

# Impact of Sulfate Salinity on the Uptake and Metabolism of Sulfur in Chinese Cabbage

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**Abstract** Increasing soil salinity is a major threat to crop production in many agricultural areas throughout the world. Although sodium chloride (NaCl) is one of the most abundant salts in soils, others viz. sulfate salts may also be present in high concentrations in some soil types. Sulfate salts, e.g. Na<sub>2</sub>SO<sub>4</sub>, are still widely under-represented amongst salt stress studies and the mechanism of its toxicity is poorly understood. Exposure of Chinese cabbage to Na<sub>2</sub>SO<sub>4</sub> already reduced growth at levels  $\geq 20$  mM, accompanied by an increase in the total sulfur content of both roots and shoots, which in the shoot for a greater part could be ascribed to an accumulation of sulfate. Moreover, there was an increase in the total water-soluble non-protein thiol content (glutathione) in roots and shoots. Enhanced sulfur metabolite levels (sulfate, glutathione) would down-regulate the expression and activity of the sulfate transporters and APS reductase (glutathione). Indeed, Na<sub>2</sub>SO<sub>4</sub> exposure resulted in a down-regulation of the sulfate uptake capacity of the roots at  $\geq 5$  mM, whereas the transcript level of the sulfate transporters Sultr1;2 and Sultr4;1 and APS reductase in the roots was reduced at  $\geq 20$  mM. Apparently in the shoot this regulatory signal transduction pathway was overruled by the toxic effects of Na<sub>2</sub>SO<sub>4</sub>, since in contrast to the roots, the transcript levels of Sultr4;1 and APS reductase were enhanced in the shoot at  $\geq 30$  mM and  $\geq 5$  mM Na<sub>2</sub>SO<sub>4</sub>, respectively.

Salt tolerance of plants and its improvement is one of the most prominent topics in crop research due to both the acuteness of the threats of salinity for agriculture and the complex physiology that underlies salt tolerance in plants (Flowers 2004; Parida and Das 2005; Peleg et al. 2011). Sulfur metabolism may have significance in the tolerance of plants to salinity. For instance, salt stress may result in an enhanced glutathione level, which presumably has adaptive significance in the protection of

