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RESEARCH PAPER

Low antioxidant concentrations impact on multiple signalling pathways in *Arabidopsis thaliana* partly through NPR1

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Abstract

Production of reactive oxygen species (ROS) is linked to signalling in both developmental and stress responses. The level of ROS is controlled by both production and removal through various scavengers including ascorbic acid and glutathione. Here, the role of low ascorbic acid or glutathione concentrations was investigated on ozone-induced cell death, defence signalling, and developmental responses. Low ascorbic acid concentrations in *vtc1* activated expression of salicylic acid (SA)-regulated genes, a response found to be dependent on the redox-regulated transcriptional co-regulator NPR1. In contrast, low glutathione concentrations in *cad2* or *pad2* reduced expression of SA-regulated genes. Testing different responses to jasmonic acid (JA) revealed the presence of at least two separate JA signalling pathways. Treatment of the *vtc1* mutant with JA led to hyper-induction of *MONODEHYDROASCORBATE REDUCTASE3*, indicating that low ascorbic acid concentrations prime the response to JA. Furthermore, NPR1 was found to be a positive regulator of JA-induced expression of *MDHAR3* and *TAT3*. The *vtc1* and *npr1* mutants were sensitive to glucose inhibition of seed germination; an opposite response was found in *cad2* and *pad2*. Overall, low ascorbic acid concentrations mostly led to opposite phenotypes to low glutathione concentrations, and both antioxidants interacted with SA and JA signalling pathways.

Key words: Ascorbic acid, defence signalling, glutathione, jasmonic acid, ozone, salicylic acid.

Introduction

For successful survival, plants have to adapt and acclimatize to the surrounding environment. One common consequence of exposure to abiotic stress conditions, for example extreme temperatures or high light levels, is the production of reactive oxygen species (ROS). Far from being only damaging agents, ROS are also used by plants as signalling molecules in a variety of process ranging from defence against pathogens to root development (Apel and Hirt, 2004; Foyer and Noctor, 2005*a*; Jaspers and Kangasjärvi, 2010; Torres, 2010). It has become increasingly clear that signalling pathways in plants are not organized into linear pathways; instead, defence signalling is better described as a web of interactions. Not even individual ROS give uniform responses; instead, separate molecules (ozone, hydrogen peroxide, superoxide, and singlet oxygen), acting at different subcellular locations give rise to unique changes in gene expression (Gadjev *et al.*, 2006; Wrzaczek *et al.*, 2010). ROS production and scavenging are intimately linked, and the balance between them will determine defence signalling output as well as damage and cell-death responses. An extensive repertoire of enzymatic ROS scavengers (including catalase, superoxide dismutase, and ascorbate peroxidase) and low-molecular-weight scavengers (including ascorbic acid, glutathione, and α-tocopherol) protect plants from excessive ROS production. Ascorbic acid and glutathione are connected through the ascorbic acid-glutathione cycle (Noctor and Foyer, 1998) and are essential for plants; *Arabidopsis thaliana* mutants with very

Abbreviations: AAPH, 2,2′-azobis-2-methyl-propanimidamide; GSH, reduced glutathione; GSSG, oxidized glutathione; JA, jasmonic acid; MeJA, methyl jasmonate; ORAC, oxygen radical absorbance capacity; ROS, reactive oxygen species; SA, salicylic acid; WT, wild type.

low concentrations of either compound have severe developmental defects (Vernoux *et al.*, 2000; Dowdle *et al.*, 2007).

In addition to ROS scavenging and redox chemistry, ascorbic acid also has a role in regulating the cell cycle (Potters *et al.*, 2004) and is a substrate or cofactor of many enzymes (Arrigoni and De Tullio, 2002). *Arabidopsis* mutants with low ascorbic acid concentration were initially isolated based on increased sensitivity to the air pollutant ozone (Conklin *et al.*, 2000). Further characterizations of these mutants have been instrumental in defining ascorbic acid biosynthesis in plants (Linster and Clarke, 2008) and have also shown a role for ascorbic acid in several conditions including pathogen defence and senescence (Veljovic-Jovanovic *et al.*, 2001; Pastori *et al.*, 2003; Barth *et al.*, 2004).

Similar to ascorbic acid, the tri-peptide glutathione has a role in redox chemistry and in addition has multiple roles in plant development, cell-cycle regulation, heavy-metal detoxification, and regulation of protein activity (Rouhier *et al.*, 2008; Vivancos *et al.*, 2010). The *Arabidopsis cad2* (*cadmium sensitive2*) and *pad2* (*phytoalexin deficient 2*) mutants were originally isolated based on sensitivity to cadmium and impaired biosynthesis of the secondary metabolite camalexin, respectively (Cobbett *et al.*, 1998; Parisy *et al.*, 2007). Both mutants have low glutathione concentrations and contain mutations in the same gene, γ-glutamylcysteine synthetase, the first step of glutathione biosynthesis. A third allelic mutant, *rax1* (*regulator of ASCORBATE PEROXIDASE2-1*), was isolated based on constitutive expression of an antioxidant marker gene *APX2* (Ball *et al.*, 2004). These mutants contain contrasting amounts of glutathione (Parisy *et al.*, 2007), which leads to somewhat different phenotypes; for example, *rax1* and *cad2* have different gene-expression patterns (Ball *et al.*, 2004).

Ascorbic acid and glutathione are central components in regulating the redox balance of the plant cell. The stress hormone salicylic acid (SA) activates defence signalling pathway(s) through NON-EXPRESSOR OF PR-PROTEINS1 (NPR1), which is one of the few known redox-regulated signalling proteins in plants. Inactive NPR1 is present in a cytosolic oligomer complex through intermolecular disulfide bonds (Mou *et al.*, 2003). SA signalling activates thioredoxin TRX-h5 leading to reduction in NPR1, thus converting it to active monomers that are translocated from the cytosol into the nucleus activating defence gene expression (Tada *et al.*, 2008). In *vtc1* grown under non-stressed control conditions (but not the wild type), a NPR1-GFP fusion can be detected in the nucleus, indicating that the altered redox status of *vtc1* constitutively activates the NPR1 signalling pathway (Pavet *et al.*, 2005). Consistent with this, *vtc1* and *vtc2* have a high expression of *PATHOGENESIS RELATED1* (*PR-1*) (Colville and Smirnoff, 2008; Mukherjee *et al.*, 2010). In contrast, *PR-1* expression in *cad2* is lower than the wild type; indicating that plants with low glutathione concentrations to some extent have opposite phenotypes to plants with low ascorbic acid concentrations (Ball *et al.*, 2004). These contrasting phenotypes are also seen in response to infection with *Pseudomonas syringae* where *vtc1* and *vtc2* are more tolerant, while *rax1*, *cad2*, and *pad2* are more sensitive (Ball *et al.*, 2004; Pavet *et al.*, 2005; Parisy *et al.*, 2007). Defence-related phenotypes of mutants with low ascorbic acid and glutathione concentrations are summarized in Table 1. The linkage between ROS production and scavenging, and the role of ROS, ascorbic acid, and glutathione as signalling molecules themselves, makes it challenging (if even possible) to determine the exact role of individual molecules in plant defence responses. Hence, the

