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RESEARCH PAPER

Early leaf senescence is associated with an altered cellular redox balance in *Arabidopsis cpr5/old1* mutants

H.-C. Jing[1,3,*], R. Hebeler[2,*], S. Oeljeklaus[2], B. Sitek[2], K. Stühler[2], H. E. Meyer[2], M. J. G. Sturre[1], J. Hille[1], B. Warscheid[2] & P. P. Dijkwel[1,4]

1 Department of Molecular Biology of Plants, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Haren, The Netherlands
2 Medizinisches Proteom-Center, Zentrum fuer Klinische Forschung, Ruhr-Universitaet Bochum, Bochum, Germany
3 Centre for Sustainable Pest and Disease Management, Department of Plant Pathology and Microbiology, Rothamsted Research, Harpenden, UK
4 Institute of Molecular BioSciences (IMBS), Massey University, Palmerston North, New Zealand

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Cell death; *CPR5/OLD1;* leaf senescence; reactive oxygen species; redox balance.

Correspondence
P. P. Dijkwel, Institute of Molecular Biosciences (IMBS), Massey University, Private Bag 11222, Palmerston North, New Zealand.
E-mail: p.dijkwel@massey.ac.nz

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K. Krupinska

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* Joint first authors

ABSTRACT

Reactive oxygen species (ROS) are the inevitable by-products of essential cellular metabolic and physiological activities. Plants have developed sophisticated gene networks of ROS generation and scavenging systems. However, ROS regulation is still poorly understood. Here, we report that mutations in the *Arabidopsis CPR5/OLD1* gene may cause early senescence through deregulation of the cellular redox balance. Genetic analysis showed that blocking stress-related hormonal signalling pathways, such as ethylene, salicylic acid, jasmonic acid, abscisic acid and sugar, did not affect premature cell death and leaf senescence. We took a bioinformatics approach and analysed publicly available transcriptome data of presymptomatic *cpr5/old1* mutants. The results demonstrate that many genes in the ROS gene network show at least fivefold increases in transcripts in comparison with those of wild-type plants, suggesting that presymptomatic *cpr5/old1* mutants are in a state of high-cellular oxidative stress. This was further confirmed by a comparative, relative quantitative proteomics study of *Arabidopsis* wild-type and *cpr5/old1* mutant plants, which demonstrated that several Phi family members of glutathione s-transferases significantly increased in abundance. In summary, our genetic, transcriptomic and relative quantitative proteomics analyses indicate that *CPR5* plays a central role in regulating redox balance in *Arabidopsis*.

INTRODUCTION

Aerobic life depends on oxygen. However, oxygen atoms and oxygen-containing molecules can act as highly reactive molecules, commonly known as reactive oxygen species (ROS), when unpaired electrons are present. ROS include triplet and singlet oxygen, superoxide, nitric oxide and hydroxyl radicals, which can potentially damage and deteriorate various cellular components, such as proteins, DNA and lipids, by oxidation. Thus, oxygen is inherently detrimental to aerobic organisms. The dual effect of oxygen on shaping life-history traits and evolution of aerobic organisms is generally known as the 'Oxygen Paradox' (Davies 1995). ROS can be generated by internal organelles and other cellular components, including mitochondria, chloroplasts and microbodies, as well as by external stress conditions, such as ozone exposure and UV-B radiation. To cope with the danger imposed by ROS, cells have evolved sophisticated ROS scavenging systems. These include antioxidative enzymes such as catalase, superoxide dismutase and ascorbate peroxidase, as well as non-enzymatic antioxidants such as ascorbate, the tripeptide glutathione, tocopherol and carotenoids (Apel & Hirt 2004). In addition to being a 'wear-and-tear' force, ROS are important cellular signalling molecules in the cell cycle, hormonal signalling, programmed cell death, growth and development and responses to biotic and abiotc stresses (Finkel 2003; Foyer & Noctor 2005;

Pitzschke *et al.* 2006; Moller *et al.* 2007). Thus, ROS exert effects on life either directly by oxidative damage or by acting as signals.

Because of their photosynthetic activity, chloroplasts are the major source of ROS generation in the light (Baier & Dietz 2005; Asada 2006; Rhoads *et al.* 2006) and hence tight control of ROS generation in chloroplasts is essential for whole cell redox balance (Murgia *et al.* 2004; Kariola *et al.* 2005; Perez-Ruiz *et al.* 2006; Liu *et al.* 2007). Mitochondria are the primary source of ROS in the dark or in tissues without photosynthetic machineries (Puntarulo *et al.* 1988) and recent evidence also points to the importance of mitochondria in cellular redox balance (Dutilleul *et al.* 2003; Noctor *et al.* 2007). Furthermore, cytosolic redox machineries seem to be a central component of the ROS network (Davletova *et al.* 2005; Miller *et al.* 2007). Thus, redox balance is controlled through complex cross-talk between the nucleus, chloroplast and mitochondria ROS generation and scavenging systems (Rhoads & Subbaiah 2007; Wormuth *et al.* 2007). The complexity is well-illustrated by identification of the ROS gene network, which consists of over 150 genes (Mittler *et al.* 2004).

Leaf senescence is a nutrient recycling process, and chloroplast disruption is one of the paramount events of the senescence syndrome. During senescence, ROS are actively produced, which, in turn, causes constant changes in redox regulating components (Vanacker *et al.* 2006; Zimmermann *et al.* 2006). Genes whose expression is induced by ozone and wounding overlap substantially with the so-called senescence-associated genes (*SAG*s) that are upregulated during developmental senescence (Navabpour *et al.* 2003; Le Deunff *et al.* 2004). ROS-associated genes are an important part of the transcriptomes of developmental and dark-induced senescence (Navabpour *et al.* 2003; Zimmermann & Zentgraf 2005; Gregersen & Holm 2007). Early studies indicated that mitochondria remain functional until very late during senescence (Thomas & Stoddart 1980). This is considered necessary for energy supply during the nutrient remobilisation process, which argues that mitochondria cannot be the primary trigger of senescence. In plant cells, ROS derived from mitochondria and NADPH oxidases are directly involved in triggering programmed cell death during plant–pathogen interactions (Tada *et al.* 2004; Torres *et al.* 2005; Hofius *et al.* 2007). However, recent evidence indicates that mitochondrial dysfunction is also tightly linked to senescence. Similar to antimycin A, salicylic acid and hydrogen peroxide were found to disrupt mitochondrial electron transport and induce the expression of pathogen-related and senescence-associated genes in cultured tobacco cells (Maxwell *et al.* 2002). A convincing demonstration of the involvement of mitochondria in senescence came from the isolation and characterisation of *Arabidopsis NOS1*, which encodes a mitochondria-targeted protein to inhibit ROS production (Guo & Crawford 2005). The *nos1* mutants showed enhanced ROS production and dark-induced leaf senescence. Furthermore, virus-induced silencing of a prohibitin gene, which controls mitochondrial biogenesis in *Nicotiana benthamiana*, induced elevated ROS production and premature senescence (Ahn *et al.* 2006). A more recent report showed that chloroplast-localised ACD2 regulates mitochondrial ROS production provoked by bacterial infection (Yao & Greenberg 2006). Thus, ROS generated from both chloroplasts and mitochondria can be important for senescence and programmed cell death in general.

