Tocopherol deficiency reduces sucrose export from salt-stressed potato leaves independently of oxidative stress and symplastic obstruction by callose

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Abstract

Tocopherol cyclase, encoded by the gene SUCROSE EXPORT DEFECTIVE1, catalyses the second step in the synthesis of the antioxidant tocopherol. Depletion of SXD1 activity in maize and potato leaves leads to tocopherol deficiency and a ‘sugar export block’ phenotype that comprises massive starch accumulation and obstruction of plasmodesmata in paraveinal tissue by callose. We grew two transgenic StSXD1:RNAi potato lines with severe tocopherol deficiency under moderate light conditions and subjected them to salt stress. After three weeks of salt exposure, we observed a strongly reduced sugar exudation rate and a lack of starch mobilization in leaves of salt-stressed transgenic plants, but not in wild-type plants. However, callose accumulation in the vasculature declined upon salt stress in all genotypes, indicating that callose plugging of plasmodesmata was not the sole cause of the sugar export block phenotype in tocopherol-deficient leaves. Based on comprehensive gene expression analyses, we propose that enhanced responsiveness of SnRK1 target genes in mesophyll cells and altered redox regulation of phloem loading by SUT1 contribute to the attenuation of sucrose export from salt-stressed SXD:RNAi source leaves. Furthermore, we could not find any indication that elevated oxidative stress may have served as a trigger for the salt-induced carbohydrate phenotype of SXD1:RNAi transgenic plants. In leaves of the SXD1:RNAi plants, sodium accumulation was diminished, while proline accumulation and pools of soluble antioxidants were increased. As supported by phytohormone contents, these differences seem to increase longevity and prevent senescence of SXD:RNAi leaves under salt stress.

Key words: Oxidative stress, potato, salt stress, SnRK1 signalling, starch accumulation, sucrose export defective, tocopherol, tuber yield.

Introduction

Tocopherols are amphiphilic antioxidants exclusively synthesized by photosynthetic organisms and are present in all plant organs (Asensi-Fabado and Munné-Bosch, 2010). They are composed of a hydrophobic prenyl side chain and a polar chromanol ring system that is differently methylated in α-, β-, γ-, and δ-tocopherol. The four tocopherol species differ in the degree and position of these methyl groups at the chromanol ring. Together with tocotrienols, tocopherols are collectively known as tocochromanols or vitamin E. Tocopherols are considered to play an essential antioxidant role in plants based on their excellent antioxidant activity in vitro, which is conferred by the capacity of the chromanol ring to donate a phenolic hydrogen to lipid peroxyl radicals (Liebler, 1993; Kamal-Eldin and Appelqvist, 1996). Thus, an important function of tocopherols in photosynthetic organisms is protection from lipid peroxidation (Sattler et al., 2004; Maeda et al., 2005). Furthermore, tocopherols can quench and scavenge singlet oxygen, which is a threat under photo-oxidative stress conditions (Trebst et al., 2002; Munné-Bosch et al., 2005; Kobayashi and DellaPenna, 2008). Due to their antioxidant
activity and membrane-stabilizing properties (Hincha, 2008), tocopherols are considered to play a major role in the protection of the photosynthetic apparatus.

Since the isolation of the vte1 mutant by Porfirova et al. (2002), functional studies in Arabidopsis italiana have led to a better understanding of tocopherol function. The vte1 mutant is deficient in the second step of tocopherol biosynthesis, involving tocopherol cyclase (TC), and is completely devoid of tocopherols, but still accumulates the amphiphilic precursor 2,2-dimethyl-5-phytyl-benzoquinone (DMPBQ; Porfirova et al., 2002; Sattler et al., 2003). Exposure of the vte1 mutant to high light slightly reduced its chlorophyll content and photosynthetic quantum yield in comparison to the wild type (Porfirova et al., 2002). A later study has shown that compensatory mechanisms, such as an increase in non-photochemical quenching, contribute to the prevention of excess photooxidative damage in the vte1 mutant under high light conditions (Havaux et al., 2005). The Arabidopsis homogenitase phytyltransferase (HPT) mutant vte2 is devoid of the committed step in tocopherol biosynthesis, the prenylation of homogentisic acid by phytyl diphosphate, and does not accumulate any intermediates like DMPBQ (Sattler et al., 2003, 2004). The Arabidopsis vte2 mutant showed impaired seed longevity and seedling growth during germination, as well as increased expression of many defence-related genes (Sattler et al., 2004, 2006), while tocopherol-deficient transgenic tobacco plants silenced for HPT exhibited accelerated senescence (Abbasi et al., 2009). All three effects can be connected to the antioxidant role of tocopherols.

A striking effect of tocopherol deficiency is the accumulation of soluble sugars and starch in source leaves of the maize TC mutant sxd1 (sucrose export defective; Russian et al., 1996; Botha et al., 2000) and transgenic StSXD1:RNAi-silenced potato plants (Hofius et al., 2004), which is caused by callose occlusion of plasmodesmata in phloem-associated cells in both systems. The link between photoassimilate export and tocopherol function is still unclear, given that tocopherol-deficient Arabidopsis (Maeda et al., 2006) and tobacco (Abbasi et al., 2009) plants did not exhibit this sugar export block phenotype under normal growth conditions. Among a range of abiotic stresses, only cold stress was able to trigger sugar and starch accumulation as well as callose deposition in the phloem tissue of vte2 source leaves, and to a lesser extent in vte1 source leaves (Maeda et al., 2006). Altered polyunsaturated fatty acid (PUFA) metabolism in the endoplasmic reticulum rather than photoinhibition and lipid peroxidation accounted for this cold stress-inducible sugar export block in vte2 mutants (Maeda et al., 2008). These results suggest that tocopherols can influence extra-plastidial processes independent of their antioxidant function, which supports a role for tocopherols in intracellular signalling (Provencher et al., 2001; Munné-Bosch and Falk, 2004). In line with this, studies on animals have proven that tocopherols can modulate membrane-associated signalling and gene expression (Azzi et al., 2002; Brigelius-Flohé et al., 2002). More recently, it has been found that accumulation of γ- instead of α-tocopherol in the vte4 mutant of Arabidopsis influences nuclear gene expression of the ethylene signalling pathway (Cela et al., 2011).

