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Proteomic and Biochemical Analyses Show a Functional Network of Proteins Involved in Antioxidant Defense of the *Arabidopsis anp2anp3* Double Mutant

Tomáš Taká[†], Olga Šamajová[†], Pavol Vadovi[†], Tibor Pechan[‡], Petra Košútová[†], Miroslav Ovečka[†], Alexandra Husíková[§], George Komis[†], and Jozef Šamaj^{†,*}

[†]Centre of the Region Haná for Biotechnological and Agricultural Research, Department of Cell Biology, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic

[‡]Institute for Genomics, Biocomputing & Biotechnology, Mississippi State University, Mississippi State, Mississippi 39762, United States

[§]Centre of the Region Haná for Biotechnological and Agricultural Research, Department of Biophysics, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic

Abstract

Disentanglement of functional complexity associated with plant mitogen-activated protein kinase (MAPK) signaling has benefited from transcriptomic, proteomic, phosphoproteomic, and genetic studies. Published transcriptomic analysis of a double homozygous recessive *anp2anp3* mutant of two MAPK kinase kinase (MAPKKK) genes called *Arabidopsis thaliana* *Homologues of Nucleus- and Phragmoplast-localized Kinase 2* (ANP2) and 3 (ANP3) showed the upregulation of stress-related genes. In this study, a comparative proteomic analysis of *anp2anp3* mutant against its respective Wassilevskaja ecotype (Ws) wild type background is provided. Such differential proteomic analysis revealed overabundance of core enzymes such as FeSOD1, MnSOD, DHAR1, and FeSOD1-associated regulatory protein CPN20, which are involved in the detoxification of reactive oxygen species in the *anp2anp3* mutant. The proteomic results were validated at the level of single protein abundance by Western blot analyses and by quantitative biochemical determination of antioxidant enzymatic activities. Finally, the functional network of proteins

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*Corresponding Author: jozef.samaj@upol.cz. Phone: 00420585634978. Fax: 00420585634936. Web: <http://www.cr-hana.eu/cellbiol>.

ASSOCIATED CONTENT

Supporting Information

Figure S1 - Prediction of functional networks affected in *anp2anp3* mutant compared to wild type using STRING 9.0 applied on proteomic data. Figure S2 - Identification of SOD isozymes on native PAGE gels on the basis of inhibitor sensitivity. Table S1 - Data pertinent to proteins and peptides identified in the *anp2anp3* mutant. Table S2 - Proteome Discoverer datasheet export related to protein and peptide identification in the *anp2anp3* mutant. Table S3 - Proteome Discoverer datasheet export of matched peptides. Table S4 - KEGG-based classification of proteins identified in *Arabidopsis*, ecotype Ws seedlings. Table S5 - KEGG-based classification of proteins identified in the *Arabidopsis anp2anp3* mutant. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

involved in antioxidant defense in the *anp2anp3* mutant was physiologically linked with the increased resistance of mutant seedlings against paraquat treatment.

Graphical Abstract

Arabidopsis *anp2anp3* mutant

Keywords

mitogen-activated protein kinase kinase kinase; ANP2; ANP3; Arabidopsis; proteomics; signaling antioxidant defense; oxidative stress

INTRODUCTION

Mitogen-activated protein kinase (MAPK) signaling lies at the core of plant growth, development, environmental perception and stress responses.^{1,2} It is characterized by cross-talk, redundancy and complexity exemplified by the presence of 20 MAPKs (EC 2.7.11.24), 10 MAPKKs (EC 2.7.12.2), and 60–80 MAPKKKs (EC 2.7.11.25) in the *Arabidopsis* genome and their involvement in heavily interconnected pathways.³

A well characterized example of cross-talk in plant MAPK signaling can be found in pathways initiated by a MAPKKK family called *Arabidopsis* nucleus- and phragmoplast-localized kinase 1 (ANP1), 2 (ANP2), and 3 (ANP3). These are homologous to the tobacco NPK1,⁴ a MAPKKK targeting cytokinetic phragmoplast progression.⁵ Similarly, all three members of the *Arabidopsis* ANP family regulate cytokinesis and cell expansion by cortical microtubule organization of growing or differentiating plant cells.^{4,6,7} While single knockout mutants of any ANP member show no phenotype changes, double mutants and particularly *anp2anp3* display aberrations related to both cytokinetic defects and cortical microtubule misorganization^{4,6,7} affecting the overall vegetative growth. Thus, *anp2anp3*

double mutant seedlings are reduced in size and fresh weight, and they show irregular hypocotyl⁴ and root⁷ cell outlines resulting from radial cell swelling.

The full pathway regulating cytokinetic progression downstream of ANPs (MAPKKKs) is mediated by the MKK6 (MAPKK) and results in the activation of the MPK4 (MAPK) while the activation of the entire pathway depends on the interaction of ANPs with kinesin-related protein HINKEL⁸.

However, earlier studies implicated ANPs in *Arabidopsis* stress responses, particularly under oxidative stress.⁹ In this case, oxidative-stress ANP-mediated signaling occurs via the activation of MPK3 and MPK6 in *Arabidopsis*.^{9,10} Although MPK4 is also activated by oxidative stress,¹¹ its activation rather occurs through the MEKK1-MKK1/2 pathway.^{12,13}

Therefore, the ANP family of MAPKKKs initiates two different signaling cascades that are functionally separated, while MPK4, which assumes a developmental role downstream of ANPs, is likely excluded from stress-induced ANP pathways.

MAPK cascades are important regulators of antioxidant defense. MKK5, a mitogen-activated protein kinase kinase mediates the high light-induced expression of genes of two copper/zinc SOD isoforms, namely *CuZnSOD1* and *CuZnSOD2*.¹⁴ Additionally, the expression of *CATALASE 1* is mediated by AtMKK1 in the presence of ABA and stress conditions.^{15,16} Microarray analysis of gene expression showed transcriptional upregulation of genes related to oxidative stress in *anp2anp3* mutants, suggesting negative regulation of the respective stress responses by ANP2 and ANP3.⁴ However, these results were neither correlated at the respective protein level nor validated by physiological stress responses of the mutant.

For the reasons above, and considering the involvement of ANPs in stress responses through MPK3 and MPK6,⁹ we analyzed the proteome of whole *anp2anp3* seedlings. We found that, by comparison to the Wassilevskaja ecotype (Ws) wild type, proteins involved in photosynthesis and oxidative stress were differentially regulated in the *anp2anp3* double mutant. Results from proteomic analyses suggested decreased ROS production in the mutant and increased tolerance to oxidative stress. The above assumptions were validated by appropriate biochemical and physiological approaches.

EXPERIMENTAL PROCEDURES

Plant material and cultivation

Seeds of *Arabidopsis thaliana*, ecotype Wassilevskaja (Ws) and double mutant *anp2anp3* derived from the same ecotype background⁴ were surface sterilized and placed on 1/2 Murashige-Skoog solid culture medium¹⁷ (pH 5.7) containing 1% (w/v) sucrose and 0.8% (w/v) phytigel. Seeds were stratified at 4 °C for 48 h and grown vertically at environmental conditions (16 h light/8 h dark, 22 °C). Ten days after germination, seedlings were collected for proteomic, biochemical and histochemical analyses as well as chlorophyll fluorescence imaging. Seedlings of *anp2anp3* mutant were preselected on the basis of known root and root hair phenotype.⁷ For oxidative stress resistance evaluation, 3-day-old seedlings were

transferred to a 1/2 Murashige-Skoog solid culture medium supplemented with 0.5 μM paraquat. The oxidative stress resistance was evaluated and recorded 7 days later, on 10th day of plants age. For biochemical analyses on paraquat-treated plants, 10 days old Ws and *anp2anp3* seedlings were surface treated with liquid 1/2 Murashige-Skoog media (control) or with the same media supplemented with 15 μM paraquat for 5 h. Seedlings were kept horizontally on variable speed rocker under slow shaking to prevent complete submergence of the plants. Proteome mapping of *anp2anp3* mutant was performed from four biological replicates while all other analyses were carried out in three independent biological replicates.

Proteomic analysis

Preparation of peptide digests—*Arabidopsis* wild type and *anp2anp3* mutant seedlings were homogenized in liquid nitrogen to fine powder and extracted in buffer containing 0.9 M sucrose, 0.1 M Tris-HCl, pH 8.8, 10 mM EDTA, 100 mM KCl and 0.4% v/v 2-mercaptoethanol. Total proteins were fractionated from the extract using phenol extraction and sequential precipitation in methanolic ammonium acetate (100 mM), 80% v/v aqueous acetone, 70% v/v ethanol, followed by final incubation with 80% v/v acetone.^{18,19} The final protein pellet was dissolved in 6 M urea in 100 mM Tris-HCl, pH 6.8. Prior to trypsin digestion, proteins were reduced and alkylated as previously described.¹⁸ Digested peptides were desalted on C18 cartridges (Sep Pak, Waters, Milford, MA, USA) and vacuum-dried.

2-D LC-MS/MS analysis and quantitation—Immediately prior to the 2-D LC-MS/MS analysis, tryptic digests were dissolved in 20 μL of 0.1% v/v formic acid and 5% v/v acetonitrile. The ion exchange and reverse phase online chromatography and mass spectra collection was carried out according to previously published procedure.¹⁸ Briefly, the analysis was performed using the Surveyor auto sampler and the Surveyor HPLC unit linked in-line to LCQ Deca XP Plus – ESI ion trap mass spectrometer (Thermo Scientific, Waltham, MA, USA). The HPLC included a 2-D LC separation on a strong cation exchange column (SCX BioBasic 0.32 $\times$ 100 mm), followed by a reverse phase column (BioBasic C18, 0.18 $\times$ 100 mm; both by Thermo Scientific, Waltham, MA, USA). For both columns, a flow rate of 3.0 $\mu\text{L}\cdot\text{min}^{-1}$ was used. A discontinuous gradient of 0, 10, 15, 20, 25, 30, 35, 40, 45, 50, 57, 64, 90, and 700 mM ammonium acetate in 5% v/v acetonitrile and 0.1% v/v formic acid was applied for SCX column. The reverse phase column was subjected to a 59 min gradient of acetonitrile (in 0.1% v/v formic acid) as follows: 5%–30% v/v for 30 min, 30%–65% v/v for 9 min, 95% v/v for 5 min, 5% v/v for 15 min. The mass spectra were collected in the data dependent mode, with dynamic exclusion applied, in four scan events: one MS scan (m/z range: 300–1700) followed by three MS/MS scans for the three most intense ions detected in MS scan. Other critical parameters were set as given here: Normalized collision energy: 35%, AGC (automatic gain control) “on” with MSn Target 4×10^4 , isolation width (m/z): 3.0, capillary temperature 170 $^{\circ}\text{C}$, spray voltage 2.7 kV, maximum injection time 50 and 400 ms for MS and MSMS mode, respectively.

