RESEARCH PAPER

Redox and reactive oxygen species network in acclimation for salinity tolerance in sugar beet

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Abstract

Fine-tuned and coordinated regulation of transport, metabolism and redox homeostasis allows plants to acclimate to osmotic and ionic stress caused by high salinity. Sugar beet is a highly salt tolerant crop plant and is therefore an interesting model to study sodium chloride (NaCl) acclimation in crops. Sugar beet plants were subjected to a final level of 300 mM NaCl for up to 14 d in hydroponics. Plants acclimated to NaCl stress by maintaining its growth rate and adjusting its cellular redox and reactive oxygen species (ROS) network. In order to understand the unusual suppression of ROS accumulation under severe salinity, the regulation of elements of the redox and ROS network was investigated at the transcript level. First, the gene families of superoxide dismutase (SOD), peroxiredoxins (Prx), alternative oxidase (AOX), plastid terminal oxidase (PTOX) and NADPH oxidase (RBOH) were identified in the sugar beet genome. Salinity induced the accumulation of Cu-Zn-SOD, Mn-SOD, Fe-SOD3, all AOX isoforms, 2-Cys-PrxB, PrxQ, and PrxIIF. In contrast, Fe-SOD1, 1-Cys-Prx, PrxIIB and PrxIIE levels decreased in response to salinity. Most importantly, RBOH transcripts of all isoforms decreased. This pattern offers a straightforward explanation for the low ROS levels under salinity. Promoters of stress responsive antioxidant genes were analyzed in silico for the enrichment of cis-elements, in order to gain insights into gene regulation. The results indicate that special cis-elements in the promoters of the antioxidant genes in sugar beet participate in adjusting the redox and ROS network and are fundamental to high salinity tolerance of sugar beet

Key words: Alternative oxidase, antioxidant defense, NADPH oxidase, peroxiredoxin, RBOH, salinity stress, sugar beet, superoxide dismutase.

Introduction

Sugar beet (Beta vulgaris L.) has become an important source for sugar production in temperate areas of the world. It is not only used in the food industry but also for the production of bioethanol as a source of renewable energy (Magaña et al., 2011). Sugar beet is considered to be a cash crop and requires careful agronomical practices and breeding for adaptation to...
biotic and abiotic stresses. It is cultivated in different climates in Europe, North America, and increasingly in Asia, South America and recently North Africa. This suggests that bred cultivars are able to cope with different environments and growth conditions. Particular traits of interest for improving sugar beet production include its ability to acclimate to biotic and abiotic stresses, such as cold in temperate climates as well as drought, heat and salinity (Vastarelli et al., 2013).

Drought and salinity are among the most serious threats for crop production and limit agricultural productivity around the world (Horie and Schroeder, 2004; Munns and Tester, 2008). Saline soils are widespread, which can in part be attributed to the common global issue of water deficiency (Turan et al., 2009). Enhanced salinity tolerance will enable more productive use of saline soil and hence mechanisms involved in this ability are important areas of plant research (Horie and Schroeder, 2004; Hussain et al., 2008; Katori et al., 2010). High salt concentrations in rooting medium induce ionic and osmotic imbalances and oxidative damage, which results in growth retardation, wilting or death (Parida et al., 2004). Successful acclimation includes physiological and biochemical changes (Taij et al., 2004). Selective ion uptake, exclusion and compartmentalization are required to maintain a proper K\(^+\)/Na\(^+\)-balance while synthesis of compatible solutes, such as glycine, betaine and proline, is also needed (Yeo, 1998; Hamouda et al., 2016). Analysis of the signaling pathways and regulatory mechanisms involved indicates that hormones, Ca\(^{2+}\), and redox cues function as central players in acclimation (Zhang et al., 2008). Sugar beet tolerates salinity of up to 500 mM sodium chloride (NaCl) for 7 d without losing viability (Yang et al., 2012). The genome sequence of sugar beet was recently reported (Dohm et al., 2014), making sugar beet an excellent model for studying plant response and tolerance to salinity stress (Yang et al., 2012).

Salinity stress affects cellular reactive oxygen species (ROS) generator systems, such as photosynthetic electron transport, photosynthetic hydrogen peroxide (H\(_2\)O\(_2\)) release, respiratory electron transport, and enzyme activities including glucose oxidase, xanthine oxidase and in particular, plant peroxidases and NADPH oxidase. One of the most important cellular ROS generating systems is the plasma membrane-bound NADPH oxidase RBOH (Keller et al., 1998), which decisively controls cellular redox homeostasis under salinity stress (Hossain and Dietz, 2016). ROS accumulation is a common denominator under conditions of stress (Foyer et al., 1994).

Complementary to the generator systems are enzymatic and non-enzymatic antioxidants that regulate ROS and redox homeostasis and counteract metabolic imbalances, damage to cell structures, cell death and stress adaptation (Foyer et al., 2009). Superoxide dismutases (SODs) constitute the first line of defense against superoxide (O\(_2^-\)), which is produced in most cellular compartments (Elstner, 1991). SODs are therefore found in all subcellular compartments (Alscher et al., 2002). H\(_2\)O\(_2\) is decomposed by thiol peroxidases, in particular glutathione peroxidases (GPXs) and peroxiredoxins (Prxs), and by type III heme peroxidases, ascorbate peroxidase (APX) and catalase (Mittler and Poulos, 2005). GPXs and Prxs decompose alkyl hydroperoxides in addition to H\(_2\)O\(_2\) and employ a thiol mechanism to react with peroxide (Dietz, 2016). Following oxidation by the peroxide substrate, thiol peroxidases are regenerated by electron donors such as thioredoxins, glutaredoxins and glutathione (Navrot et al., 2006; Koh et al., 2007; Dietz, 2011). Prxs display a stable TRX-fold and are grouped into four subfamilies: 1-CysPrx, 2-CysPrx, PrxQ and PrxII-like proteins (Horling et al., 2003; Muthumalingam et al., 2009). Prxs and GPXs play important roles as thiol antioxidants, modulators of cell signalling and redox sensors (Dietz et al., 2006; Navrot et al., 2006).

