were eluted with the following gradient: 0 min – 5% B, 10 min – 10% B; 65 min – 30% B; 100 min – 60%; 120 min – 100% B; 122 min – 100% B; 123 min – 5% B with a re-equilibration time of 9 min. The mass spectrometer conditions were as follows. For the electrospray ionization source, the nitrogen gas temperature was 300 °C and gas flow rate was 4 L min^{-1}. The capillary voltage was 1850 V with all analyses being carried out in positive ion mode. The fragmentor voltage was 175 V and the skimmer 70 V. Scanning was performed using the auto-MS/MS function at 5 scans s^{-1} with sloped collision energy of 3.7 V/100 Da and an offset of –2.5 V. The data were loaded directly into Progenesis LC-MS software, which deconvoluted m/z-retention time features (peptides), aligned them across all samples and normalized abundances in each sample to the median abundance of all features. MS/MS spectra of the deconvoluted peptides were exported to SpectrumMill MS Proteomics Workbench software (Agilent Technologies) or Mascot (http://www.matrixscience.com) for peptide identification. The following data extraction and peptide identification settings were used: two maximum missed cleavages; fixed modification carbamidomethylation (C); variable modifications acetylation (K), oxidized methionine (M), pyroglutamic acid (N-term Q), deaminated (N), N-acetylglucosamine (S); peptide precursor m/z 300–1600 and charge +2 to +15 (+8 with Mascot); minimum MS signal/noise ratio 25; precursor ion mass tolerance ±20 ppm, product ion mass tolerance ±50 ppm; minimum scored peak intensity 50%, peptide filter score 6 and % scored peak intensity > 60, protein filter score >11 (to remove false positives). Peptides were identified with the Arabidopsis proteome database (TAIR10 release, http://www.arabidopsis.org/). The information on identified peptides was re-imported into Progenesis LC-MS software where their relative normalized abundances in each sample were calculated. Peptides were bundled into proteins and the overall abundance of each protein calculated. Significantly different proteins were identified by two-way analysis of variance (ANOVA) across strains and treatments using Progenesis LC-MS. Predicted subcellular localization of proteins was obtained from TAIR (http://www.arabidopsis.org/) and UniProtKB (http://www.uniprot.org/) with additional information from Signal P (http://www.cbs.dtu.dk/services/SignalP/; Bendtsen *et al*. 2004), Target P (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson *et al*. 2007), DictyOGlyc (http://www.cbs.dtu.dk/services/DictyOGlyc/; Gupta *et al*. 1999), NetOGlyc (http://www.cbs.dtu.dk/services/NetOGlyc/) and NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/).

Peroxidase assay

Peroxidase activity was measured in an assay mixture consisted of 230 μL of 50 mM HEPES (pH 7.0) containing 1 mM EDTA, 20 mM pyrogallol and 10 μL of protein extract. The reaction was initiated with 5 μL of 150 mM H_2O_2. The oxidation of pyrogallol was followed in a 96-well plate with a Tecan Infinite reader (Tecan, Reading, UK) at 430 nm using an extinction coefficient of 1.953 mM^{-1}.

Cell wall proline and hydroxyproline

Leaves (1 g fresh weight) were harvested from 6-week-old plants and washed thoroughly with deionized water. Tissue was homogenized in a pestle and mortar with 5 mL of 5 mM potassium acetate buffer pH 4.6 containing 0.4 M sucrose and 1 mM EDTA. Polyvinylpolypyrrolidone was then added in a 1:10 ratio with plant material and incubated at 4 °C for 30 min followed by centrifugation at 1000 *g* for 10 min and the pellet collected. The pellet was suspended in 5 mM potassium acetate buffer, pH 4.6. A total of five washes with acetate buffer were performed and the final pellet was carried forward. The crude cell wall material was suspended in 6 M HCl containing 10 mM phenol and sealed under nitrogen before being incubated at 110 °C for approximately 20 h to hydrolyse proteins. The resulting mixture was cooled to room temperature, centrifuged to remove insoluble material and the supernatant collected, dried under a stream of nitrogen and redissolved in 0.1 mL water. Proline and hydroxyproline were measured by LC-ESI-MS/MS. This was performed using an Agilent 6420B triple quadruple (QQQ) mass spectrometer in multiple reaction monitoring (MRM) mode coupled with an Agilent 1200 series Rapid Resolution HPLC system (Agilent Technologies). Chromatography was performed on a ZIC-HILIC column (particle size 3.5 μm, porosity 100 Å, internal diameter, 2.1 mm, length, 150 mm; Merck Millipore, Billerica, MA, USA) at 35 °C. 2 μL of hydrolysate was injected. Mobile phase A contained 95% acetonitrile and 0.01% formic acid. Mobile phase B contained 5% acetonitrile, 10 mM ammonium acetate and 0.1% formic acid. The flow rate was 0.25 mL min^{-1} with 5% B at 0 min, 50% B at 10 min, 90% B from 15–20 min and 5% B at 25 min. The QQQ was operated in positive ion mode and ESI source conditions were nitrogen gas temperature 350 °C, drying gas flow rate 9 L min^{-1}, nebulizer pressure 35 psig and capillary voltage 4 kV. Data were acquired in MRM mode with a dwell time of 50 ms. For proline, the precursor ion m/z was 116.1, fragmentor voltage 66 V, collision energy 20 V and the product ion m/z was 86. For hydroxyproline, the precursor ion m/z was 132, fragmentor voltage 66 V, collision energy 13 V and the product ion m/z was 86. Quantification was by comparison of peak areas with standards.