The .raw files were searched using the SEQUEST algorithm of the Proteome Discoverer 1.1.0 (Thermo Scientific, Waltham, MA, USA) software. The workflow option was utilized with selection of parameters: Lowest and highest charge: +1 and +3, respectively; minimum

and maximum precursor mass: 300 and 6,000 Da, respectively; minimum S/N ratio: 3; enzyme: trypsin; maximum missed cleavages: 2; LCQ default (according to manufacturer) precursor and fragment mass tolerance: 2.5 and 0.8 Da, respectively; FDR 0.01; dynamic modifications: cysteine carbamidomethylation (+57.021), methionine oxidation (+15.995), methionine dioxidation (+31.990).

The spectral data were matched against target and decoy databases. The NCBI (www.ncbi.nlm.nih.gov) *Arabidopsis* genus taxonomy referenced protein database (67,924 entries as of November 2012) served as the target database, while its reversed copy served as a decoy database. The Proteome Discoverer results files (.msf) were uploaded to ProteoIQ 2.1 (NuSep) software for further filtering. Only proteins detected with at least three spectral counts, FDR 1%, 95% probability and listed as “Top” proteins (defined by ProteoIQ “Within a protein group, each and every respective peptide could be matched to the top protein”) are considered as high confidence matches and are presented in the results.

Label-free quantitative analysis was carried out as previously published.¹⁸ Briefly, the unfiltered TurboSEQUENT result files were uploaded to ProtQuant software²⁰ for analysis based on sum of TurboSEQUENT cross correlation factors (Xcorr) of all identified peptides in three replicates of each biological sample. One-way ANOVA analysis was used to identify statistically significant ($p < 0.05$) differences in protein amount.

Protein extract preparation for immunoblotting, native electrophoresis and enzyme activity assays—Liquid nitrogen powders of wild type and *anp2anp3* mutant seedlings were homogenized with 50 mM sodium phosphate buffer (pH 7.8) containing 1 mM EDTA, 10% v/v glycerol and “Complete” EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). Subsequently, the extract was centrifuged at 12,000 g for 15 min at 4 °C and the protein concentration of the supernatant was measured using the Bradford assay.²¹ The extract was used for superoxide dismutase (SOD) isozyme activity detection as well as immunoblotting on native polyacrylamide gels and spectrophotometric measurements of dehydroascorbate reductase (DHAR) and ascorbate peroxidase (APX) activities. For immunoblot analysis on SDS PAGE gels, the extracts containing equal amount of proteins were supplemented with 4 times concentrated Laemmli SDS buffer (to reach final concentration of 10% v/v glycerol, 60 mM Tris/HCl pH 6.8, 2% w/v SDS, 0.002% w/v bromophenol blue and 5% v/v β -mercaptoethanol), boiled for 5 min at 95 °C and the insoluble particles were removed by centrifugation.

Immunoblot analysis—Protein extracts were separated either by SDS-PAGE or native PAGE (MINI-Protean Tetra Cell, Biorad, Hercules, CA, USA). Identical protein amounts were loaded for each sample. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Little Chalfont, United Kingdom) in a wet tank unit (Bio-Rad) at 100 V for 1.5 h. For immuno-detection of protein bands, the membrane was blocked with 5% w/v low-fat dry milk in Tris-buffer-saline (TBS, 100 mM Tris-HCl; 150 mM NaCl; pH 7.4) for 1 h, and subsequently incubated with anti-FeSOD1 and anti-MnSOD primary antibodies (Agrisera, Vännäs, Sweden) diluted 1:3,000 and 1:2,000 respectively in TBS-T (TBS; 0.1% v/v Tween 20) containing 1% w/v low-fat dry milk at 4 °C overnight. After washing in TBS-T, the membrane was incubated at room temperature for 1.5 h with a

horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:5,000 in TBS-T containing 1% w/v BSA. Following at least three washing steps in TBST, proteins were detected by incubating the membrane in Clarity Western ECL substrate (Biorad). Luminescence was detected using Chemidoc MP documentation system (Biorad).

Analysis of SOD isozymes—Isozymes of SOD were separated on 10% native PAGE at constant 10 mA/gel. To differentiate activities of different SOD isoforms²², samples were loaded on three separate gels. One of the gels was preincubated for 15 min in 5 mM hydrogen peroxide (H_2O_2), in 50 mM sodium phosphate buffer (pH 7.8), which inhibits CuZnSOD and FeSOD but does not affect the MnSOD isozymes. The second gel was preincubated in 2 mM KCN (in 50 mM sodium phosphate buffer, pH 7.8) for 15 min, which inhibits CuZnSOD, but has no effect on FeSOD and MnSOD activity. The last gel was preincubated for the same period of time in 50 mM sodium phosphate buffer, pH 7.8 without the addition of an inhibitor. Afterward, the gels were stained according to modified method of Beauchamp and Fridovich²³ by two-step incubation in dark in a solution containing 0.6 mM nitroblue tetrazolium (NBT) in 50 mM sodium phosphate buffer (pH 7.8), followed by incubation in a solution containing 0.06 mM riboflavin, 5 mM EDTA and 0.25% v/v TEMED in 50 mM sodium phosphate buffer (pH 7.8). Finally, the gels were exposed to light until the SOD activity appeared as translucent bands on a dark background. The bands intensities were measured using Image Lab software (Biorad). Statistical evaluation of data (3 replicates) was carried out using Student's *t* test. As a loading control equal amounts of protein were resolved by SDS-PAGE as described above and stained with colloidal coomassie blue.

Spectrophotometric measurement of APX and DHAR activities, and ascorbate content—APX activity was measured following the H_2O_2 -dependent oxidation of ascorbic acid at 290 nm (extinction coefficient $2.8\text{ mM}^{-1}\cdot\text{cm}^{-1}$) as described by Amako et al²⁴. DHAR was measured by following the increase in absorbance at 265 nm due to glutathione dependent production of ascorbate.²⁵ The ascorbate content was measured in trichloroacetic acid-extracted samples using an α - α' -bipyridyl-based colorimetric assay as described by Gillespie and Ainsworth.²⁶

Measurement of NADPH oxidase activity—NADPH oxidase activity was measured in membrane fraction according to Saghi and Fluhr.²⁷ For extraction of membrane fraction, seedlings were homogenized in ice-cold extraction buffer (50 mM HEPES, pH 7.2, 0.1 mM $MgCl_2$, 3 mM EDTA, 0.25 M sucrose, 1 mM DTT, 0.6% w/v poly(vinylpyrrolidone), 3.6 mM L-cysteine, “Complete” EDTA free protease inhibitor cocktail from Roche). The homogenate was centrifuged at 10,000 g for 45 min at 4 °C, and the membrane fraction was isolated by ultracentrifugation of the supernatant at 203,000 g for 5 min. The resulting pellet was resuspended with 10 mM TrisHCL pH 7.4. The NADPH-dependent superoxide ($O_2^{\bullet-}$) - generating activity was determined spectrophotometrically by following the reduction of sodium 3'-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT) at 492 nm for 5 min. The rate of NADPH dependent $O_2^{\bullet-}$ generation was calculated using an extinction coefficient of $0.26\text{ mM}\cdot\text{cm}^{-1}$.

Measurement of $O_2^{\bullet-}$ production—Production of $O_2^{\bullet-}$ was measured as described by Able et al.²⁸ by monitoring the reduction of XTT. Equal amount of fresh material for both wild type and mutant seedlings was homogenized with ice-cold 50 mM TrisHCl buffer (pH 7.5) and centrifuged at 10,000 g for 10 min. The reaction mixture contained 50 mM TrisHCl buffer (pH 7.5), 30 μ L of sample and 0.5 mM XTT. The reduction of XTT was determined at 470 nm for 1 h. Corrections were made for the background absorbance in the presence of 100 units of commercial SOD (Sigma S4636).

Measurement of H_2O_2 levels—The levels of H_2O_2 were measured spectrophotometrically using xylenol orange assay based on the oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) ions in the presence of soluble peroxides.²⁹ *Ws* and *anp2anp3* seedlings (100 μ g in fresh weight) were homogenized by liquid nitrogen and incubated for 15 min on ice after the addition of cold distilled water (300 μ L). Following centrifugation (13,000 g for 5 min), 40 μ L of the extract was used for H_2O_2 level estimation. The reaction mixture contained 250 μ M ammonium sulfate, 250 μ M ferrous sulfate, 100 μ M sorbitol, 1% (v/v) ethanol and 100 μ M xylenol orange in 25 mM sulfuric acid. The difference in absorbance between 550 and 800 nm generated after 15 min was used for estimation of H_2O_2 level according to calibration curve.

Histochemical detection of $O_2^{\bullet-}$ and H_2O_2 production—Seedlings of wild type and *anp2anp3* mutant were used for visualization of $O_2^{\bullet-}$ production using NBT staining.³⁰ After a brief vacuum infiltration (3 $\times$ 30 s) in NBT solution (4.3 mM) in 10 mM potassium phosphate buffer (pH 7.8), whole seedlings were stained for 30 min in the dark. Stained seedlings were boiled in clearing solution containing 20% v/v acetic acid, 20% v/v glycerol and 60% v/v ethanol for 5 min and stored in mixture of 20% glycerol v/v and 80% v/v ethanol. Reduced NBT was visualized as a dark blue-colored formazan deposit.