Only a little is known about the role of tocopherols in salt stress. Tocopherol-depleted HPT:RNAi tobacco plants showed a decreased tolerance to salt stress and enhanced lipid peroxidation upon salt challenge (Abbasi et al., 2007). However, HPT-silenced tobacco did not exhibit a sugar export block either under control or salt-stress conditions. Here, we used two tocopherol-deficient SXD1:RNAi transgenic potato lines silenced for TC that were generated in a previous study and are known to exhibit a sugar export block when grown at a moderate PFD of 500 μE m⁻² s⁻¹ (Hofius et al., 2004). We grew these plants at a lower PFD of 250 μE m⁻² s⁻¹, in which no sugar export block occurs. To investigate the connection between oxidative and ionic stress caused by salt treatment, tocopherol deficiency, and carbohydrate export, we challenged SXD1:RNAi plants with salt stress by watering with 150 mM NaCl. We found impaired sugar export from source leaves in salt-stressed SXD1:RNAi potato plants, which resulted in a stronger reduction of tuber yield in the transgenic plants compared to the wild type. However, no excessive callose deposition in the phloem could be observed in salt-stressed transgenic plants. Moreover, these plants did not show higher oxidative stress or lipid peroxidation than wild-type plants, but a reduced salt uptake that was probably caused by diminished photosynthetic gas exchange compared to the wild type. Based on our data, we present a model explaining how reduced sugar export is caused in tocopherol-deficient SXD:RNAi potato upon salt stress.

Materials and methods

Plant material, growth conditions, and sampling

Solanum tuberosum L. var. Solara (potato) was obtained from Bioplant (Ebstorf, Germany). Tocopherol-deficient transgenic lines StSXD1:RNAi-21 and -22 had been generated in a previous study (Hofius et al., 2004) by constitutively expressing an intron-spliced hairpin RNA (RNAi) construct targeted at StSXD1, which encodes TC.

All potato plantlets used for our experiments were vegetatively propagated from stem cuttings in tissue culture under a 16-h light/8-h dark regime (150 μmol m⁻² s⁻¹) at 21°C and 50% relative humidity on Murashige and Skoog medium (Sigma, St Louis, USA) containing 2% (w/v) sucrose. Rooted plantlets were subsequently grown in the greenhouse in individual 4-l pots at 50% humidity, with supplementary light (250 μmol m⁻² s⁻¹) under a 16-h day/8-h night (22°C/18°C) regime. The salt treatment started 38 days after transfer to soil, and consisted of irrigation with tap water without (control plants) and with additional salt (150 mM NaCl). Plants were irrigated with 200 ml of the corresponding solution every other day for the first 10 days of treatment. From day 11 to day 39, control plants received 250 ml daily water application until they reached maturity at day 39 after the onset of treatment. To avoid waterlogging, salt-treated plants were irrigated daily with 125 ml saline solution (150 mM NaCl) from day 11 to day 20, and irrigated every other day with 125 ml 150 mM NaCl from day 21 to day 39. At day 39 after the onset of treatment, salt application was stopped and salt-treated plants were irrigated further with 100 ml tap water every other day until the harvest of tubers at day 58. At the time of harvest, NaCl accumulation in the soil was comparable for pots with wild-type Solara (17.6 ± 1.2 mg NaCl mg⁻¹ soil dry weight), SXD21:RNAi (19.5 ± 1.3 mg NaCl mg⁻¹ soil dry weight), and SXD22:RNAi (17.6 ± 2.1 mg NaCl mg⁻¹ soil dry weight).
After 2 weeks of treatment, a reduction in plant size was evident in salt-treated plants compared to their respective controls. Therefore, leaf gas exchange rate and PSII quantum efficiency were assessed as described below. At 19 days of treatment, before the appearance of senescence in salt-treated plants, fully illuminated leaf samples were collected at three whorl positions to perform physiological measurements: top (young leaves, leaf 3), middle (fully expanded leaves, leaf 8), and bottom (leaf 11, just above the lowest leaf). At the time of sampling, the leaf water potential of salt-stressed middle and bottom leaves was comparable between all genotypes (Supplementary Figure S3). While leaf water potential was not substantially increased upon salt stress in middle and bottom leaves, salt-stressed top leaves exhibited a 5-fold increase in water potential compared to the controls. Leaves for biochemical analyses were sampled towards the end of the photoperiod (12h into the light period), snap frozen in liquid nitrogen and kept at −80°C until measurement. In addition, middle leaves were collected at the end of the subsequent night period for sugar and starch measurements. Aerial and tuber biomass were determined at 20 days of treatment with one subset of plants, while tuber biomass was measured again at the end of the plant life cycle, 58 days after the onset of treatment.

Quantification of tocopherol, soluble sugars, starch, and free amino acids
Tocopherol, soluble sugar, starch, and free amino acid contents were determined from aliquots of 20–30 mg leaf tissue as described in Abbasi et al. (2007). While tocopherol and amino acid contents were determined after HPLC separation by fluorescence detection, soluble sugars and starch were quantified using a spectrophotometric assay.

Measurement of leaf sugar exudation rate
Fully expanded source leaves (leaf 9) were excised at the base of the petiole 5 h after the onset of the light period. Submerged petioles were recut and subsequently placed in 15 ml 20 mM EDTA solution, while leaves were kept at growth light conditions. The exudate from the first 30 min was discarded. Then, the collection tube was changed at 1 h intervals for assessment of changes in exudation rate over a time course of 3 h. Sucrose concentration in the exudates was determined spectrophotometrically as described by Abbasi et al. (2009). Sugar exudation rate was calculated on a leaf area basis after correcting for differences in transpiration between the sampled leaves.

Gas-exchange and photosynthetic performance measurements
Photosynthetic parameters (A, E, ETR, and Fv/Fm) were determined at a PFD of 400 μmol m−2 s−1 with a combined gas exchange/chlorophyll imaging system (GFS-3000 and MINI-Imaging-PAM chlorophyll fluorometer, Walz, Effeltrich, Germany) at 350 ppm CO2, 13 000 ppm H2O, and a leaf temperature of 22°C as described by Horst et al. (2008).

Elemental analysis
Leaf samples were oven dried, and 50 mg dry tissue was acid digested with 1 ml 70% HNO3 and 0.5 ml 30% H2O2 (Baker Instr grade) in closed Teflon vessels at 90°C overnight. Samples were then mixed with 20 ml ultrapure H2O, and sodium (Na), potassium (K), and calcium (Ca) were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) with a Perkin Elmer Optima 3200RL spectrometer (Waltham, USA). Sample solutions showing an Na concentration below the ICP-OES detection limit (3 ppm) were also measured by atomic absorption spectrometry using a Varian AA240FS spectrometer (Palo Alto, USA).

Measurement of leaf osmolality
Potato leaf discs of 0.6 cm² were homogenized and, after centrifugation at 14 000 rpm for 3 min, 5 to 10 μl supernatant were mixed with ultrapure H2O up to a final volume of 100 μl. Solutions were measured using a freezing-point micro-osmometer (Vogel OMS15, Giessen, Germany).