Table 1. Stress-related phenotypes of *Arabidopsis* mutants with low ascorbic acid or glutathione concentrations

	vtc1	***vtc2-1***	***vtc2 vtc5***	***rax1***	***cad2***	***pad2***	***rml1***	**References**
Ascorbic acid content compared with WT (%)	≈30	≈20–30	≈0	ND	WT	ND	ND	Conklin *et al*., 2000 Veljovic-Jovanovic *et al*., 2001 Pavet *et al*., 2005 Dowdle *et al*., 2007 Colville and Smirnoff, 2008
Glutathione content compared with WT (%)	WT	WT	ND	≈38	≈30	≈22	≈3	Pavet *et al*., 2005 Vernoux *et al*., 2000 Parisy *et al*., 2007 Colville and Smirnoff, 2008
Ozone	Sensitive	Sensitive	ND	ND	WT	WT	ND	Conklin *et al*., 2000; this study
Virulent bacteria	Resistant	Resistant	ND	Sensitive	Sensitive	Sensitive	ND	Ball *et al*., 2004 Pavet *et al*., 2005 Parisy *et al*., 2007
PR-1 gene expression	Increased	Increased	ND	Decreased	Decreased	ND	ND	Pastori et al., 2003 Ball *et al*., 2004 Pavet *et al*., 2005 Colville and Smirnoff, 2008
Miscellaneous			Growth arrest after germination				Defective root growth	Vernoux *et al*., 2000 Dowdle *et al*., 2007

ND, not determined; WT, wild-type phenotype.

terminology 'oxidative signalling' has been proposed to encompass the response to ROS, ascorbic acid, and glutathione (Foyer and Noctor, 2005*a*, *b*).

Here, the properties of the oxidative signalling network are explored further using mutants with low ascorbic acid (*vtc1*) or glutathione (*cad2*, *pad2*) concentration. NPR1 is one of the few known redox-regulated defence signalling proteins, and the concentrations of SA, glutathione and H_2O_2 are proposed to be co-regulated (Mateo *et al.*, 2006). Thus, double mutants *npr1 vtc1*, *npr1 cad2*, and *npr1 pad2* were constructed to determine the contribution of NPR1 in oxidative signalling. Also, a second stress hormone, jasmonic acid (JA), is shown to interact with ascorbic acid and glutathione in the regulation of the defence-gene expression.

Materials and methods

Plant material

Wild type *Arabidopsis* accession Columbia-0 (Col-0) was used as an ozone-tolerant control plant. Mutant seeds were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC; http://arabidopsis.info/) or were gifts from Dr Patricia Conklin (*vtc1-1*) and Dr Christopher S. Cobbett (*cad2-1*). Double mutants were generated using *npr1-1* as the pollen acceptor. Double mutants were identified using PCR-based markers: *npr1-1* (TGCTCTTCATTTCGCTGTTG and GAGTGCGGTTCTACCTTCCA, NlaIII cuts WT), *pad2* (TCTATTTGCGAATTCCCCTTT and CAAATGGTAGCATTCCTGTGC, DdeI cuts WT), *cad2-1* (mutant for primer CATTATGAGAAACTACATGCTTGG, WT for primer GAGAAACTACATGCCGAAAGTTGG, rev primer GGCAATGGTTAGTCAAAATCG), and *vtc1-1* (TGCATTTTCAGGAAAAGGAGTT and TTAGCAAAATCAACAAGGGGCCTTG, StyI cuts WT). All genotypes were confirmed in the F_3 generation.

Seeds were vernalized for 3 d and then grown on 1:1 v/v mixture of peat and vermiculite with subirrigation. Growth conditions were 23°C/19°C (day/night), 70%/90% relative humidity, under a 12 h photoperiod with 200 µmol m^{-2} s^{-1} irradiance in controlled growth chambers (Weiss Bio1300; Weiss Umweltstechnik, Reiskirchen-Lindenstruth, Germany). For fresh-weight and flower-time experiments, plants were grown in growth rooms under the same growth conditions.

Ozone and MeJA treatment

Three-week-old *Arabidopsis* plants were exposed to 375 nl l^{-1} ozone for 6 h. Samples were harvested at 8 h after the onset of the ozone treatment. Cell death was quantified by ion leakage as previously described (Overmyer *et al.*, 2000). Samples for RNA isolation were isolated from 3-week-old plants sprayed with 0.5 mM methyl jasmonate (MeJA) in 0.01% Triton-X100, and controls were sprayed with 0.01% Triton-X100. After 8 h, leaves were harvested, frozen in liquid nitrogen, and stored at –80°C until RNA was isolated.

Total antioxidant assay

Plants were grown as above for 3 weeks, harvested into 2 ml screw-cap tubes containing glass beads and sand, and frozen in liquid nitrogen. Extracts were prepared by adding ice-cold 50% acetone and shaking at 6800 rpm for 2×20 s in a Precellys 24 (Bertin Technologies, Montigny le Bretonneux, France). The oxygen radical absorbance capacity (ORAC) assay was performed as described by Gillespie *et al.* (2007). A Victor 1420 Wallac plate reader (Perkin-Elmer, Waltham, MA, USA) was used to measure the fluorescence, and the data were analysed in WorkOut 2.5 (Dazdaq, Brighton, UK).

Real-time quantitative RT-PCR

RNA isolation and cDNA synthesis were performed as previously described (Wrzaczek *et al.*, 2010). The reverse-transcription reaction was diluted to a final volume of 60 µl, and 1 µl was used as template for the PCR using LightCycler 480 SYBR Green I master mix (Roche Diagnostics, Basel, Switzerland). PCR was performed in triplicate on a LightCycler 480 (Roche Diagnostics) with the following cycling conditions: 1 cycle for 10 min at 95 °C, 55 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s followed by a melting-curve analysis. The primers used for PCR are listed in Supplementary Table S1 at *JXB* online. The raw threshold cycle (Ct) values were normalized against a reference gene (At4g34270) selected from Czechowski *et al.* (2005) and used to compare the results from WT with mutant, or mock treated control samples with treated samples as previously described (Wrzaczek *et al.*, 2010). Amplification efficiency was calculated for each primer pair from standard curves with serially diluted cDNA.

MeJA root growth

Age-matched seeds were sterilized and planted on ½ MS plates, vernalized for 2 d at +4 °C and grown for 3 d at 200 µmol m^{-2} s^{-1} for 16/8 h light/dark, at 23 °C. Subsequently, seedlings were transferred to half-strength MS plates supplemented with 10 µM or 50 µM MeJA (dissolved in ethanol), or the corresponding amount of ethanol for the control. The root length was measured after 5 d. The *coi1-16* mutant (a gift from Dr John Turner; Ellis and Turner, 2002) was used as a JA-insensitive control.