Here, we identified some of the central components of the cellular antioxidant defense system in sugar beet, and followed their transcriptional response during acclimation to 300 mmol L\(^{-1}\) NaCl. We tested the hypothesis that the ROS and redox network participates in the extraordinarily high salinity tolerance of sugar beet. The transcriptional response pattern was placed into the broad framework of the cellular redox state. This study addressed an additional layer of redox and ROS homeostasis, namely the activation of safety mechanisms, as there are terminal oxidases present in different subcellular compartments. Mitochondrial alternative oxidase (AOX) (Considine et al., 2002; Hossain and Dietz, 2016) and plastid terminal oxidase (PTOX) (Stepien and Johnson, 2009; Hossain and Dietz, 2016) help dissipate excess reducing power.

Materials and methods

Plant materials and NaCl treatment

Seeds of sugar beet (Beta vulgaris subsp. vulgaris), cultivar KWS2320 were sterilized with 70% (v/v) ethanol, 0.1% (w/v) HgCl\(_2\) and 0.2% (w/v) thiram, placed in germination pots in vermiculite and perlite mixture and soaked in water in darkness for one week. The tray was then placed in growth chambers with 10 h light with an intensity of 100 µmol m\(^{-2}\) s\(^{-1}\) at 21 °C and 14 h darkness at 18 °C with 55% relative humidity for another week. The growth condition was adjusted according to the temperate climate, as the cultivar is adapted to temperate regions. After 14 d, uniformly grown seedlings were transferred to hydroponic containers with Hoagland solution (Ghoulam et al., 2002). All plants grew under control conditions for 35 d and up-salting started from day 36 in the case of salinity-stressed plants. Salt concentration was gradually increased to 300 mM NaCl in 50 mM\(^{-1}\) increments (Fig. 1A). Tissue was harvested from the fully expanded third to sixth leaves and the whole root after the point of first branching, from five independent experiments at time points indicated. Tissue was then frozen in liquid nitrogen and stored at −80 °C.

Determination of photosystem II quantum yield and CO\(_2\) fixation rate

The steady-state quantum yield (F\(_{V}/F\(_{M}\)) of photosystem II (PSII) was measured using the Mimi-PAM Fluorometer (Walz, Germany) under light conditions as described by Oelze et al. (2012). CO\(_2\) fixation of sugar beet leaves under stressful and control conditions was measured with a portable gas exchange system (GFS-3000, Heinz Walz GmbH, Effeltrich, Germany). The CO\(_2\) assimilation rate was measured at a light intensity of 100 µmol photons m\(^{-2}\) s\(^{-1}\), a relative humidity of 50% and at 22 °C.

Determination of osmotic potential and sodium, potassium and chloride content

Osmotic potential was measured by using an automatic cryoscopic osmometer (Knauer, Berlin, Germany), following calibration
The sugarbeet ROS network under salinity between 0 and 300 mosmol kg\(^{-1}\). The osmotic potential is given as mosmol kg\(^{-1}\). Sodium and potassium contents were determined from tissue sap using a flame photometer (Model 410; Sherwood Scientific Ltd, Cambridge, UK) calibrated between 0 and 10 ppm. Chloride content was determined in tissue sap using a Chloride Analyzer (Model 926S; Sherwood Scientific Ltd, Cambridge, UK).

Determination of antioxidant and non-protein thiols content

Ascorbate (AA) and dehydroascorbate (DHA) content were determined as described by Horling et al. (2003). Glutathione (GSH) and oxidized glutathione (GSSG) content were quantified with an enzyme cycling assay based on sequential oxidation of GSH by 5,5'-dithiobis(2-nitrobenzoate) (DTNB) and reduction by NADPH in the presence of glutathione reductase (GR) (Griffith, 1980). Tissue weighing 200 mg was extracted in 1 ml 0.1 M HCl and 0.1 mM EDTA. For total GSH content, 200 µL neutralized supernatant was incubated with 6 mM DTNB for 5 min followed by a 15 min incubation with 5 µL 2-vinylpyridine. After centrifugation, total GSH content was determined from the supernatant. The reaction was started with the addition of GR. Changes in 5'-thio-2-nitrobenzoic acid absorbance were spectrophotometrically monitored at 412 nm. To determine GSSG content, the neutralized supernatant was incubated first with 2-vinylpyridine for 15 min, then with 6 mM DTNB for 5 min and subsequently GR and NADPH were added. The difference between total glutathione and GSSG content is presented as GSH content. Non-protein thiols content was determined using 0.1 M phosphate buffer at pH 7.0, 0.5 mM EDTA and 1 mM DTNB (Del Longo et al., 1993). Absorbance was measured at 412 nm using a microplate reader (KC4, BITEK Instruments, Bad Friedrichshall, Germany). Values were corrected for the absorbance of a blank without extract and determined with a glutathione standard curve.

Determination of protein, malondialdehyde and hydrogen peroxide content

Total protein (mg g\(^{-1}\) FW) content was determined using bovine serum albumin as a standard, according to Bradford (1976). \(\text{H}_2\text{O}_2\) content was quantified by chemiluminescence with luminol (Perez and Rubio, 2006). Malondialdehyde (MDA) content was measured colorimetrically according to Stewart and Bewley (1980) with some modification. Leaf tissue weighing 100 mg was homogenized in 0.1% trichloroacetic acid (TCA) on ice. Following centrifugation, 1.5 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA) was added to 500 µl supernatant and incubated at 95 °C for 30 min. Following cooling and centrifugation, absorption was measured at 532 nm and the amount of MDA calculated, with \(\varepsilon=155 \text{mM}^{-1}\text{cm}^{-1}\).