We are interested in understanding the regulation of leaf senescence. Using *Arabidopsis* as a model, we have isolated a large collection of *old* mutants (Jing *et al.* 2002). Recently, *OLD1* was shown to be allelic to *CPR5*, which has pleiotropic functions in regulating multiple biological processes (Jing *et al.* 2007). Amongst various *cpr5* mutant phenotypes is cell death associated with spontaneous lesion formation and premature senescence (Bowling *et al.* 1997; Jing *et al.* 2002; Yoshida *et al.* 2002). In this paper, we report efforts to dissect the primary causes of this cell death phenotype in *cpr5* mutants. We constructed various double mutants between *cpr5* and mutants blocking the signalling of cell death-related hormonal signalling. In addition to the analysis of transcriptome data, we quantitatively compared the soluble proteomes of presymptomatic *cpr5* mutant and wild-type plants by two-dimensional difference in gel electrophoresis (2-D DIGE) using fluorescent dyes. Our results provide a link between ROS and cell death processes, which prompts us to propose *CPR5* as an important player in regulating cellular redox balance.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana accessions L*er*-0 and Col-0 were the wild types used. The mutant alleles and transgenic plants used were *old1-1* (renamed *cpr5-l1*) (Jing *et al.* 2007), *cpr5-1* (Bowling *et al.* 1997), *cpr5-2* (Boch *et al.* 1998), *npr1-1* (Cao *et al.* 1997), *ein2-1* (Guzman & Ecker 1990), *ctr1-1* (Kieber *et al.* 1993), *jar1-1* (Staswick *et al.* 1992) and *abi4-1* (Finkelstein *et al.* 1998) and *Arabidopsis* plants expressing the bacterial *nahG* gene (Delaney *et al.* 1994) were grown under standard conditions (22 °C, 60–70% relative humidity and 16 h light). Depending on the specific phenotypes and identities of the particular mutations, a variety of screening methods employing hormonal responses and PCR-based marker assays were used to isolate double mutants.

Plants were grown in an rich organic soil (TULIP PROFI No.4, BOGRO B.V., Hardenberg, The Netherlands) or in Murashige and Skoog medium containing 0.8% agar under the conditions as described (Jing *et al.* 2002).

Transcriptome data

The transcriptome data were obtained from the Genevestigator public database (Zimmermann *et al.* 2004). The datasets were provided by Xinnian Dong's lab. Detailed

experimental conditions were described in Experiment 175 (Expression profiling of *cpr5*, *cpr5npr1*, *cpr5scv1*, *cpr5npr1svi1* and *npr1* lines). The tissue used for microarray profiling was aboveground parts of 2-week-old seedlings. At this stage, no cell death symptoms were visible and we defined it as the presymptomatic stage. The transcription data were downloaded from the Genevestigator website (http://www.genevestigator.ethz.ch/) and the values were averaged for all comparisons.

Comparative proteomics analysis of the *cpr5/old1* mutant and wild-type plants

For differential analysis of the soluble proteomes of the *cpr5* mutant and wild-type plants, 16-day-old L*er*-0 and *cpr5-l1* mutant plants were used. Plants were grown as described by Hebeler *et al.* (2008). Identification of changes in protein abundance based on DIGE minimal labelling (Unlü *et al.* 1997; Marouga *et al.* 2005), followed by mass spectrometry (MS) was performed as described by Hebeler *et al.* (2008), with slight modifications. Protein extracts were prepared according to Giavalisco *et al.* (2003). Briefly, approximately 500 mg of frozen *A. thaliana* plants were supplied with 0.125 parts (w/w) of solution 1 [one tablet Complete™ protease inhibitor (Roche Diagnostics, Mannheim, Germany) dissolved in 2 ml of 100 mM KCl, 20% (v/v) glycerol and 50 mM Tris, pH 7.1] and 0.05 parts (w/w) of solution 2 [1 mM pepstatin A (Serva, Heidelberg, Germany) and 1.4 μM PMSF (AppliChem, Darmstadt, Germany) dissolved in ethanol]. Plants were ground to a fine powder in a mortar placed in liquid nitrogen. After centrifugation of the homogenate (30 min at 226,000 *g* and 4 °C), the supernatant was collected. Proteins extracted from wild-type and mutant plants were subsequently differentially labelled with the fluorescent CyDyes Cy3 and Cy5. In addition, an internal standard allowing for normalisation of spot intensities and comprising equal amounts of protein from each sample was labelled with Cy2. Labelling reactions were performed as described in detail by Hebeler *et al.* (2008). Differentially labelled samples were then combined in equal ratios and concomitantly subjected to 2-D PAGE. In total, six independent experiments were performed.

2-D-PAGE employing isoelectric focusing (IEF) in the first, and SDS–PAGE in the second, dimension was performed following the protocol of Klose (1975); IEF was carried out according to Klose & Kobalz (1995) using tube gels (20 cm × 1.5 mm). Following IEF, the tube gels were ejected and incubated in equilibration buffer [125 mM Tris, 40% (w/v) glycerol, 3% (w/v) SDS, 65 mM DTT, pH 6.8] for 15 min. Gels were then washed three times with SDS–PAGE running buffer (25 mM Tris, 192 mM glycine, 0.2% SDS), placed on top of polyacrylamide gels (20 cm × 30 cm × 1.5 mm; 15.0% total acrylamide, 1.3% bisacrylamide), and fixed with 1.0% (w/v) agarose containing 0.01% (w/v) bromphenol blue (Riedel de-Haen, Seelze, Germany). SDS–PAGE was performed at 15 °C at a constant current flow of 75 mA for 15 min, followed by a constant current flow of 200 mA for 6–7 h.

Following image analysis using the ImageQuant™ software (version 5.2, Amersham Biosciences/GE Healthcare, Piscataway, NJ, USA), statistical data analysis and relative protein quantification were performed using the DeCyder™ software, version 6.0 (Amersham Biosciences/GE Healthcare). Spot intensities were normalised based on the internal standard labelled with Cy2. A protein spot was considered as significantly regulated if it showed an average factor of ±1.5, as well as a Student's *t*-test value of $P < 0.05$. Average abundance ratios were calculated by dividing fluorescent intensities of wild-type protein spots by those of mutant protein spots.

For identification of protein spots exhibiting significant differences in fluorescence intensities, a preparative gel (400 μg protein loaded) was prepared and stained with colloidal Coomassie Brilliant Blue (Neuhoff *et al.* 1990). Spots were excised and destained by alternately incubating them with 20 μl of 10 mM ammonium hydrogen carbonate (NH_4HCO_3) and 20 μl of 5 mM NH_4HCO_3/50% acetonitrile (ACN) for 10 min each. Gel pieces were dried *in vacuo* and subjected to proteolytic digestion with trypsin (Promega, Mannheim, Germany) dissolved in 10 mM NH_4HCO_3 (pH 7.8) at a final concentration of 0.03 $\mu g \cdot \mu l^{-1}$. Subsequent to tryptic digestion at 37 °C overnight, proteolytic peptides were extracted twice with 10 μl of ACN and 5% FA mixed 1:1 (v/v). Extracts were combined, ACN was removed *in vacuo* and samples were acidified by addition of 5% FA to a final volume of 20 μl.

For MS analyses of tryptic digests, a Dionex LC Packings system (Dionex LC Packings, Idstein, Germany) coupled to a QSTAR XL instrument (Applied Biosystems, Foster City, CA, USA) was used. Peptide mixtures were separated by online RP capillary HPLC as described by Schaefer *et al.* (2004). The MS instrument was equipped with a nanoelectrospray ion source (SCIEX, Toronto, Canada) and distal-coated SilicaTips (FS360–20–10-D; New Objective, Woburn, MA, USA); parameters were set as detailed by Hebeler *et al.* (2008).