Analysis of publicly available gene-expression data

A list of genes encoding ascorbic acid and glutathione biosynthesis genes and ascorbic acid–glutathione cycle genes was compiled from AraCyc pathways (ftp://ftp.arabidopsis.org/home/tair/Pathways/). In addition, genes used in the qPCR analysis were added to the list. Publicly available experiments using the Affymetrix ATH1-121501 platform were obtained from several data sources: NASC Arrays http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl (ABA—NASCARRAYS-176; SA—NASCARRAYS-192; BTH—NASCARRAYS-392; Senescence experiment 1 NASCARRAYS-52; Senescence experiment 2—NASCARRAYS-150). ArrayExpress http://www.ebi.ac.uk/microarrayas/ae/ (MeJA—EATMX-13; Paraquat—E-ATMX-28). Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/ (H_2O_2—GSE5530; Salt—GSE5623; Heat—GSE19603; High light—GSE7743; *sid2*—GSE9955; *lht1*—GSE19109; *Pseudomonas syringae* ES4326—GSE18978; *sni1*- GSE6827; *csn3*, *csn4* and *csn5*—GSE9728; *cs26* long day—GSE19241; SA 24 h—GSE14961; *siz1*—GSE6583; *mkk1mkk2*—GSE10646; Ethylene—GSE14247; *npr1*—GSE13833; *Botrytis cinerea* infection—GSE5684; Ozone—GSE5722; vtc1 and vtc2—GSE19257; oas-a1—GSE19245). The Integrated Microarray Database System http://ausubellab.mgh.harvard.edu/imds (experiment names: NPR1 direct targets full genome and local and systemic responses to *Trichoplusia ni* feeding). Raw data for *acd11* (Palma *et al.*, 2010) were obtained from John Mundy. The raw.cel files were robust mulitiarray average normalized, and for each experiment the $\log_2$-base fold changes of treatment versus control, or mutant versus wild type, were computed. The preprocessed data were clustered using Bayesian hierarchical clustering as described in Wrzaczek *et al.* (2010).

Flower time and fresh weight

Plants were germinated and grown as described above with 12 individuals of each genotype, one plant per pot (6×6cm pot), with the pots placed randomly on three different trays in the growth

room. Plant positions were randomized every 3 days. Fresh weight was measured on 3-week-old plants. For flower time, the plants were monitored daily, and the day of bud emergence was recorded.

Germination on sugar plates

Age-matched seeds were sown on plates with half-strength MS-Agar and supplemented with 0 mM, 300 mM glucose or 300 mM mannitol and vernalized for 3 ds. Plates were transferred to growth room with 23/19 °C (day/night), 70/90% relative humidity, under a 12 h photoperiod with an irradiance of 200 μmol m^{-2} s^{-1}. Germination was scored after 7 d.

Statistics

Statistics were performed in GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). qPCR statistis was performed with Relative Expression Software Tool 2009 (Qiagen, Hilden, Germany). Ion-leakage data were analysed with a linear model and computing contrasts with Bonferroni corrections implemented in R (Bretz *et al.*, 2010).

Results

Ozone-induced cell death

A short, high-concentration exposure to ozone causes visible lesions and cell death in sensitive plants (Wohlgemuth *et al.*, 2002). Ascorbic acid is required for ozone defence (Conklin and Barth, 2004), but the role of glutathione is less clear. Wild-type Col-0 (WT) plants and mutants defective in ascorbic acid or glutathione biosynthesis and corresponding double mutants with *npr1* were exposed to increasing amounts of ozone. Under our growth conditions, no visible damage was seen in any of the genotypes when exposed up to 300 nl l^{-1} ozone. After 375 nl l^{-1} ozone for 6 h, visible lesions were frequently observed only in *npr1 vtc1*. The magnitude of cell death was quantified with ion leakage (Fig. 1). Increased cell death was measured in all genotypes, which was further significantly increased in the double *npr1 vtc1*. The reduced concentration of glutathione in *cad2* and *pad2* did not seem to affect ozone sensitivity. The *vtc1* mutant was originally isolated as an ozone-sensitive mutant. However, this ozone sensitivity was observed at higher doses and after a longer exposure (Conklin *et al.*, 2000) than used in this study.

The ozone sensitivity of *vtc* mutants has been suggested to be a result of low ascorbic acid concentration leading to reduced scavenging of ozone derived ROS. The low ascorbic acid concentration in *vtc1* and glutathione concentration in *cad2* or *pad2* could potentially be compensated by increases in other classes of antioxidants allowing the plant to maintain ROS-scavenging capacity. Measurements of glutathione in *vtc* mutants and ascorbic acid in *cad2* have yielded conflicting results but often show the WT concentration of these compunds (Table 1; Veljovic-Jovanovic *et al.*, 2001; Pavet *et al.*, 2005; Colville and Smirnoff, 2008). Rather than measuring individual antioxidants, the total antioxidant capacity was measured based on ORAC (Fig. 2). This assay compares the capacity of a plant extract to prevent radical [2,2′-azobis-2-methyl-propanimidamide

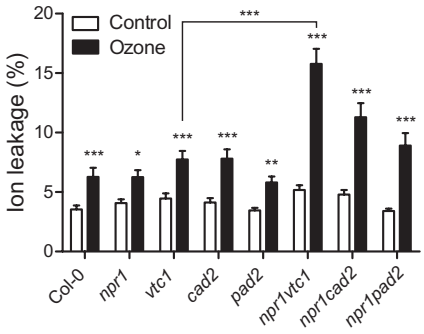

Fig. 1. Ozone sensitivity of antioxidant mutants. Plants of the indicated genotypes were exposed to a single 6-h pulse of 375 nl l^{-1} of ozone and cell death was monitored as ion leakage at 8 h after the beginning of the exposure. The presented data are the average and ±SE of five biological repeats of this experiment, with each repeat consisting of five samples. Mean values of ozone treatment that differed significantly from controls were identified with a linear model and Bonferroni correction (*P <0.05, **P <0.01, ***P <0.001).

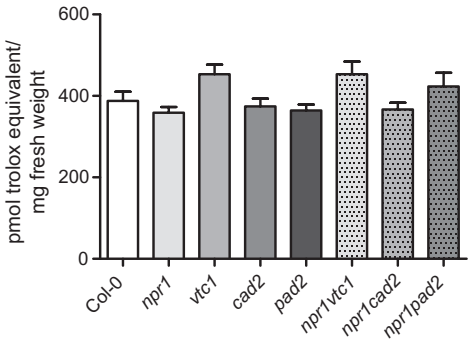

Fig. 2. Antioxidant status of antioxidant mutants. The total antioxidant capacity of 22-d-old plants was determined by the ORAC assay. Values are the mean ±SE of seven biological repeats (n=35). No statistical significances were found in the one-way ANOVA and Tukey post-hoc test.

(AAPH)] induced destruction of a fluorescent reporter (fluorescein) with a reference, a water-soluble vitamin E analogue—Trolox (Gillespie *et al.*, 2007). There was a trend towards a higher scavenging capacity of *vtc1* and *npr1 vtc1*; however, no statistically significant differences were detected between any of the mutants and WT. Thus, the low ascorbic acid or glutathione concentrations in *vtc1*, *cad2*, *pad2*, and the double mutants might be compensated by an increase in other (unidentified) antioxidants.