SDS-PAGE and immunoblotting

SDS-PAGE and western blotting were performed as in Ströher et al. (2009). Binding of the first rabbit antibodies was achieved overnight in 1% skimmed milk in Tris-buffered saline with 0.1% Tween 20 (TBST) at the following dilutions: anti-At2-CysPrx, PrxQ and CuZn SOD2 at 1:3000; anti-Avena sativa D1 at 1: 5000. After secondary antibody binding, proteins were detected using chemiluminescence.
Antioxidant enzyme activities

Tissue weighing 200 mg was homogenized in 1 ml of 0.1 M phosphate buffer at pH 6.5. The supernatant was used to determine the activity of enzymes according to Urbanek et al. (1991) and protein content as above (Bradford, 1976). The reaction mixture for catalase consisted of 100 μl extract in 3 ml phosphate buffer at pH 6.8 (Urbanek et al., 1991). The detoxification of H2O2 was measured at 240 nm (Cary 300 Bio UV/VIS, Varian, Middelburg, Netherland), 

\[ \varepsilon = 43.6 \text{ M cm}^{-1} \] 

SOD activity was quantified according to Sen Gupta et al. (1993) and peroxidase according to Malik and Singh (1980), with 

\[ \varepsilon = 2.8 \text{ M cm}^{-1} \] 

GR activity was measured by following the increase in absorbance in the presence of GSSG and DTNB (Sairam et al., 2002), with 

\[ \varepsilon = 13.6 \text{ M cm}^{-1} \] 

Determination of plasma membrane NADPH oxidase activity

NADPH-dependent O2− generating activity in isolated microsomal membrane vesicles was determined by following the O2−-dependent reduction of XTT (Na 3´-[1-[phenylaminocarbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid) (Kaundal et al., 2012). The assay mixture contained 50 mM Tris-HCl at pH 7.5, 0.5 mM XTT, 0.1 mM NADPH and the membrane fraction. Tissue was ground in liquid nitrogen and 0.5 g of the powder was weighed out in empty pre-chilled Falcon tubes. 6 ml of ice-cold protein extraction buffer was added on ice and vortexed at room temperature. Homogenized tissue was filtered through four layers of cheesecloth and the flow through transferred to 2 ml microcentrifuge tubes on ice. After centrifugation at 10 000g for 4°C for 45 min, the supernatant was transferred to ultra centrifuge tubes. Microsomal membranes were pelleted from the supernatant by centrifugation at 50 000g for 30 min. The pellet was suspended in 0.33 M sucrose, 3 mM KCl, and 5 mM potassium phosphate at pH 7.8. The plasma membrane fraction was isolated by adding the microsomal suspension to an aqueous two-phase polymer system to give a final composition of 6.2% (w/w) Dextran T500, 6.2% (w/w) polyethylene glycol (PEG) 3000, 0.33 M sucrose, 3 mM KCl, and 5 mM potassium phosphate at pH 7.8 with protease inhibitors. After three rounds of partitioning the resulting upper phase was diluted 5-fold in ice cold 10mM Tris-HCl dilution buffer at pH 7.4, containing 0.25 M sucrose, 3 mM EDTA, 1 mM DTT, 3.6 mM L-cysteine, 0.1 mM MgCl2 and protease inhibitors. The fractions were centrifuged at 120000g for 30 min. The pellets were resuspended in 1 ml of 10 mM Tris-HCl at pH 7.4 for the activity assay. All procedures were carried out at 4 °C. The reaction was initiated with NADPH. In the presence of O2−, XTT generates a yellow formazan that was quantified spectrophotometrically at 492 nm and calculated with 

\[ \varepsilon = 21 \ 600 \text{ M cm}^{-1} \] 

Transcription quantification

RNA isolation and cDNA synthesis were performed according to Wormald et al. (2006) with a few modifications. Semi-quantitative RT-PCR analysis was carried out as previously described at the individually optimized cycle number (Finkemeier et al., 2005), using the given primer combinations (see Supplementary Table S1 at JXB online). Primers were designed with Primer3Plus software (www.bioinformatics.nl/cgi-bin/ primer3plus). Loading was normalized with actin. qRT-PCR was carried out in the iCycler ™ Thermal Cycler (Bio-Rad, USA) with the iQ™SYBR Green Supermix (Bio-Rad, USA) in a final volume of 20 μl according to the manufacturer’s instructions. The iCycler was programmed to 95 °C for 1 min; 45x (95 °C for 30s, 58 °C for 40s, 72 °C for 45 s), 72 °C for 10 min, followed by a melting curve program of 55–95 °C in increasing steps of 0.5 °C. Efficiencies of each reaction were calculated using LinRegPCR software (Ruijter et al., 2009). Signal values were subsequently derived from the threshold cycles, with the average background subtracted, using the equation provided by Pfaffl (2001).

Sequence alignment and construction of phylogenetic trees

Sugar beet genes homologous to known Arabidopsis thaliana SOD, Prx, AOX and RBOH genes were searched for using FASTA and WU-BLAST2 programs. The amino acid sequences were aligned using CLUSTALW 2.1 (Larkin et al., 2007) with the default configuration. Their phylogenetic relationships were determined using the maximum likelihood (ML) algorithm incorporated in the program MEGA version 5 (Tamura et al., 2011). Bootstrap analyses with 500 replicates were performed to assess the robustness of the branches. Pairwise sequence alignment was done by blasting the isoforms of each gene group and sequence identity was retrieved (see Supplementary Table S2).

Cis-regulatory elements analysis

Promoters of stress-responsive genes in sugar beet were analyzed in silico for putative cis-elements using available genome sequences and our transcriptomic data. The Plant Promoter Analysis Navigator (PlantPAN 2.0; http://PlantPAN2.itps.ncku.edu.tw) (Chow et al., 2015) and The AthaMap (AthaMap 8; http://www.athaemap.de) (Steffens et al., 2005) databases were used to identify cis-regulatory elements for 1000 bp upstream promoters of differentially regulated genes of B. vulgaris under salinity stress, with A. thaliana as reference (Table 3, Supplementary Tables S3–S5).