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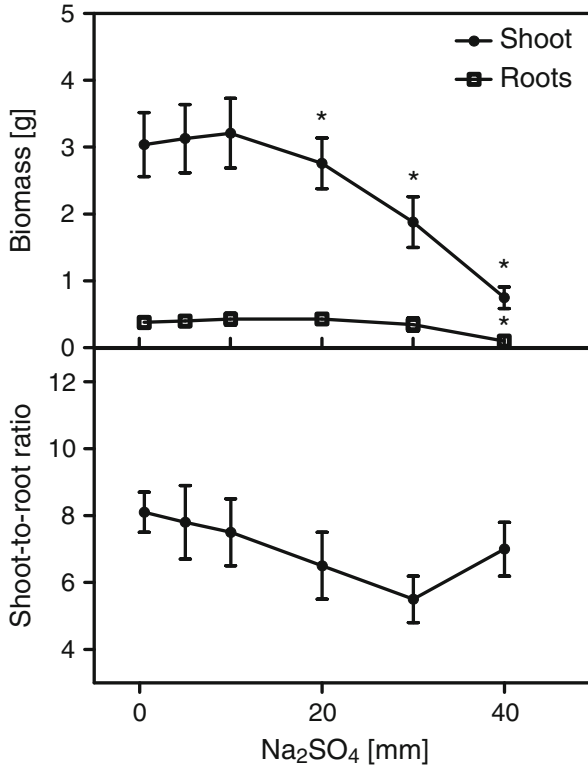
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plants against reactive oxygen species (Noctor et al. 1998; Mittler 2002; Tausz et al. 2004; Szalai et al. 2009). The production of reactive oxygen species may be increased if  $\text{Na}^+$  accumulates in other cell compartments than the vacuole (Zhu et al. 2007). The enhanced glutathione levels appeared to be coupled to increased levels of  $\text{Na}^+$  in the cytosol, since an enhanced level of glutathione (and cysteine) was absent in transgenic *Brassica napus* that over-expressed a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter upon  $\text{NaCl}$  exposure (Ruiz and Blumwald 2002). However, the most important mechanisms in plants to avoid  $\text{Na}^+$  toxicity are the so-called includer/excluder strategies where  $\text{Na}^+$  is actively transported either back to the outside of the cell/plant and/or into the vacuole in order to prevent cytosolic  $\text{Na}^+$  accumulation (Blumwald 2000; Munns and Tester 2008). These strategies also evolved in halophytes, accompanied by anatomical adaptations such as succulence (increased cell size by salt accumulation in vacuoles) or specialized organs for salt exclusion via the leaves (salt glands). Another crucial factor is the cellular  $\text{K}^+/\text{Na}^+$  ratio, which needs to be kept high in order to prevent an inhibition of enzymes regulated by  $\text{K}^+$  (Maathuis and Amtmann 1999; Tester and Davenport 2003; Chen et al. 2005; Zhu 2007; Cuin et al. 2008). In addition to  $\text{NaCl}$  plants also may have to deal with  $\text{Na}_2\text{SO}_4$  salinity (Garcia and Hernandez 1996) and many areas are even dominated by sulfate salts (Chang et al. 1983; Keller et al. 1986). Although salt stress is usually mainly attributed to  $\text{Na}^+$  toxicity many studies showed that the accompanying anion might change the severity of the toxicity (e.g. Renault et al. 2001). In many species it had been shown that sulfate salinity might be more toxic than chloride salinity (Eaton 1942; Paek et al. 1988; Bilski et al. 1988; Datta et al. 1995; Renault et al. 2001). The physiological basis of the toxic effects of sulfate salinity has still to be resolved. In this study the impact of  $\text{Na}_2\text{SO}_4$  salinity on the uptake, distribution and assimilation of sulfate was studied in Chinese cabbage.

Chinese cabbage (*Brassica pekinensis* (Lour.) Rupr. cv. Kasumi F1 (Nickerson-Zwaan, Made, The Netherlands)) was germinated in vermiculite. Ten day-old seedlings were pre-grown on a 10 % Hoagland nutrient solution for 2 days and subsequently grown on a 25 % Hoagland nutrient solution (pH 5.9; for composition see Koralewska et al. 2007) at  $\text{Na}_2\text{SO}_4$  concentrations of 0.5, 5, 10, 20, 30 and 40 mM in 30 l containers (ten sets per container, three plants per set) which were placed in a climate controlled room for 11 days. Day and night temperatures were 21 and 17 °C ( $\pm 1$  °C), respectively, relative humidity was 60–70 % and the photoperiod was 14 h at a photon flux rate of  $230 \pm 20 \mu\text{mol m}^{-2} \text{s}^{-1}$  (within the 400–700 nm range) at plant height, supplied by Philips HPI-T (400 W) lamps. After 11 days of exposure plants were harvested and shoot and root fresh weight were determined. For determination of the dry matter content, fresh plant tissue was dried at 80 °C for 24 h. For anion analysis, roots were rinsed in ice-cold de-mineralized water (for  $3 \times 20$  s) to remove sulfate from the free space. Shoots and roots were separated, weighed, frozen in liquid  $\text{N}_2$  and stored at  $-20$  °C until further analysis. Anions were extracted from frozen plant material and determined refractometrically after separation by HPLC (Shahbaz et al. 2010). Water-soluble non-protein thiols were extracted from freshly harvested plant tissue (Shahbaz et al. 2010) and the total water-soluble non-protein content was determined colorimetrically according to De Kok et al. (1988). For