Hormonal profiling
Levels of ABA (abscisic acid), ACC (the ethylene precursor, 1-amino-cyclopropane-1-carboxylic acid), SA (salicylic acid), JA (jasmonic acid), IAA (indole-3-acetic acid), IPA (isopentenyladenosine), 2-IP (isopentenyladenin), Z (zeatin), ZR (zeatin riboside), DHZ (dihydrozeatin), and DHZR (dihydrozeatin riboside) were simultaneously analysed by UPLC-ESI/MS/MS using deuterium-labelled hormone analogues as internal standards as described by Müller and Munné-Bosch (2011). In short, leaf samples (50 mg) were extracted in a final volume of 400 μl methanol:isopropanol:glacial acetic acid mixture, 40:59:1 (v/v/v), including a re-extraction. After filtration through a 0.2 μm PTFE filter (Waters, Milford, MA, USA), fresh extracts were injected into the UPLC–ESI/MS/MS system. Chromatography was performed using an Acquity UPLC System (Waters, Milford, MA, USA) with a HALO C18 (Advanced Materials Technology, Inc., Wilmington, USA) column (2.1 × 75 mm, 2.7 μm). ESI/MS/MS detection was carried out using an API 3000 triple quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada).
Gene expression analysis

Transcript amounts of the SnRK1 target genes ASNI, SnRKα, SnRKγ, SuSy2, SENI, TPS11, and UGE were determined by qRT-PCR exactly as described by Debast et al. (2011). Primers used for transcript quantitation of SnSUTI (Riesmeier et al., 1993) were qStSUT1fw 5′-TGT CTT GGC AAA TGC TTT GTA-3′ and qStSUT1rev 5′-TTT TAC CAA CCC AAA GGA CC-3′. The primers for qRT-PCR quantitation of the SnSUTI interactors SnSnakin1 and StPDI (Krügel et al., 2012; Krügel and Kühn, 2013) were qStSnakin1fw 5′-GTA ATT CAA AGT GCA AGC TGA GAT G-3′, qStSnakin1rev 5′-GTC CCT ATA ACA AGG ACA TAC TTC ATG-3′, qStPDIfw 5′-GCA AAC CTT GAT GCC GAT AA-3′ and qStPDirrev 5′-TCT CGG CCA CCA TCA TAA TC-3′. The potato homologue of the Arabidopsis XTH5 gene was quantified with the primers qStXTH5fw 5′-GGA CCC ATT GGA ACA AGT TGT AAA C-3′ and qStXTH5rev 5′-GCCCTGAATCTTTTCATGGCCATT-3′, while the closest homologue of Arabidopsis vacuolar proton-coupled pyrophosphatase AtPVP1 was assessed with the primers qStPVP1fw 5′-GGA TTT GCT ATT GGT TCT GCT GCA-3′ and qStPVP1rev 5′-GTC CCT ATA ACA AGG ACA TTC ATG-3′. In all cases, potato ubiquitin was used as an internal reference gene, as described by Debast et al. (2011).

Results

Tocopherol-deficient potato source leaves exhibit sugar export deficiency and impaired nocturnal starch mobilization under salt stress

Knockdown of TC by constitutive expression of an RNAi construct targeted at the StSXD1 TC gene resulted in tocopherol-deficient potato lines (Hofius et al., 2004). Among the transgenic lines analysed in our previous study (Hofius et al., 2004), StSXD1::RNAi-22, -27, and -21 showed the strongest reduction in foliar tocopherol levels, containing 0.7, 2.1, and 2.0% of wild-type α-tocopherol, respectively. At a PFD of 500 µmol quanta m⁻² s⁻¹, only StSXD1::RNAi-22 and -27, with <2.1% of wild-type α-tocopherol, displayed a clear sucrose export-deficient phenotype (Hofius et al., 2004).

We first assessed whether the reduction in tocopherol content of StSXD1::RNAi-21 and -22 was still comparable to the previous study after nine years of vegetative propagation of the lines on axenic media. In the present study, young three-week-old plantlets of StSXD1::RNAi-22 and -21 exhibited 1.2±0.2% and 1.1±0.2% of wild type α-tocopherol content, respectively, and therefore these two lines were selected for further experiments.

Tocopherol content in stressed and control leaves at different whorl positions was further analysed in 57-day-old plants, 19 days after the onset of salt treatment (Fig. 1). Total tocopherol was determined as the sum of α-, γ- and δ-tocopherol (β-tocopherol was below the detection limit). α-tocopherol accounted for 95–98% of total tocopherol content in wild-type leaves, and for >99% of the total tocopherol in leaves of transgenic plants. In control conditions, the reduction in tocopherol content in middle and bottom leaves of both transgenic lines was similar to that of the initially screened plantlets (see Materials and Methods). Overall, tocopherol contents did not change significantly upon salt stress in all genotypes. However, salt-stressed bottom leaves of the transgenic plants exhibited the strongest tocopherol deficiency (1.3% of the wild type in line 21 and 1.5% of the wild type in line 22).

Previously, it was demonstrated that StSXD1-silenced potato lines with less than ~2% residual tocopherol content showed an impaired photoassimilate export when grown at a PFD of 500 µmol quanta m⁻² s⁻¹, leading to a strong accumulation of sugars and starch in source leaves (Hofius et al., 2004). In the present study, plants were grown at a lower PFD of 250 µmol quanta m⁻² s⁻¹. Compared to the previous study, a less pronounced accumulation of total soluble sugars was observed in fully expanded source leaves of both tocopherol-deficient plant lines in control conditions at the end of the light period (Fig. 2). In contrast, we could not observe a significant accumulation of starch in the transgenic plants under control conditions compared to the wild type (Fig. 2). In fully expanded control leaves of SXD::RNAi plants, accumulation of soluble sugars was increased almost 3-fold compared to wild-type control leaves, while bottom leaves of transgenic

![Fig. 1. Total tocopherol content of StSXD1-silenced potato leaves. Total tocopherol levels were calculated on a fresh weight (FW) basis, for StSXD1::RNAi-21 and -22 potato lines and the wild type (WT), at top (leaf 3, left panel), middle (leaf 8, middle panel), and bottom (leaf 11, right panel) whorl positions. Samples were collected 19 days after the onset of treatments, consisting of irrigation either with 150 mM NaCl (salt stress, black bars) or water (control, white bars). Data represent the mean ± SE of four individual plants.](image-url)
Salt stress provokes a strong reduction in starch content of both middle and bottom wild-type leaves to 8–12% of the levels observed in control conditions (Fig. 2). In contrast, the starch content of tocopherol-deficient plants did not decrease by >20% upon salt stress. As a result, starch content was increased 7- to 13-fold in middle and bottom leaves of transgenic potato plants compared to stressed wild-type leaves of the respective whorl position. In salt-stressed middle leaves of the wild type, sucrose content was increased by 75% compared to control conditions, while sucrose content was comparable in the transgenic plants in stress and control conditions. Unlike the wild type, tocopherol-deficient plants showed a decrease in total soluble sugar content upon salt stress, (Fig. 2, middle panel). Taken together, source leaves of transgenic plants retained higher soluble sugar levels than the wild type under salt stress, but the differences to the wild type were smaller in stress than in control conditions.