Statistical analysis

Pairwise comparisons were performed with Students t-test. Statistical grouping of the data was achieved by applying Fisher’s LSD, with P<0.05, using InfoStat statistical software.

Results

Effect of salinity on growth and redox state of sugar beet

The NaCl concentration in the hydroncrops solution was increased stepwise to 300 mM. The plants were then kept at this level for 14 d for detailed kinetic analysis (Fig. 1A). This regime was chosen after comparing different NaCl concentrations, where growth was still measurable at 450 mM but ceased at 600 mM. Morphological changes were observed after salinity treatment (Fig. 1B–C). The high final salt level of 300 mM NaCl still enabled significant growth. The plant response was scrutinized by following the level of H2O2 and SOD activity during up-salting (Fig. 1D). H2O2 levels increased in roots and leaves after the first salt step and until the second step but did not increase further during subsequent up-salting steps. Instead levels started to decline at the highest salt concentrations in leaves. Total SOD activity roughly followed H2O2 levels indicating that sugar beet acclimated to salinity with no effects on fresh weight-related protein content, which was considered important for subsequent analyses (see Supplementary Fig. S1).

Photosynthetic quantum yield, CO2 fixation, ionic and osmotic state

The quantum yield of photosystem II (ΦPSII) was unchanged between salt-treated and control plants during up-salting and at different time points at 300 mM NaCl (Fig. 2C, Supplementary Fig. S2A, B). CO2 fixation rates decreased under salt treatment with little difference between 3 h and
The sugar beet ROS network under salinity

14 d at 300 mM NaCl (Fig. 2C). This is despite the strong increase in osmotic potential, and sodium, chloride and potassium content (see Supplementary Fig. S2C–F).

Oxidative stress and antioxidant defense

H$_2$O$_2$ accumulation, as a recognized marker of oxidative stress, was quantified in leaves during the 14-day period, after reaching 300 mM NaCl (Fig. 2A). The basal steady state level of H$_2$O$_2$ was around 1.7 and 1.2 nmol mg$^{-1}$ FW in unsalted control and up-salted sugar beet, respectively. The H$_2$O$_2$ content in sugar beet leaves decreased during aging but importantly it was always significantly lower in salt-treated tissue compared with controls (Fig. 2A). MDA levels in salt-stressed leaves were also below control samples indicating less lipid peroxidation and membrane damage (Fig. 2B). Non-protein thiols (NPTs) were lower in stressed plants than in controls (Fig. 2D). Ascorbate and glutathione pools were analyzed as these low molecular mass antioxidants are linked to redox homeostasis and signaling and also function as reductants in the water–water cycle (Oelze et al., 2012). Glutathione levels dropped in stressed plants from 3 h to 14 d after up-salting (Table 2). Significantly lower GSH levels were observed at 14 d of salt stress (Fig. 5A–F). Up-regulation was greatest for SOD2 with approximately a 13-fold difference between 300 mM and 0 mM NaCl (Fig. 5B). Mitochondrial MnSOD1 transcript levels were unchanged at 3 h but significantly increased at 14 d (Fig. 5D). The order

Regulation of peroxiredoxin and superoxide dismutase proteins

The abundance of 2-cysteine peroxiredoxin (2-CysPrx) protein was examined via western blotting using control and salt-stressed plant samples (Fig. 3A, Supplementary Fig. S3). 2-CysPrx amounts increased with aging and under salt stress. Chloroplast peroxiredoxin Q (PrxQ) levels were unchanged in salt-treated plants compared with controls at 3 h but were increased after 14 d (Fig. 3B). CuZnSOD showed the strongest response to salt stress with a 3-fold increase after 14 d (Fig. 3C, Supplementary Fig. S3A–C).

Sugar beet SOD gene family members and their transcriptional regulation

The accumulation of 2-Cys-Prx, PrxQ and CuZnSOD proteins under salinity prompted us to use bioinformatics to identify important antioxidant genes in the sugar beet genome and to quantify their transcriptional response by qPCR. Three CuZnSOD genes, one MnSOD gene and two FeSOD genes were identified (Table 1). The construction of a phylogenetic tree showed that the SOD family, with 14 available accessions in the databanks of B. vulgaris and A. thaliana, segregated into three clearly separated groups based on sequence similarity (Fig. 4A). Group I encompassed FeSOD genes that included two isoforms in sugar beet. MnSOD isoforms were related to FeSOD genes but separated into a distinct cluster. The three CuZnSOD genes in sugar beet were grouped in pairs with their cognate homologs in Arabidopsis. Relatedness was confirmed by sequence alignment (Supplementary Fig. S4). With the exception of FeSOD1, which was strongly down-regulated under salinity, all other SOD transcripts revealed significantly higher levels at 14 d of salt stress (Fig. 5A–F). Up-regulation was greatest for SOD2 with approximately a 13-fold difference between 300 mM and 0 mM NaCl (Fig. 5B).
of transcript up-regulation was: CuZnSOD2 > CuZnSOD3 > CuZnSOD1 > FeSOD3 > MnSOD.

Transcriptional regulation of Prx genes

The sugar beet genome was searched for genes encoding peroxiredoxins (Dietz, 2011). While ten Prx genes were identified in Arabidopsis, only six were identified in sugar beet and were used to construct a phylogenetic tree (Fig. 4B). Sugar beet encodes only one cytosolic Prx, PrxIIIB, while Arabidopsis encodes three and one homologous pseudo-gene At-PrxIIA. The sugar beet genome contains a single copy of mitochondrial PrxIIF, nuclear 1-CysPrx, 2-CysPrx and PrxQ. Among these thiol peroxidases, 1-CysPrx, PrxQ and PrxIIIE transcripts were lower in salt-treated plants at 3 h after up-salting (Fig. 5G–L). At the end of the experiments only transcripts encoding the two chloroplast Prxs, 2-CysPrx and PrxQ, were increased in salt-stressed plants, while cytosolic PrxIIIB transcript levels were decreased and all others were similar between both treatments. Relatedness was confirmed by sequence alignment (Supplementary Fig. S5). In order to complement these data, tissue distribution was analyzed (Supplementary Fig. S6) and revealed rather ubiquitous expression, with the exception of FeSOD1 and CuZnSOD2 that were scarcely expressed in seeds. Likewise PrxQ was absent from seeds and 1-CysPrx expression was very low in leaves (Supplementary Fig. S6).