Seedlings of wild type and *anp2anp3* mutant were used for *in situ* detection of H_2O_2 with 3,3'-diaminobenzidine (DAB).³¹ Briefly, seedlings were after brief vacuum infiltration in a solution containing 4.7 mM DAB, Tween 20 (0.05% v/v) and 10 mM sodium phosphate buffer (pH 7.0) placed in the dark for 8 h. The stained seedlings were cleared by boiling into 20% v/v acetic acid, 20% v/v glycerol and 60% v/v ethanol for 15 min and stored in the mixture of 20% glycerol v/v and 80% v/v ethanol. Production of H_2O_2 was visualized as a brown precipitate of oxidized DAB.

The stained seedlings were observed with a Leica M165FC stereomicroscope (Leica Microsystems, Wetzlar, Germany). For detailed images acquisition, a light microscope Zeiss Axio Imager M2 equipped with DIC optics (Carl Zeiss, Jena, Germany) was used. Settings were identical for all the pictures in the experiment. The ImageJ software was used to assess the mean staining intensity of labeled cotyledons and leaves. The data were statistically evaluated by Student's *t* test.

Chlorophyll fluorescence imaging—The chlorophyll fluorescence was monitored using a FluorCam 700 MF imaging system (Photon Systems Instruments, Czech Republic). All measurements were performed in 12–15 replicates. To measure fluorescence signal, short, several microseconds-lasting flashes of red light were applied in 20 ms intervals. The

fluorescence signal was calculated by pulse amplitude modulation (the signals measured before the flashes were subtracted from the signal measured during the flashes). Overall integral light intensity of the measuring flashes was low enough to avoid the closure of the reaction centers of photosystem II (PSII). The minimum chlorophyll fluorescence yield (F_0) was determined after 30 min of dark adaptation by application of the measuring flashes. For the determination of maximum chlorophyll fluorescence (F_M), a saturating pulse of 1.6 s (white light, $2,500 \mu\text{mol m}^{-2} \text{s}^{-1}$) was applied in dark adapted state. To determine the maximum fluorescence yield during light adaptation (F_M'), plants were exposed to red actinic light ($230 \mu\text{mol m}^{-2} \text{s}^{-1}$) after next 2 min of dark adaptation. After 3 s, the actinic light was accompanied by series of saturating pulses applied in 24 s intervals for 2 min and additional 8.5 min in 70 s intervals.

The maximum quantum efficiency of PSII photochemistry (F_V/F_M) was calculated as $(F_M - F_0)/F_M$; the effective quantum yield of electron transport (Φ_{PSII}) as $(F_M' - F_t)/F_M'$; the nonphotochemical quenching of chlorophyll fluorescence (qN) as $1 - (F_M' - F_0')/(F_M - F_0)$ and the excitation pressure on PSII ($1 - q_p$) as $1 - (F_M' - F_t)/(F_M' - F_0')$, where F_t is the fluorescence yield measured just prior the application of saturating pulse and F_0' is the minimal fluorescence for light adapted state calculated as $F_0/(F_V/F_M) + (F_0/F_M')^{32}$.

The two-way t test was performed for statistical analyses using the OriginPro 8.5.1 (OriginLab Corporation, Northampton, MA, USA).

RESULTS

General overview of the differential proteome of *anp2anp3* mutant compared to wild type

Global proteome analysis of *anp2anp3* seedlings resulted in the identification of 630 proteins. Detailed information pertinent to peptide and protein identification is publicly available at http://www.igbb.msstate.edu/journal_data/Anp_Arabidopsis.html, http://www.igbb.msstate.edu/journal_data/ANP_matched_peptides.csv, and http://www.igbb.msstate.edu/journal_data/ANP_protein&peptide_results.csv, as well as in Tables S1–S3.

Identified proteins in Ws and *anp2anp3* were functionally classified using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) web-based application,³³ which allows functional grouping of proteins according to KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (Figure 1). Functional classes containing less than 0.3% of the identified proteins in both proteomes are not included in the chart. Complete outputs of the KEGG-based classification of Ws and *anp2anp3* proteomes are presented in Table S4 and Table S5. The most abundant functional classes referred to metabolic pathways (24% in both proteomes), biosynthesis of secondary metabolites (11.2% in both proteomes), ribosome assembly (6% in both proteomes) and photosynthesis (3.85% in Ws and 4.5% in *anp2anp3*). Additionally, some protein classes appear to be overrepresented either in the wild type or in the *anp2anp3* mutant. For example, proteins involved in glucosino-late biosynthesis were identified only in the *anp2anp3* mutant. Next, functional classes such as proteins involved in galactose metabolism and glycerolipid metabolism were more than 3-

fold more abundant in the *anp2anp3* mutant as compared to Ws. On the other hand, functional classes of proteins involved in phenylalanine, tyrosine and tryptophan biosynthesis as well as in tyrosine metabolism and RNA transport were underrepresented in the mutant, containing 5-, 4-, and 3-fold less proteins, respectively, as compared to Ws.

Statistically significant quantitative results were obtained via comparative proteomic analysis of *anp2anp3* double mutant vs wild type *Arabidopsis* plants. Twenty-five differentially abundant proteins were detected (Table 1). These proteins were categorized into seven different functional classes (Table 1). An overview of functional protein networks affected in the *anp2anp3* mutant as suggested by present comparative proteomic analysis is shown in Figure S1. Proteins differentially abundant in the *anp2anp3* mutant vs wild type seedling plants are involved in calcium ion signaling (e.g., calmodulin isoform CAM4³⁴ and chaperonin 20³⁵), vesicular trafficking and cytokinesis (e.g., RabEld³⁶), photosynthesis, and finally oxidative stress (see next section). Notably, lipoxygenase 2 (LOX2) was the most upregulated protein in the mutant (14.57-fold) when compared to the wild type. LOX2 is a 13S linolenic acid peroxidase involved in wound-induced jasmonic acid biosynthesis.³⁷ Jasmonic acid signaling pathway is interconnected with MAPK signaling via the MKK3/MPK6 pathway.³⁸ Our data indicate that perturbed function of ANPs in the *anp2anp3* double mutant leads to LOX2 accumulation, and therefore, ANPs likely play a negative role in the regulation of jasmonic acid signaling.

Upregulation of ROS detoxifying enzymes in the *anp2anp3* mutant—Four proteins with essential roles in ROS detoxification were upregulated in the *anp2anp3* mutant when compared to wild type (Table 1). Among them, the abundance of FeSOD1 was more than 2-fold increased. This plastid-localized enzyme is responsible for the decomposition of O₂^{•−} radicals.³⁹ In addition, chaperonin 20 (CPN20), a recently identified regulator of FeSOD1 activity,³⁵ was also increased. The MnSOD isozyme (At3gl0920) was also upregulated (2.36-fold), however, the confidence in this result is not satisfactory (*p* = 0.19). Furthermore, DHAR1 was upregulated as well. DFIAR is an enzyme involved in ascorbate-glutathione (asc-glu) cycle and is responsible for maintenance of cellular reduced ascorbate pool which is crucial for the favorable redox state in the cell.^{40,41}

Finally, a protein with GDP-D-mannose 3',5'-epimerase activity was also more abundant and it is known to be involved in ascorbate biosynthesis.⁴² Together, these proteomic data indicated that *anp2anp3* mutant may have increased capacity to decompose ROS eventually leading to better tolerance of oxidative stress conditions.

***anp2anp3* mutant show accelerated antioxidant defense**—We performed immunoblot analysis in order to validate the increased FeSOD1 and MnSOD abundances in *anp2anp3* seedlings compared to wild type. This approach consistently showed upregulation of FeSOD1 and MnSOD in the mutant (Figure 2). To examine whether the higher FeSOD1 and MnSOD abundance resulted also in increased activity of these enzymes in the *anp2anp3* mutant, we performed a native PAGE followed by specific SOD activity staining. Based on the sensitivity of individual SOD isozymes to KCN and H₂O₂ (Figure S2), we have detected three isozymes of SOD in both wild type and *anp2anp3* mutant corresponding to MnSOD, FeSOD and CuZnSOD accordingly (Figure 3A, B). Comparison between the wild type and

the *anp2anp3* mutant revealed substantial increase in the total FeSOD and MnSOD activities in the mutant seedlings, which was supported also by quantification of the band densities (Figure 3C). Immunoblotting following native PAGE was carried out in order to correlate the activity of FeSOD and MnSOD with their abundance. This analysis showed, that the FeSOD1 and MnSOD levels (Figure 3D – I) correlated with total FeSOD and MnSOD activities visualized on native PAGE gels (Figure 3A – C).

The observed increase in MnSOD and FeSOD activities and abundances implies that the *anp2anp3* has a higher ability to remove $O_2^{\bullet-}$ radicals. The examination of SOD activities and abundances after short-term paraquat treatment (15 μ M for 5 min) showed significant decrease in FeSOD and MnSOD isozyme activities in Ws, while they remained at the same level in *anp2anp3* seedlings (Figure 3). These results show that *anp2anp3* mutant possesses increased capacity to remove $O_2^{\bullet-}$ radicals under oxidative stress conditions.

Concomitantly to the upregulation of SOD levels and activities, our proteomic analysis revealed the significant upregulation of enzymes involved in ascorbate metabolism and regeneration in the *anp2anp3* mutant (Table 1). The enzymatic activity of DHAR was addressed spectrophotometrically,²⁵ in order to validate the significance of DHAR1 upregulation in the *anp2anp3* mutant identified by proteomic analysis. In this case, DHAR activity in the mutant was increased by 1.3-fold compared to the wild type (Figure 4A). Consistently with a role of increased ascorbate turnover to scavenge H_2O_2 ¹⁴ in the *anp2anp3* mutant, we found a similarly increased enzymatic activity of APX when compared to the wild type (Figure 4B). Next, upregulation of GDP-D-mannose 3',5'-epimerase (GME; Table 1), an enzyme involved in ascorbate biosynthesis,⁴³ suggested elevated ascorbate levels in the *anp2anp3* mutant. For this reason, ascorbate levels were quantitated in both wild type and mutant seedlings.²⁶ The spectrophotometric assay of ascorbate levels validated the increased efficiency of ascorbate production by GME in the *anp2anp3* mutant (Figure 4C).