In order to clarify whether the transitory starch pool in stressed transgenic plants is not mobilized in the dark due to a sugar export block or whether starch synthesis during the light period remains high in the transgenic plants, middle source leaves were analyzed at the end of the dark period (Fig. 3). In control conditions, the turnover of starch was similar between the genotypes, but soluble sugars showed a higher diurnal turnover in tocopherol-deficient plants than in the wild type, suggesting that sugar export was hampered in the transgenic plants during the light phase. In the salt-stressed wild type, the soluble sugar content declined by a quarter during the dark period, which was similar to control conditions. In contrast, soluble sugar content remained constant between the two time points in stressed leaves of the two transgenic lines. Similarly, nocturnal starch mobilization was virtually absent in stressed SXD1:RNAi leaves, while it was elevated in stressed wild-type plants compared to control conditions (87% starch turnover vs 69% starch turnover in control and stress conditions, respectively). Taken together, salt stress was able to reduce soluble sugar accumulation in SXD1-RNAi source leaves, while nocturnal starch mobilization was apparently abolished in the transgenic plants.

Salt stress does not cause callose accumulation in the phloem, but leads to decreased sugar export and diminished tuber yield in tocopherol-deficient potato plants

The apparent absence of nocturnal starch mobilization in tocopherol-deficient source leaves indicates that major carbohydrate metabolism is disturbed upon salt stress. As outlined in the introduction, previous studies have commonly found that sugar export deficiency of tocopherol-depleted source leaves was a consequence of callose deposition in the vascular tissue, which prevented sucrose loading into the phloem. We therefore analyzed whether this was also the case in salt-stressed SXD:RNAi potato plants. Quantitative measurements of callose content in fully expanded and bottom source leaves showed that callose levels in both StSXD1-RNAi lines were increased ~2-fold compared to the wild type plants in control conditions (Fig. 4A). Salt stress caused a marked decrease in callose content in all three genotypes. Microscopic observation of aniline blue-stained leaves confirmed that callose deposition was mainly located in the vasculature of tocopherol-deficient control leaves, while it was barely detectable in wild-type control leaves (Fig. 4B). In agreement with the quantitative results, microscopic analysis showed that callose deposition in salt-stressed, tocopherol-deficient plants was less prominent compared to control conditions. Therefore, the impact of salt stress on the sugar export capacity of source leaves was assessed by determining...
the sugar exudation rate of fully expanded source leaves (Fig. 5). Sucrose exudation rate was reduced by 27% in StSXD1:RNAi-21 and by 36% in StSXD1:RNAi-22 leaves compared to wild-type plants in control conditions. The sucrose exudation rate was not significantly altered in wild-type plants under salt stress, while tocopherol-deficient plants exhibited a >80% decrease in export rate. Thus, sugar export rate was strongly diminished in salt-stressed SXD:RNAi leaves, despite diminished callose deposition in the vasculature.

As a consequence of decreased sugar export in SXD:RNAi source leaves under salt stress, a 60% decline in tuber weight of the transgenic plants compared to the wild type was observed early during tuberization at 20 days post-stress initiation (dpi) (Table 1). In control conditions, the tuber biomass of the transgenic plants was diminished by only 48% compared to the wild type at the same time point. The decrease in tuber yield of the transgenic plants became more pronounced at the end of the life cycle. SXD:RNAi plants exhibited a yield penalty of ~79–83% under stress vs 42% in control conditions at final harvest (Table 1). Tuber starch content remained comparable between all genotypes within the same treatment, both at 20 and 58 days after the onset of stress (Supplementary Figure S1). Nevertheless, tuber starch content decreased 2-fold in stress compared to control conditions in all genotypes. In contrast, aerial biomass, as measured 20 days after the onset of treatment, was similar in the three genotypes in control and salt-stress conditions (it was even higher in StSXD1:RNAi-21 than in wild-type controls). As a consequence, the shift in favour of aerial versus tuber biomass was more pronounced in the transgenic plants under salt-stress conditions (Table 1).

Tocopherol-deficient potato leaves exhibit less sodium accumulation, but a higher soluble antioxidant capacity during salt challenge

Since the negative impact of tocopherol deficiency on sugar export and tuber yield was exacerbated under salt treatment, we assessed whether the physiology of tocopherol-deficient leaves was more sensitive to salt challenge and whether an elevated degree of oxidative stress might explain the altered sugar response of transgenic leaves. The CO₂ assimilation rate of SXD1:RNAi source leaves was significantly reduced by 40–60% in the transgenic plants under salt-stress conditions (Table 2). The transpiration rate was diminished in salt-stressed transgenic plants in comparison to stressed wild-type plants (Table 2), and concomitantly the contents of xylem-mobile Na⁺ and Ca²⁺ were 2- to 3-fold reduced in SXD1:RNAi source leaves (Fig. 6). Consequently, the K⁺/Na⁺ ratio was higher in both middle and bottom leaves of salt-stressed transgenic plants, indicating that the foliar ionic balance was less disturbed in SXD1:RNAi leaves compared to the wild type (Supplementary Figure S2). However, leaf osmolality in middle and bottom source leaves did not exhibit significant changes across genotypes and treatments (Supplementary Figure S3).

The pool sizes of the major water-soluble antioxidants, ascorbate and glutathione, were elevated in middle and bottom source leaves of the transgenic plants compared to the wild type, in control conditions already (Fig. 7). Salt stress caused a decrease in total glutathione and, to a greater extent, total ascorbate content of middle wild-type leaves, while total glutathione and ascorbate contents were comparable in stressed and non-stressed bottom wild-type leaves. Ascorbate and glutathione pool sizes were also diminished in tocopherol-deficient plants upon salt treatment, but commonly both pools remained larger compared to wild-type controls (Fig. 7). The redox state of the ascorbate and glutathione pools were very similar across genotypes and treatments, with glutathione showing values higher than 90%, while ascorbate redox state ranged around 80% (Supplementary Figure S4). MDA levels were not significantly different between the
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genotypes irrespective of treatment and, in addition, MDA levels were not increased upon salt stress in the transgenic plants, indicating the absence of excessive lipid peroxidation in salt-stress conditions (Supplementary Figure S4).

Furthermore, transgenic plants survived longer than wild-type plants under salt stress, as shown in Supplementary Figure S5, which represents plants 42 days after the onset of treatment. At that time, stressed wild-type plants were dead, while all tocopherol-deficient plants, despite showing severe wilting symptoms, still exhibited a few pale green leaves (see arrows in Supplementary Figure S5).

In summary, SXD1:RNAi potato plants were more tolerant towards salt stress compared to the wild type, which can be accounted for by reduced Na⁺ accumulation in leaves and elevated soluble antioxidant pools. However, the transgenic plants exhibited a much more pronounced yield penalty when challenged with saline conditions compared to the wild type.