For peptide and protein identification, uninterpreted peptide ESI-MS/MS spectra were correlated with the *A. thaliana* EBI protein sequence database (http://www.ebi.ac.uk, ipi.ARATH.v3.17.fasta) using the search algorithm SEQUEST (Eng *et al.* 1994; Ducret *et al.* 1998) (TURBOSEQUEST v.27). Search parameters and criteria for accepting a protein as identified were as reported in Hebeler *et al.* (2008).

RESULTS

Blocking of hormonal signalling does not prevent the early onset of *cpr5*-induced leaf senescence

Mutations in *CPR5* resulted in defective responses to multiple hormonal and other signalling molecules. We constructed double mutants between *cpr5* and mutants defective in the sensing and/or signalling of ethylene,

jasmonic acid, salicylic acid, abscisic acid and sugar. The double mutants were used to examine whether the cell death and senescence phenotypes could be blocked by perturbations in these stress-related hormonal pathways. As shown in Fig. 1, 30 days after germination all the double mutants exhibited premature cell death and leaf senescence in the 1st and/or 2nd rosette leaf pairs, comparable to *cpr5* mutants. In contrast, the wild-type and the individual hormonal mutants had no signs of cell death and senescence on these leaves (results not shown). We therefore concluded that impairing ethylene, salicylic acid, jasmonic acid, abscisic acid or sugar signalling does not prevent the earlier onset of cell death and senescence induced in *cpr5* mutants.

Expression of developmental senescence marker genes in presymptomatic *cpr5* mutants

To further validate the roles of the stress-related hormones in *cpr5*-induced cell death and senescence, we took advantage of publicly available whole genome transcriptome data. Microarray gene expression profiling for *cpr5* mutants was carried out by Xinnian Dong's lab and the data available at the Genevestigator website (Zimmermann *et al.* 2004). Two-week-old seedlings, which did not show any signs of cell death or senescence, were used for the profiling. We expect that this presymptomatic stage profiling is meaningful in dissecting cellular alterations leading to the death and senescence of *cpr5* mutants.

We compared the expression levels of several *SAGs* known to be up-regulated in developmentally regulated senescence (Fig. 2A). Four of the seven *SAGs* exhibited similar expression levels in *cpr5* mutants and wild-type seedlings, including *SAG12* (*AT5G45890*), *SAG18* (*AT1G71190*) and *SAG101* (*AT5G14930*). These genes were identified as *sensu stricto* developmental senescence marker genes (Lohman *et al.* 1994; Noh & Amasino 1999; He *et al.* 2001). To our surprise, *SAG13* (*AT2G29350*) had over 20-fold higher amounts of transcripts in *cpr5* mutants than in wild-type seedlings. *SAG13* is a well known molecular marker induced by ozone, UV-B, abscisic acid as well as senescence (A-H-Mackerness *et al.*

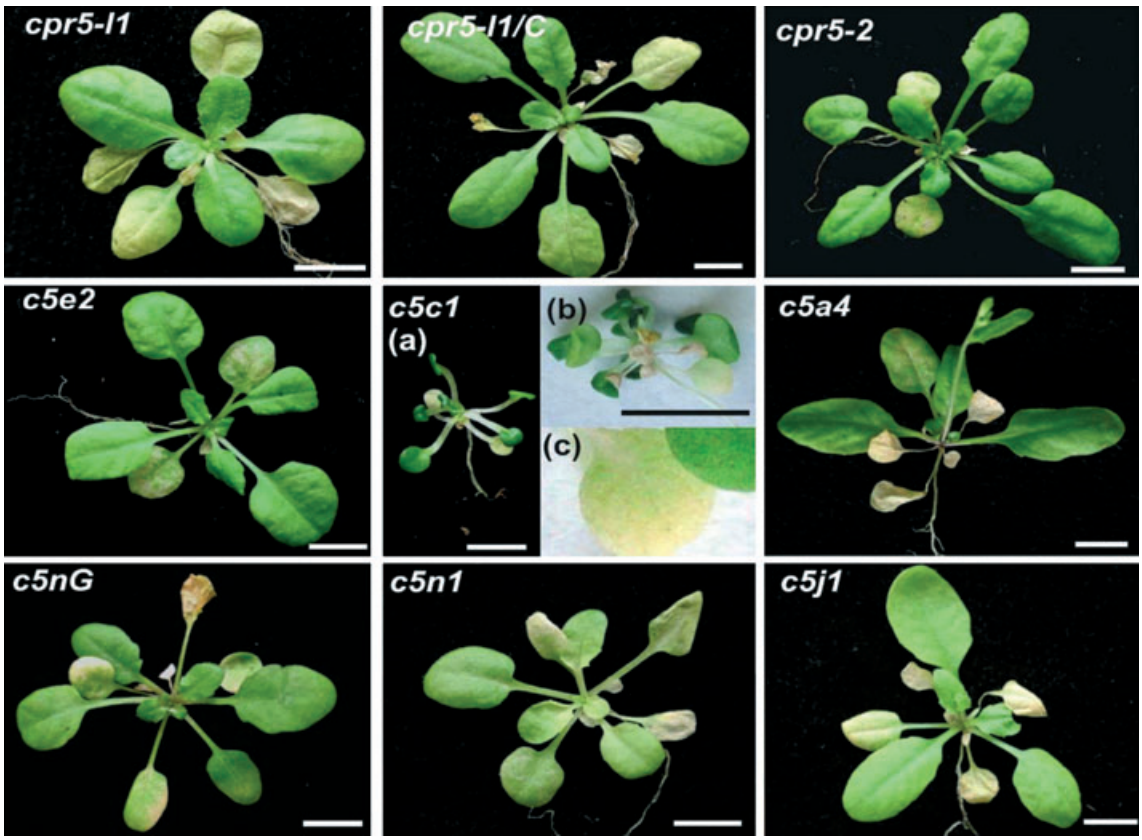

Fig. 1. Senescence phenotypes of *cpr5* mutants and double mutants. Plants were grown on soil under the conditions described in Material and Methods for 30 days and representative plants were selected and photographed. All plants had a similar amount of rosette leaves except *c5c1* plants, which grew slower than others as envisaged by the lower numbers of leaves (a). A representative 35-day-old *c5c1* plant with similar leaf numbers was taken and photographed upside down (b). An enlarged view of a *c5c1* rosette leaf is shown (c). Double mutant abbreviations: *c5e2* = *cpr5-l1/ein2-1*; *c5c1* = *cpr5-l1/ctr1-1*; *c5a4* = *cpr5-l1/abi4-1*; *c5nG* = *cpr5-l1/NahG*; *c5n1* = *cpr5-l1/npr1-1*; *c5j1* = *cpr5-l1/jar1-1*. Bars represent 1 cm.