Determination of ROS levels in the *anp2anp3* seedlings—The increased antioxidant capacity in the *anp2anp3* mutant should be reflected in alleviated levels of ROS. To prove this hypothesis, we aimed to monitor the production of $O_2^{\bullet-}$ and H_2O_2 , both in whole seedlings according to standard histochemical detection (Figures 5 and 6). We also analyzed the activity of NADPH oxidase (generating $O_2^{\bullet-}$) as well as the levels of $O_2^{\bullet-}$ and H_2O_2 spectrophotometrically (Figure 7).

The production of $O_2^{\bullet-}$ was examined directly in wild type and *anp2anp3* seedlings through the reduction of nitroblue tetrazolium (NBT) to formazan precipitate.³⁰ NBT staining revealed decreased formazan accumulation in cotyledons but increased accumulation in leaves, thus reflecting lower $O_2^{\bullet-}$ production in cotyledons but higher in leaves of the *anp2anp3* seedlings as compared to the wild type ones (Figure 5A, B, E). Next, the *anp2anp3* seedlings accumulated significantly lower levels of H_2O_2 in their cotyledons and leaves in comparison to wild type seedlings, as determined by histochemical DAB staining (Figure 5C, D, F).

NBT staining in wild type leaves was weak and rather diffuse with prevalent localization at the vasculature (Figure 6A). However, strong NBT staining of *anp2anp3* leaves was observed in clustered pattern restricted to stomatal complexes and mesophyll cells surrounding the stomatal pore (Figure 6B inset, arrowhead). In all cases, the most prominent NBT staining of mesophyll cells in both wild type and *anp2anp3* seedlings showed plastidic localization while the apoplast was also stained (Figures 6C, D, arrows).

DAB staining of H_2O_2 followed similar pattern in leaves of both wild type and *anp2anp3* seedlings (Figures 6E, F), differing only in staining intensity. Again, chloroplasts were the most prominent sites where DAB deposits accumulated (Figures 6G, H). Leaf cells of *anp2anp3* seedlings were much bigger by comparison to those of Ws seedlings (Figures 6C – H) due to disturbed cell growth and development caused by microtubule-related defects.⁷

By assaying the $O_2^{\bullet-}$ production rate spectrophotometrically, it was found that its levels increased by 2.5-fold in the *anp2anp3* mutant compared to the wild type (Figure 7A).

This increase was further corroborated by direct measurement of NADPH oxidase activity, one of the main sources of $O_2^{\bullet-}$ production, which was nearly 2-fold increased in the *anp2anp3* mutant (Figure 7B). These results suggest that the increased $O_2^{\bullet-}$ level in the *anp2anp3* mutant is, at least partially, linked to higher NADPH oxidase activity.

We assayed H_2O_2 levels by using a method based on xylenol orange.²⁹ Consistent with the histochemical observations, spectrophotometric assessments showed lower levels of H_2O_2 in mutant seedlings (Figure 7C). The short-term paraquat treatment had no significant effect on H_2O_2 levels in neither Ws nor *anp2anp3* mutant seedlings (Figure 7C).

Increased paraquat resistance of *anp2anp3* mutant—The question of whether the increased abundance and activity of antioxidant enzymes would affect the response of *anp2anp3* mutants to oxidative stress was addressed by comparing the growth of *anp2anp3* and wild type seedlings in medium containing paraquat, a methyl viologen inducing the production of $O_2^{\bullet-}$ radicals in photosynthetic tissues.⁴⁴ The effects of paraquat on growth and viability were recorded after 7 days of continuous exposure of seedlings of *anp2anp3* and wild type (seedlings were 10 days old). Exposure to paraquat caused chlorophyll bleaching in wild type seedlings, which was not the case in the mutant (Figure 8). Paraquat treatment hampered seedling growth more effectively in the case of wild type, while *anp2anp3* mutants exhibited significant resilience to continuous paraquat presence (Figure 8 C, D).

Downregulation of several photosynthetic proteins in the *anp2anp3* mutant—ROS production is intimately linked to aerobic metabolism, particularly during the photosynthesis.⁴⁵ Meanwhile, the differential proteomic analysis of *anp2anp3* and Ws seedlings revealed significant downregulation of photo-synthetic proteins in the mutant (Table 1). For example, ferredoxin 2, a protein responsible for the electron delivery to various substrates, was substantially downregulated. The DNA-damage-repair/tolerance protein 112, otherwise known as plastocyanin 2, a photosynthetic electron carrier participating in electron transfer between P700 and the cytochrome b_6f complex in

photosystem I,⁴⁶ was also downregulated in the mutant seedlings. The decreased abundance of constituents of photosystems I and II, such as PsBP-1 and 2, and LHCB5, may relate to their possible instability in the mutant seedlings.⁴⁷ On the other hand, a major subunit of photosystem I (PSAD1)⁴⁸ was upregulated. The data above indicate that double mutation of *ANP2* and *ANP3* genes lead to the lower abundance of crucial photosynthetic proteins in *Arabidopsis thaliana*.

In order to prove, whether the decreased abundance of photosynthetic proteins lead to lower photosynthetic activity in the *anp2anp3* mutant, we performed a chlorophyll fluorescence imaging analysis. Measurement of chlorophyll fluorescence induction did not reveal significant differences between *anp2anp3* mutant and wild type in either maximum quantum efficiency of photosystem II (PSII) photochemistry in the dark adapted plants (F_v/F_m) (Figure 9A) nor in parameters of the light adapted state such as the effective quantum yield of electron transport through PSII (Φ_{PSII}) and nonphotochemical quenching of chlorophyll fluorescence (qN) (Figure 9B). The above results indicate that the *anp2anp3* mutant exhibits similar photosynthetic efficiency as the wild type.

DISCUSSION

Differential proteomic studies on MAPK mutants may help to elucidate MAPK functions leading to adaptation of plants to prolonged or continuous environmental stresses.⁴⁹

Although there have been many studies addressing proteome-wide responses following the exposure of *Arabidopsis* or crop plants to prolonged abiotic stresses,^{50–53} there has not been a complementary work linking such responses to specific signaling pathways. Here, we addressed the proteome of *anp2anp3* mutant in the light of the previously undescribed role of ANP MAPKKKs in reaction to oxidative stress. In this respect, the present study provides such quantitative information on the differential protein abundance between the double MAPKKK mutant *anp2anp3* and the respective wild type, and it complements previous knowledge on the proteome-wide effects of stress conditions such as oxidative,⁵⁴ UVB,⁵⁵ salt⁵⁶ and heavy metal⁵⁰ stresses that involve MAPK signaling.

Oxidative stress tolerance of the *anp2anp3* mutant

Oxidative stress through the metabolic accumulation of ROS is an unavoidable consequence of aerobic metabolism and particularly photosynthesis,⁵⁷ but it is also related to various abiotic stress conditions including heavy metal toxicity, salinity, ultraviolet irradiation and temperature stresses, all of which culminate in the accumulation of various ROS.⁵⁸ Although ROS may have signaling attributes with developmental and stress priming consequences,⁵⁹ their overproduction may lead to toxic effects including lipid peroxidation⁶⁰ and protein oxidation,⁶¹ ultimately leading to challenging of cellular integrity and finally to cell death.⁶²

To cope with these harmful effects of ROS, plants exposed to oxidative stress (through exposure to high irradiance or high energy UV illumination,⁶³ excessive salinity,⁶⁴ or heavy metals in the rhizosphere⁶⁵) show upregulation of stress-responsive genes encoding proteins with protective and ROS-detoxifying functions.⁵⁹

In this sense, the protein complement, the biochemical and physiological properties of *anp2anp3* mutant are suggestive of a constitutive antioxidative response and imply a negative role of the ANP family in the regulation of oxidative stress tolerance. Similar traits were reported for *mpk3* and *mpk6* knockout mutants against UV-B irradiation⁶⁶ and oxidative stress⁶⁷. It is interesting that deactivation of ozone-induced MPK3 and MPK6 activity by the MAPK phosphatase MKP2 has also a positive regulatory role in antioxidant defense since knocking out MKP2 results in constitutive overactivation of MPK3 and MPK6 and hypersensitization against ozone.⁶⁸ Notably, a recent study¹⁰ reported decreased capacity of *anp2anp3* mutant to generate elicitor-induced oxidative burst and elevated sensitivity against the necrotrophic fungus *Botrytis cinerea*.

Increased activity and abundance of FeSOD and MnSOD

In addition to other changes, the comparative proteome analysis of the *anp2anp3* mutant essentially unveiled the upregulation of proteins involved in ROS detoxification (Table 1), which is consistent with the oxidative stress tolerance of *anp2anp3* seedlings.

First, we observed increased capacity for $O_2^{\bullet-}$ removal in the mutant, which is an immediate consequence of the activity of three isozymes of SODs, differing in metal cofactor.⁶⁹ Proteomic analysis showed more than 2-fold increase in the abundance of FeSOD1 in the mutant as compared to the wild type. FeSOD is a chloroplastic SOD, mostly responsible for the removal of $O_2^{\bullet-}$ produced during photosynthesis. The increased abundance of FeSOD1 in *anp2anp3* seedlings was validated by immunoblot analysis. Consistently, the FeSOD1 abundance positively correlated with the total FeSOD activity in the *anp2anp3* seedlings, showing almost 2-fold increase as revealed by native PAGE SOD activity staining. The heterologous overexpression of *Arabidopsis* FeSOD1 confers increased oxidative stress tolerance to transgenic maize, suggesting that the upregulation of FeSOD 1 expression⁷⁰ may suffice to explain the increased tolerance of *anp2anp3* seedlings to oxidative stress. Moreover, a chloroplast-localized CPN20 was recently identified as FeSOD1 interacting partner and regulator of FeSOD1 activation. Alongside FeSOD1, CPN20 was also found to be upregulated in the *anp2anp3* mutant. On the basis of this knowledge we propose, that the increased FeSOD activity in the *anp2anp3* mutant might be a combined consequence of increased FeSOD1 and CPN20 protein levels. In addition, proteomics also showed increased abundance of mitochondrial MnSOD isozyme decomposing $O_2^{\bullet-}$ originating from the respiratory electron transport chain.⁷¹ This was validated by immunoblot analysis using anti-MnSOD antibody, as well as by MnSOD specific activity staining on native PAGE gels. Importantly, *anp2anp3* mutant showed increased SOD activities also under oxidative stress conditions.