Fig. 5. Changes in sucrose exudation rate of source leaves upon salt treatment. Samples were taken from middle leaves (leaf 9) and were collected 19 days after the onset of treatments (salt stress, black bars; control, white bars). Data represent the mean ± SE of four individual plants. Data were analysed by t-test; significant differences between the transgenic lines and the wild type within a treatment are indicated by a black asterisk (control treatment) or a white asterisk (stress treatment), while diamonds indicate significant differences between control and salt stress within a genotype (P < 0.05). WT, wild type.
Central carbon and amino acid metabolism and sucrose-dependent gene expression are altered in salt-stressed SXD1:RNAi

Despite the observed lower Na⁺ uptake rates and increased antioxidant capacity, increased accumulation of compatible solutes could also account for the elevated tolerance of SXD:RNAi plants towards salt stress. We had already found that the contents of osmotically active soluble sugars were increased by about 40% in stressed SXD:RNAi leaves compared to the wild type (Fig. 1). Furthermore, Pro accumulation in stressed transgenic plants was 2- to 3-fold higher compared to stressed wild-type plants (Fig. 8). Pro accounted for up to 70% of the total foliar free amino acid pool in SXD:RNAi leaves, which corresponds to a 1.5- to 2-fold increase of Pro relative to the wild type.

Likewise, the contents of the major free amino acids Gln, Asn, and Asp were elevated in SXD1:RNAi leaves compared to the wild type in both control and stress conditions (Fig. 8). While the accumulation of these major amino acids was more significant in salt-stressed transgenic plants, the contents of most TCA cycle intermediates dropped more prominently compared to wild-type leaves (Supplementary Figure S6), which may reflect elevated carbon flux from carboxylate precursors into the amino acid pool. Asparagine contents were increased most prominently in SXD:RNAi leaves in comparison to the wild type (Fig. 8). Asn content was elevated 2-3 times in SXD:RNAi source leaves in control conditions and even 6-15 times higher in salt-stressed transgenic plants.

As the asparagine synthase gene ASN1 is known to represent a SnRK1 target gene, the altered effect of salt stress on free Asn content in SXD1:RNAi leaves prompted us

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**Table 1. Aerial and tuber biomass of tocopherol-deficient potato plants at two time points during salt-stress exposure**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Genotype</th>
<th>Control</th>
<th>Salt stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerial biomass (g) 20 dpi</td>
<td>Wild type</td>
<td>52.0 ± 2.0a</td>
<td>53.2 ± 4.5a</td>
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<td></td>
<td>SXD1:RNAi 21</td>
<td>58.7 ± 3.8b</td>
<td>56.6 ± 7.7a</td>
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<td></td>
<td>SXD1:RNAi 22</td>
<td>51.5 ± 4.3a</td>
<td>53.8 ± 5.5a</td>
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<td>Tuber biomass (g) 20 dpi</td>
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<td>39.3 ± 2.6a*</td>
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<td>41.5 ± 1.7b</td>
<td>15.8 ± 3.6b*</td>
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<td>28.2 ± 3.6c</td>
<td>15.6 ± 2.2b*</td>
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<td>Tuber biomass (g) 58 dpi</td>
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<td>Shoot / Tuber ratio 20 dpi</td>
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<td>SXD1:RNAi 21</td>
<td>1.42 ± 0.10b</td>
<td>3.46 ± 0.06b*</td>
</tr>
<tr>
<td></td>
<td>SXD1:RNAi 22</td>
<td>1.84 ± 0.16c</td>
<td>3.54 ± 0.80b*</td>
</tr>
</tbody>
</table>

---

**Table 2. Gas-exchange and PSII efficiency parameters of StSXD1-silenced potato plants after 14-days’ exposure to salt stress**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Genotype</th>
<th>Control</th>
<th>Salt stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (μmol CO₂ m⁻² s⁻¹)</td>
<td>Wild type</td>
<td>5.70 ± 1.80a</td>
<td>2.40 ± 0.59a*</td>
</tr>
<tr>
<td></td>
<td>SXD1:RNAi 21</td>
<td>4.43 ± 2.28a</td>
<td>0.85 ± 0.13b*</td>
</tr>
<tr>
<td></td>
<td>SXD1:RNAi 22</td>
<td>4.96 ± 1.62a</td>
<td>1.38 ± 0.64b*</td>
</tr>
<tr>
<td>E (mmol m⁻² s⁻¹)</td>
<td>Wild type</td>
<td>1.36 ± 0.68a</td>
<td>0.43 ± 0.12a*</td>
</tr>
<tr>
<td></td>
<td>SXD1:RNAi 21</td>
<td>1.12 ± 0.91a</td>
<td>0.20 ± 0.05b*</td>
</tr>
<tr>
<td></td>
<td>SXD1:RNAi 22</td>
<td>1.05 ± 0.43a</td>
<td>0.27 ± 0.11b*</td>
</tr>
<tr>
<td>ETR</td>
<td>Wild type</td>
<td>69.1 ± 2.3a</td>
<td>59.3 ± 5.5a*</td>
</tr>
<tr>
<td></td>
<td>SXD1:RNAi 21</td>
<td>71.2 ± 6.4a</td>
<td>51.0 ± 6.4a*</td>
</tr>
<tr>
<td></td>
<td>SXD1:RNAi 22</td>
<td>71.5 ± 7.2a</td>
<td>56.5 ± 8.5a*</td>
</tr>
<tr>
<td>Fv/Fm</td>
<td>Wild type</td>
<td>0.797 ± 0.016a</td>
<td>0.789 ± 0.017a</td>
</tr>
<tr>
<td></td>
<td>SXD1:RNAi 21</td>
<td>0.784 ± 0.011a</td>
<td>0.759 ± 0.075a</td>
</tr>
<tr>
<td></td>
<td>SXD1:RNAi 22</td>
<td>0.806 ± 0.024a</td>
<td>0.794 ± 0.026a</td>
</tr>
</tbody>
</table>

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*a* Aerial biomass was measured one day after leaf sampling (20 dpi) and tuber biomass was measured at 20 dpi and at the end of the experiment (58 dpi). Data represent the means ± SD of 4–5 individual plants. Data were analysed by t-test: significant differences among genotypes within a treatment are indicated by different letters for each parameter, and significant differences between control and salt stress within a genotype are indicated by an asterisk (*P* < 0.05).