determination of the total sulfur and nitrogen contents, oven-dried plant material was pulverized by a Retsch Mixer-Mill (type MM2; Haan, Germany). Total sulfur content was determined with the barium sulfate precipitation method (Koralewska et al. 2008) and total nitrogen content was determined according to a modified Kjeldahl method (Barneix et al. 1988). Sulfate uptake capacity was determined as described by Koralewska et al. (2007). Three sets of plants (three plants per set) per treatment were transferred to 25 % Hoagland solution labeled with  $^{35}\text{S}$ -sulfate ( $2 \text{ MBq l}^{-1}$ ) and incubated for 30 min at  $30^\circ\text{C}$ , at  $0.5 \text{ mM Na}_2\text{SO}_4$ . Subsequently, plants were removed and roots rinsed in ice-cold non-labeled nutrient solution for  $3 \times 20 \text{ s}$ . Roots and shoots were separated and digested in  $1 \text{ N HCl}$  at room temperature for 7 days. The extracts were filtered through one layer of Miracloth and  $100 \mu\text{l}$  of the filtrate was mixed with  $1 \text{ ml}$  Emulsifier Scintillator Plus (Perkin Elmer, Boston, MA, USA). Radioactivity was measured with a liquid scintillation counter (TRI-CARB 2000 CA Liquid Scintillation Analyzer, Perkin Elmer, Waltham, MA, USA). Total RNA from roots and shoots was isolated by a method based on Verwoerd et al. (1989), which involved an additional phenol-chloroform-isoamyl alcohol extraction of the aqueous phase after the first centrifugation, or by using TRI REAGENT™ (SIGMA), a mixture of guanidine thiocyanate and phenol in a mono-phase solution. The final air-dried pellet was dissolved in an appropriate volume of diethyl pyrocarbonate-treated water. The quality of the RNA preparations was checked by electrophoresis of a  $2 \mu\text{g}$  aliquot on a  $1 \%$  (w/v) Tris-acetate/agarose gel. The concentration was calculated from the absorbance at  $260 \text{ nm}$  in water. Determination of the expression of sulfate transporter was carried out according to Church and Gilbert (1984), with pre-hybridization and hybridization at  $65$  and  $60^\circ\text{C}$ , respectively. Ten  $\mu\text{g}$  of total RNA per slot was separated on a  $1.2 \%$  (w/v) agarose/formaldehyde gel and blotted onto a positively charged nylon membrane (Hybond-N+). Sequence diversity, especially in the 3' non-coding region, allowed the use of partial cDNA fragments for gene-specific hybridization to the respective Brassica sulfate transporter mRNA. The cDNA fragments were labeled with  $^{32}\text{P}$ -dCTP and used as hybridization probes. After hybridization with probes for sulfate transporters, membranes were washed at  $65^\circ\text{C}$  twice with  $2 \times \text{SSC}$ ,  $0.1 \%$  SDS for 5 and 30 min, once with  $1 \times \text{SSC}$ ,  $0.1 \%$  SDS and twice with  $0.1 \times \text{SSC}$ ,  $0.1 \%$  SDS for at least 30 min each, and exposed to Kodak BioMax MS film or to Cyclone MultiPurpose Phosphor Screen (Perkin Elmer, UK). Statistical analysis was performed with an unpaired Student's t-test.

Although in natural ecosystems Brassicaceae species occur in dry and saline habitats and even in extreme sulfur-enriched gypsum-bearing soils (Ernst 1990; Dixon 2007), some of the modern cultivated hybrids and cultivars appear to be very sensitive to sulfate salinity. The results of the present study showed that the shoot growth of Chinese cabbage was already inhibited at  $20 \text{ mM Na}_2\text{SO}_4$  (Fig. 1). The shoot growth was slightly more susceptible to salt stress than root growth and the latter was only significantly reduced at  $40 \text{ mM Na}_2\text{SO}_4$ , resulting in a slight decrease in the shoot to root ratio at  $\geq 30 \text{ mM Na}_2\text{SO}_4$ . Dry matter content of shoots was enhanced at  $\text{Na}_2\text{SO}_4$  concentrations  $\geq 30 \text{ mM}$ , up to twofold at  $40 \text{ mM}$ , whereas that of roots was only slightly enhanced at  $40 \text{ mM Na}_2\text{SO}_4$  (Fig. 2).