Therefore, the massive ozone-induced cell death observed in *npr1 vtc1* (Fig. 1) and of *vtc* mutants (Conklin *et al.*, 2000) may not be a result of deficient ROS scavenging per se but rather a consequence of an altered balance of defence and cell-death signalling pathways brought about by low ascorbic acid concentrations. Alternatively, the specific localization of the antioxidant may be important. The ORAC measurements were performed on whole leaf extracts, and previously the ascorbic acid concentration of the apoplast has been suggested to be a determinant of

ozone sensitivity (Yoshida *et al.*, 2006; Foyer and Noctor, 2009).

Gene expression under control conditions in antioxidant mutants

If a low ascorbic acid or glutathione concentration alter defence-signalling pathways, this should be visible as changes in the expression of defence-related genes. Indeed, previous results from gene-expression analysis using microarrays in *vtc1, rax1*, and *cad2* indicated that SA-responsive genes had a higher expression in *vtc1* and lower expression in *rax1* and *cad2* (Pastori *et al.*, 2003; Ball *et al.*, 2004). Marker genes were selected based on previous array analysis (Pastori *et al.*, 2003; Ball *et al.*, 2004; Ahlfors *et al.*, 2009) and from public microarray data from experiments using MeJA or the SA analogue benzothiadiazole (https://www.genevestigator.com/). Plants were grown for 22 d and gene expression analysed with real-time quantitative reverse transcription-PCR (qPCR). Several SA-regulated genes, *LECTIN-LIKE PROTEIN* (*LLP*), *Oxygenase*, *SENESCENCE ASSOCIATED 21* (*SAG21*), *PR-1*, and *PR-2*, showed a higher expression in *vtc1* (Fig. 3). Furthermore, the low expression of these genes in *npr1* indicated that they were regulated through the NPR1 signalling pathway. The elevated expression of these genes was missing in the double mutant *npr1 vtc1*, suggesting that signals resulting in altered gene expression in *vtc1* are

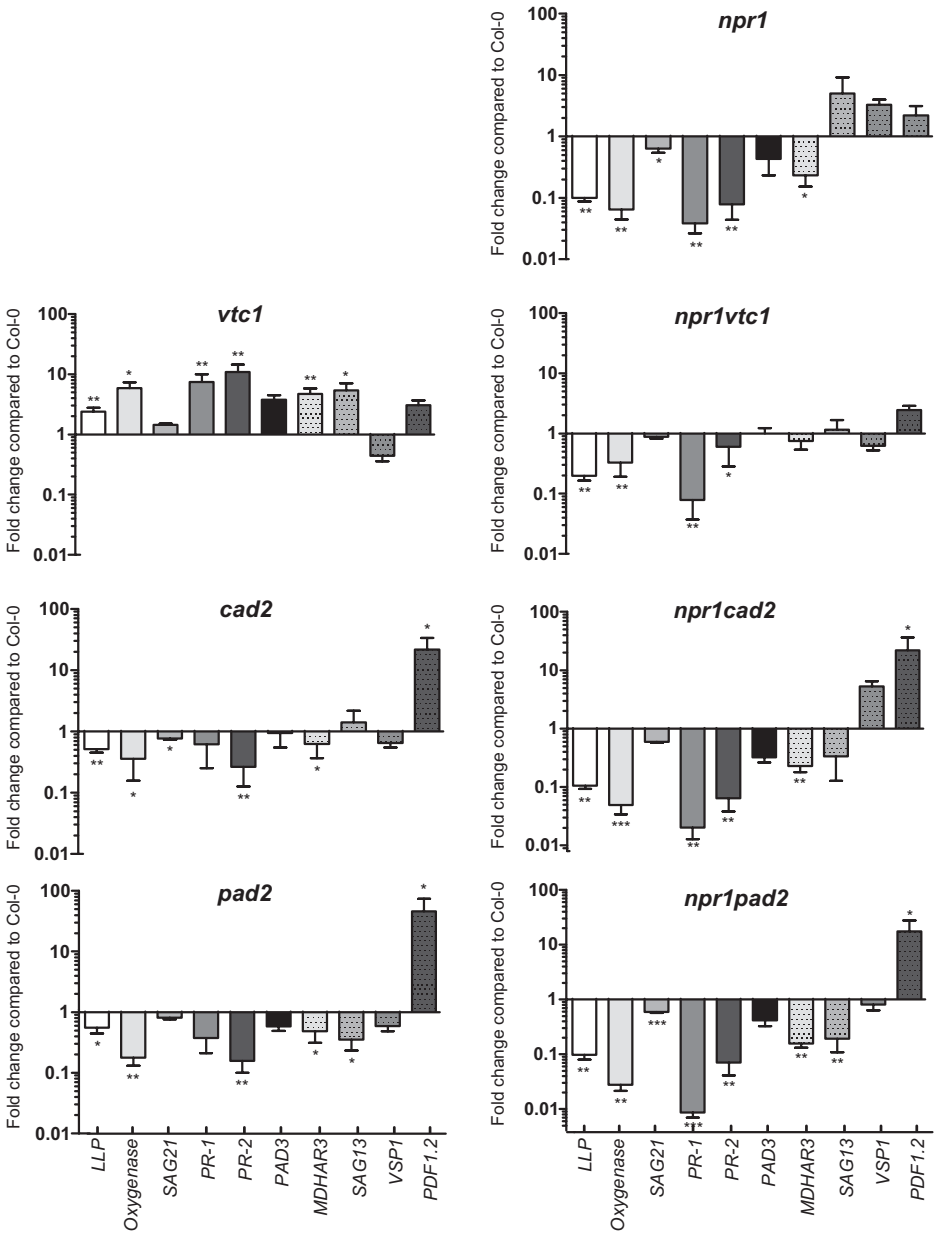

Fig. 3. Gene expression in antioxidant mutants. Expression of SA and JA marker genes was analysed by qPCR in 3-week-old antioxidant mutants. Transcript levels were normalized to the reference gene and expressed relative to Col-0 grown under control conditions. Values are the mean ±SE of five biological repeats. Statistical significance was calculated using Relative Expression Software Tool 2009 (**P* <0.05; ***P* <0.01; ****P* <0.001).

mediated through SA and NPR1. The *cad2* and *pad2* mutants had a lower expression of the SA marker genes and thus resembled *npr1*, suggesting that the low glutathione concentration of *cad2* and *pad2* could lead to a more inactive oligomeric state of NPR1. *MONODEHYDROASCORBATE REDUCTASE3—MDHAR3* represents a marker gene regulated by SA or JA as well as multiple other stress treatments (Wrzaczek *et al.*, 2010). This gene had a higher expression in *vtc1* and lower in *npr1*, thus resembling the SA marker genes. However, in the *npr1 vtc1* double mutant, this gene showed intermediate expression, suggesting that other signalling pathways in addition to SA signalling were activated in *vtc1*.