The increased $O_2^{\bullet-}$ production in the *anp2anp3* mutant

Histochemical and spectrophotometric assays showed significant increase of $O_2^{\bullet-}$ production in the mutant seedlings. Obviously, $O_2^{\bullet-}$ is produced constitutively in photosynthetic reactions by direct photoreduction of O_2 by reduced electron transport components. This constitutively produced $O_2^{\bullet-}$ is effectively scavenged by SODs in chloroplast (Cu/Zn SOD and FeSOD), serving as an alternative electron acceptor in photoprotection.⁵⁹ Generation of $O_2^{\bullet-}$ during photosynthesis is substantially increased under

photoinhibition of photosynthesis induced by abiotic stimuli such as light and cold stress.⁷² Another source of $O_2^{\bullet-}$ is a plasma membrane localized NADPH oxidase, which generates extracellular $O_2^{\bullet-}$ using NADPH as an electron donor. NADPH oxidase activity is induced by pathogen infection,⁷³ exogenous abscisic acid,⁷⁴ or heavy metal stress.⁷⁵ It is also required for developmental processes such as the ABA-induced stomatal closure⁷⁶ or for cell expansion.⁷⁷

Judging from the similar photosynthetic activity in the *anp2anp3* mutant and in the wild type, it is unlikely that increased $O_2^{\bullet-}$ levels in the *anp2anp3* reflect differences in photosynthesis. Since the increase in total $O_2^{\bullet-}$ level was similar to the increase of NADPH oxidase, we suggest that the increased total $O_2^{\bullet-}$ production in *anp2anp3* mutant accounts for plasma membrane NADPH oxidase, which has elevated activities in this mutant. Similarly, elevated $O_2^{\bullet-}$ levels were detected also in *Arabidopsis mkk4* mutant.⁷⁸ MKK4 is a MAP kinase kinase which was suggested to act downstream of ANP1,⁷⁹ and it was proposed to be redundantly targeted by both ANP2 and ANP3. MKK4/MKK5 are upstream activators of MPK3/MPK6 in response to external H_2O_2 .⁹ ANP1, ANP2 and ANP3 were shown to be redundant in MPK3/MPK6 activation in response to external H_2O_2 .⁹ Therefore, we hypothesize that ANPs may negatively affect NADPH oxidase activity in *Arabidopsis*, likely through MKK4-dependent activation of MPK3 and MPK6. Similar regulation was recently proposed for MPK8, acting downstream of MKK3, which suppresses the expression of *RbohD* gene, encoding NADPH oxidase.⁸⁰

Accelerated ascorbate-glutathione cycle in the *anp2anp3* mutant

The *anp2anp3* mutant exhibited upregulation of DHAR1 at the protein level (Table 1). The higher abundance of DHAR1 was also reflected in its increased activity in the *anp2anp3* mutant. This enzyme is responsible for recycling of the ascorbate.⁸¹ DHAR overexpression leads to elevated foliar ascorbate levels⁸² and more efficient protection against ROS.⁸³

The reduction of dehydroascorbate to ascorbate by DHAR occurs within the asc-glu cycle.⁸⁴ Within this cycle, the reduced ascorbate serves as electron donor for efficient removal of H_2O_2 by APX. The increased activities of APX and DHAR in the *anp2anp3* mutant found by the present study support the idea of an accelerated asc-glu cycle resulting in rapid H_2O_2 decomposition. We measured also the total ascorbate content, showing increased levels in the mutant. This is consistent with the increased abundance of GME, involved in ascorbate synthesis,⁸⁵ which was found by our proteomic analysis. Except for the important role of ascorbate in the maintenance of redox homeostasis, this abundant nonenzymatic antioxidant itself has an ability to scavenge ROS such as $O_2^{\bullet-}$ and hydroxyl radical.⁸⁴

The histochemical and spectrophotometric examination showed decreased levels of H_2O_2 in *anp2anp3* mutant. The major mechanism of H_2O_2 production involves the dismutation of the $O_2^{\bullet-}$, which can either be catalyzed by SODs or occur spontaneously.⁸⁶ At high concentrations, H_2O_2 causes serious damaging effects leading to cell death.⁸⁷ The lower levels of H_2O_2 detected by DAB staining and spectrophotometric measurement in *anp2anp3* mutant are in agreement with the accelerated antioxidative defense and redox homeostasis.

Together, our proteomic and biochemical analyses suggest that *anp2anp3* double mutant possesses a functional network of proteins (Figure 10), ensuring favorable cellular redox conditions as well as accelerated defense against overproduction of ROS.

There is some previous evidence suggesting an increased oxidative stress resistance in plants overexpressing either CuZnSOD, MnSOD, or FeSOD but also H₂O₂ scavenging enzyme APX.^{70,88–90} Consistently with these reports, the accelerated antioxidant defense represented by FeSOD, MnSOD, APX, and DHAR as well as ascorbate confers oxidative stress resistance to the *anp2anp3* mutant. The MAPK signaling toward antioxidant enzymes (CuZnSOD and catalase) was shown to be mediated by a H₂O₂-inducible MAPK cascade consisting of MEKK1 (MAPKKK), MKK1, MKK2, and MKK5 (MAPKK) and downstream MPK3 and MPK6.^{14–16} Our results indicate involvement of ANP2 and ANP3 in the regulation of redox homeostasis and antioxidant defense.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Rodriguez MCS, Petersen M, Mundy J. Mitogen-activated protein kinase signaling in plants. *Annu. Rev. Plant Biol.* 2010; 61:621–649. [PubMed: 20441529]
2. Šamajová O, Plíhal O, Al-Yousif M, Hirt H, Šamaj J. Improvement of stress tolerance in plants by genetic manipulation of mitogen-activated protein kinases. *Biotechnol. Adv.* 2013; 31:118–128. [PubMed: 22198202]
3. Andreasson E, Ellis B. Convergence and specificity in the *Arabidopsis* MAPK nexus. *Trends Plant Sci.* 2010; 15:106–113. [PubMed: 20047850]
4. Krysan PJ, Jester PJ, Gottwald JR, Sussman M. R An *Arabidopsis* mitogen-activated protein kinase kinase gene family encodes essential positive regulators of cytokinesis. *Plant Cell.* 2002; 14:1109–1120. [PubMed: 12034900]
5. Sasabe M, Soyano T, Takahashi Y, Sonobe S, Igarashi H, Itoh TJ, Hidaka M, Machida Y. Phosphorylation of NtMAP6S-I by a MAP kinase down-regulates its activity of microtubule bundling and stimulates progression of cytokinesis of tobacco cells. *Genes Dev.* 2006; 20:1004–1014. [PubMed: 16598040]
6. Takahashi Y, Soyano T, Kosetsu K, Sasabe M, Machida Y. HINKEL kinesin, ANP MAPKKKs and MKK6/ANQ MAPKK, which phosphorylates and activates MPK4 MAPK, constitute a pathway that is required for cytokinesis in *Arabidopsis thaliana*. *Plant Cell Physiol.* 2010; 51:1766–1776. [PubMed: 20802223]
7. Beck M, Komis G, Muller J, Menzel D, Šamaj J. *Arabidopsis* homologs of nucleus- and phragmoplast-localized kinase 2 and 3 and mitogen-activated protein kinase 4 are essential for microtubule organization. *Plant Cell.* 2010; 22:755–771. [PubMed: 20215588]

8. Sasabe M, Machida Y. Regulation of organization and function of microtubules by the mitogen-activated protein kinase cascade during plant cytokinesis. *Cytoskeleton* (Hoboken). 2012; 69:913–918. [PubMed: 23027702]
9. Kovtun Y, Chiu WL, Tena G, Sheen J. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc. Natl. Acad. Sci. U. S. A.* 2000; 97:2940–2945. [PubMed: 10717008]
10. Savatin DV, Bisceglia NG, Marti L, Fabbri C, Cervone F, Lorenzo GD. The Arabidopsis Nucleus-And Phragmoplast-Localized Kinase-Related Protein Kinases Are Required for Elicitor-Induced Oxidative Burst and Immunity. *Plant Physiol.* 2014; 165:1188–1202. [PubMed: 24812107]
11. Gawronski P, Wito D, Vashutina K, Bederska M, Betliński B, Rusaczek A, Karpiński S. Mitogen-Activated Protein Kinase 4 Is a Salicylic Acid-Independent Regulator of Growth But Not of Photosynthesis in Arabidopsis. *Mol. Plant.* 2014; 7:1151–1166. [PubMed: 24874867]
12. Pitzschke A, Djamei A, Bitton F, Hirt H. A major role of the MEKK1-MKK1/2-MPK4 pathway in ROS signalling. *Mol. Plant.* 2009; 2:120–137. [PubMed: 19529823]
13. Nakagami H, Soukupová H, Schikora A, Zárský V, Hirt H. A Mitogen-activated protein kinase kinase mediates reactive oxygen species homeostasis in *Arabidopsis*. *J. Biol. Chem.* 2006; 281:38697–38704. [PubMed: 17043356]
14. Xing Y, Cao Q, Zhang Q, Qin L, Jia W, Zhang J. MKK5 regulates high light-induced gene expression of Cu/Zn superoxide dismutase 1 and 2 in *Arabidopsis*. *Plant Cell Physiol.* 2013; 54:1217–1227. [PubMed: 23677921]
15. Xing Y, Jia W, Zhang J. AtMEK1 mediates stress-induced gene expression of CAT1 Catalase by triggering H₂O₂ production in *Arabidopsis*. *J. Exp. Bot.* 2007; 58:2969–2981. [PubMed: 17728292]
16. Xing Y, Jia W, Zhang J. AtMKK1 mediates ABA-induced CAT1 expression and H₂O₂ production via AtMPK6-coupled signaling in *Arabidopsis*. *Plant J. Cell Mol. Biol.* 2008; 54:440–451.
17. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 1962; 15:473–497.
18. Taká T, Pechan T, Richter H, Müller J, Eck C, Böhm N, Obert B, Ren H, Niehaus K, Šamaj J. Proteomics on brefeldin A-treated *Arabidopsis* roots reveals profilin 2 as a new protein involved in the cross-talk between vesicular trafficking and the actin cytoskeleton. *J. Proteome Res.* 2011; 10:488–501. [PubMed: 21090759]
19. Hurkman WJ, Tanaka C. K Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. *Plant Physiol.* 1986; 81:802–806. [PubMed: 16664906]
20. Bridges SM, Magee GB, Wang N, Williams WP, Burgess SG, Nanduri B. ProtQuant: a tool for the label-free quantification of MudPIT proteomics data. *BMC Bioinformatics.* 2007; 8(Suppl 7):S24. [PubMed: 18047724]
21. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976; 72:248–254. [PubMed: 942051]
22. Pan SM, Yau YY. Characterization of superoxide dismutase in *Arabidopsis*. *Plant Cell Physiol.* 1998; 37:58–66.
23. Beauchamp G, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* 1971; 44:276–87. [PubMed: 4943714]
24. Amako K, Chen GX, Asada K. Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. *Plant Cell Physiol.* 1994; 35:497–504.
25. Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. *Plant Cell Physiol.* 1981; 22:867–880.
26. Gillespie KM, Ainsworth EA. Measurement of reduced, oxidized and total ascorbate content in plants. *Nat. Protoc.* 2007; 2:871–874. [PubMed: 17446888]
27. Sagi M, Fluhr R. Superoxide production by plant homologues of the gp91 (phox) NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection. *Plant Physiol.* 2001; 126:1281–1290. [PubMed: 11457979]