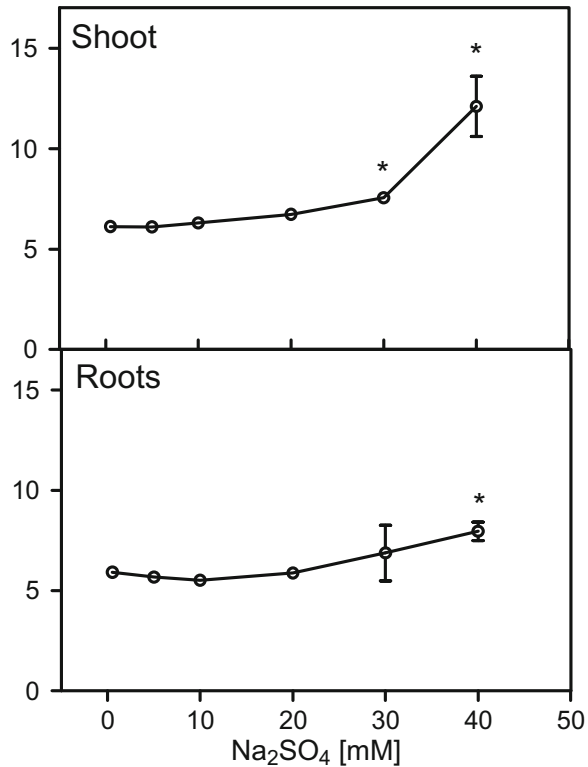


**Fig. 1** The impact of Na<sub>2</sub>SO<sub>4</sub> salinity on growth and shoot to root ratio of Chinese cabbage. Data represent the mean of two experiments with 15–19 measurements with three plants in each ± SD (\* =  $p < 0.01$ ; unpaired Student's t-test)

Exposure of plants to Na<sub>2</sub>SO<sub>4</sub> salinity resulted in an increase in the total sulfur content of both roots and shoots (Fig. 3), which in the shoot for a greater part could be ascribed to an accumulation of sulfate (Fig. 4). The sulfate content increased gradually with the Na<sub>2</sub>SO<sub>4</sub> concentration, but it was strongly enhanced at 40 mM Na<sub>2</sub>SO<sub>4</sub> and its content in roots and shoots was increased 1.5-fold and fourfold, respectively. Apparently, the regulatory control of the uptake of sulfate by the roots was overruled at Na<sub>2</sub>SO<sub>4</sub> concentrations exceeding 30 mM. In both shoots and roots, total nitrogen and nitrate decreased with the Na<sub>2</sub>SO<sub>4</sub> concentration, indicating that the uptake and assimilation of nitrate was negatively affected by sulfate salinity (Figs. 3 and 4). Similar to previous observations there was apparently no direct linkage between the uptake and assimilation of sulfate and nitrate (Stulen and De Kok 2012).