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**Fig. 6.** Effects of salt treatment on sodium and calcium content in source leaves. Sodium (top panels) and calcium contents (bottom panels) are depicted on a dry weight (DW) basis. Left panels represent middle leaves (leaf 8); right panels represent bottom leaves (leaf 11). Samples were collected 19 days after the onset of treatments (salt stress, black bars; control, white bars) and data represent the mean ± SE of four individual plants. Data were analysed by t-test: significant differences between the transgenic lines and the wild type (WT) within a treatment are indicated by a black asterisk (control treatment) or a white asterisk (stress treatment), while diamonds indicate significant differences between control and salt stress within a genotype (*P* < 0.05).
to analyse SnRK1-mediated regulation of metabolism. In Fig. 9, the transcript accumulation of six SnRK1 target genes is shown: \textit{ASN1}, \textit{UGE}, \textit{SuSy2}, \textit{XTH5}, \textit{TPS11}, and \textit{SnRKα} (coding for sugar-regulated asparagine synthetase, UDP-glucose epimerase, sucrose synthase, xyloglucan endotransglucosylase-hydrolase, and trehalose 6-phosphate synthase isoforms, respectively (see Debast et al., 2011). The \textit{Arabidopsis} homologues of \textit{ASN1}, \textit{SuSy2}, \textit{SnRKα}, and \textit{XTH5} were found to be regulated via the transcription factor bZIP11 (Hanson et al., 2008). A strong upregulation of \textit{ASN1} and \textit{SuSy2} transcript amounts was observed in all genotypes upon salt stress, and \textit{ASN1} and \textit{SuSy2} transcript levels were induced much more strongly in stressed SXD1:RNAi than in wild-type plants (Fig. 9). \textit{UGE} was induced in tocopherol-deficient plants upon salt exposure, but not in wild-type plants. In contrast, \textit{XTH5}, \textit{TPS11}, and \textit{SnRKα} transcripts did not show significant differences across treatments or genotypes (Fig. 9). Furthermore, the content of trehalose-6-phosphate, which is thought to correlate with cellular sucrose availability (Lunn et al., 2006), was elevated about 2-fold in SXD1:RNAi compared to wild-type source leaves in all conditions (Supplementary Figure S6). Taken together, our data indicate a discrepancy between individual indicators of cellular sucrose availability, i.e. SnRK target gene expression and trehalose-6-phosphate content.

Fig. 7. Effects of salt treatment on the pool size of foliar soluble antioxidants in source leaves of \textit{StSXD1}-silenced potato plants. Total glutathione (top panels) and total ascorbate content (bottom panels) are depicted. Left panels represent middle leaves (leaf 8); right panels represent bottom leaves (leaf 11). Samples were collected 19 days after the onset of treatments (salt stress, black bars; control, white bars) and data represent the mean ± SE of four individual plants. Data were analysed by \( t \)-test; significant differences between the transgenic lines and the wild type (WT) within a treatment are indicated by a black asterisk (control treatment) or a white asterisk (stress treatment), while diamonds indicate significant differences between control and salt stress within a genotype (\( P < 0.05 \)).

Fig. 8. Content of free amino acids in source leaves of tocopherol-deficient plants. (A) Amino acid contents in middle leaves (leaf 8). (B) Amino acid contents in bottom leaves (leaf 11). Total amino acid levels represent the sum of single free amino acid contents determined by HPLC. Samples were collected 19 days after the onset of treatments (salt stress, black bars; control, white bars) and data represent the mean ± SE of four individual plants. Data were analysed by \( t \)-test; significant differences between the transgenic lines and the wild type within a treatment are indicated by a black asterisk (control treatment) or a white asterisk (stress treatment), while diamonds indicate significant differences between control and salt stress within a genotype (\( P < 0.05 \)).
We therefore assessed whether the regulation of sucrose export from source leaves is also altered in SXD1:RNAi. The transcript level of the single copy H⁺/sucrose cotransporter SUT1, which controls apoplastic phloem loading (Riesmeier et al., 1993), was strongly diminished upon salt stress, but there were no consistent differences between tocopherol replete and depleted genotypes (Fig. 9). In contrast, the SUT1-interacting protein Snakin-1 (Kruegel et al., 2012) was induced at the transcript level in SXD:RNAi leaves under all conditions (Fig. 9). Cell wall-bound and soluble invertase activity, which can also efficiently counteract phloem loading (Rolland et al., 2006), were not significantly altered between wild-type and transgenic plants in control and salt-stress conditions (Supplementary Figure S7). Similarly, transcript amounts of the vacuolar H⁺-pyrophosphatase, which might prevent sucrose utilization and phloem loading (Lerchl et al., 1995), were diminished to a similar extent in all genotypes under salt stress (Fig. 9). Our data leave Snakin-1 as the only potential candidate that may affect the rate of sucrose export from SXD:RNAi source leaves upon salt stress.

ACC, SA, and cytokinin levels are altered in source leaves of tocopherol-deficient potato

In order to gain more insight into the altered physiology of tocopherol-deficient plants, the levels of a range of hormones related to stress responses as well as to plant growth and development were measured. ABA accumulation was elevated upon salt stress only in bottom leaves, which was more pronounced in the wild type compared to SXD:RNAi leaves (Fig. 10). While the ethylene precursor ACC displayed a 5- and 3-fold increase in middle and bottom leaves of wild-type plants exposed to salt stress, respectively, transgenic plants showed a less dramatic increase in ACC content, reaching 40% (middle leaves) and 20% (bottom leaves) under salt stress (Fig. 10). In parallel, SXD1:RNAi lines contained 5- to 8-fold more SA than wild-type plants in control conditions. Salt stress did not cause a substantial decrease in SA levels in wild-type leaves, while a 40–65% decline of SA content was found in stressed middle and bottom leaves of transgenic plants compared to control conditions. As a result, the difference in SA levels between wild-type and transgenic plants was lowered upon...
Tocopherol deficiency and salt stress

Salt treatment, although they remained significantly higher in the transgenic plants. JA levels were low compared to the above-mentioned stress hormones, and differences were not found between the three genotypes (Supplementary Figure S8). Levels of the cytokinins IPA and ZR were higher in bottom leaves of stressed transgenic plants compared to wild-type plants (Fig. 10). Diminished ACC and SA as well as elevated cytokinin contents all point towards delayed senescence in salt-stressed SXD1:RNAi leaves, which might simply be caused by a diminished hexose/sucrose ratio in stressed transgenic plants (also observed in Lara et al., 2004; Kocal et al., 2008).

Discussion

SXD:RNAi potato lines as a tool to study the potential causes of a sugar export block

In previous work by Hofius et al. (2004), it was observed that tocopherol-deficient SXD:RNAi potato plants exhibited a photoassimilate export-defective phenotype that coincided with callose deposition in the phloem of source leaves, similar to what had been observed for the maize tocopherol deficient sxd1 mutant previously (Botha et al., 2000; Provencher et al., 2001). On the other hand, tocopherol-deficient tobacco and Arabidopsis plants did not exhibit such a sugar export block in standard growth conditions at regular PFD (Maeda et al., 2006; Abbasi et al., 2009). This difference between species can be explained by the fact that potato and maize exhibit a high rate of sugar export from source leaves due to the presence of strong systemic carbon sinks, while Arabidopsis and tobacco source leaves have lower sugar export rates per leaf area (see also Abbasi et al., 2009).

When growing the same tocopherol-deficient SXD:RNAi potato lines at a photon flux density (PFD) of 250 μmol m⁻² s⁻¹ (this study) instead of 500 μmol m⁻² s⁻¹ (as in Hofius et al., 2004), starch accumulation in unstressed source leaves of the transgenic plants was absent, and only a 2.5-fold accumulation of soluble sugars and limited callose accumulation in the vasculature were observed compared to the wild type. This suggests that callose plugging in the vasculature of SXD:RNAi potato may depend on incident light intensity. We aimed at exploiting these permissive growth conditions to examine whether the disturbance of foliar physiological processes by salt stress can induce a sugar export block in tocopherol-deficient potato plants.