RESULTS

Peroxidase activity is increased in *vtc* mutants and by high light

To extract samples enriched in ionically bound cell wall proteins, the pellet from homogenized leaves was eluted with $CaCl_2$. This treatment displaces ionically (non-covalently) bound proteins from the pellet producing a supernatant that is enriched in cell wall enzymes such as type III peroxidase (Colville & Smirnoff 2008). Peroxidase activity using pyrogallol as substrate was significantly higher in *vtc2-1*,

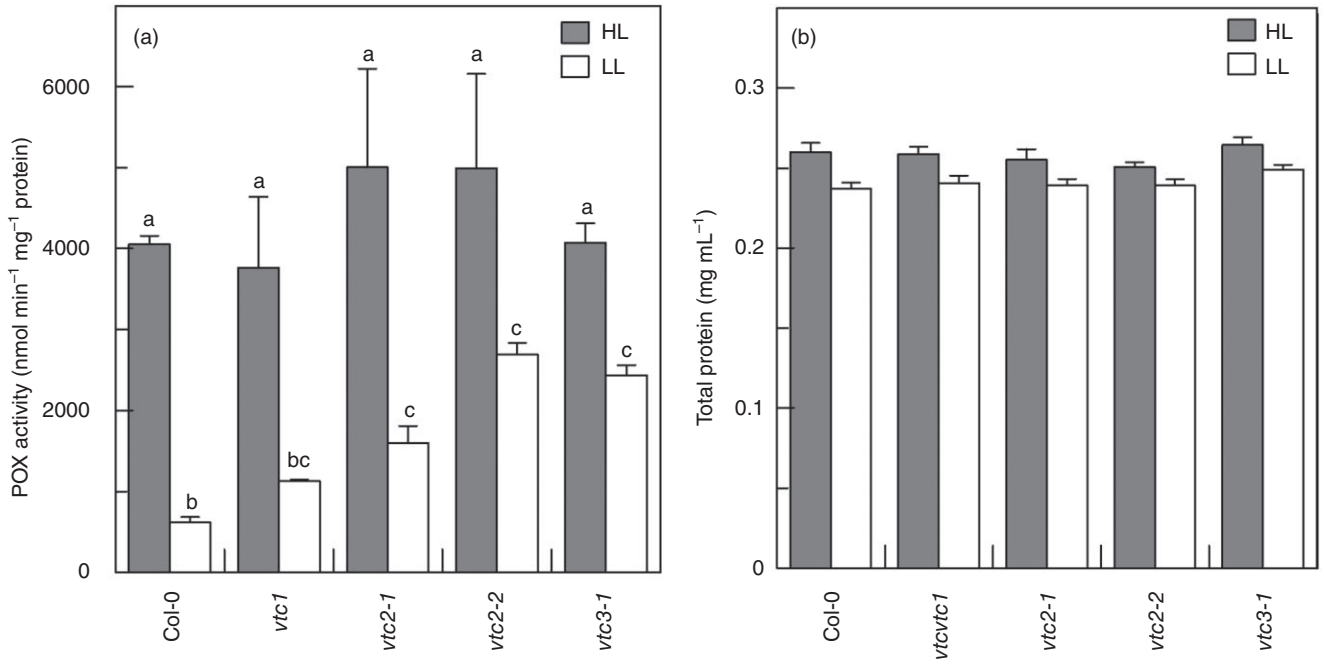

Figure 1. Ionically bound peroxidase activity (a) and total protein content (a) in leaf cell wall extracts of wild type (Col-0) and *vtc Arabidopsis thaliana* mutants. Plants were acclimated to HL (550–650 μmol m^{-2} s^{-1}) and LL (100 μmol m^{-2} s^{-1}) for 4 d prior to analysis. Data are mean values with standard error ($n = 3$ biological replicates). Columns labelled with different letters are significantly different [analysis of variance (ANOVA), $P < 0.05$].