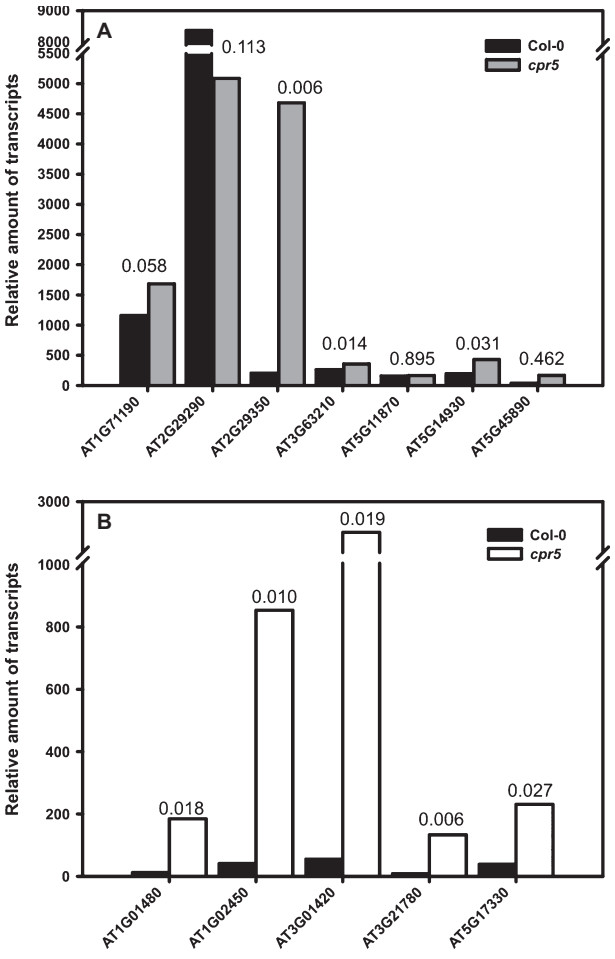

Fig. 2. Expression of senescence-associated and stress-related genes. (A) Expression of senescence-associated genes, known to be induced by developmentally regulated senescence. (B) Expression of several stress-related genes. See text for details. Data were obtained from Experiment 175 of the Genevestigator database and analysed as described in Material and Methods. Bars represent the average of two measurements. The numbers are the P values calculated by a one-way ANOVA of the differences between the means of wild-type and *cpr5* transcripts at the $\alpha = 0.05$ significance level.

1999, 2001; Miller *et al.* 1999; Brodersen *et al.* 2002) and the increased *SAG13* levels indicate that *cpr5* mutants are highly stressed.

We explored the expression profiles of over 100 genes known to be involved in the biosynthesis, perception, signalling and responses of ethylene, jasmonic acid, salicylic acid, abscisic acid and sugar. Only five genes showed an over fivefold increase in transcript levels in the *cpr5* mutant as compared to the wild type (Fig. 2B). In the ethylene pathway, only *AT1G01480* was highly expressed in *cpr5* mutants, and encodes a member of the 1-aminocyclopropane-1-carboxylate synthase gene family (*ACS2*). *ACS2* is constitutively expressed in most tissues and its expression is induced by wounding and IAA, (Tsuchisaka & Theologis 2004). In the salicylic acid pathway, *NIMIN1* (*AT1G02450*) and *AT3G01420* were highly expressed. NIMIN1 interacts with NPR1 and regulates the transcription of TGA factors (Weigel *et al.* 2005), whereas *AT3G01420* encodes an alpha-dioxygenase and is induced by salicylic acid, cell death, jasmonic acid, lipoxygenease activity, fatty acid alpha-oxidation and oxidative stress (De Leon *et al.* 2002). *AT3G21780* and *AT5G17330* encode UDP-glycosyltransferase and glutamate decarboxylase, respectively. Both genes were responsive to oxidative stress induced under various abiotic stresses, including sodium chloride and abscisic acid (Skopelitis *et al.* 2006).

Taken together, in the presymptomatic *cpr5* mutants the expression profiles of the majority of genes induced by developmental senescence and the genes involved in stress hormone and sugar signalling were not different from those in wild-type seedlings. However, presymptomatic *cpr5* mutants did show signs of stress, as manifested by the high-expression level of *SAG13* and the up-regulation of stress-associated genes.

ROS-related gene expression in presymptomatic *cpr5* mutants

The elevated expression levels of *SAG13* and other hormonally regulated stress-related genes in presymptomatic *cpr5* mutants prompted us to examine the expression profiles of the ROS gene network. Recently, microarray data were analysed that were collected from eight different experiments in which ROS were induced (Mittler *et al.* 2004; Gadjev *et al.* 2006). ROS were induced after treatment of wild-type plants with ozone, the catalase inhibitor 3-aminotriazole (AT), *Alternaria alternata* (AAL) toxin or the herbicide methyl viologen (MV). Furthermore, oxidative stress was induced in plants carrying a mutation in the *FLU* gene (Meskauskiene *et al.* 2001) or in genes encoding the antioxidant enzymes catalase 2, cytosolic ascorbate peroxidase or superoxide dismutase. Gadjev *et al.* (2006) identified five genes which can be considered as hallmarks for the general oxidative stress response, as at least seven out of the eight ROS-inducing treatments caused a fivefold or higher increase in their expression. In addition, many genes were identified that were up-regulated by one or more than one ROS-inducing treatment. Table 1 lists those genes whose expression increased at least fivefold in *cpr5* mutants, in addition to being induced by at least three of the ROS-inducing treatments. Three of the five universal ROS hallmarks were up-regulated more than fivefold in *cpr5* mutants. Of the 27 transcripts that were up-regulated in six of the eight ROS-inducing treatments, 16 transcripts showed a more than fivefold increase in *cpr5* mutants. However, amongst 66 transcripts specifically up-regulated by ozone, MV herbicide treatment and in *flu* mutants, only seven transcripts were up-regulated more than fivefold (Table 1). These results clearly suggest that *cpr5* mutants are experiencing high-oxidative stress. Therefore, we specifically examined the expression profiles of genes related to the

Table 1. ROS network genes upregulated in *cpr5* mutants.[a]

gene class	AGI code	ratio (*cpr5*/Col-0)	P-value	description
Transcripts up-regulated	AT1G57630	31.1	0.027	disease resistance protein RPP1-WsB
in at least seven of the	AT2G43510	12.2	0.002	defensine-like protein
eight experiments	AT1G05340	5.0	0.022	expressed protein
Transcripts up-regulated	AT2G29470	53.7	0.234	glutathione s-transferase
in six of the eight experiments	AT1G26420	40.9	0.016	FAD-linked oxidoreductase
	AT3G11340	37.2	0.297	UDP-glucoronosyl/UDP-glucosyl transferase
	AT4G39670	34.7	<0.001	expressed protein
	AT5G13080	28.6	0.155	AtWRKY75
	AT3G28210	13.1	0.030	zinc finger protein (AN1-like)
	AT1G13340	10.2	0.008	expressed protein
	AT2G29460	8.9	0.024	glutathione s-transferase
	AT2G29490	7.6	0.199	glutathione s-transferase
	AT4G37370	7.5	0.010	cytochrome P450-like
	AT4G37990	7.2	0.042	mannitol dehydrogenase ELI3-2
	AT2G41380	6.8	0.034	putative embryo-abundant protein
	AT3G26830	6.8	0.166	cytochrome P450 71B15
	AT4G01870	6.4	0.024	TolB protein-related
	AT1G26380	6.4	0.029	FAD-linked oxidoreductase
	AT2G32190	6.0	0.078	(unknown protein)
Transcripts up-regulated specifically	AT3G13610	54.0	0.158	oxidoreductase, 2OG-Fe(II) oxygenase
in CAT2HP1, AT and AAL experiments	AT1G21310	37.0	0.283	extensine-like protein
	AT3G10320	25.0	0.057	expressed protein
	AT3G26440	6.8	0.012	expressed protein
Transcripts up-regulated specifically	AT3G25610	10.1	0.024	ATPase II, putative
in *flu*, O3 and MV experiments	AT5G52750	8.5	0.002	heavy metal-associated domain-containing protein
	AT1G28480	7.4	0.003	glutaredoxin, putative
	AT3G10930	7.2	0.027	expressed protein
	AT3G23250	6.4	0.073	AtMYB15
	AT1G07000	6.3	0.014	leucine zipper protein, putative
	AT5G59820	5.5	0.038	ZAT12 transcription factor
ROS scavenging transcripts	AT1G32350	15.5	0.034	Mitochondrial NADPH oxidase-like alternative oxidase
	AT3G09940	8.3	0.002	Cytosolic monodehydroascorbate reductase (MDAR2)