JA responses of antioxidant mutants

The JA-responsive marker gene *PLANT DEFENSIN 1.2* (*PDF1.2*) exhibited a high expression in *cad2* and *pad2* (Fig. 3), suggesting that the response to JA might be altered in the antioxidant mutants. Furthermore, JA is a proposed regulator of ascorbic acid and glutathione biosynthesis (Xiang and Oliver, 1998; Sasaki-Sekimoto *et al.*, 2005; Suza *et al.*, 2010). MeJA inhibition of root growth, a classical JA assay used in several JA mutant screens, was used to test a general response of the mutants to JA. The roots of *vtc1* and *npr1 vtc1* were significantly smaller than WT under control conditions (Fig. 4), a growth response that is not dependent on ascorbic acid (Barth *et al.*, 2010). Root growth of *cad2*, *pad2*, and *npr1* single and corresponding double mutants was inhibited by MeJA to the same extent as WT.

Although one general response to JA (root growth) appeared to be intact in the antioxidant mutants; there could still be other JA pathways with altered function. To investigate the connection between JA and antioxidants further, WT and mutants were treated with 0.5 mM MeJA for 8 h, and samples were taken for gene-expression analysis. Marker genes were selected to represent different classes of JA-responsive genes, *VEGETATIVE STORAGE PROTEIN1* (*VSP1*), a marker gene used to test JA responses (Ellis and Turner, 2001), *MDHAR3* encoding a protein central for the ascorbic acid–glutathione cycle and regulated by JA, SA, and ozone (Sasaki-Sekimoto *et al.*, 2005; Wrzaczek *et al.*, 2010), *LIPOXYGENASE4* (*LOX4*)—a marker for JA biosynthesis and *TYROSINE AMINOTRANSFERASE 3* (*TAT3*), a marker for wounding and JA (Yan *et al.*, 2007). The several hundred-fold increased expression of *VSP1* indicated effective induction of JA responsive signalling pathways (Fig. 5). *MDHAR3* and *TAT3* showed markedly higher MeJA-induced expression in *vtc1* compared with WT, suggesting that this mutant could be primed to respond to JA. This effect was not seen for *VSP1* or *LOX4*, suggesting that at least two parallel JA

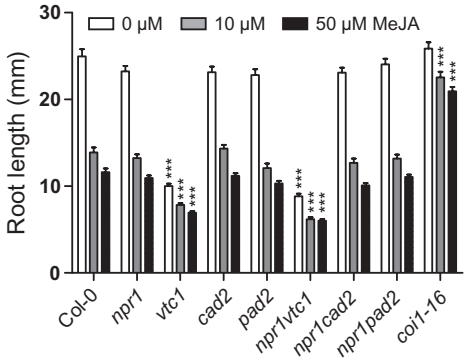

Fig. 4. MeJA inhibition of root growth: root growth of antioxidant mutants on control media, 10 µM MeJA and 50 µM MeJA. The *coi1-16* mutant was included as a JA-insensitive mutant. Values are the mean ± SE of four biological repeats; *n*=50–70. Mean values that differed significantly from the WT were identified by the one-way ANOVA and Tukey's post-hoc test (***P <0.001).

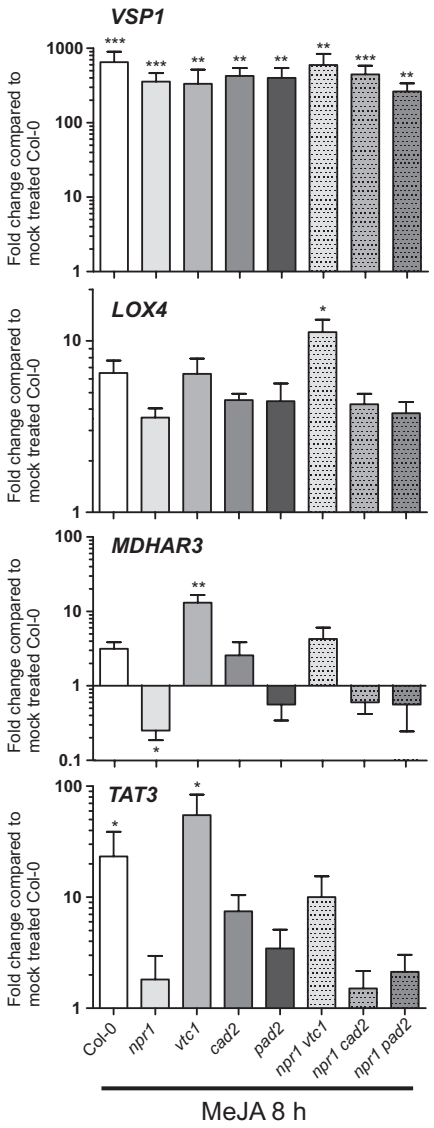

Fig. 5. MeJA-induced gene expression. Expression of marker genes in response to 8 h of 0.5 mM MeJA was analysed by qPCR in the antioxidant mutants. Transcript levels were normalized to the reference gene and expressed relative to mock treated Col-0. Values are the mean ±SE of four biological repeats. Statistical significance was calculated using Relative Expression Software Tool 2009 (*P <0.05; **P <0.01; ***P <0.001).

response pathways were acting in *vtc1*. NPR1 is traditionally considered a negative regulator of JA responses (Gfeller *et al.*, 2010). MeJA induction of *MDHAR3* and *TAT3* was abolished in *npr1* (Fig. 5), indicating that NPR1 also acted as a positive regulator for some JA-responsive genes.

Regulation of glutathione and ascorbic acid biosynthesis, and the ascorbate–glutathione cycle at the gene-expression level

To further explore the interconnection between antioxidants, SA, JA, and stress, genes from the ascorbate–glutathione cycle and biosynthesis of ascorbic acid and glutathione were clustered using public array data from the Affymetrix ATH1 chip (Fig. 6). Experiments were selected to include hormone treatments, abiotic and biotic stress, senescence and mutants with constitutive activation of defence signalling (see Materials and methods for a complete description of experiments). The marker genes used in qPCR were also included in the analysis. All qPCR marker genes clustered together (cluster II) and were strongly induced by abiotic and biotic stress, in senescence and constitutive defence mutants, and by SA and the SA-analogue BTH (benzothiadiazole). The only genes from the ascorbate–glutathione cycle in this cluster were *MDHAR2* and *MDHAR3*. Cluster I genes also had increased expression levels in response to biotic stress, some abiotic stresses (ozone and high light), and by SA, BTH, and ethylene. Genes of interest in this cluster include both steps of glutathione biosynthesis (*GSH1*, *GSH2*), *GLUTATHIONE REDUCTASE1* and *APX2*. MeJA has previously been shown to increase *GSH1* and *GSH2* expression, which was also seen in cluster I (Xiang and Oliver, 1998). Additionaly ethylene increased expression of *GSH1* and *GSH2*. Cluster III genes had decreased expression in response to biotic stress, senescence, BTH and ethylene. Whereas glutathione biosynthesis genes had increased expression by stress, the response of ascorbic acid biosynthesis genes was far more complex, probably related to several biosynthesis routes. In the main L-galactose pathway there are ten steps, where the last four steps are specific for ascorbic acid biosynthesis (Linster and Clarke, 2008). The first committed step catalysed by GDP-L-galactose phosphorylase, encoded by *VTC2* and *VTC5*, only the latter gene appeared stress responsive with increased expression by biotic and abiotic stress. In contrast, the next step of the pathway (*VTC4*), had strongly decreased expression by biotic stress, ethylene and senescence and to a lesser extent by abiotic stress. The *vtc1* and *vtc2* mutants clustered together and had increased expression of SA marker genes, which was more prominent in *vtc1*. The knockout of a cytosolic *O*-acetylserine(thiol)lyase, which reduced cysteine and glutathione biosynthesis (López-Martín *et al.*, 2008), had decreased expression of the SA qPCR marker genes, similar to the results from *cad2* and