28. Able AJ, Guest DI, Sutherland MW. Use of a new tetrazolium-based assay to study the production of superoxide radicals by tobacco cell cultures challenged with avirulent zoospores of *Phytophthora parasitica* var *nicotianae*. *Plant Physiol.* 1998; 117:491–499. [PubMed: 9625702]
29. Cheeseman JM. Hydrogen peroxide concentrations in leaves under natural conditions. *J. Exp. Bot.* 2006; 57:2435–2444. [PubMed: 16766599]
30. Ramel F, Sulmon C, Bogard M, Couée I, Gouesbet G. Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced tolerance in *Arabidopsis thaliana* plantlets. *BMC Plant Biol.* 2009; 9:28. [PubMed: 19284649]
31. Daudi A, Cheng Z, O'Brien JA, Mammarella N, Khan S, Ausubel FM, Bolwell GP. The apoplastic oxidative burst peroxidase in *Arabidopsis* is a major component of pattern-triggered immunity. *Plant Cell.* 2012; 24:275–287. [PubMed: 22247251]
32. Oxborough K, Baker N. R Resolving chlorophyll a fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components-calculation of qP and F_v'/F_m' without measuring F_o' . *Photosynth. Res.* 1997; 54:135–142.
33. Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey G, Muller J, Doerks T, Julien P, Roth A, Simonovic M, Bork P, von Mering C. STRING 8-a global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Res.* 2009; 37:D412–D416. [PubMed: 18940858]
34. McCormack E, Tsai Y-G, Braam J. Handling calcium signaling: *Arabidopsis* CaMs and CMLs. *Trends Plant Sci.* 2005; 10:383–389. [PubMed: 16023399]
35. Kuo WY, Huang GH, Liu AG, Cheng GP, Li SH, Chang WC, Weiss C, Azem A, Jinn TL. CHAPERONIN 20 mediates iron superoxide dismutase (FeSOD) activity independent of its co-chaperonin role in *Arabidopsis* chloroplasts. *New Phytol.* 2013; 197:99–110. [PubMed: 23057508]
36. Camacho L, Smertenko AP, Perez-Gomez J, Hussey PJ, Moore I. *Arabidopsis* Rab-E GTPases Exhibit a Novel Interaction with a Plasma-membrane Phosphatidylinositol-4-phosphate 5-kinase. *J. Cell Sci.* 2009; 122:4383–4392. [PubMed: 19903693]
37. Seltmann MA, Stingl NE, Lautenschlaeger JK, Kriskche M, Mueller MJ, Berger S. Differential impact of lipoxygenase 2 and jasmonates on natural and stress-induced senescence in *Arabidopsis*. *Plant Physiol.* 2010; 152:1940–1950. [PubMed: 20190093]
38. Takahashi F, Yoshida R, Ichimura K, Mzouguchi T, Seo S, Yonezawa M, Maruyama K, Yamaguchi-Shinozaki K, Shinozaki K. The mitogen-activated protein kinase cascade MKK3-MPK6 is an important part of the jasmonate signal transduction pathway in *Arabidopsis*. *Plant Cell.* 2007; 19:805–818. [PubMed: 17369371]
39. Pilon M, Ravet K, Tapken W. The biogenesis and physiological function of chloroplast superoxide dismutases. *Biochim. Biophys. Acta BBA-Bioenerg.* 2011; 1807:989–998.
40. Gallie D. R The Role of L-ascorbic acid recycling in responding to environmental stress and in promoting plant growth. *J. Exp. Bot.* 2013; 64:433–443. [PubMed: 23162122]
41. Smirnoff N. Ascorbate biosynthesis and function in photo-protection. *Philos. Trans. R. Soc. London, B: Biol. Sci.* 2000; 355:1455–1464. [PubMed: 11127999]
42. Wolucka BA, Van Montagu M. GDP-mannose 3',5'-epimerase forms GDP-L-gulose, a putative intermediate for the *de novo* biosynthesis of vitamin C in plants. *J. Biol. Chem.* 2003; 278:47483–47490. [PubMed: 12954627]
43. Valpuesta V, Botella MA. Biosynthesis of L-ascorbic acid in plants: new pathways for an old antioxidant. *Trends Plant Sci.* 2004; 9:573–577. [PubMed: 15564123]
44. Bus JS, Aust SD, Gibson JE. Superoxide- and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. *Biochem. Biophys. Res. Commun.* 1974; 58:749–755. [PubMed: 4365647]
45. Karpiński S, Szechyńska-Hebda M, Wituszyńska W, Burdiak P. Light acclimation, retrograde signalling, cell death and immune defenses in plants. *Plant Cell Environ.* 2013; 36:736–744. [PubMed: 23046215]
46. Pesaresi P, Scharfenberg M, Weigel M, Granlund I, Schroder WP, Finazzi G, Rappaport F, Masiero S, Furini A, Jahns P, Leister D. Mutants, overexpressors, and interactors of *Arabidopsis* plastocyanin isoforms: revised roles of plastocyanin in photosynthetic electron flow and thylakoid redox state. *Mol. Plant.* 2009; 2:236–248. [PubMed: 19825610]

47. Allahverdiyeva Y, Suorsa M, Rossi F, Pavesi A, Kater MM, Antonacci A, Tadini L, Pribil M, Schneider A, Wanner G, Leister D, Aro EM, Barbato R, Pesaresi P. *Arabidopsis* plants lacking PsbQ and PsbR subunits of the oxygen-evolving complex show altered PSII super-complex organization and short-term adaptive mechanisms. *Plant J.* 2013; 75:671–684. [PubMed: 23647309]
48. Ihnatowicz A, Pesaresi P, Varotto G, Richly E, Schneider A, Jahns P, Salamini F, Leister D. Mutants for photosystem I subunit D of *Arabidopsis thaliana*: effects on photosynthesis, photosystem I stability and expression of nuclear genes for chloroplast functions. *Plant J.* 2004; 37:839–52. [PubMed: 14996217]
49. Sinha AK, Jaggi M, Raghuram B, Tuteja N. Mitogen-activated protein kinase signaling in plants under abiotic stress. *Plant Signal. Behav.* 2011; 6:196–203. [PubMed: 21512321]
50. Li G, Peng X, Xuan H, Wei L, Yang Y, Guo T, Kang G. Proteomic analysis of leaves and roots of common wheat (*Triticum aestivum* L.) under copper-stress conditions. *J. Proteome Res.* 2013; 12:4846–4861. [PubMed: 24074260]
51. Hossain Z, Khatoon A, Komatsu S. Soybean proteomics for unraveling abiotic stress response mechanism. *J. Proteome Res.* 2013; 12:4670–4684. [PubMed: 24016329]
52. Singh R, Jwa NS. Understanding the responses of rice to environmental stress using proteomics. *J. Proteome Res.* 2013; 12:4652–4669. [PubMed: 23984864]
53. Amme S, Matros A, Schlesier B, Mock HP. Proteome analysis of cold stress response in *Arabidopsis thaliana* using DIGE-technology. *J. Exp. Bot.* 2006; 57:1537–1546. [PubMed: 16574749]
54. Wang H, Wang S, Lu Y, Alvarez S, Hicks LM, Ge X, Xia Y. Proteomic analysis of early-responsive redox-sensitive proteins in *Arabidopsis*. *J. Proteome Res.* 2012; 11:412–424. [PubMed: 22050424]
55. Du H, Liang Y, Pei K, Ma K. UV radiation-responsive proteins in rice leaves: a proteomic analysis. *Plant Cell Physiol.* 2011; 52:306–316. [PubMed: 21172955]
56. Jiang Y, Yang B, Harris NS, Deyholos MK. Comparative proteomic analysis of NaCl stress-responsive proteins in *Arabidopsis* roots. *J. Exp. Bot.* 2007; 58:3591–3607. [PubMed: 17916636]
57. Foyer GH, Noctor G. Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. *Antioxidants Redox Signal.* 2009; 11:861–905.
58. Suzuki N, Koussevitzky S, Mittler R, Miller G. ROS and Redox Signalling in the Response of Plants to Abiotic Stress. *Plant Cell Environ.* 2012; 35:259–270. [PubMed: 21486305]
59. Apel K, Hirt H. REACTIVE OXYGEN SPECIES: Metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 2004; 55:373–399. [PubMed: 15377225]
60. Farmer EE, Mueller MJ. ROS-mediated lipid peroxidation and RES-activated signaling. *Annu. Rev. Plant Biol.* 2013; 64:429–450. [PubMed: 23451784]
61. Jacques S, Ghesquière B, Van Breusegem F, Gevaert K. Plant proteins under oxidative attack. *Proteomics.* 2013; 13:932–940. [PubMed: 23172756]
62. Wang Y, Lin A, Loake GJ, Chu G. H₂O₂-induced leaf cell death and the crosstalk of reactive nitric/oxygen species. *J. Integr. Plant Biol.* 2013; 55:202–208. [PubMed: 23331502]
63. Hideg E, Jansen MA, Strid A. UV-B exposure, ROS, and stress: inseparable companions or loosely linked associates? *Trends Plant Sci.* 2013; 18:107–115. [PubMed: 23084465]
64. Jaspers P, Kangasjärvi J. Reactive oxygen species in abiotic stress signaling. *Physiol. Plant.* 2010; 138:405–413. [PubMed: 20028478]
65. Hossain Z, Komatsu S. Contribution of proteomic studies towards understanding plant heavy metal stress response. *Front. Plant Sci.* 2013; 3:310. [PubMed: 23355841]
66. González Besteiro MA, Bartels S, Albert A, Ulm R. *Arabidopsis* MAP kinase phosphatase 1 and its target MAP kinases 3 and 6 antagonistically determine UV-B stress tolerance, independent of the UVR8 photoreceptor pathway. *Plant J.* 2011; 68:727–737. [PubMed: 21790814]
67. Lee JS, Ellis BE. *Arabidopsis* MAPK phosphatase 2 (MKP2) positively regulates oxidative stress tolerance and inactivates the MPK3 and MPK6 MAPKs. *J. Biol. Chem.* 2007; 282:25020–25029. [PubMed: 17586809]
68. Xing Y, Jia W, Zhang J. AtMKK1 mediates ABA-induced CAT1 expression and H₂O₂ production via AtMPK6-coupled signaling in *Arabidopsis*. *Plant J.* 2008; 54:440–451. [PubMed: 18248592]