[a] The classification of ROS gene network was from Gadjev *et al.* (2006). The expression data were taken from experiment 175 (Expression profiling of *cpr5*, *cpr5npr1*, *cpr5scv1*, *cpr5npr1svi1* and *npr1* lines) from the Genevestigator website (https://www.genevestigator.ethz.ch/) and analysed as described in Materials and Methods. Only genes with at least fivefold increases in transcripts are shown. The P values indicate one-way ANOVA of the differences between the means of wild-type and *cpr5* transcripts at $\alpha = 0.05$ significance level.

ROS scavenging network (Mittler *et al.* 2004). Out of 70 genes examined, only two genes exhibited a more than fivefold increase in transcript level (Table 1). These two genes are mitochondrial alternative oxidase (AOX, *AT1G32350*) and cytoplasmic monodehydroascorbate reductase (MDAR2, *AT3G09940*). Interestingly, none of the NADH and NADPH oxidase members showed increased transcription in presymptomatic *cpr5* mutants, suggesting the existence of non-cytosolic sources of ROS production.

We further examined the expression profiles of the ROS-dependent putative transcription factors that were identified by Gadjev *et al.* (2006) (Table 2). Interestingly, one third of the transcription factors increased at least fivefold in presymptomatic *cpr5* mutants in comparison with the wild type. Amongst the most increased transcripts are the NAM transcription factor *AT3G04070*, GT-1-like (*AT2G38250*) and *WRKY25* (*AT2G30250*). Although many members of the same gene families were up-regulated after one or more than one ROS-inducing treatment, only the *WRKY25* and *WRKY53* transcripts were significantly increased. The transcripts coding for the ethylene responsive transcription factors ERF1, ERF2 and ERF6 remained relatively constant. Since only a subset of the ROS-induced transcription factors were increased, not all ROS pathways may be involved in ROS generation in *cpr5* mutants, or not all ROS pathways may have been induced at the presymptomatic stage. Nevertheless, our analysis of ROS transcriptional footprints in the *cpr5* mutant demonstrate that *cpr5* is experiencing high-oxidative stress at presymptomatic stages.

Table 2. ROS responsive transcription factors which are up-regulated in *cpr5* mutants.[a]

AGI code	ratio	P-value	description
At2g38340	30.2	0.094	DREB transcription factor
At5g13080	28.6	0.155	AtWRKY75
At3g04070	18.8	0.006	NAM transcription factor
At2g38250	9.5	0.037	GT-1-like transcription factor
At2g30250	9.5	0.002	AtWRKY25
At1g52890	7.4	0.028	NAM transcription factor
At3g23250	6.4	0.073	AtMYB15
At3g15500	6.0	0.042	AtNAC3
At5g59820	5.5	0.038	ZAT12
At1g43160	5.2	0.153	ERF/AP2 transcription factor
At3g50260	3.9	0.023	DREB transcription factor
At2g43000	3.4	0.083	NAM transcription factor
At4g23810	3.2	0.032	AtWRKY53
At4g18880	2.7	0.024	AtHsfA4A
At2g40140	2.6	0.015	CCCH-type zinc finger
At1g10585	2.3	0.145	bHLH transcription factor
At4g17500	2.2	0.304	AtERF1
At4g17230	1.9	0.073	scarecrow-like transcription factor
At5g63790	1.9	0.032	NAM transcription factor
At1g62300	1.9	0.164	AtWRKY6
At1g27730	1.9	0.094	ZAT10
At1g80840	1.8	0.141	AtWRKY40
At2g38470	1.8	0.266	AtWRKY33
At5g05410	1.6	0.171	DREB2A
At1g18570	1.6	0.249	AtMYB51
At5g04340	1.5	0.318	C2H2 zinc finger
At4g27410	1.3	0.274	NAM transcription factor
At5g47220	1.3	0.741	AtERF2
At2g26150	1.3	0.737	AtHSFA2
At1g25560	1.2	0.355	AP2 transcription factor
At1g77450	1.1	0.599	NAM transcription factor
At4g17490	0.6	0.732	AtERF6

[a] The classification of ROS transcription factors was from Gadjev *et al.* (2006). The expression data were taken from experiment 175 (Expression profiling of *cpr5*, *cpr5npr1*, *cpr5scv1*, *cpr5npr1svi1* and *npr1* lines) from the Genevestigator website (https://www.genevestigator.ethz.ch/) and analysed as described in Materials and Methods. The P values indicate one-way ANOVA of the differences between the means of wild-type and *cpr5* transcripts at $\alpha = 0.05$ significance level.

Enhanced glutathione s-transferase protein expression in presymptomatic *cpr5* mutants

In order to gain reliable information on differences in protein abundance between presymptomatic *cpr5-l1* mutant and wild-type plants, we performed a comparative, relative quantitative study of the soluble proteomes employing DIGE technology (Unlü *et al.* 1997; Marouga *et al.* 2005). Figure 3 shows the distribution of Cy3-labelled wild-type (A) and Cy5-labelled mutant proteins/protein spots (B) after separation on a 2-D gel. Computational image analysis revealed that 23 out of approximately 900 protein spots consistently showed significant differences in fluorescent intensities. MS analysis of these spots (marked in Fig. 3) led to the identification of 17 different proteins or protein subunits (listed in Table 3). Eight spots (number 58, 60, 62, 63, 64, 73, 79 and 84) contained more than one protein. In these cases, it was not possible to provide accurate abundance ratios and regulation factors for each individual protein present in the spot.

All proteins unambiguously down-regulated in the *cpr5* mutant are plastidic: RuBisCO large subunit (identified in the spots highlighted in the upper rectangles and insets in Fig. 3A,B), an NADP(H) oxidoreductase, a superoxide dismutase, a peroxiredoxin and an oxygen-evolving enhancer protein. In addition, the proteomics data suggest that the RuBisCO small subunits 1A and 2B are also down-regulated. This is in agreement with observations made by Hebeler *et al.* (2008) in a previous proteomics study of the *cpr5-l1* mutant. These results indicate that, in contrast to wild-type plants, a disruption of chloroplasts and degradation of chloroplast-residing proteins has already been initiated in the presymptomatic *cpr5* mutants.

Except for a quinone reductase, all proteins clearly exhibiting increased abundance in *cpr5* mutants belonged to the large family of glutathione s-transferases (GSTs), which are generally known as detoxifying enzymes. AtGSTF2 (*AT4G02520*), AtGSTF6 (*AT1G02930*), AtGSTF7 (*AT1G02920*) and AtGSTF9 (*AT2G30860*) all belonging to the class Phi subfamily, were two- to 14-fold up-regulated. Although AtGSTF8 (*AT2G47730*), another GST isoform of class Phi, co-migrated with a second protein on the 2-D gel, we presume that it is also up-regulated. This assumption is supported by Hebeler *et al.* (2008).