vtc2-2 and *vtc3-1* than wild type (Col-0) when the plants were grown in low light (LL, Fig. 1). Activity was not significantly higher in *vtc1-1* in the current experiments although it has been previously reported to be higher in this mutant (Veljovic-Jovanovic *et al*. 2001; Colville & Smirnoff 2008). Under these growth conditions, apoplastic ascorbate was significantly lower in *vtc1*, *2* and *3* (Supporting Information Fig. S3). Growth in high light (HL) increased peroxidase activity in all genotypes by approximately twofold and abolished differences between genotypes. Protein concentration of the ionically bound protein fraction did not differ between genotypes and was marginally greater in HL. Peroxidase activity is therefore higher in *vtc* mutants when grown under normal laboratory lighting but this effect is swamped under light intensities closer to the natural environment. The Arabidopsis genome encodes 73 type III peroxidases, which are largely predicted to be targeted to the apoplast or vacuole (Valério *et al*. 2004). Therefore, a mass spectrometry-based proteomics approach was taken to discover which of these proteins are responsible for the increased peroxidase activity in ascorbate-deficient plants.

The Arabidopsis leaf cell wall glycoproteome

Proteins were isolated from pellets of fully expanded rosette leaf homogenates by eluting with $CaCl_2$ and LiCl followed by affinity purification of the eluate by ConA affinity chromatography. This method should provide a fraction enriched in glycoproteins that are ionically bound to the cell wall (Minic *et al*. 2007). Approximately 5187 peptides were detected in Col-0, *vtc1* and *vtc2-2* after deconvolution by Progenesis LC-MS (Supporting Information Table S1). Of these, 2430 provided MS/MS spectra for peptide identification from which a total of 128 unique proteins (including 19 intracellular, 27 undefined and 82 secreted proteins) represented by two or more peptides, were identified from Col-0, *vtc1* and *vtc2-2* grown under LL and HL (Table 1). Their predicted intracellular locations were determined and, overall, 67% were predicted to be extracellular, secreted or associated with the endomembrane system. Therefore, this method provides significant enrichment in cell wall proteins. The 63 glycoproteins identified are shown in Table 2 (and Supporting Information Table S1). The identified proteins are mostly predicted to be N-glycoslyated and are dominated by hydrolases, proteases, peroxidases, lectins, FLAs and receptor-like kinases.

Cell wall proteins differentially expressed between Col-0 and *vtc2*

A detailed quantitative proteomic investigation focused on a comparison of Col-0 and *vtc2*-2. The relative expression of identified peptides, based on their LC-MS peak areas normalized to the median of all peptides was calculated using Progenesis LC-MS software. Peptides were bundled into their cognate proteins and the sum of the peaks areas of unique peptides for each protein was used to calculate relative protein abundance. Overall, 937 ($P < 0.05$) and 403 ($P < 0.01$) peptides were significantly different across strain and light comparisons (Supporting Information Table S1). Twelve cell wall proteins were identified as being significantly

Light	Strain	Total identified	% Secreted	% Intracellular	% Undefined
HL	Col-0	71	73	18	18
	vtc1	95	65	31	15
	vtc2-2	62	73	15	8
LL	Col-0	111	63	34	24
	vtc1	110	55	48	23
	vtc2-2	54	76	13	6
	Mean	84	67	27	16

Table 1. Predicted subcellular localization of ionically bound proteins purified from *Arabidopsis thaliana* Col-0 and *vtc* mutant leaves by ConA affinity chromatography

The plants were acclimated to low light (LL, 100 μmol m^{-2} s^{-1}) and high light (HL, 550 μmol m^{-2} s^{-1}) for 4 d prior to extraction.

different ($P < 0.05$) between Col-0 and *vtc2-2* (Table 3; Supporting Information Table S2). Of these, five were consistently more abundant in *vtc2-2* than in Col-0 in both LL and HL – a lectin (At5g03350), two type III peroxidases (PRX33/At3g49110 and PRX34/At3g4912), a cysteine-rich receptor-like kinase (CRRK, At5g48540) and an aspartyl protease (At1g03220). Among the proteins with lower abundance in *vtc2-2* were three FLAs, FLA1 (At5g55730), FLA2 (At4g12730) and FLA8 (At2g45470). A germin-like protein (GLP1/At1g72610) was decreased in HL acclimated wild type and *vtc2-2* plants but was higher in the Col-0 compared with *vtc2* in L and HL. The abundance of individual peptides for the closely related PRX33 and PRX34 are shown in Table 4. Since only one peptide is specific to PRX33 and six peptides are common to both proteins, it is not possible to conclude which of these two are more affected in *vtc2-2* (Fig. 2).

Proline and hydroxyproline content of leaf cell wall proteins

Many cell wall proteins contain hydroxyproline which provides a site for glycosylation (Basu *et al*. 2013). Ascorbate is required for maintaining the activity of prolyl hydroxylases during post-translational proline hydroxylation (De Tullio *et al*. 1999), so it is possible that glycoprotein abundance is affected by a limitation on proline hydroxylation in the *vtc* mutants. To assess this possibility, proline and hydroxyproline were measured after hydrolysing crude cell wall preparations. 34–42% of proline residues were hydroxylated, reflecting the postulated large proportion of hydroxyproline-rich proteins in the cell wall. There was no significant difference between strains (Table 5).