Tocopherol-deficient Arabidopsis vte2 mutants showed a sugar export block during cold acclimation, which was dependent on fatty acid desaturation in the ER-derived phospholipid pool by FAD2 (fatty acid desaturase2) (Maeda et al., 2008). As this stress-inducible sugar export block in vte2 was independent of light intensity, the degree of photoinhibition, or lipid peroxidation, it may thus be linked to non-antioxidant functions of tocopherols (Maeda et al., 2008). Since increased synthesis of PUFAs is important for acclimation to both cold and salt stress in plants (e.g. Maeda et al., 2008; Zhang et al., 2012), salt challenge of SXD:RNAi potato

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Fig. 10. Phytohormone contents in source leaves of tocopherol-deficient potato plants exposed to 19 days’ salt treatment. Leaf content of ABA, ACC, SA, and of the cytokinins IPA and ZR are shown from top to bottom. Left panels represent middle leaves (leaf 8); right panels represent bottom leaves (leaf 11). Black bars, salt-stress treatment; white bars, control treatment. Data represent the mean ± SE of four individual plants. Data were analysed by t-test; significant differences between the transgenic lines and the wild type within a treatment are indicated by a black asterisk (control treatment) or a white asterisk (stress treatment), while diamonds indicate significant differences between control and salt stress within a genotype (P < 0.05).
plants was thought to provide information as to whether non-oxidant functions of tocopherol are important for salt-stress acclimation in potato leaves.

**The reduction in sugar export rate in tocopherol-deficient potato leaves is not caused by excessive callose plugging**

The content of soluble sugars was higher in SXD:RNAi source leaves compared to wild-type plants in salt stress and, in addition, starch mobilization in transgenic plants was abolished under salt-stress conditions. However, soluble sugars and starch contents of stressed transgenic plants did not exceed the level found in control conditions. Therefore, the response of major leaf carbohydrates in SXD transgenic plants to salt stress does not compare to what is described as a sugar export block in previous reports (Botha et al., 2000; Hofius et al., 2004; Maeda et al., 2006). In salt stress, there is neither an excessive accumulation of leaf carbohydrates, nor pronounced plugging of plasmodesmata in the leaf vasculature of the transgenic plants. Even if tocopherol-deficiency had caused limited phospholipid desaturation in salt-stressed SXD:RNAi leaves as described for cold-stressed Arabidopsis vte2 mutants (Maeda et al., 2008), this did not lead to a sugar export block phenotype. Therefore, sugar export in the potato transgenic plants must be impaired by a different mechanism.

Sugar export in tocopherol-deficient source leaves was diminished by 80% upon salt stress and nocturnal sugar and starch levels were higher than in stressed wild-type plants, indicating an elevated retention of carbohydrates in SXD:RNAi source leaves. Furthermore, the substantial sugar export deficiency of salt-stressed transgenic plants was evident through a massively diminished exudation rate and caused a substantial tuber yield penalty. Thus, we tried to explain this phenomenon at the molecular level. First, we assessed whether reduced apoplastic phloem loading could explain the marked decline in sugar export in salt-stressed, tocopherol-deficient plants by analysing the transcript abundance of the sugar transporter SUT1 that is responsible for phloem loading in potato (Riesmeier et al., 1993) and represents a single-copy gene. SUT1 was strongly downregulated upon salt stress in all three genotypes, although SUT1 transcript levels remained significantly higher in the transgenic plants compared to the wild type. Once an increased SUT1 transcript abundance is unlikely to result in less apoplastic phloem loading in salt-challenged SXD:RNAi leaves, we looked at the SUT1-interacting proteins PDI and Snakin1 (Krügel et al., 2012), which are thought to have the potential to modulate SUT1 activity by integrating redox and/or defence signals (Krügel and Kühn, 2013). However, direct experimental evidence in support of these assumed roles is lacking. Snakin1 (SN1) transcript amounts were increased 2-fold in SXD:RNAi leaves under all conditions, while PDI transcript amounts remained unaltered across genotypes and conditions (not shown). Strong overexpression of the small cysteine-rich SN1 protein in potato had only minor effects on morphology and primary metabolism in the absence of abiotic stress (Nahirnak et al., 2012). Notably, proline contents were found to be increased in SN1 overexpressors compared to controls (Nahirnak et al., 2012), which might indicate increased carbon allocation into this osmoprotectant that may occur at the expense of sucrose export. It seems valuable to test this assumption by studying salt- and drought-stress tolerance of SN1-overexpressing potato plants.

We also investigated other key functions that can influence sucrose export. Both cell wall invertase (cw-INV) and soluble invertase can counteract apoplastic loading by cleaving sucrose in the apoplast or by stimulating sucrose import and cleavage in the vacuole, respectively (Rolland et al., 2006). Both activities seem to be diminished upon salt stress, but consistent differences were absent between the genotypes.

An induction of vacuolar H+-pyrophosphatase is observed in the halophyte Thalungiella halophila in saline conditions (Sun et al., 2010). Although the Arabidopsis homologue AVP1 is not induced upon salt stress, overexpression of AVP1 homologues from wheat, Arabidopsis and T. halophila were able to increase salt-stress tolerance in plants (e.g. Gaxiola et al., 2001; Gao et al., 2006). Elevated tonoplast H+-pyrophosphatase is thought to increase the tonoplast membrane potential to drive the import of potassium into the vacuole by proton-coupled uptake systems (Blumwald et al., 2000). It is reasonable to assume that the cytosol might be deprived of pyrophosphate by elevated H+-pyrophosphatase activity, thereby hampering phloem loading of sucrose in companion cells that rely on low cytosolic pyrophosphatase activity to efficiently utilize sucrose (Lerchl et al., 1995). However, the potato homologue of AVP1 was transcriptionally repressed by salt stress to a similar extent in all three genotypes, disproving this attractive hypothesis.

Once we could not identify substantial differences in these major players of sucrose export, we checked whether an imbalance in cellular sugar signalling might account for reduced sucrose export in salt-stressed SXD:RNAi source leaves.