Transcript regulation of alternative and NADPH oxidases

To address the regulation of redox and ROS homeostasis, the sugar beet genome was searched for homologs of important safety valves, namely alternative oxidases (AOX) in mitochondria, plastid terminal oxidase (PTOX) as well as the ROS generation systems of NADPH oxidases, including the respiratory burst oxidase homologue RBOH. Phylogenetic analysis revealed three sequences in sugar beet related to Arabidopsis AtAOX1 and AtAOX2 sequences and two with similarity to At-PTOX (Fig. 4C, Table 1, Supplementary Figs S7, S8). Each of the BvAOX and BvPTOX transcripts was significantly up-regulated in salt-treated sugar beet at 3 h and 14 d (Fig. 6A–E). The search for RBOH genes in the sugar beet genome generated seven hits. Phylogenetic analysis revealed one homolog each for AtRBOHA/B/C/D/G-, E-, F/I, H/J-like, namely BvRBOHB, BvRBOHE, BvRBOHF, and BvRBOHH (Fig. 4D). One BvRBOH isoform was particularly different and named BvRBOHK (Fig. 4D). With the exception of BvRBOHB that was up-regulated 3 h after salt treatment, all other BvRBOH isoforms were significantly suppressed under salinity both at 3 h and 14 d (Fig. 6F–J).

Cis-regulatory element analysis

The promoters of the above identified stress-responsive genes of sugar beet were investigated for the presence of cis-regulatory elements. This was carried out to gain some insight into gene regulation and plant signaling under stress conditions. The occurrence of special cis-elements appears to play important roles in the differential regulation of salinity-induced transcripts in B. vulgaris as compared with A. thaliana. Over-representation or exclusive occurrence in B. vulgaris was observed for GATA, WRKY, bHLH, TCP, DOF, ZF-HD, NF-YB, TALE, TBP, dehydrin and BES1 cis-elements (Supplementary Table S3–S5). Over-represented cis-elements vary within B. vulgaris, with GATA and WRKY
Table 1. Gene annotation of SODs, Prxs, AOXs and RBOHs in B. vulgaris subsp. vulgaris genotype KWS2320 and Arabidopsis thaliana

The table depicts the protein names and genome annotations, putative localization, protein length and calculated molecular mass. Abbreviations: cp, chloroplast; ecm, extracellular matrix, apoplast; mt, mitochondrion; per, peroxisome; vac, vacuole; cyt, cytoplasm; nuc, nucleus; pl, plastid; plast, plastoglobule; pm, plasma membrane; cw, cell wall.

| Enzyme and reaction | Protein/gene name | Reference sequence | Localization | Length (amino acids) and mol. wt (kDa) |
|---------------------|-------------------|-------------------|-------------|--------------------------------------|
| Superoxide dismutase (SOD) | FeSOD1 FeSOD1 | XM_010678412.1 AT4G25100.3 | cp, mt | 324/36.26 212/23.78 |
| O₂+O₂→2H⁺→H₂O₂+O₂ | — FeSOD2 | — AT5G51100.3 cp | — | 305/34.66 |
| — FeSOD3 | — AT5G23310.1 cp | — | 262/30.25 263/30.35 |
| Cu/ZnSOD1 Cu/ZnSOD1 | XM_010687302.1 AT1G08830.1 cyt, ecm, nuc | — | 152/15.25 152/15.10 |
| Cu/ZnSOD2 Cu/ZnSOD2 | XM_010696513.1 AT5G18100.1 cyt, cp, ecm, per, vac | — | 228/23.13 216/22.24 |
| — Cu/ZnSOD3 | — XM_010689254.1 | — | — |
| MnSOD1 MnSOD1 | XM_010672327.1 AT3G10920.1 mt | — | 230/25.58 231/25.44 |
| — Fe-MnSOD | — AT3G56350.1 mt | — | 241/26.89 |
| Peroxiredoxin (Prx) | 1-Cys-Prx 1-Cys-Prx | XM_010669313.1 AT1G48130.1 cyt, nuc | — | 219/24.16 216/24.08 |
| 2P-SH+H₂O₂→P-S-S-P+2H₂O | — 2-Cys-Prx | — AT3G11630.1 ecm, cp | — | 266/29.09 266/29.09 |
| 2P-SH+H₂O₂→P-S-S-P+2H₂O | 2-Cys-Prx 2-Cys-PrxB | XM_010685313.1 AT5G06290.1 ecm, cp, mt | — | 271/29.74 273/29.77 |
| — 2-Cys-Prx1IF | — AT5G06050.1 mt | — | 174/18.66 201/21.44 |
| — PrxQ | XM_010689254.1 AT3G26060.1 cp, plast | — | 214/23.65 216/23.67 |
| — — Prx8A | — AT5G59699.1 cyt, nuc | — | 553/62.65 |
| — Prx1B | XM_010693075.1 AT1G65980.1 cp, cyt, pm | — | 162/17.51 162/17.42 |
| — — Prx1C | — AT1G65970.1 cyt | — | 162/17.41 |
| — — Prx1D | — AT1G60740.1 cyt, pm | — | 162/17.47 |
| — Prx1E | XM_010685588.1 AT3G52960.1 cp, pl, cw | — | 227/24.07 234/24.68 |
| — — Prx1E | — AT3G52960.1 cp, pl, cw | — | 227/24.07 234/24.68 |
| IAlternative oxidase (AOX) | AOX1A AOX1A | XM_010679200.1 AT3G22370.1 mt | — | 353/39.62 354/39.98 |
| 2e⁻+2H⁺→O₂H₂O | AOX1B AOX1B | XP_010677502.1 AT3G22360.1 mt | — | 102/11.19 325/37.43 |
| — AOX1C | — AT3G27620.1 mt | — | 329/37.82 |
| — AOX1D | — AT1G32350.1 mt | — | 318/36.20 |
| — AOX2 | XM_010692188.1 AT5G64210.1 mt | — | 329/37.69 353/40.09 |
| — PTOX1 | XM_010688453.1 AT4G22260.1 cp | — | 363/41.77 351/40.57 |
| — PTOX2 | — XP_010686755.1 — cp | — | 596/65.40 |
| NADPH oxidase (RBOH) | — RBOH A | — AT5G07390.1 pm | — | 902/102.94 |
| NADPH⁺e⁻+O₂→NADP⁺+O₂⁻+H⁺ | — RBOH B | XM_010675737.1 AT1G09090.2 pm | — | 887/100.94 843/96.39 |
| — RBOH C | — AT5G51060.1 pm | — | 905/102.52 |
| — — RBOH D | — AT5G47910.1 pm | — | 921/103.91 |
| — RBOH E | XM_010689256.1 AT1G19230.1 pm | — | 945/106.04 934/105.55 |
| — RBOH F | XM_010678094.1 AT1G64060.1 pm | — | 947/107.72 948/108.42 |
| — — RBOH G | — AT4G25090.1 pm | — | 849/96.86 |
| — RBOH H | XM_010687568.1 AT5G06010.1 pm | — | 868/100.63 |
| — — RBOH I | — AT4G11230.1 pm | — | 941/106.95 |
| — — RBOH J | — AT3G45810.1 pm | — | 912/102.94 |
| — RBOH K | XM_010696961.1 — pm | — | 797/91.11 |