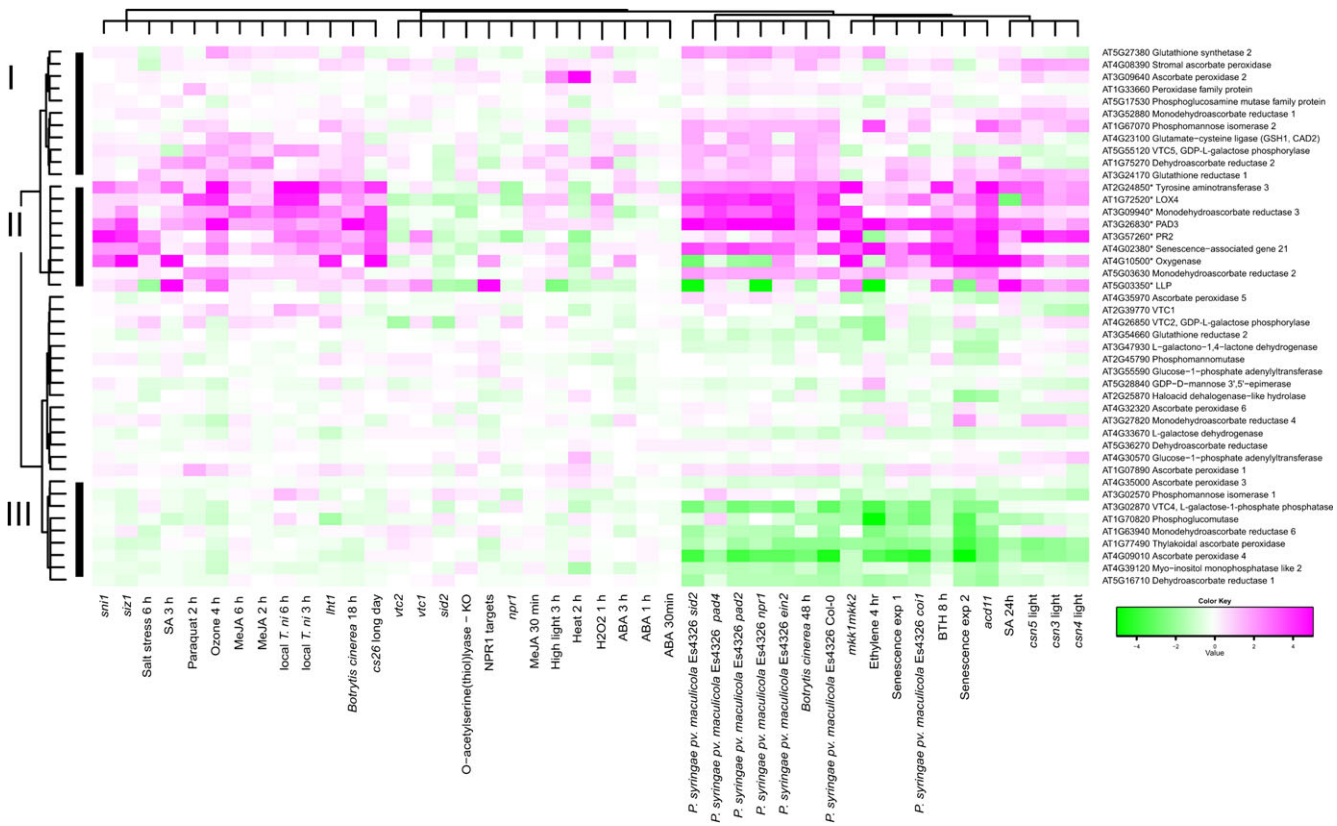

Fig. 6. Expression of ascorbate–glutathione cycle, ascorbic acid, and glutathione biosynthesis genes during abiotic and biotic stress, during hormone treatments, and in constitutive defence mutants. Bootstrapped Bayesian hierarchical clustering of genes is shown in plants subjected to stress treatments compared with normal growth conditions, or in mutant versus wild type. Magenta and green indicate increased and decreased expression compared with untreated or wild-type plants, respectively.

pad2 (Fig. 3). Overall, the clustering reinforces that ROS, antioxidants, hormone and stress responses are highly interconnected, and by altering any of these, the output in gene expression will change.

Developmental responses of antioxidant mutants

The gene-expression analysis (Figs 3, 5) indicated that low ascorbic acid or glutathione concentration altered both SA and JA signalling. Both of these hormones are important regulators of ROS induced cell death (Overmyer *et al.*, 2003) which could contribute to increased cell death of *vtc1, npr1 vtc1* and *npr1 pad2* (Fig. 1). Plant hormones are not only regulators of stress responses; they are also intimately involved in plant development. To gain further insight into the consequence of low ascorbic acid or glutathione concentration the antioxidant mutants for three developmental responses, flower time, size and glucose inhibition of germination, were investigated, where low ascorbic acid or glutathione concentration have been shown to lead to altered responses (Ogawa *et al.*, 2001; Pavet *et al.*, 2005; Kotchoni *et al.*, 2009).

Mutants with low ascorbic acid concentration have been described as either early or late flowering in different studies, possibly a consequence of different light levels (Pavet *et al.*, 2005; Kotchoni *et al.*, 2009). Under the conditions of this experiment (12/12 h light/dark, 250 μmol photons m^{-2} s^{-1}), *vtc1* and *npr1 vtc1* were to some extent late flowering (Fig. 7A). Low glutathione concentrations, either in the *cad2* mutant or by treatment with a glutathione biosynthesis inhibitor, delayed flowering (Ogawa *et al.*, 2001). Under our growth conditions, the *npr1* and *npr1 cad2* mutants were early flowering, but *cad2*, *pad2* or *npr1 pad2* were not, suggesting that a specific balance between glutathione concentration and NPR1 influences flowering.

The *vtc1* mutant was smaller than WT (Fig. 7B) and has smaller cells (Pavet *et al.*, 2005). The small size could be a result of a lower amount of precursors available for cell-wall biosynthesis (the ascorbic acid biosynthesis pathway shares intermediates with cell-wall polysaccharide synthesis), or due to constitutive activation of SA-dependent defences (Pavet *et al.*, 2005). When compared with *vtc1*, *npr1 vtc1* was larger, indicating that constitutive activation of SA defences through NPR1 in *vtc1,* at least partially, decreases its size.