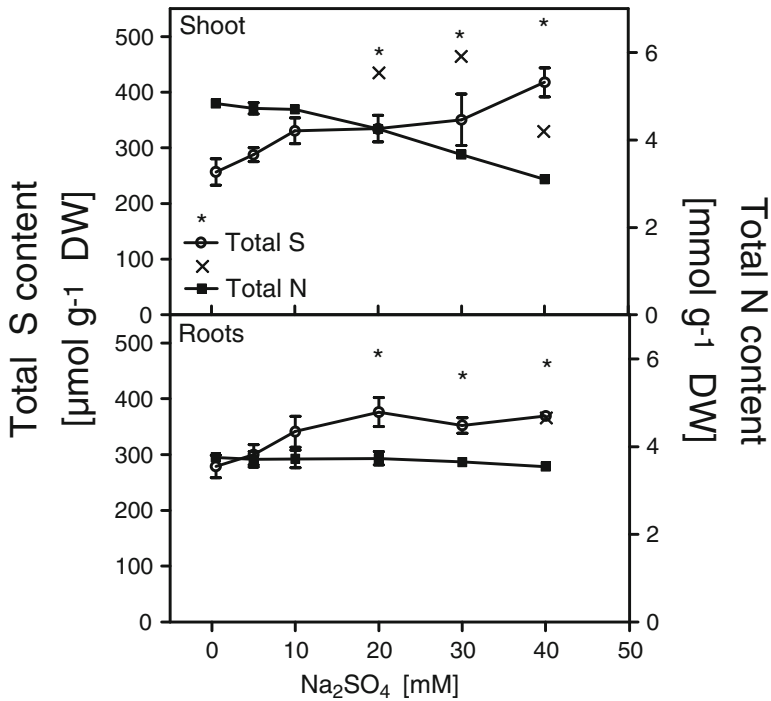
Similar to observations with NaCl (Ruiz and Blumwald 2002), Na<sub>2</sub>SO<sub>4</sub> salinity resulted in an increase in the total water-soluble non-protein thiol content (presumably GSH) in roots and shoots at concentrations  $\geq 20$  mM (Fig. 5). Evidently this increase remained relatively low and only at toxic Na<sub>2</sub>SO<sub>4</sub> concentrations, and at

**Fig. 2** The impact of  $\text{Na}_2\text{SO}_4$  salinity on dry matter content of shoot to roots of Chinese cabbage. Data represent the mean of two experiments with three measurements with three plants in each  $\pm$  SD (\* =  $p < 0.01$ ; unpaired Student's t-test)



sulfate levels in shoots fourfold higher than that of the control, a substantial increase of thiol content (twofold) occurred. From the present data, the increased thiol/glutathione level appears to be a consequence of the excessive sulfate accumulation and not an adaptive protective response against salinity.

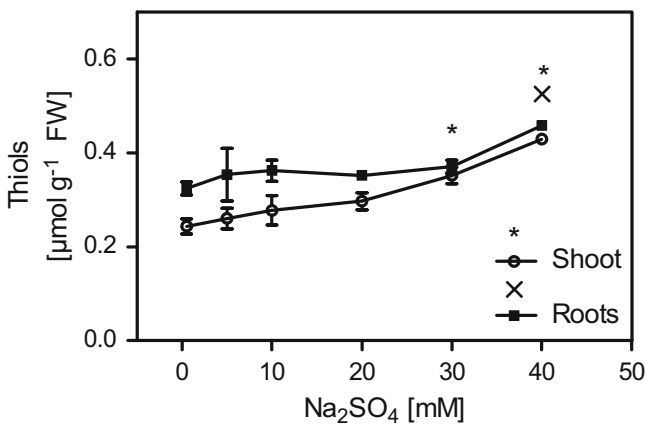
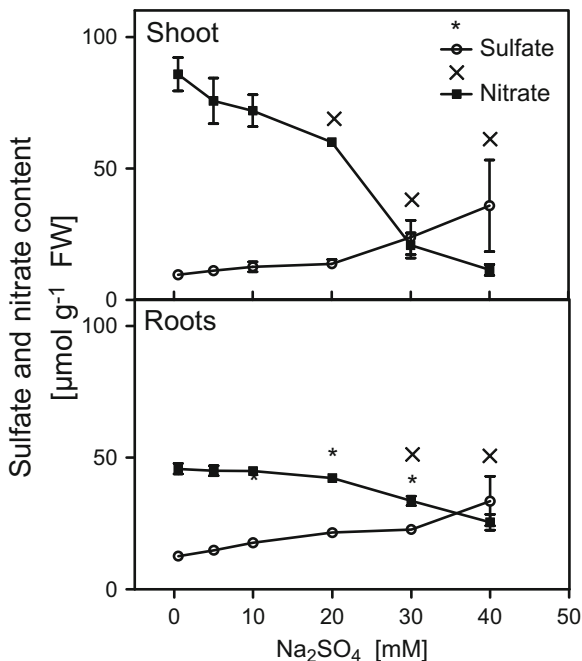
Sulfate uptake plays a major role in the control of plant sulfur homeostasis (Vauclare et al. 2002). The uptake and distribution of sulfate is mediated by distinct sulfate transporters, which activity may be controlled at a transcriptional, translational and/or post-translational level, and is regulated by the plant sulfur requirement for growth (Hawkesford and De Kok 2006; De Kok et al. 2011). Sulfate salinity had a substantial effect on the expression and activity of the sulfate transporters and the expression of APS reductase of Chinese cabbage (APR; Fig. 6). However, there were considerable differences in response of the different sulfate transporters and APS reductase between roots and shoots of Chinese cabbage. The Group 1 transporters are responsible for the primary uptake of sulfate by the root and in sulfate-sufficient Brassica species only Sultr1;2 is expressed (Hawkesford and De Kok 2006; Koralewska et al. 2007, 2008, 2009; De Kok et al. 2011). Also upon  $\text{Na}_2\text{SO}_4$  salinity Sultr1;2 was the sole Group 1 sulfate transporter expressed in roots and the transcript levels of Sultr1;1 were negligible (Fig. 6).  $\text{Na}_2\text{SO}_4$  salinity resulted in a decreased expression of Sultr1;1 in the roots at  $\geq 30$  mM, whereas the