more abundant in promoters regions of down-regulated transcripts and bHLH, TCP, dehydrin and BES1 more abundant in up-regulated transcripts (Supplementary Tables S3, S4).

Antioxidant enzyme activities

Activities of SOD, catalase, GR, APX and peroxidase (POD) were determined in leaves from salt-stressed and control plants in order to assess the state of the antioxidant systems (Fig. 7). Higher SOD activity was detected in stressed leaves, with the highest activity at 14 d with a 5-fold increase compared with controls (Fig. 7A). GR activity (Fig. 7E) was unaffected by saline growth conditions. However all other antioxidant enzyme activities were stimulated under salinity, with total APX activity showing the least increase, followed by total POD activity and catalase (Fig. 7B-D). Finally, NADPH oxidase activity was determined in plasma membrane-enriched membrane fractions of sugar beet leaves (Fig. 7F). Interestingly, NADPH oxidase was constant in control leaves at 3 h and 14 d, while it decreased by ~40% in salt-stressed plants at 3 h after up-salting up to 300 mM NaCl and by >60% at 14 d.
Discussion

Sugar beet is able to grow in saline soils (Ghoulam et al., 2002; Yang et al., 2012; Yang et al., 2013). In line with previous studies our results revealed efficient growth of sugar beet in medium with 300 mM NaCl. This was indicated by unchanged \( \Phi_{\text{PSII}} \) and maintained growth of ~50% of control conditions. Most notably, accumulated \( \text{H}_2\text{O}_2 \) levels were lower under salinity than control conditions. This response attracted our attention since salt stress usually stimulates ROS accumulation (Miller et al., 2010). Concomitantly, levels of non-protein thiols, oxidized glutathione and ascorbate were decreased in salinized sugar beet. Sugar beet therefore appears to adjust the cellular redox milieu to a lower level of oxidative stress in high NaCl conditions than under control conditions. This regulatory mechanism contrasts the response of other plant species that display salt stress-induced enhancement of ROS accumulation, such as maize (AbdElgawad et al., 2016), rice (Wutipraditkul et al., 2015) and Arabidopsis (Ben Rejeb et al., 2015).

The time-dependent increases in sodium and chloride content as well as osmotic potential (Supplementary Fig. S2) indicate the strong impact of salinity stress and precludes exclusion strategies in sugar beet. Maintenance of 50% photosynthetic CO\(_2\) fixation and unaltered \( \Phi_{\text{PSII}} \) demonstrates the high acclimation ability of sugar beet to strongly raised NaCl levels. The reduced growth rate quantitatively mirrored the reduced CO\(_2\) fixation rate. Efficient non-photochemical quenching may contribute to lowered ROS burden under salinity. Increased osmotic potential and transient ROS accumulation in halophyte roots are suggested to be instrumental...
in short-term activation of antioxidant defense, as well as in triggering expression of transcription factors important during long-term salinity (Ellouzi et al., 2014). ROS accumulation was found in the glycohyte Arabidopsis during short- and long-term salt stress (Ellouzi et al., 2011; Ellouzi et al., 2014). Osmotic shock triggered by salinity stress may activate initial ROS production for subsequent activation of defense mechanisms (Choudhury et al., 2013).