High glucose concentrations reduce germination and growth of *Arabidopsis* seeds on MS medium. A complex network of signals is involved in this response, including general metabolism and the plant hormones abscisic acid and ethylene (Ramon *et al.*, 2008). After 7 d of growth on 300 mM glucose, three different classes of seedlings were observed: seeds that did not germinate, white seedlings, and green seedlings (Fig. 8A). The growth of *npr1*, *vtc1*, and particularly *npr1 vtc1* was inhibited by glucose. Interestingly, growth of *npr1 cad2* and *npr1 pad2* double mutants was not inhibited as in *npr1*, indicating that all three components ascorbic acid, glutathione, and NPR1 were involved in sugar signalling. No major changes were observed on growth in the osmotic stress control mannitol (Fig. 8B).

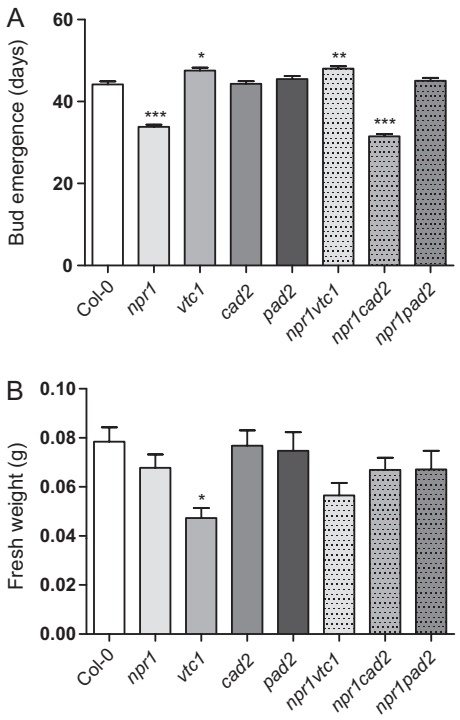

Fig. 7. Flower time and fresh weight of antioxidant mutants. (A) Flower time, determined as bud emergence in a short day (12/12 h light/dark). (B) Fresh weight, determined on 3-week-old plants. The data represent the means ±SE of four biological repeats. n=35 for flower time and n=54–59 for fresh weight. Mean values that differed significantly from the WT were identified with one-way ANOVA and Tukey's post-hoc test (*P <0.05; **P <0.01; ***P <0.001).

Discussion

The correct integration of internal developmental signals with external cues from the environment is essential for plant survival. ROS are signalling intermediates for both development and abiotic and biotic stress responses. Ozone exposure leads to a controlled production of ROS in the apoplast, similar to the ROS burst produced in the hypersensitive response elicited in plants in response to pathogen infection (Wohlgemuth *et al.*, 2002; Kangasjärvi *et al.*, 2005; Ahlfors *et al.*, 2009). Thus, ozone can be used to study the role of apoplastic ROS as signalling molecules without the added complexity of any other manipulation of the plant or infection with pathogens. Plants with low ascorbic acid concentrations are ozone-sensitive, which traditionally has been considered to be a consequence of decreased ROS detoxification (Conklin and Barth, 2004), but it is likely that ascorbic acid concentration of a specific compartment, for example, the apoplast (Yoshida *et al.*, 2006), could be more important than the plant's total ascorbic acid concentration or total antioxidant capacity. A model for ROS-induced cell death has previously been

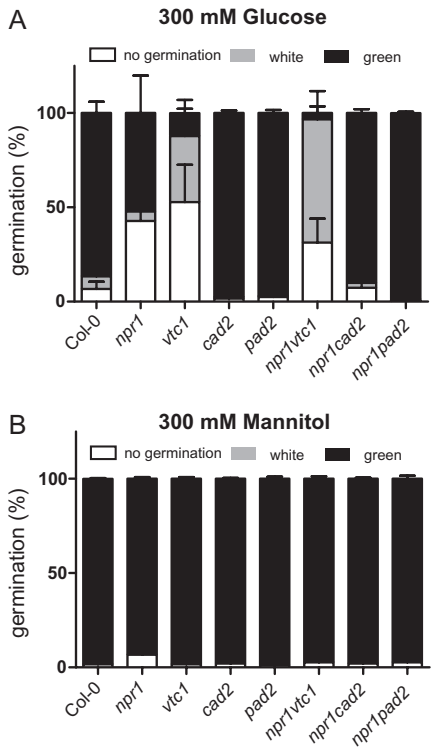

Fig. 8. Sugar inhibition of germination. The germination frequency of seeds from antioxidant mutants grown on MS media supplemented with 300 mM glucose (A) or 300 m M mannitol (B) for 7 d. Seeds were scored in three different categories: no germination, white seedlings, and green seedlings. Values are the mean ±SE of three biological repeats. n=150–300.

suggested where initial ROS production activates biosynthesis of SA, JA, and ethylene. Subsequently, the balance of these hormones determines the extent of cell death (Overmyer *et al.*, 2003). The *vtc1* mutant accumulates high concentrations of SA and SA glucoside, indicating a primed state that is able to respond faster to stress (Mukherjee *et al.*, 2010). Thus, a pulse of apoplastic ROS could tip the balance of signalling pathways towards cell death in *vtc1*. This process appears to be under partial control of NPR1, since cell death was further significantly increased in *npr1 vtc1*. In contrast when the *npr1* mutation was introduced into the ozone-sensitive mutant *rcd1*, it led to increased ozone tolerance (Overmyer *et al.*, 2005). Thus, NPR1 appears to be at the intersection of several signalling pathways, and even in response to a single stress (i.e. ozone), absence of NPR1 can promote either cell death or survival.

To gain further insight into which pathways could be altered in mutants with low ascorbic acid or glutathione concentrations, a gene-expression analysis was performed using a set of stress-related marker genes. Constitutive activation of defences in *vtc1* has been proposed to be regulated by NPR1 based on the movement of GFP-NPR1 into the nucleus of *vtc1* (Pavet *et al.*, 2005). High expression of SA related marker genes in *vtc1* was fully suppressed by *npr1* (Fig. 3), and the concentration of SA glucoside was reduced in *vtc1 npr1* and double mutants affecting SA accumulation *vtc1 pad4* and *vtc1 eds5* (Mukherjee *et al.*, 2010), strongly suggesting that primed defence responses in *vtc1* are indeed regulated though SA and NPR1. In contrast, SA marker genes had a lower expression in *cad2* and *pad2*; thus, these mutants resemble *npr1* (Fig. 3). Low expression of SA marker genes has also been observed in plants treated with a glutathione biosynthesis inhibitor (Colville and Smirnoff, 2008) and was also seen in a mutant for cytosolic *O*-acetylserine(thiol)lyase with reduced cysteine and glutathione biosynthesis (Fig. 6). This suggests that glutathione itself could act as a signalling molecule, and less glutathione in *cad2* or *pad2* might impact directly on defence responses (Ball *et al.*, 2004; Senda and Ogawa, 2004). The ratio of reduced (GSH) to oxidized (GSSG) glutathione may also be an important factor, feeding GSH but not GSSG to leaves induced expression of *PR-1* (Gomez *et al.*, 2004). Consistent with this, a *gr1* mutant (which lacks GLUTATHIONE REDUCTASE1, and thus has increased concentrations of GSSG) had lower SA concentrations and decreased PR-1 expression in a catalase-deficient mutant background (Mhamdi *et al.*, 2010). NPR1, as well as one of its interacting transcription factors, TGA1, is under redox control (Despres *et al.*, 2003; Mou *et al.*, 2003); thus the low expression of SA marker genes in *cad2* and *pad2* could be a result of low glutathione concentrations, or the redox balance in these mutants could lead to increased sequestering of NPR1 in inactive oligomer complexes (Mou *et al.*, 2003).