69. Kliebenstein DJ, Monde RA, Last RL. Superoxide dismutase in *Arabidopsis*: an eclectic enzyme family with disparate regulation and protein localization. *Plant Physiol.* 1998; 118:637–650. [PubMed: 9765550]
70. Van Breusegem F, Slooten L, Stassart JM, Moens T, Botterman J, Van Montagu M, Inzé D. Overproduction of *Arabidopsis thaliana* FeSOD confers oxidative stress tolerance to transgenic maize. *Plant Cell Physiol.* 1999; 40:515–523. [PubMed: 10427774]
71. Morgan MJ, Lehmann M, Schwarzlander M, Baxter CJ, Sienkiewicz-Porzucek A, Williams TCR, Schauer N, Fernie AR, Fricker MD, Ratcliffe RG, et al. Decrease in manganese superoxide dismutase leads to reduced root growth and affects tricarboxylic acid cycle flux and mitochondrial redox homeostasis. *Plant Physiol.* 2008; 147:101–114. [PubMed: 18337490]
72. Hodgson RA, Raison JK. Superoxide production by thylakoids during chilling and its implication in the susceptibility of plants to chilling-induced photoinhibition. *Planta.* 1991; 183:222–228. [PubMed: 24193624]
73. Torres MA, Dangl JL, Jones JDG. *Arabidopsis* gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proc. Natl. Acad. Sci. U. S. A.* 2002; 99:517–522. [PubMed: 11756663]
74. Jiang M, Zhang J. Involvement of plasma-membrane NADPH oxidase in abscisic acid- and water stress-induced antioxidant defense in leaves of maize seedlings. *Planta.* 2002; 215:1022–1030. [PubMed: 12355163]
75. Hao F, Wang X, Chen J. Involvement of plasma-membrane NADPH oxidase in nickel-induced oxidative stress in roots of wheat seedlings. *Plant Sci.* 2006; 170:151–158.
76. Desikan R, Cheung M-K, Bright J, Henson D, Hancock JT, Neill SJ. ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. *J. Exp. Bot.* 2004; 55:205–212. [PubMed: 14673026]
77. Foreman J, Demidchik V, Bothwell JHF, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee G, Jones JDG, et al. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature.* 2003; 422:442–446. [PubMed: 12660786]
78. Kim S-H, Woo D-H, Kim J-M, Lee S-Y, Chung WS, Moon Y-H. *Arabidopsis* MKK4 mediates osmotic-stress response via its regulation of MPK3 activity. *Biochem. Biophys. Res. Commun.* 2011; 412:150–154. [PubMed: 21806969]
79. Colcombet J, Hirt H. *Arabidopsis* MAPKs: a complex signalling network involved in multiple biological processes. *Biochem. J.* 2008; 413:217–226. [PubMed: 18570633]
80. Takahashi F, Mizoguchi T, Yoshida R, Ichimura K, Shinozaki K. Calmodulin-Dependent Activation of MAP Kinase for ROS Homeostasis in *Arabidopsis*. *Mol. Cell.* 2011; 41:649–660. [PubMed: 21419340]
81. Yoshida S, Tamaoki M, Shikano T, Nakajima N, Ogawa D, Ioki M, Aono M, Kubo A, Kamada H, Inoue Y, Saji H. Cytosolic dehydroascorbate reductase is important for ozone tolerance in *Arabidopsis thaliana*. *Plant Cell Physiol.* 2006; 47:304–308. [PubMed: 16361320]
82. Chen Z, Young TE, Ling J, Chang S-G, Gallie DR. Increasing vitamin C content of plants through enhanced ascorbate recycling. *Proc. Natl. Acad. Sci. U. S. A.* 2003; 100:3525–3530. [PubMed: 12624189]
83. Chen Z, Gallie D. R Dehydroascorbate Reductase Affects Leaf Growth, Development, and Function. *Plant Physiol.* 2006; 142:775–787. [PubMed: 16891549]
84. Foyer GH, Noctor G. Ascorbate and Glutathione: The Heart of the Redox Hub. *Plant Physiol.* 2011; 155:2–18. [PubMed: 21205630]
85. Conklin PL, Norris SR, Wheeler GL, Williams EH, Smirnov N, Last RL. Genetic evidence for the role of GDP-mannose in plant ascorbic acid (vitamin C) biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* 1999; 96:4198–4203. [PubMed: 10097187]
86. Van Breusegem F, Vranova E, Dat JF, Inzé D. The role of active oxygen species in plant signal transduction. *Plant Sci.* 2001; 161:405–414.
87. Yao N, Tada Y, Park P, Nakayashiki H, Tosa Y, Mayama S. Novel evidence for apoptotic cell response and differential signals in chromatin condensation and DNA cleavage in victorin-treated oats. *Plant J.* 2001; 28:13–26. [PubMed: 11696183]

88. Gupta AS, Webb RP, Holaday AS, Allen RD. Overexpression of Superoxide Dismutase Protects Plants from Oxidative Stress (Induction of Ascorbate Peroxidase in Superoxide Dismutase-Overexpressing Plants). *Plant Physiol.* 1993; 103:1067–1073. [PubMed: 12232001]
89. Kwon SY, Jeong YJ, Lee HS, Kim JS, Cho KY, Allen RD, Kwak SS. Enhanced tolerances of transgenic tobacco plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against methyl viologen-mediated oxidative stress. *Plant, Cell Environ.* 2002; 25:873–882.
90. Camp WV, Capiou K, Montagu MV, Inze D, Slooten L. Enhancement of Oxidative Stress Tolerance in Transgenic Tobacco Plants Overproducing Fe-Superoxide Dismutase in Chloroplasts. *Plant Physiol.* 1996; 112:1703–1714. [PubMed: 8972606]
91. Eng J, McCormack A, Yates J. R An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass. Spectrom.* 1994; 5:976–989. [PubMed: 24226387]

Figure 1. Classification of proteins identified in the *Arabidopsis anp2anp3* double mutant and Ws according to KEGG pathways.

Figure 2.

Immunoblotting analysis of superoxide dismutase (SOD) isozymes in *anp2anp3* mutant and wild type (Ws) seedlings. (A, B) Immunoblot detection (A) and band optical density quantification (B) of FeSOD1 (mean $\pm$ SD, $N = 3$); (C, D) Immunoblot detection (C) and band optical density quantification (D) of MnSOD (mean $\pm$ SD, $N = 3$). The right panels in A and C show Ponceau S staining of respective PVDF membranes. * in B, D indicates statistical significance of optical density difference at $p < 0.05$.

Figure 3.

Isozyme pattern and immunoblotting on native PAGE gels of superoxide dismutase (SOD) in wild type (Ws) and *anp2anp3* double mutant in control conditions (-PQ) and after paraquat treatment (+PQ). (A) SOD specific activity staining and (B) respective loading control represented by colloidal coomassie blue-stained SDS PAGE gels. Arrows in A indicate MnSOD and FeSOD, and the bracket shows CuZn SOD isozymes. (C) Quantification of the band densities in A (mean $\pm$ SD, $N = 3$). Asterisks indicate a statistically significant difference between Ws and *anp2anp3* double mutant in control

conditions as well as after paraquat treatment and between Ws in control conditions and after paraquat treatment as revealed by Student's *t* test (* indicates statistical significance at $p < 0.05$, ** indicates statistical significance at $p < 0.01$). (D and G) Immunoblots of FeSOD1 and MnSOD prepared on native PAGE gels using anti FeSOD1 and anti MnSOD antibodies. (E, H) Ponceau S staining of proteins (respective to D and G) transferred to PVDF membrane. (F, I) Quantification of the band densities in D and G (mean $\pm$ SD, $N = 3$). Asterisks indicate statistically significant differences in optical densities as revealed by Student's *t* test ($p < 0.05$) between Ws and *anp2anp3* double mutant in control conditions, as well as between Ws in control conditions and after paraquat treatment.

Figure 4.

Quantitative demonstration of enzymatic activities within the ascorbate-glutathione cycle and ascorbate content in *anp2anp3* double mutant. (A) Specific activity of dehydroascorbate reductase (DHAR; mean $\pm$ SD, $N = 3$). (B) Specific activity of ascorbate peroxidase (APX, mean $\pm$ SD, $N = 3$) and (C) total ascorbate content (mean $\pm$ SD, $N = 3$). * in A indicates statistical significance of enzymatic activities at $p < 0.05$, ** in B and C indicates statistical significance at $p < 0.01$.