The increase in abundance of members of the GST superfamily at the protein level prompted us to analyse expression profiles of this gene family, which has been divided into seven subfamilies in *Arabidopsis*. Of the 53 genes examined, seven showed significant increases in transcripts in *cpr5* mutants, of which five were from the Tau and two from the Phi family (Table 4). The expression of three Tau family members, *AT2G29479*, *AT2G29460* and *AT2G29490*, were also substantially increased under most of the ROS-inducing conditions. Data on the expression levels of the remaining four Tau and four Phi family members were not included in the ROS marker gene list. Thus, the increased gene and protein expression levels of a number of GST isoforms in presymptomatic *cpr5* mutants further substantiate the notion that *cpr5* mutants experience high-oxidative stress.

DISCUSSION

Here, we report our genetic, transcriptomic and relative quantitative proteomics analyses of expression profiles of genes and soluble proteins in presymptomatic *cpr5* mutants in comparison with wild-type seedlings. *CPR5* is a gene with pleiotropic functions and has been shown to be involved in the regulation of many processes, including sensitivities to hormones and sugar, trichome development, defence responses, spontaneous cell death and

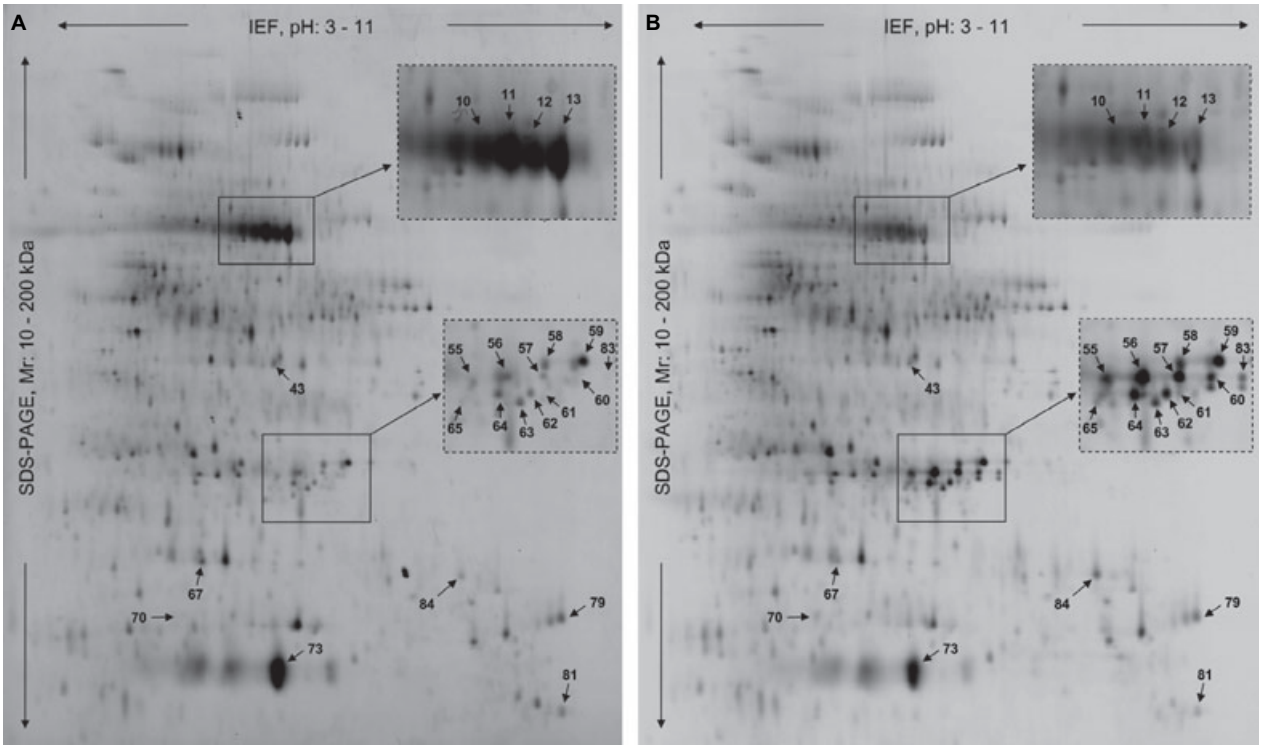

Fig. 3. Distribution of protein spots derived from *A. thaliana* wild-type L*er*-0 and *cpr5-l1/old1-1* mutant plants on a representative 2-D DIGE gel. A and B reflect false colour images of the corresponding fluorescent images of Cy3-labelled wild-type and Cy5-labelled mutant proteins, respectively. For 2-D DIGE analysis, differentially labelled wild-type and mutant proteins were mixed in equal ratio and separated as described in Materials and Methods. Numbers indicate protein spots showing altered fluorescent abundance in the wild-type and the *cpr5* mutant plants on the basis of statistical data analysis of six independent biological experiments (Table 3) that were further subjected to MS analysis for protein identification. Rectangles indicate areas with protein spots exhibiting major differences in protein abundance; insets zoom into these areas. The protein underlying the spots in the upper inset was identified as the large subunit of RuBisCO; proteins identified in the lower inset predominantly belonged to the family of GSTs.

senescence (Bowling *et al.* 1997; Kirik *et al.* 2001; Jing *et al.* 2002; Yoshida *et al.* 2002). We believe that it is important to examine gene and protein expression profiles before any visible phenotypes becomes apparent in order to dissect cellular alterations responsible for the various defects in *cpr5* mutants. Our analyses provide substantial evidence indicating the importance of *CPR5* in regulating cellular redox balance.

cpr5 mutants are under high-cellular oxidative stress

Presymptomatic *cpr5* mutants displayed several distinct characteristics in transcriptomic profiles. (i) SAG genes that are up-regulated by developmentally induced senescence, had similar or slightly increased amounts of transcripts in *cpr5* mutants as compared to the wild type. This was not true for the *SAG13* marker gene, but this gene has been shown to also be induced by oxidative stress (Miller *et al.* 1999; Navabpour *et al.* 2003). (ii) Perturbations of hormonal signalling pathways demonstrated that the onset of *cpr5*-induced cell death and leaf senescence is independent of ethylene, salicylic acid, jasmonic acid, sugar or abscisic acid. These results imply that cellular alterations other than these hormonal signals are responsible for accelerated cell death and senescence in *cpr5* mutants. Moreover, the majority of the genes involved in hormonal biosynthesis, perception, signalling and responses were not altered. The five genes whose transcripts were over fivefold increased were tightly associated with stress responses, particularly oxidative burst. (iii) Many genes in the ROS gene network had increased transcripts. All these lines of evidence strongly suggest that *cpr5* mutants experience high-cellular oxidative stress that is not induced by hormonal changes.