**Fig. 3** The impact of  $\text{Na}_2\text{SO}_4$  salinity on total sulfur and nitrogen content of shoots and roots of Chinese cabbage. Data represent the mean of three measurements with 12 plants in each  $\pm$  SD (\*/ $\times$  =  $p < 0.01$ ; unpaired Student's t-test)

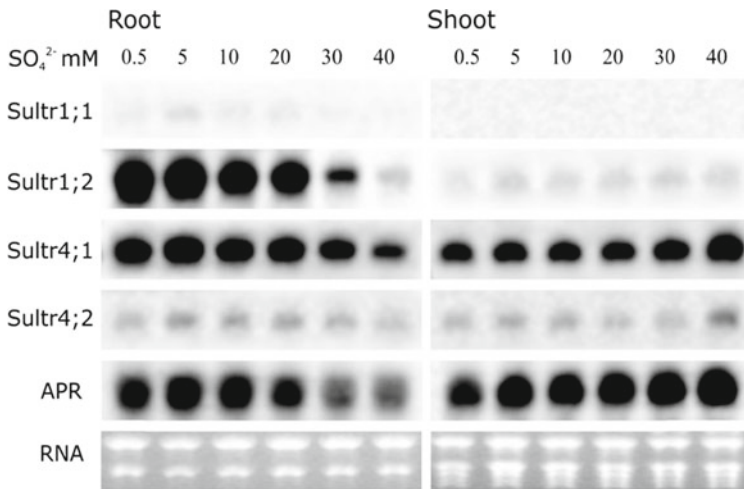
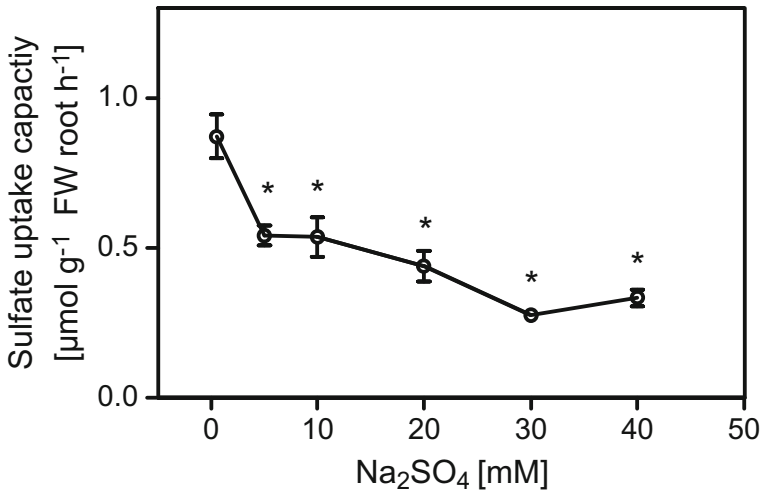
sulfate uptake capacity was already decreased  $\geq 5$  mM. The latter further decreased with the  $\text{Na}_2\text{SO}_4$  concentration and was reduced more than 2.5-fold at 40 mM (Fig. 6). Apparently,  $\text{Na}_2\text{SO}_4$  salinity affected the regulation of the sulfate transporters in the roots already at lower concentrations at translational and/or post translational than at transcriptional level. The Group 4 transporters are involved in the vacuolar efflux of sulfate (Hawkesford 2003; Kataoka et al. 2004; Hawkesford and De Kok 2006; De Kok et al. 2011) and there was also a decrease in the transcript level of Sultr4;1 in roots at 40 mM  $\text{Na}_2\text{SO}_4$ , whereas Sultr4;2 was hardly expressed at all. The latter was in agreement with previous observations that Sultr1;1 and Sultr4;2 were only expressed in sulfate-deprived Brassica tissue (Koralewska et al. 2009; De Kok et al. 2012; Shahbaz et al. 2014). The transcript level of APR, the key regulating enzyme in the sulfate reduction pathway (Hawkesford and De Kok 2006; De Kok et al. 2011) was also reduced in the roots at  $\geq 30$  mM (Fig. 6). Sultr1;1 was hardly and Sultr4;2 was only slightly expressed in both roots and shoots. Sulfate salinity only resulted in an increased transcript level of Sultr4;1 and Sultr4;2 at 40 mM  $\text{Na}_2\text{SO}_4$ , whereas that of APR was increased at  $\geq 5$  mM  $\text{Na}_2\text{SO}_4$  (Fig. 6). Of the Group 1 and 4 transporters, only Sultr4;2 was substantially expressed in the shoot of Chinese cabbage (Fig. 5). Contrary to the observations in the root, its