DISCUSSION

Composition of the Arabidopsis leaf glycoproteome

Considering the large number of peptides detected but not identified in this study, it is clear that the 63 proteins identified are the most abundant and the coverage is not complete. Concanavalin A affinity purification was successful at enriching the sample for glycoproteins. Although the glycoproteome of fully expanded Arabidopsis leaves has not been previously documented, comparison with other studies of cell wall proteins shows that similar coverage was achieved. The most closely comparable studies are the glycoproteome of Arabidopsis stems (Minic *et al*. 2007) and etiolated hypocotyls (Zhang *et al*. 2011). These studies identified 102 and 126 proteins, respectively, using ConA and boronic acid affinity chromatography and a combination of 2D gel electrophoresis/MALDI-ToF and LC-ion trap MS/MS. The 63 proteins in the current study is comparable but was achieved by LC-QToF auto-MS/MS alone. Surprisingly, only 24 proteins were common to each study (Supporting Information Table S3). However, the types of protein in terms of function were similar in both studies suggesting that stem and leaf cells may express different isoforms.

Differentially expressed cell wall glycoproteins in *vtc2-2*

The *vtc2-2* mutant used in this study has ~20% of normal ascorbate in its leaves, comparable to *vtc2-1* (Conklin *et al*. 2000). It is affected in GDP-L-galactose phosphorylase, the first dedicated enzyme of the L-galactose ascorbate biosynthesis pathway, and has an amino substitution that results in an inactive enzyme (Jander *et al*. 2002; Dowdle *et al*. 2007). The quantitative proteomic analysis showed that four glycoproteins were higher in abundance in *vtc2*-2 cell walls. PRX33 and 34 have very similar sequences and so cannot easily be distinguished. The others were a lectin-like protein, a cysteine-rich receptor kinase (CRRK) and an aspartyl protease (AP). Inspection of the transcriptomes of *vtc1* and *vtc2-1* (Kerchev *et al*. 2011) using the Genevestigator tool (https://www.genevestigator.com; Zimmermann *et al*. 2004) showed that transcript levels encoding all these proteins were higher in both mutants compared with Col-0, this being statistically significant except for CRRK and AP in *vtc2-1* (Supporting Information Fig. S1). Therefore, in the case of these genes, the transcriptional profile of two ascorbate-deficient mutants is reflected in cell wall protein content. *VTC1* encodes GDP-mannose pyrophosphorylase, another ascorbate biosynthesis enzyme (Conklin *et al*. 1999), suggesting that the effect on transcript and protein expression was caused specifically by ascorbate deficiency. Among the seven proteins that were decreased in *vtc2-2*, the presence of three FLAs was striking. Inspection of the transcriptome data showed that their transcript levels were all decreased in *vtc1* and *vtc2-2* (Supporting Information Fig. S1). The other

Table 2. Glycoproteins identified from the ionically bound cell wall protein fraction of *Arabidopsis thaliana* Col-0, *vtc1* and *vtc2-2* after ConA chromatography