This is further supported by our proteomics study. Both transcriptomics and proteomics profiling resulted in the identification of several GST isoforms as significantly up-regulated in the *cpr5* mutant, and glutathione s-transferases are important components for cellular redox regulation (Edwards *et al.* 2000; Wagner *et al.* 2002). Strikingly, all five GSTs belong to the Phi subfamily. For three isoforms (ATGSTF1, ATGSTF2 and ATGSTF7), the increases in protein abundance correlate with increased transcript levels. However, for ATGSTF8 and ATGSTF9,

Table 3. Proteins found to be differentially regulated in *cpr5-l1/old1-1* mutant plants as revealed by 2-D DIGE experiments.

spot number	protein name	UniProtKB/Swiss-Prot Name	IPI accession	AGI code	Mr (kDa)	Seq. cov. (%)	Av. ratio (±SD)	RF (±CV in %)
10	RuBisCO large subunit	RBL	IPI00535114.1	ATCG00490	52.9	28.0	1.94 (±0.39)	−1.9 (±20.1)
11	RuBisCO large subunit	RBL	IPI00535114.1	ATCG00490	52.9	29.4	1.75 (±0.54)	−1.8 (±30.9)
12	RuBisCO large subunit	RBL	IPI00535114.1	ATCG00490	52.9	30.1	2.37 (±0.61)	−2.4 (±25.7)
13	RuBisCO large subunit	RBL	IPI00535114.1	ATCG00490	52.9	33.6	2.94 (±1.07)	−2.9 (±36.4)
43	NADP(H) oxidoreductase	Q9LNS9	IPI00539484.1	AT1G20020	38.8	27.9	1.62 (±0.23)	−1.6 (±14.2)
55	Quinone reductase	Q9LSQ5	IPI00529112.1	AT5G54500	21.8	33.8	0.30 (±0.10)	+3.3 (±33.3)
56	Glutathione s-transferase PM24 (*At*GSTF2)	GSTF4	IPI00535149.2	AT4G02520	24.0	54.5	0.13 (±0.04)	+7.7 (±30.8)
57	Glutathione s-transferase PM24 (*At*GSTF2)	GSTF4	IPI00535149.2	AT4G02520	24.0	54.5	0.10 (±0.03)	+10.0 (±30.0)
58	Glutathione s-transferase (*At*GSTF9)	O80852	IPI00538125.1	AT2G30860	24.1	25.6	0.43 (±0.10)	+2.3 (±23.3)
	Glutathione s-transferase PM24 (*At*GSTF2)	GSTF4	IPI00535149.2	AT4G02520	24.0	24.4		
59	Glutathione s-transferase (*At*GSTF9)	O80852	IPI00538125.1	AT2G30860	24.1	13.0	0.51 (±0.06)	+2.0 (±11.8)
60	Ribosome recycling factor, chloroplast (Precursor)	RRFC	IPI00545948.1	AT3G63190	30.4	20.0	0.11 (±0.02)/	+9.1 (±18.2)/
	Glutathione s-transferase 11 (*At*GSTF7)	GST11	IPI00530258.1	AT1G02920	23.5	29.7	0.13 (±0.03)[a]	+7.7 (±23.1)
	Glutathione s-transferase PM24 (*At*GSTF2)	GSTF4	IPI00535149.2	AT4G02520	24.0	14.2		
61	Glutathione s-transferase PM24 (*At*GSTF2)	GSTF4	IPI00535149.2	AT4G02520	24.0	10.4	0.34 (±0.08)	+2.9 (±23.5)
62	Glutathione s-transferase 1 (*At*GSTF6)	GSTF1	IPI00548409.1	AT1G02930	23.5	32.7	0.25 (±0.04)	+4.0 (±16.0)
	Glutathione s-transferase PM24 (*At*GSTF2)	GSTF4	IPI00535149.2	AT4G02520	24.0	14.2		
63	Superoxide dismutase [Mn], mitochondrial	SODM	IPI00546447.1	AT3G10920	25.4	16.0	0.53 (±0.07)	+1.9 (±13.2)
	Glutathione s-transferase 6 (*At*GSTF8)	GSTF6	IPI00536062.1	AT2G47730	24.1	7.4		
64	Glutathione s-transferase 1 (*At*GSTF6)	GSTF1	IPI00548409.1	AT1G02930	23.5	36.1	0.22 (±0.13)/	+4.5 (±59.1)/
	Glutathione s-transferase PM24 (*At*GSTF2)	GSTF4	IPI00535149.2	AT4G02520	24.0	29.4	0.33 (±0.05)[a]	+3.0 (±15.2)
65	Glutathione s-transferase 1 (*At*GSTF6)	GSTF1	IPI00548409.1	AT1G02930	23.5	26.4	0.27 (±0.06)	+3.7 (±22.2)
67	Superoxide dismutase (Cu-Zn), chloroplast	SODCP	IPI00520632.1	AT2G28190	22.2	13.0	1.57 (±0.30)	−1.6 (±19.1)
70	Peroxiredoxin type 2	Q9LF96	IPI00533612.1	AT3G52960	24.7	27.8	1.95 (±0.40)	−2.0 (±20.5)
73	RuBisCO small subunit 2B	RBS2B	IPI00523477.1	AT5G38420	20.3	41.4	2.10 (±0.54)	−2.1 (±25.7)
	RuBisCO small subunit 1A	RBS1A	IPI00539020.1	AT1G67090	20.2	40.6		
79	Peroxiredoxin Q	Q9LU86	IPI00548978.1	AT3G26060	23.7	54.2	1.69 (±0.67)	−1.7 (±39.6)
	RuBisCO small subunit 1A	RBS1A	IPI00539020.1	AT1G67090	20.2	23.9		
	Photosystem I reaction center subunit IV A	PSAE1	PI00532440.1	AT4G28750	15.0	13.3		
81	Oxygen-evolving enhancer protein 3-1, chloroplast	PSBQ1	IPI00532582.1	AT4G21280	23.8	33.6	2.32 (±1.46)	−2.3 (±62.9)
83	Glutathione s-transferase 11 (*At*GSTF7)	GST11	IPI00530258.1	AT1G02920	23.5	29.7	0.07 (±0.02)	+14.3 (±28.6)
84	Peroxiredoxin Q	Q9LU86	IPI00548978.1	AT3G26060	23.7	21.3	0.39 (±0.07)	+2.6 (±17.9)
	RuBisCO small subunit 1A	RBS1A	IPI00539020.1	AT1G67090	20.2	19.4		

Plants were harvested after 16 days of growth; proteins were extracted, differentially labelled with CyDyes and separated by 2-D PAGE. Following image analysis, protein spots indicating differential expression were excised and subjected to MS analysis as described in Materials and Methods. Average (Av.) abundance ratios were calculated by dividing fluorescence intensities of wild-type proteins by those of mutant proteins. Data represent means of six independent experiments. Spot numbers correspond to numbering in Fig. 3. For the GST isoforms, the terms according to the current nomenclature for plant GSTs are given as well. RuBisCO = ribulose 1,5-bisphosphate carboxylase/oxygenase; Mr = theoretical molecular weight rounded to one decimal place; Seq. cov. = sequence coverage by amino acid count; SD = standard deviation; RF = regulation factor indicating the regulation of the protein in mutant *versus* wild-type plants; CV = coefficient of variation.

[a] Protein spots could not be resolved in the preparative 2-D gel stained with colloidal coomassie blue and used for MS-based protein identification.