JA regulates expression of multiple genes in ascorbic acid and glutathione biosynthesis and ROS detoxification (Xiang and Oliver, 1998; Sasaki-Sekimoto *et al.*, 2005; Suza *et al.*, 2010). In the MeJA root inhibition assay, *cad2* and *pad2* had WT responses indicating a normal response to MeJA (Fig. 4). The poor growth of *vtc1* roots makes it difficult to use this assay to estimate its JA response. However, a complex response was seen for the mutants in gene-expression analysis. At least two separate JA signalling pathways were active based on MeJA hyperinduction of *MDHAR3* and *TAT3* in *vtc1* and, in contrast, normal induction of *VSP1* and *LOX4* (Fig. 5). This contrasting gene-expression response illustrates that to fully explore SA and JA responses, the selection of marker genes should be extended to include additional genes other than only the classical *PR-1* (SA marker) and *VSP1* (JA marker). NPR1 is a required signalling component for many SA responses and is usually described as a negative regulator of JA signalling (Gfeller *et al.*, 2010). The use of separate JA marker genes showed that NPR1 can act as a positive regulator of at least one JA signalling pathway; MeJA-induction of *MDHAR3* and *TAT3* was impaired in *npr1* (Fig. 5). Interestingly, in systemic induced resistance NPR1 also acts as a positive regulator downstream from JA (Ramirez *et al.*, 2010), indicating that NPR1 is recruited to multiple steps in SA and JA signalling.

Antagonistic interaction among plant hormones is a common phenomenon and is frequently observed between SA and JA where NPR is a central regulator (Spoel *et al.*, 2003). The JA-responsive gene *PDF1.2* is highly sensitive to

SA inhibition, and this antagonistic action has been proposed to act through redox changes in glutathione, as application of a glutathione biosynthesis inhibitor removes the antagonistic action of SA on JA (Koornneef *et al.*, 2008). A critical role for the GSH:GSSG ratio in expression of JA-related genes was seen in the effect of the *gr1* mutation, which leads to repression of JA-responsive genes including *LOX* and *TAT3* (Mhamdi *et al.*, 2010). Interestingly, in *cad2* and *pad2*, the *PDF1.2* expression was significantly higher than in WT (Fig. 3), thus further supporting a role for glutathione in regulating JA responses.

ROS detoxification via the ascorbate–glutathione cycle is a multi-gene network where many enzymes are encoded by multiple genes (Mittler *et al.*, 2011). To gain further insight into how this network at the gene-expression level responds to stress, ROS, and plant hormones, array data from selected experiments were analysed by the Bayesian hierarchical clustering method (Fig. 6). The list of genes used in the analysis included ascorbate–glutathione cycle, ascorbic acid and glutathione biosynthesis genes, and the marker genes used in qPCR analysis. Genes with very high induced expression by multiple stresses and hormones (cluster II) were all the qPCR marker genes and two genes from the ascorbate–glutathione cycle *MDHAR2* and *MDHAR3* (the latter is the qPCR marker gene in Figs 3 and 5). Genes with increased expression (cluster I) included both steps of glutathione biosynthesis (*GSH1* and *GSH2*) and one of two genes encoding GDP-L-galactose phosphorylase (*VTC5*). Genes with reduced expression included several ascorbic acid biosynthesis genes *VTC1*, *VTC2*, *VTC4, GDP-Man-3,5-epimerase*, and *Phosphomannomutase* (*PMM*). In addition to JA and SA, also ethylene was found to induce *GSH1* and *GSH2* expression. The similar gene-expression profiles of several biotic stresses (*P. syringae* ES4326 and *B. cinerea*), senescence, ethylene, the SA analogue benzothiadiazole *S*-methyl ester (BTH), and mutants undergoing spontaneous cell death *acd11* and *mkk1mkk2* (Gao *et al.*, 2008; Qiu *et al.*, 2008; Palma *et al.*, 2010), indicate that these treatments elicit signalling pathways that eventually converge at the promoters of genes in biosynthesis of ascorbic acid and glutathione, and in the ascorbate–glutathione cycle. Identification of these transcription factors could help unravel the ROS-antioxidant signalling network.

For many stress-related phenotypes, low ascorbic acid concentration and low glutathione concentration lead to opposite phenotypes and gene-expression profiles (Table 1, Fig. 3). This opposite effect was clearly evident in the sugar inhibition of seed germination, especially in the *npr1* background (Fig. 8). The *npr1* mutant had decreased germination, which was enhanced in the double mutant *npr1 vtc1* and was suppressed in *npr1 cad2* and *npr1 pad2*, again indicating that glutathione or the redox balance influences NPR1 signalling.

For some defence-related phenotypes differing glutathione concentrations lead to phenotypic variation, exemplified by different gene-expression patterns in *rax1* and *cad2* (Ball *et al.*, 2004). In our data, the most striking difference between *cad2* and *pad2* was observed in the flowering time of the double mutants with *npr1*, where *npr1 cad2* was early flowering, but *npr1* pad2 was not. Since the difference in glutathione concentration of *cad2* and *pad2* is rather minor (20 vs 30% of WT; Parisy *et al.*, 2007), this indicates that very small changes in redox balance can induce major changes in developmental fate. That these small changes can have such a dramatic effect could also explain the differences in phenotypes between different laboratories; even a slight change in growth condition could alter the balance of a redox signalling pathway.

Both ROS and antioxidants are clearly important in plant development and stress responses, and can be used by the plant as signalling molecules (Foyer and Noctor, 2005*a*, *b*; Potters *et al.*, 2010). However, testing the role of the 'cellular redox state' or 'oxidative signalling' represents a fundamental challenge. The diverse chemical nature of various ROS, redox-active components, and ROS scavenging enzymes makes it difficult to measure more than a few representative species per sample. To probe the complexity of oxidative signalling, the application of new tools could provide novel insights, including *in vivo* probes for ROS and redox measurements, e.g. HyPer (Costa *et al.*, 2010) and redox-sensitive GFP (Meyer *et al.*, 2007), measurements of antioxidant concentrations in specific subcellular compartments (Queval *et al.*, 2011), mutants with specific defects in redox balance or ROS signalling, double mutants to position the order of redox events in a signalling pathway, and a broader range of marker genes in gene-expression analysis reflecting the full range of hormone signalling, including responses to SA and JA.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table 1. Primer sequences and amplification efficiency used in real-time quantitative PCR.

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