AGI number	Glycosylation	Predicted location	Description
Enzyme activity/metabolism			
At4g01130	N	ER	Acetylesterase, similar to GDSL-motif lipase/hydrolase family protein
At3g56310	N	CW	α-galactosidase/melibiase/α-D-galactoside galactohydrolase
At1g65590	N and O	PM	β-hexosaminidase
At3g13790	N	CW	β-fructofuranosidase
At1g68560	N and O	CW/apoplast	Bifunctional α-L-arabinofuranosidase/β-D-xylosidase
At4g01700	N	CW	Chitinase, putative
At1g66970	N	PM	Glycerophosphoryl diester phosphodiesterase family protein
At5g20950	N and O	CW	Glycosyl hydrolase family 3 protein
At3g07320	N	CW	Glycosyl hydrolase family 17 protein
At1g28600	N	CW	GDSL-motif lipase
At3g14210	N	CW	GDSL-motif lipase
At5g25980	N and O	Apoplast	Myrosinase (thioglucoside glucohydrolase)
At4g25900	N and O	CW/CM	Aldose 1-epimerase family protein
At3g47800	N	CW	Aldose 1-epimerase family protein
At1g72610	N	CW	Germin-like protein (GLP1)
At5g20630	N	CW	Germin-like protein
At1g03220	N and O	CW	Aspartyl protease family protein
At1g03230	N	CW	Aspartyl protease family protein
At1g09750	N and O	CW	Aspartyl-like protease protein
At3g52500	N and O	CW	Aspartyl protease family protein
At3g10410	N	Vacuole	Serine carboxypeptidase-like 49 (SCPL 49)
At4g36195	N	CW	Serine carboxypeptidase S28 family protein
At5g23210	N and O	CW	Serine carboxypeptidase 34 (SCPL34)
At2g33530	N and O	CW	Serine carboxypeptidase-like 46 (SCPL 46)
At3g49110	N	CW	Class III peroxidase 33
At3g49120	N	CW	Class III peroxidase 34
At4g08770	N	Apoplast	Class III peroxidase 37
At4g08780	N	CW	Class III peroxidase 38
At1g71695	N	Vacuole	Class III peroxidase 12
At4g21960	N	EM	Class III peroxidase 42
At2g06850	N and O	CW	Endoxyloglucan transferase (EXGT-A1)
At4g37800	N	CW	Xyloglucosyl transferase 7 (XTH7)
Signalling			
At4g23300	N	EM	Cysteine-rich receptor-like kinase
At5g48540	N and O	CW	Cysteine-rich receptor-like kinase 55
At1g56340	N	ER	Calreticulin 1 (CRT1)
At1g09210	N and O	ER	Calreticulin 2 (CRT2)
At1g08450	N	ER	Calreticulin 3 (CRT3)
At2g27060	N	PM	ATP binding /threonine kinase
At1g33590	N and O	CW	Disease resistance protein-related/LRR protein-related
At1g33600	N	CW	Leucine-rich repeat protein
At3g20820	N and O	CW	Leucine-rich repeat protein
At5g12940	N and O	CW	Leucine-rich repeat protein
At3g12145	N	CW	Leucine-rich repeat protein
At4g13340	N	CW	Leucine-rich extensin like protein
At1g78980	N	TM	Strubbelig-receptor family 5 (SRF 5)
Fasciclin-like arabinogalactans			
At2g04780	N and O	PM	Fasciclin-like arabinogalactan protein 7 (FLA7)
At5g55730	N and O	CW	Fasciclin-like arabinogalactan protein 1 (FLA1)
At4g12730	N and O	PM	Fasciclin-like arabinogalactan protein 2 (FLA2)
At2g45470	N and O	CW	Fasciclin-like arabinogalactan protein 8 (FLA8)
At3g60900	N and O	CW	Fasciclin-like arabinogalactan protein 10 (FLA10)
Lectins			
At1g78820	N & O	Apoplast	Curculin-like (mannose-binding) lectin
At1g78830	N	CW	Curculin-like (mannose-binding) lectin
At3g15356	N and O	CW	Legume lectin family protein
At5g03350	N and O	CW	Legume lectin family protein
At3g16530	N and O	CW/apoplast	Lectin-like protein
Protein folding			
At1g21750	N	ER/vacuole	Disulfide isomerase-like (PDIL) protein
At1g77510	N and O	ER	Disulfide isomerase-like (PDIL) protein
Other			
At1g17100	N	PM/vacuole	SOUL heme-binding family protein
At4g12420	N and O	CW	SKU5 copper oxidase-like
At3g08030	N	CW	DUF642 unknown function
At5g25460	N and O	CW	DUF642 unknown function (responsive to L-GalL)
At5g11420	N and O	CW	DUF642 unknown function (galactose-binding)
At4g32460	N and O	CW	DUF642 unknown function (galactose-binding)

Predicted subcellular localization and glycosylation pattern are shown.
O, O-glycosylation; N, N-glycosylation; PM, plasma membrane; CW, cell wall; EM, endomembrane; ER, endoplasmic reticulum.

Table 3. Leaf cell wall glycoproteins that are differentially expressed between Col-0 and *vtc2-2 Arabidopsis thaliana*

AGI number	Description	Peptides	Score	*P*	Mean normalized abundance			
					Col-0 HL	Col-0 LL	*vtc2-2* HL	*vtc2-2* LL
Higher in *vtc2-2*								
At3g49120	Peroxidase 34	11 (3)	294.9	0.0001	13.10	12.80	25.70	22.90
At3g49110	Peroxidase 33	6 (1)	88.7	0.0044	0.15	0.16	0.26	0.27
At5g48540	Cysteine-rich receptor-like kinase	2	28.6	0.0045	0.12	0.12	0.17	0.22
At1g03220	Aspartyl protease-like protein	8	192.2	0.0003	2.25	3.24	3.26	4.74
At5g03350	Lectin-like protein	6 (5)	164.2	0.0014	2.84	7.34	4.21	15.20
Lower in *vtc2-2*								
At5g55730	Fasciclin-like arabinogalactan protein 1	3	64.0	0.0019	1.72	1.19	0.93	0.91
At4g12730	Fasciclin-like arabinogalactan protein 2	4	56.0	0.0012	1.57	1.58	1.02	1.20
At2g45470	Fasciclin-like arabinogalactan protein 8	6	120.6	0.0001	4.14	5.78	2.22	3.26
At1g72610	Germin-like protein (GLP1)	2	45.9	0.0054	5.20	13.80	3.86	7.90
At3g08030	DUF642 Unknown function	4	58.2	0.0300	3.19	1.84	0.99	1.88
At4g08780	Peroxidase 38	4 (1)	86.1	0.0300	0.78	0.92	0.43	0.71
At1g28590	GDSL-like lipase	2	30.5	0.0007	1.55	0.87	1.04	0.72