Efficient response of SOD in salt-stressed sugar beet

Sugar beet contains homologs of each SOD form typically present in plants such as A. thaliana supporting the assumed conserved functions. High SOD activity is needed for rapid detoxification of $O_2^{•−}$, for example to minimize lipid peroxidation and peroxinitrate formation, if nitric oxide is formed concomitantly. Peroxynitrite (ONOO$^{−}$) is highly reactive with many cellular constituents and reacts with various amino acid residues in proteins, in particular with Phe, Trp, Tyr, His, Met and Cys residues (reviewed by Mock and Dietz, 2016). SOD, which is present in multiple subcellular compartments, is considered a first line of defense against ROS (Alscher et al., 2002). With the exception of FeSOD1 transcript levels, which were down-regulated after 3 h and 339 h of salt stress, transcripts of CuZnSOD2, CuZnSOD3, CuZnSOD1, FeSOD3 and MnSOD1 were highly up-regulated under long-term salinity stress. CuSOD2 transcripts showed a small but significant increase after 3 h. As expected, based on the phylogenetic relatedness of SOD genes and the deduced amino acid sequences, MnSOD and FeSOD genes were highly related in sugar beet with sequence identity between both types of 93.6%. In contrast CuZnSOD genes have a very low sequence similarity with FeSOD and MnSOD genes. They separated into a unique cluster (Fig. 4a, Supplementary Fig.
S4, Supplementary Table 2) and probably evolved separately in eukaryotes (Alscher et al., 2002). Alscher et al. (2002) suggested that the spatial and temporal function of SOD isozymes is defined by their subcellular location and conditional expression regulated by upstream sequences in their promoters. The pattern of salt-induced transcript responses in sugar beet differed from that reported in Arabidopsis, where CuSOD genes were scarcely responsive while FeSOD genes responded the most (Mittler et al., 2004). The focus of this study was directed towards acclimation to salinity and less to the immediate response to up-salting. Importantly, total SOD activity was up 7-fold at 3 h and 5-fold at 339 h (Fig. 7A). SOD activity determined during up-salting revealed first a parallel increase with H$_2$O$_2$ and then stabilized SOD activity with decreasing H$_2$O$_2$ at the end of the up-salting period (Fig. 1D). Up-regulation of SOD participates in adjusting the low ROS state. Produced H$_2$O$_2$ must be detoxified; this is facilitated by increased activities of catalase and APX (Fig. 7) and increased amounts of 2-CysPrx and PrxQ (Fig. 3). Stimulation of the ascorbate glutathione cycle is suggested to be an important mechanism of salinity tolerance (Stepien and Klobus, 2005).

**Fig. 6.** Transcript levels of AOX/PTOX and RBOH in sugar beet from stressed and non-stressed plants. Samples were taken at 3 h and 14 d after reaching a concentration of 300 mM NaCl as shown in Fig. 1. Transcript amounts were quantified by qPCR and calculated relative to actin levels. qPCR data represent the average of three independent experiments with two technical replicates each. The significant difference was calculated using Student's t-test and labelled with different letters after analysis with Fisher LSD test, with P<0.05, using InfoStat statistical software. C, control; S, salinity.
The role of peroxiredoxins in sugar beet

In higher plants, the minimum set of Prx is six isoforms: one plastidal (PrxIIE), one mitochondrial (PrxIIF), one cytosolic type II Prx (PrxIIB), one cytosolic/nuclear 1-CysPrx, one plastid 2-CysPrx and one plastidal PrxQ (Dietz et al., 2006). Arabidopsis and rice express additional isoforms, ten and nine, respectively. Interestingly our search of the sugar beet genome conforms with the predicted minimum set of six Prx genes. The enzymatic activity of the different plant Prx isoforms has been well explored but the

Table 2. Characterization of glutathione and ascorbate status of control and salt-stressed sugar beet plants

The first analysis was done 3 h after reaching a concentration of 300 mM NaCl. Data are means ±SD of n=5 experiments with two technical replicates for glutathione and ascorbate. The significant difference was calculated using Student’s t-test and labelled with different letters after analysis with Fisher LSD test, with P<0.05, using InfoStat statistical software.

| Time (h) | GSH (µmol g⁻¹ FW) | GSSG (µmol g⁻¹ FW) | ASC (µmol g⁻¹ FW) | DHA (µmol g⁻¹ FW) |
|----------|--------------------|---------------------|-------------------|-------------------|
|          | Control            | Stress              | Control           | Stress            |
| 3        | 1.83 ± 0.27 a      | 1.77 ± 0.31 a       | 0.39 ± 0.12       | 0.32 ± 0.12       |
| 27       | 1.70 ± 0.28 a      | 1.69 ± 0.31 a       | 0.46 ± 0.11       | 0.39 ± 0.08       |
| 51       | 1.59 ± 0.38 a      | 1.57 ± 0.34 a       | 0.38 ± 0.12       | 0.44 ± 0.11       |
| 123      | 1.60 ± 0.26 a      | 1.41 ± 0.27 a       | 0.34 ± 0.15       | 0.32 ± 0.09       |
| 171      | 1.63 ± 0.29 a      | 1.33 ± 0.21 a       | 0.31 ± 0.18       | 0.37 ± 0.11       |
| 339      | 1.65 ± 0.16 a      | 1.21 ± 0.11 b       | 0.29 ± 0.12       | 0.25 ± 0.09       |

Fig. 7. Activities of antioxidant enzymes in sugar beet leaves under salinity stress and control conditions: SOD (A), CAT (B), peroxidase (POD) (C), ascorbate peroxidase (APX) (D), glutathione reductase (GR) (E) and NADPH oxidase (RBOH) (F). Data are means ±SD of n=5 experiments with three measurements each. The significant difference was calculated using Student’s t-test and labelled with different letters after analysis with Fisher LSD test, with P<0.05, using InfoStat statistical software. C, control; S, salinity.
precise function within the redox regulatory network of the cell is still elusive. Functions as peroxidases, proximity peroxidases, ROS sensors, interaction partners and chaperones have all been proposed (König et al., 2012). 2-CysPrxB and PrxQ transcripts and protein levels were slightly increased during long-term salinity stress, while all other transcript levels decreased or were unaltered. The unresponsiveness of the single cytosolic PrxIIIB gene contrasted results from Arabidopsis where AtPrxIIIB, C and D showed strong responses to salinity and other stresses (Horling et al., 2002; Horling et al., 2003). This difference suggests that gene duplication in Arabidopsis allowed for diversification in stress response, while BvPrxIIIB adopts a function more similar to housekeeping. These results indicate that 2-CysPrx contributes to the protection of chloroplast structures against oxidative damage by participating in detoxification processes (Baier and Dietz, 1999a,b). Furthermore, König et al. (2002) proposed that 2-Cys-Prx acts not only in the water–water cycle pathway for energy dissipation in photosynthesis but also in peroxide detoxification in plastids during the dark phase. 1-CysPrx transcripts strongly but transiently decreased during salt stress. 1-CysPrx in Arabidopsis is highly abundant in seeds compared with root tissues but was not expressed in leaf tissues or only in some cells like petiole junctions (Stacy et al., 1996; Haslekas et al., 1998; Stacy et al., 1999). 1-CysPrx in A. thaliana and B. vulgaris comprise 216 and 219 amino acids, respectively, possess a C-terminal extension with a putative nuclear signal, and as shown for Arabidopsis is localized in the nucleus and cytosol (Rouhier and Jacquot, 2002).