**Sugar signalling might attenuate sucrose export in tocopherol-deficient source leaves**

In plants, SnRK1s are highly conserved protein kinases closely related to SNF1 in yeast and AMPK in animals (Hallford et al., 2003), which play a key role in the regulation of carbon metabolism and energy balance. Upon energy depletion caused by abiotic stress situations, darkness, or nutrient deprivation, SnRK1s trigger extensive transcriptional activation of major catabolic pathways to provide alternative sources of energy and metabolites (Baena-González et al., 2007). To assess whether SnRK1 signalling is altered in SXD:RNAi leaves, we determined the transcript levels of several genes that were shown to be potential SnRK1 targets in potato tubers (Debast et al., 2011). Three of the eight target genes, AsnS1, SuSy2, and UDPglcE, showed higher transcript levels in tocopherol-deficient plants than in wild-type plants under salt stress. Despite a very substantial induction of ASN1 upon salt stress, transcripts of all three genes were <2-fold elevated in SXD:RNAi leaves compared to the wild type under stress conditions. Three other SnRK1 targets, XTH5, TPS11, and
SnRKα (Fig. 9), as well as SnRKγ and SEN1 (not shown), were not differentially expressed across genotypes or conditions. This either indicates that SnRK1 signalling does not play a role in salt-stressed potato leaves, or that SnRK1 targets may differ between leaf and tubers (see Debast et al., 2011). In support of a role of SnRK1 signalling during salt stress, it has been shown that SnRK1 is engaged in the regulation of vacuolar potassium transport during salt challenge in the ice plant *Mesembryanthemum crystallinum* (Chiang et al., 2013), that the SnRK1 target transcription factor bZIP11 is induced in salt-stressed *Arabidopsis* leaves (Weltmeier et al., 2009), and that SnRK1 is activated in other abiotic stress conditions like drought, cold, or hypoxia (see O’Hara et al., 2013 for a compilation), which also influence the cellular osmotic potential. While the accumulation of sugars and the signalling metabolite T6P declined in SXD:RNAi leaves upon salt stress, *AsnS1*, *SuSy2*, and *UDPglcE* were increased about 2-fold more in the transgenic plants than in the wild type. Thus, the response of these three SnRK1 target genes is consistent with a decline in sugar and T6P upon salt stress in the transgenic plants.

Based on our results, we propose a chain of events that can explain the strong reduction in sucrose export in SXD:RNAi leaves during salt stress. The extensive and continuous accumulation of sugars in the vacuole of salt-stressed leaf cells probably leads to a drop in cytosolic sucrose concentration, which in turn engages SnRK1 signalling to relay carbon shortage. Tocopherol-deficient leaves apparently produce a stronger SnRK1 signal than the wild type, which might result in a more pronounced attenuation of sucrose export from SXD:RNAi source leaves. While the transcriptional repression of SUT1 as a key player of phloem loading is similar in all salt-stressed genotypes, SUT1 activity might be diminished in the transgenic plants due to interaction with other proteins like Snakin-1. This hypothesis seems especially attractive, since the cysteine-rich protein Snakin-1 has the potential to integrate redox signals (Krügel and Kühn, 2013), which might already be altered in unstressed SXD:RNAi leaves due to increased pools of the soluble antioxidants ascorbate and glutathione. Increased retention of sugars in salt-stressed SXD:RNAi leaf cells does not ameliorate sugar availability, but rather dampens photosynthetic carbon assimilation. In addition, nocturnal starch mobilization may also be abrogated in the transgenic plants as a consequence of altered SnRK1 signalling (as summarized in O’Hara et al., 2013).

It remains a challenge for future studies to resolve how tocopherol deficiency effects increased sensitivity of SXD:RNAi plants to SnRK1 signalling during salt-stress adaptation.

**SXD:RNAi leaves remain more virescent under salt stress**

In our study, major differences between tocopherol-deficient and wild-type potato plants were displayed under salt-stress conditions. Because tocopherol is a well-known antioxidant, with a major role in protecting unsaturated fatty acids and the photosynthetic apparatus from oxidative damage, it would be expected that leaves of tocopherol-deficient plants suffer from enhanced oxidative stress upon salt challenge. However, salt-stressed SXD:RNAi leaves showed unaltered photochemical efficiency of PSII and MDA levels as well as comparable ascorbate and glutathione redox states relative to wild-type plants. However, tocopherol deficiency in SXD1:RNAi plants was compensated by an increase in ascorbate and glutathione pools during salt stress. A similar compensatory response was found by Kanwischer et al. (2005) in *Arabidopsis vte1* mutants. Taken together, this all indicates that leaves of tocopherol-deficient plants did not suffer from more severe oxidative stress than wild-type plants under saline conditions, as was also evident from the phenotype of the stressed plants. An analysis of phytohormone contents supports the macroscopic observation that leaf senescence is delayed in salt-stressed transgenic plants compared to the wild type. It is well known that SA can accumulate in damaged or necrotic tissue during leaf senescence, but SA contents were reduced by 50% in salt-stressed leaves of SXD1:RNAi in comparison to unstressed controls. Furthermore, the contents of ACC and ABA, which can both act as triggers of leaf senescence (Gan and Amasino, 1997), remained low in salt-stressed SXD:RNAi leaves, while their contents were particularly elevated in bottom leaves of salt-stressed, wild-type plants. Concomitantly, the cytokinin zeatin riboside was most pronouncedly elevated in bottom leaves of tocopherol-deficient plants under salt challenge. The increased longevity of SXD:RNAi leaves under salt stress can be explained by a synergism of increased soluble antioxidants, reduced sodium uptake as a consequence of a diminished transpiration rate, and elevated proline contents.

**Conclusion**

Our study has led to two major results. First, it has revealed that sugar export from tocopherol-deficient leaves can be reduced in the absence of excessive callose plugging of plasmodesmata in the vasculature. Our data favour the idea that an increased sensitivity to SnRK1 signalling impedes sucrose export in SXD:RNAi leaves. Second, despite a severe reduction in tuber yield, tocopherol-deficient potato leaves did not exhibit physiological signs of elevated oxidative stress upon salt challenge. The delayed senescence of the salt-stressed transgenic plants can be explained by reduced sodium uptake, and by enhanced accumulation of the osmoprotectant proline and soluble antioxidants.

It will remain a challenge for the future to entirely unravel the molecular basis of reduced sucrose export in these salt-stressed, tocopherol-deficient potato plants.

**Supplementary material**

**Supplementary Figure S1.** Tuber starch content of tocopherol-deficient potato plants at two time points during salt-stress exposure.

**Supplementary Figure S2.** Effects of salt treatment on potassium content and the potassium/sodium ratio in source leaves.
Supplementary Figure S3. Leaf osmolality of salt-treated and control plants.

Supplementary Figure S4. Effects of salt treatment on the redox state of foliar soluble antioxidants and lipid peroxidation in source leaves of StSXD1-silenced potato plants.

Supplementary Figure S5. Phenotype of 11-week-old tocopherol-deficient potato plants compared to the wild type after the end of 19 days of salt stress.

Supplementary Figure S6. Contents of intermediates of central carbon metabolism in source leaves of SXD1 RNAi transgenic potato plants upon salt exposure.

Supplementary Figure S7. Soluble and cell-wall bound invertase activity in StSXD1-silenced potato plants after 19 days of salt stress.

Supplementary Figure S8. Phytohormone contents in source leaves of tocopherol-deficient potato plants exposed to 19 days salt treatment.

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References


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