Plants were acclimated to HL (550–650 μmol m^{-2} s^{-1}) and LL (100 μmol m^{-2} s^{-1}) for 4 d before purification of ionically bound cell wall proteins by ConA affinity chromatography. Peptides were analysed by LC-QToF MS/MS and quantified by Progenesis LC-MS software. The values are mean normalized protein relative abundance across all peptides (n = 3 biological replicates). Peptide numbers in parentheses are unique peptides used for quantification. Significance values (ANOVA) for strains are shown. Light did not have a significant effect.

proteins did not have a consistent expression pattern. To further interrogate the functions of these proteins, the expression pattern of their transcripts was compared across experimental perturbations and mutants using a publicly available microarray database (https://www.genevestigator.com; Zimmermann *et al*. 2004). Hierarchical clustering of the differentially expressed proteins produced two distinct clusters of proteins with increased or decreased expression which are distinguished by particular patterns of gene expression across the microarray database (Supporting Information Figs S1 & S2). In particular, PRX33/34 (which share the same Affymetrix probe), the cysteine-rich RLK and aspartyl kinase form a cluster and respond with increased expression to pathogen challenge, SA, elictors (e.g. Flg22), hydrogen peroxide, ozone and UV-B. They also all show strong increase in the *flu* mutant, which generates increased singlet oxygen in the light (op den Camp *et al*. 2003). FLA1, 2 and 8 as well as germin-like protein (GLP1) and At3g08030 (unknown function) cluster together. In contrast, they have decreased expression in response to pathogen challenge, SA, elicitors (e.g. Flg22), ozone and UB-B radiation, as well as lower expression in *flu*. PRX38 is an exception because it clusters more strongly with PRX34 than with the other decreased proteins; however, it was quantified using one unique peptide. In addition, the lectin-like protein formed a distinct cluster on its own but, correspondingly to its protein level,

Table 4. Abundances of individual peptides of Peroxidase 34 (At3g49120) and Peroxidase 33 (At3g49110) in wild type and *vtc2-2*

Sequence	Score	Mass	*z*	Mean normalized abundance			
				Col-0 HL	Col-0 LL	*vtc2* HL	*vtc2* LL
Peroxidase 34 (At3g49120)							
DAFGNANSAR	29.5	1021.46	2	14.7	13.5	24.5	21.9
IAASILR	38	742.471	2	31.3	32.9	62.7	68.9
NVGLDRPSDLVALSGGHTFGK	22.4	2139.1	3	48	45.2	99.9	92.7
TPTVFDNK	11	920.452	2	45.3	42.1	67.5	64.7
Peroxidase 33 (At3g49110)							
NVGLDRPSDLVALSGAHTFGK	5.1	2153.12	3	1.5	1.6	2.6	2.7
Common to PRX33 and 34							
GFPVIDR	10.1	802.435	2	23.8	25.1	58	52.5
MGNITPTTGTQGQIR	29.4	1573.79	2	4.7	5.1	9.5	11.1
NQCQFILDR	55.5	1192.57	2	14.8	15.1	26.1	31.8
SALVDFDLR	54.9	1034.54	2	37.5	40.7	89.7	71.3
VPLGR	24.1	540.339	2	16.6	16.5	27.8	27.1
YYVNLK	12.6	798.429	2	24.5	27.1	52.4	54.1

Plants were acclimated to HL (550–650 μmol m^{-2} s^{-1}) and LL (100 μmol m^{-2} s^{-1}) for 4 d before purification of ionically bound cell wall proteins by ConA affinity chromatography. Peptides were analysed by LC-QToF MS/MS and quantified by Progenesis LC-MS software.