Table 4. Transcripts of Phi and Tau glutathione s-trnasferase genes in *cpr5* mutants.[a]

family	AGI code	ratio	P-value
Phi family glutathione s-transferase	AT2G02930	8.5	0.060
	AT4G02520	8.5	0.060
	AT1G02920	5.7	0.022
	AT1G02930	5.7	0.022
	AT5G17220	1.3	0.090
	AT2G30870	1.2	0.408
	AT1G02940	1.2	0.172
	AT2G47730	1.0	0.239
	AT2G30860	0.9	0.300
	AT1G02950	0.7	0.120
	AT3G03190	0.7	0.157
	AT3G62760	0.5	0.230
	AT1G49860	0.2	0.425
Tau family glutathione s-transferase	AT2g29470	53.7	0.234
	AT1g74590	47.3	0.040
	AT1g69930	9.1	0.010
	AT2g29460	8.9	0.024
	AT2g29490	7.6	0.199
	AT3g09270	5.8	0.009
	AT2g29480	5.5	0.233
	AT5g62480	3.7	0.374
	AT1g69920	2.6	0.329
	AT1g17170	2.2	0.295
	AT2g29420	1.8	0.003
	AT2g29440	1.3	0.467
	AT1g59700	1.0	0.829
	AT3g43800	1.0	0.997
	AT1g78380	1.0	0.821
	AT1g10360	0.9	0.452
	AT1g17180	0.9	0.759
	AT1g78320	0.8	0.444
	AT1g78340	0.8	0.236
	AT2g29450	0.7	0.133
	AT1g27130	0.7	0.532
	AT1g10370	0.7	0.157
	AT1g17190	0.7	0.366
	AT1g78370	0.6	0.114
	AT1g78360	0.6	0.645
	AT1g27140	0.5	0.228
	AT1g59670	0.4	0.529
	AT1g53680	0.3	0.077

[a] The classification of glutathione gene family is from the Arabidopsis Community (http://www.arabidopsis.org). The expression data were taken from Experiment 175 (Expression profiling of *cpr5*, *cpr5npr1*, *cpr5scv1*, *cpr5npr1svi1* and *npr1* lines) from the Genevestigator website (https://www.genevestigator.ethz.ch/) and analysed as described in Materials and Methods. The P values indicate one-way ANOVA of the differences between the means of wild-type and *cpr5* transcripts at $\alpha = 0.05$ significance level.

the amounts of transcripts are identical in *cpr5* mutants and wild type, suggesting that the increase in protein level may result from posttranscriptional regulation. It is also notable that several members of the GST Tau family had increased transcription, but none of these were identified as differentially regulated proteins in the proteomics analysis. GSTs of the Phi subfamily have not been reported to be senescence-associated, indicating that elevated ROS scavenging proteins in presymptomatic *cpr5* mutants are probably used to tolerate stresses other than those associated with senescence. Given the difference between our senescence-inducing system and the senescence-inducing system in cell suspension cultures described by Swidzinski *et al.* (2004), it is not surprising that the *cpr5* proteome differed markedly from that of senescing suspension cultures.

Taken together, our trancriptomic and proteomic analyses suggest that presymptomatic *cpr5* mutants are under high-cellular oxidative stress. *cpr5* mutants have various pleiotropic phenotypes and this may be the result of uncontrolled ROS generation. Considering the scope of transcriptome modifications caused by *cpr5* mutations, we propose that *CPR5* is a central regulator of cellular redox balance.

ROS induced lesion mimic cell death and senescence in *cpr5* mutants

Two different cell death processes were readily visible in *cpr5* plants: senescence-associated and lesion-mimic (Bowling *et al.* 1997; Jing *et al.* 2002; Yoshida *et al.* 2002). The results suggest that both types of cell death may occur independently in *cpr5* mutants. In presymptomatic *cpr5* mutants, the *SAG13* transcripts were over 20-fold higher. Increased *SAG13* expression in combination with unchanged *SAG12* expression may suggest that the initial cell death is not a result of age-induced senescence (Brodersen *et al.* 2002; Lin *et al.* 2007). In addition, two substantially increased transcripts code for mitochondrial and cytosolic ROS scavenging proteins, and none of the genes encoding chloroplast-localized ROS scavenging proteins showed increases in transcription. Furthermore, in post-symptom *cpr5* mutants, *SAG12* is expressed to high levels, and in *CPR5*-overexpressing plants, early leaf senescence was uncoupled from lesion formation, with a correlation between *CPR5* and *SAG12* expression levels (Jing *et al.* 2002, 2007). We also found that in *cpr5* mutants, lesion-mimic cell death occurred in the absence of ethylene signalling, whereas developmental senescence-associated cell death required a functional ethylene pathway (data not shown).

These results may imply that two distinct cell death processes occur in *cpr5* mutants and that the likely primary trigger for these two cell death processes seems to be the altered redox balance. It is not clear whether the lesion mimic cell death in *cpr5* mutants resembles that of cell death that occurs during hypersensitive responses triggered in plant–pathogen interactions. However, *cpr5* mutants exhibited enhanced resistance to virulent strains of *Pseudomonas syringae* and *Peronospora parasitica* (Bowling *et al.* 1997; Clarke *et al.* 2001), which may very

well be related to its elevated production of ROS. The association of ROS with senescence is also well established (Navabpour *et al.* 2003; Woo *et al.* 2004; Guo & Crawford 2005). In *cpr5* mutants, it appears that *cpr5*-induced ROS alteration initially causes lesion mimic cell death and then senescence. It is necessary to identify the components involved in *cpr5*-induced alteration in ROS production and scavenging before molecular links can be established on how *CPR5* regulates these two cell death processes.

CPR5 executes its early-life beneficial functions by balancing cellular redox

Our recent work characterising transgenic *Arabidopsis* plants with constitutive over-expression of *CPR5* allowed us to propose *CPR5* as a senescence regulatory gene with pleiotropic functions, as predicted by the Antagonistic Pleiotropy Theory of Senescence (Jing *et al.* 2007). We demonstrated that *CPR5* exerts its later-life detrimental effects by promoting senescence. One of the unsolved issues is how *CPR5* exerts its early-life beneficial effects. In the present paper, we provided transcriptional and proteomics evidence that in the presymptomatic *cpr5* mutants uncontrolled ROS generation and altered ROS scavenging activities are amongst the earliest altered cellular events. A recent report indicates that hexokinase 1 contributes partially to *cpr5*-induced phenotypes (Aki *et al.* 2007). This is consistent with our double mutant analysis indicating that blocking sugar signalling did not alter the onset of leaf senescence. We compared the expression profiles of 103 genes involved in sugar-sensing signalling and metabolism, including hexokinase 1 in presymptomatic *cpr5* mutants with those in wild types, and found that their fold changes were between 0.5 and 2.5. These results implicate that, at least in presymptomatic *cpr5* mutants, sugar-relating signalling and metabolism is not, or only marginally, altered. Thus, we concluded that a major function of *CPR5* lies in the regulation of ROS generation and scavenging and balancing cellular redox to achieve its early-life beneficial effects.

In summary, the 'Oxygen Paradox' is one of the key challenges that plants have to deal with. On the one hand, efficient utilisation of oxygen is a prerequisite for photosynthetic activities, which provides building blocks for all cellular components and for life on Earth. On the other hand, minimising the potential damage caused by ROS is also essential for keeping cellular machineries functioning. Thus, tackling ROS signalling and controlling ROS generation and scavenging is one of the driving forces for plant genome evolution. It is anticipated, and has been shown, that a plant genome contains genetic information coding for sophisticated redox controls (Apel & Hirt 2004; Pitzschke *et al.* 2006). It would be interesting to pursue the downstream components of *CPR5* involved in redox regulation.

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CONFLICTS OF INTEREST

The authors have declared no conflicts of interest.

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