Figure 5.

Generation and distribution of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) in leaves and cotyledons of 10-day-old *Arabidopsis* plants of wild type Ws (A, C) and *anp2anp3* double mutant (B, D). $O_2^{\bullet-}$ production was visualized as dark blue coloration by nitroblue tetrazolium (NBT) staining (A, B), and H_2O_2 production was visualized as dark brown coloration by 3,3'-diaminobenzidine (DAB) staining (C, D). Semiquantitative analysis of the intensity of NBT (E) and DAB (F) staining in cotyledons and leaves of wild type plants and *anp2anp3* mutants. ** indicates statistical difference significant at a p value < 0.01 as

determined by Student's *t* test ($n = 34$ for cotyledons and $N = 76$ for leaves in E, $N = 30$ for cotyledons and $N = 58$ for leaves in F). Bar represents 0.5 mm for A–D.

Figure 6.

Cellular pattern of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) distribution in leaves of wild type (Ws) plants (A, C, E, G) and *anp2anp3* mutants (B, D, F, H). Nitroblue tetrazolium (NBT) staining revealed generation of $O_2^{\bullet-}$ in leaf vascular tissue and uniform distribution in leaf mesophyll cells in Ws plants (A), while in leaves of *anp2anp3* mutant the staining showed a clustered pattern (B). Intensive staining in these isolated islands was localized to stomata (arrowhead), surrounding epidermal cells (inset in B), and mesophyll cells close to the stomatal cavity (B). In both Ws plants and *anp2anp3* mutants, NBT

staining in mesophyll cells was restricted mainly to chloroplasts (arrows in C, D). 3,3'-Diaminobenzidine (DAB) staining of Ws and *anp2anp3* mutant leaves (E, F) revealed generation of H₂O₂ in vascular tissue and in mesophyll cells, which was more intensive in leaves of Ws plants (E). Staining in leaf mesophyll cells was localized mainly to chloroplasts (arrows in G, H). Bar = 100 µm (A, B, E, F), 20 µm (insert in B), and 10 µm (C, D, G, H).

Figure 7.

Quantitative demonstration of superoxide ($O_2^{\bullet-}$) production and H_2O_2 level in *anp2anp3* double mutant. (A) Level of $O_2^{\bullet-}$ production in wild type (Ws) and *anp2anp3* seedlings, presented as change in absorbance at 470 nm caused by $O_2^{\bullet-}$ -induced XTT reduction (mean $\pm$ SD, $N = 3$). (B) Specific activity of NADPH oxidase in *anp2anp3* mutant and wild type seedlings (mean $\pm$ SD, $N = 3$). (C) H_2O_2 concentration in control (-PQ) and paraquat treated (+PQ) seedlings of Ws and *anp2anp3*. Asterisks indicate statistically significant difference between Ws and *anp2anp3* double mutant (* indicates statistical significance at $p < 0.05$, ** indicates statistical significance at $p < 0.01$).

Figure 8.

Effect of oxidative stress on seedlings of *anp2anp3* double mutant. (A, B) *Arabidopsis* seedlings of wild type (Ws) (A) and *anp2anp3* mutant (B) grown under control conditions (-PQ). (C, D) *Arabidopsis* seedlings of Ws (C) and *anp2anp3* double mutant (D) grown on 1/2 MS media supplemented with 0.5 μM paraquat (+PQ). Three-day-old seedlings (wild type and mutant) were transferred to control and paraquat containing media and grown for 7 days. Note inhibition of the seedling growth and development as well as chlorophyll bleaching (arrows in C) in the leaves of Ws plants in comparison to the mutant plants. Bar represents 1 cm.

Figure 9.

(A) Spatial distribution of the maximum quantum yield of photosystem II photochemistry (F_v/F_m) within *Arabidopsis* seedlings of wild type (Ws) and *anp2anp3* double mutant obtained by chlorophyll fluorescence imaging. (B) Actual quantum yield of photosystem II electron transport (Φ_{PSII} upper graph) and nonphotochemical chlorophyll fluorescence quenching (qN , bottom graph) during chlorophyll fluorescence induction in *Arabidopsis* seedlings of Ws (black, mean $\pm$ SD, $N = 3$) and *anp2anp3* mutant (red, mean $\pm$ SD, $N = 3$).

Figure 10.

Functional network of proteins/enzymes, reactive oxygen species, and redox-active compounds found by the current study to be modified in the *anp2anp3* mutant (depicted in colored boxes). Red arrow shows activation, dashed arrow means metabolic pathway. The small arrows in boxes indicate upregulation or downregulation of individual components. Abbreviations: Asc = ascorbate, APX = ascorbate peroxidase, CPN20 = Chaperonin 20, DHAR 1 = dehydroascorbate reductase, GME = GDP-D-mannose 3'S'-epimerase, FeSOD = Fe-superoxide dismutase, MDHA = monodehydroascorbate, MnSOD = Mn-superoxide dismutase.

Table 1

Proteins Differentially Abundant in the *anp2anp3* Mutant as Compared to the Ws Wild Type

Sequence ID	Protein	Protein Weight (kDa)	Protein Isoelectric Point (pI)	Total Score ^d	Total % Seq Coverage	Total Peptides	Total Spectra	Expre ^s sion in <i>anp2anp3</i> vs Ws	<i>anp2anp3</i> /Ws ratio ^b	1-way ANOVA p factor
Photosynthesis and Carbon Fixation										
NP_186799.2	CA1 (carbonic anhydrase 1)	37.41	5.67	26.42	37.46	8	45	down	0.68	0.025
NP_176291.1	FED A (ferredoxin 2)	15.51	4.18	8.26	54.05	2	32	down	0.58	0.016
NP_051084.1	photosystem II 47 kDa protein	55.98	6.43	11.92	9.06	4	8	down	0.29	0.036
NP_563852.1	2,3-bisphosphoglycerate-independent phosphoglycerate mutase 1	60.52	5.20	18.29	20.83	6	21	down	0.27	0.039
NP_172153.1	PSBP-1 (oxygen-evolving enhancer protein 2)	28.11	8.38	13.46	19.85	4	44	down	0.65	0.002
NP_180637.2	PSBP-2 (photosystem II subunit P-2)	28.15	5.88	15.87	21.07	4	82	down	0.53	6.04 × 10 ⁻⁰⁴
NP_051067.1	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	52.90	5.85	109.16	57.62	33	652	down	0.81	1.85 × 10 ⁻⁰⁵
NP_192186.1	PSAD-1 (photosystem I subunit D-1)	22.57	10.17	30.05	50.00	10	89	up	2.14	2.67 × 10 ⁻⁰⁴
NP_192772.1	LHCB5 (light harvesting complex of photosystem II 5);	30.12	5.97	23.12	40.71	8	24	down	0.13	0.0158
NP_173459.1	DRT112 (DNA-damage-repair/tolerance protein 112)	16.96	4.92	6.34	38.92	2	6	down	0.48	0.0023
Stress Response										
NP_001190417.1	GME (GDP-D-mannose 3',5'-epimerase)	42.71	5.81	15.05	18.57	6	16	up	2.03	0.0253
NP_173387.1	DHAR1 (dehydroascorbate reductase)	23.61	5.52	31.77	59.62	9	77	up	1.53	0.049
NP_181934.1	late embryogenesis abundant domain-containing protein	36.00	4.51	6.15	12.00	3	4	down	0.47	7.27 × 10 ⁻⁰⁵
NP_001189847.1	heat shock cognate 70 kDa protein 3 (HSC70-3)	71.09	4.82	36.67	32.20	14	35	down	0.51	0.014
NP_194098.1	major latex protein-related/MLP-related	17.49	5.91	22.06	50.99	7	111	down	0.66	0.014
NP_188267.1	jacalin-like lectin domain-containing protein	72.41	5.20	6.94	9.50	3	3	down	0.58	0.047
NP_194240.1	FeSOD1 (Fe superoxide dismutase 1)	21.07	6.51	18.22	46.24	5	52	up	2.21	0.019
Amino Acid Metabolism										
NP_195506.1	SHM1 (serine hydroxymethyltransferase 1)	57.35	8.28	41.30	33.27	13	67	up	1.32	0.045
NP_194927.1	ASP5 (aspartate aminotransferase 5)	50.92	7.65	6.79	6.93	2	10	up	<i>anp2anp3</i> unique	1.77 × 10 ⁻⁰⁶
NP_177876.1	O-methyltransferase family 2 protein	29.12	5.00	9.22	15.06	3	10	up	<i>anp2anp3</i> unique	1.29 × 10 ⁻⁰⁷

Sequence ID	Protein	Protein Weight (kDa)	Protein Isoelectric Point (pI)	Total Score ^a	Total % Seq Coverage	Total Peptides	Total Spectra	Epxression in <i>amp2amp3</i> vs Ws	<i>amp2amp3</i> /Ws ratio ^b	1-way ANOVA p factor
Calcium Signaling										
NP_176814.1	Atlg66410 CAM4 (calmodulin 4)	16.83	3.82	12.22	46.31	3	31	down	0.57	0.027
Protein Folding										
NP_197572.1	AtSg20720 CPN20 (chaperonin 20)	26.77	9.38	29.66	46.64	9	24	up	3.41	0.027
Transport										
NP_186777.1	At3g01280 porin, putative	29.39	9.20	8.27	20.29	3	7	up	3.84	0.009
NP_193769.1	AtRABE1b/AtRab8D (<i>Arabidopsis</i> Rab GTPase homologue E1b)	51.58	5.78	55.53	54.83	17	99	up	1.32	0.035
Lipid Metabolism										
NP_S66875.1	At3g45140 LOX2 (lipoxygenase 2)	101.96	5.33	31.99	22.99	11	24	up	14.57	3.90×10^{-05}

^aTotal score represents sum of Xcorr parameters for each protein as calculated by the SEQUEST algorithm⁹1 of the Proteome Discoverer software (Thermo Scientific, Waltham, MA, USA).

^bRatios of sums of Xcorr values for particular proteins across three replicates.