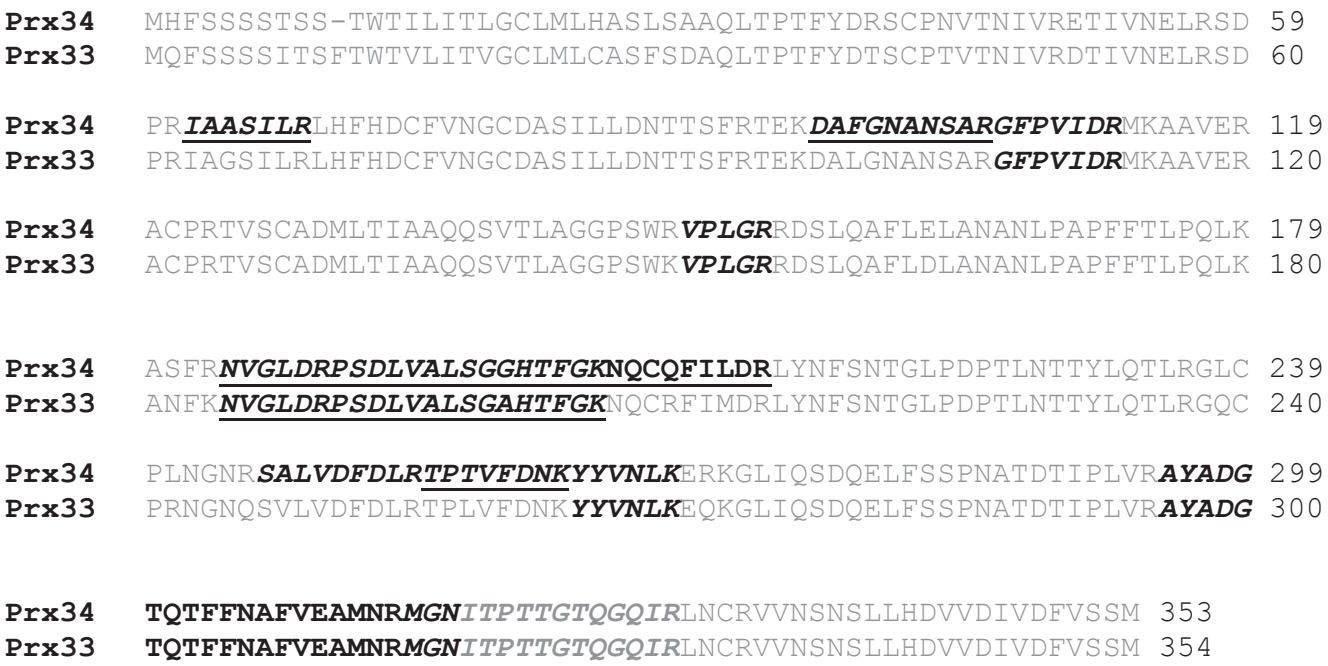

Figure 2. Alignment of peroxidase 33 and 34 amino acid sequences showing the peptides identified by LC-MS/MS. The peptides in bold black font were identified while those underlined are unique to either protein.

expression is higher in *vtc1* and *vtc2-1*. Therefore, the cell glycoproteins affected by ascorbate deficiency are also responsive to various oxidative stresses, SA and pathogen challenge.

PRX33 and PRX34, two peroxidases able to generate hydrogen peroxide are more abundant in *vtc2-2* cell walls

The results suggest that PRX33/34 contribute to increased peroxidase activity in *vtc2-2*, the approximately twofold change in abundance being close to the threefold change in peroxidase activity under low light conditions. Other proteins that were not detected in these experiments could contribute to higher peroxidase activity in high light. It is likely that these peroxidases also contribute to higher activity in the other *vtc* mutants (Veljovic-Jovanovic *et al*. 2001; Colville & Smirnoff 2008). The functions of the multiple type III peroxidase isoforms in Arabidopsis have not been fully established. In the cell wall, they have roles in catalysing hydrogen peroxide-dependent peroxidative cross linking of cell wall polymers, leading to cell wall stiffening and lignifications (Herrero *et al*. 2013). Wall peroxidases can also, under the appropriate conditions, produce hydrogen peroxide or hydroxyl radicals (Bolwell & Wojtaszek 1997; Frahry & Schopfer 1998). The latter have been implicated in scission of cell wall polysaccharides leading to wall loosening and growth (Liszkay *et al*. 2004). Interestingly, PRX33 and PRX34 are implicated in generating apoplastic ROS in response to MAMPs such as Flg22 and Elf26 peptides, derived from bacterial flagellin and elongation factor Tu, respectively. *PRX34* T-DNA insertion mutants and plants with decreased *PRX33* and *PRX34* expression due to antisense expression of a French bean homolog have a 50% decrease in MAMP-induced ROS production and are more susceptible to the biotrophic pathogen *P. syringae* pv. tomato DC3000 (Bindschedler *et al*. 2006; Daudi *et al*. 2012; O'B). The PRX33/24 knockdown plants also had decreased expression of MAMP-activated genes in response to Flg22 treatment and *P. syringae* challenge. They also had decreased basal expression of MAMP-activated genes in the absence of a challenge, suggesting that the basal level of ROS produced by peroxidase in wild-type plants maintains them in a primed state (Daudi *et al*. 2012). Since ascorbate-deficient *vtc* mutants have increased basal resistance to biotrophic pathogens such as *P. syringae* and *H. arabidopsidis* (Barth *et al*. 2004; Mukherjee *et al*. 2010), it is interesting that they also

Table 5. Comparison of the proline and hydroxyproline content and hydroxyproline as a % of total proline in cell wall protein hydrolysates from Col-0, *vtc1* and *vtc2-1 Arabidopsis thaliana* leaves