**The AOX gene family and their transcript response to salinity**

Alternative oxidases function in the dissipation of reducing power in energetic electron transport of both mitochondria (AOX) and chloroplasts (PTOX). In the sugar beet genome, three AOX genes were identified that were highly similar to AtAOX1 and AtAOX2 groups in A. thaliana and therefore named BvAOX1A, BvAOX1B and BvAOX2. Two genes coding for proteins with high homology to PTOX were identified in sugar beet and named BvPTOX1 and BvPTOX2. Efficient and coordinated up-regulation of AOX and PTOX may represent a specific feature of sugar beet during salinity stress tolerance because transcripts of all four AOX and AOX-like genes as well as PTOX were up-regulated both at 3 h and 339 h of salt stress. Up-regulation of PTOX and AOX probably stabilize the photosynthetic quantum yield of PSII under salinity despite inhibited photosynthesis. Overexpression of AtAOX1a lowers ROS formation in leaves (Smith et al., 2009). This result is in line with the hypothesis that AOX participates in cell reprogramming under salinity stress. Activation of AOX limits ROS release from the mitochondrial respiratory chain. This activation is achieved in Arabidopsis by transcriptional control and by post-translational mechanisms (Rhoads et al., 1998; Smith et al., 2009). If AOX-dependent dissipation of excess reducing power is absent, ROS accumulates and can diffuse to other cell compartments (reviewed by Dietz et al., 2016). Thus AOX contributes to mitigating oxidative stress under conditions of high salinity stress (Hanqing et al., 2010). Moreover, genetic enhancement of AOX expression reduces ROS levels in transgenic tobacco (Maxwell et al., 1999). Also, the abundance of PTOX positively correlates with salinity levels (Ivanov et al., 2012; Nawrocki et al., 2015). From these findings we conclude that transcriptional up-regulation of AOX and PTOX participates in the suppression of ROS accumulation in salt-stressed sugar beet.

**The RBOH gene family and their regulation**

Sugar beet expresses five RBOH and RBOH-like genes compared to ten in Arabidopsis, where AtRBOHD and AtRBOHF preferentially accumulate upon salt stress (Ma et al., 2012). Arabidopsis single mutants devoid of AtRboHD or AtRboHF are indistinguishable from wild type if stressed with 300 mM NaCl. Conversely, the double mutant AtRboHDF reveals reduced viability in 300 mM NaCl administered for 14 d and simultaneously accumulates less H₂O₂ (Ma et al., 2012). Interestingly in sugar beet viability remained at 100% under salt stress despite the strong decrease in RBOH activity. Ben Rejeb et al. (2015) assigned a crucial role to RBOHs in salinity-induced regulation of the antioxidant defense in Arabidopsis

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**Table 3.** Cis-elements potentially responsible for the regulation of salinity induced transcripts in B. vulgaris but absent in the same gene from A. thaliana.

| Gene name | Cis-regulating elements | Cis-regulating elements sequence | Expression matrix |
|-----------|-------------------------|---------------------------------|-------------------|
| Cu-ZnSOD1; Cu-ZnSOD2; Cu-ZnSOD3; FeSOD3; MnSOD1; 2CysPrx B; Prx Q; AOX1A; AOX2; PTOX1; RBOH B | Dehydrin (LTRECOREATCOR15) | CCGAC |
| Cu-ZnSOD1; 2CysPrx B; AOX1A; AOX2; PTOX1 | BES1 (TFmatrixID_0494) | CACGTG* |

* indicates details provided in Supplementary Table S5.
under short-term treatments. The salinity-induced activation of endosomal RBOH is suppressed in the phosphatidylinositol-3-kinase mutant pi3k (Leshem et al., 2007) resulting in less oxidative stress. Post-translational control of RBOH by cellular Ca\(^{2+}\). ROS and phosphorylation networks participates in the early responses to salinity, particularly in glycerophytes but possibly also in halophytes (Kurusu et al., 2015). Moreover, BvRBOHB levels doubled at 3 h in contrast to all other RBOH transcripts that were down-regulated both at 3 h and 339 h. Thus BvRBOHB may be involved in early acclimation responses to ionic and osmotic stress, similar to AtRBOHD and AtRBOHF in Arabidopsis (Ma et al., 2012).

**Table 3**

| Autor | Title | Year | Journal |
|-------|-------|------|---------|
| Baker | Plant Physiology | 1999 | 1407–1414. |
| Alscher | Journal of Experimental Botany | 1999 | 1331–1341. |
| Li | Physical Review Letters | 2009 | 166–168. |

**Supplementary Data**

Supplementary data are available at JXB online.

Table S1. List of primers used for qPCR analysis.
Table S2. Sequence identities between sugar beet isoforms of SODs, Prxs, AOXs, PTOXs and RBOHs.
Table S3. Occurrence of cis-elements in promoter regions of salinity stress responsive genes of *B. vulgaris*.
Table S4. Occurrence of cis-elements in promoter regions of stress responsive genes of *A. thaliana*.
Table S5. Sequence details of BES1 present in up-regulated genes of *B. vulgaris*.

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