	Proline	Hydroxyproline	% Hydroxyproline
Col-0	3.5 ± 1.7	2.5 ± 1.5	42.1 ± 5.0
vtc1	5.1 ± 1.7	3.4 ± 0.7	40.3 ± 3.9
vtc2-1	3.3 ± 1.4	1.9 ± 0.2	35.9 ± 7.1

The imino acid concentrations are expressed as nmol mL^{-1} in the protein hydrolysate from equal leaf weights. Values are mean ± SD ($n = 3$). The percentage of hydroxyproline does not differ significantly between strains ($P = 0.05$).

have higher expression of PRX33 and PRX34 protein. Investigation of double mutants showed that the increased basal resistance of *vtc1* is dependent on *PAD4*, *NPR1* and *EDS5*, which are all involved in SA-mediated defence. SA and H_2O_2 are also higher in *vtc* mutants (Mukherjee *et al*. 2010). Therefore, the priming of basal resistance by ascorbate deficiency could be maintained by apoplastic hydrogen peroxide generated by peroxidase, although ascorbate deficiency itself could also contribute to higher peroxide levels. As noted in the previous section, *PRX33/34* is induced by elicitors, pathogen, challenge and SA as well as ascorbate deficiency. NADPH oxidases also contribute to hydrogen peroxide production in response to pathogen challenge (Torres *et al*. 2002); however, unlike the peroxidases, these do not have increased transcript levels in *vtc1* and *vtc2* (Kerchev *et al*. 2011), suggesting that these are not the a source of apoplastic ROS in these mutants. Apoplastic peroxidase activity also has other effects. The PRX33/34 knockdown plants had larger leaves and delayed senescence (Daudi *et al*. 2012) but, in contrast, PRX33/34 knockdown seedlings had shorter roots while overexpression of PRX34 increased root and cell length (Passardi *et al*. 2006). These ascorbate status-responsive peroxidases could therefore influence growth as well as pathogen responses via hydrogen peroxide scavenging or production, influencing both pathogen responses, redox signalling and oxidative cross linking of cell wall proteins and polysaccharides.

A set of FLAs has decreased expression in *vtc* mutants and in response to pathogen challenge

FLAs are a subgroup of the arabinogalactan proteins (AGPs). They are are highly glycosylated proline/hydroxyproline-rich proteins often containing glycosylphosphatidylinositol (GPI)-anchors. Members of this group have proposed structural roles, cell adhesion, signalling and development (Johnson *et al*. 2003; Seifert & Roberts 2007). The three FLAs, FLA 1, 2 and 8, with lower expression in *vtc2-2* are among the most abundant of the FLAs in Arabidopsis (Johnson *et al*. 2003). Their expression is repressed by abscisic acid (Johnson *et al*. 2003) as well as the pathogen and oxidative stresses discussed in the previous section. Further analysis shows that a set of FLAs including FLA1, 2 and 8 have the same expression pattern across various perturbations (Supporting Information Fig. S1). This expression pattern suggests that expression of a subset of FLAs is decreased by ascorbate deficiency and is involved in biotic and abiotic stress responses. However, their precise role remains to be determined. FLA1 AND 8 have been identified as GPI-anchored proteins by proteomic analysis of membrane fractions suggesting possible roles in signalling (Borner *et al*. 2003). AGPs are O-glycosylated on hydroxyproline residues (Basu *et al*. 2013). The prolyl hydroxylases that form hydroxyprolyl residues post-translationally require ascorbate to maintain catalytic activity (Myllyla *et al*. 1984),so it is possible that production of AGPs and FLAs could also be limited by ascorbate supply, analogously to limitation of the synthesis of the hydroxyproline containing extracellular matrix protein collagen in mammals, which, in severe cases, results in scurvy. However, hydrolysis of cell walls from wild type and *vtc1* and *vtc2* showed similar amounts of hydroxyproline are present. This supports transcription rather than post-translational hydroxylation as the process resulting in decreased FLA1, 2 and 8 proteins.

Conclusion

The cell wall glycoproteome is influenced by ascorbate deficiency. The peroxidases PRX33/34 are more highly expressed in the cell wall in low light. Peroxidase may contribute to the increased basal resistance of *vtc* mutants to biotrophic pathogens. The decreased expression of FLA1, 2 and 8 also suggests a role for these proteins in pathogen and oxidative stress responses and further investigation may reveal novel functions for this group of cell wall proteins.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Gene expession patterns of proteins differentially expressed between Col-0 and *vtc2-2*.pdf.
Figure S2. Hierarchical clustering of differential genes v perturbations.gif.
Figure S3. Ascorbate concencentration in apoplastic fluid from Col-0 and *vtc* mutants.
Table S1. Peptides from Arabidopsis leaf proteins purified by Con A chromatography.xlsx.
Table S2. Proteins differentially expressed between Col-0 and *vtc2-2*.pdf.
Table S3. Comparison between glycoproteins identified from Arabidopsis leaves with those identified from Arabidopsis stems by Minic *et al*. xlsx.