**Fig. 4** The impact of Na<sub>2</sub>SO<sub>4</sub> salinity on sulfate and nitrate content of shoot and roots of Chinese cabbage. Data represent the mean of three measurements with three plants in each ± SD (\*/\*x = p<0.01; unpaired Student's t-test)



**Fig. 5** The impact of Na<sub>2</sub>SO<sub>4</sub> salinity on total water-soluble non-protein thiols of shoot and roots of Chinese cabbage. Data represent the mean of three measurements with three plants in each ± SD (x = p<0.01; unpaired Student's t-test)

transcript levels increased at ≥30 mM Na<sub>2</sub>SO<sub>4</sub>, together with that of APS reductase (Fig. 6). The latter needs to be further investigated. It has been suggested that sulfate itself, or reduced sulfur compounds, may have a role in the regulation of expression and activity of the sulfate transporters and APS reductase (Hawkesford and De Kok 2006; De Kok et al. 2011). For instance, high tissue levels of these compounds



**Fig. 6** The impact of Na<sub>2</sub>SO<sub>4</sub> salinity on sulfate uptake capacity and gene expression of sulfate transporters (Sultr) and APS reductase (APR; Northern blot analysis) of shoot and roots of Chinese cabbage. Equal RNA loading was determined by ethidium bromide staining of gels (shown in the *bottom* panels). Data on sulfate uptake capacity represent the mean of three measurements with three plants in each ±SD (\* = p<0.01; unpaired Student’s t-test)

would down-regulate the expression and activity of the sulfate transporters (sulfate, glutathione) and APS reductase (glutathione). Indeed, in the roots this relationship between the content of these sulfur compounds and the expression and activity of the sulfate transporters and expression of APS reductase does exist although in the shoot the toxic effects of Na<sub>2</sub>SO<sub>4</sub> salinity apparently overruled this regulatory signal transduction pathway.



Sulfate salinity was described as having a greater inhibitory effect on growth than chloride salinity in wheat (Datta et al. 1995), sugar beet and tomato (Eaton 1942), wild potato (Bilski et al. 1988), pepper (Navarro et al. 2003) and on germination in barley (Huang and Redmann 1995), alfalfa (Redmann 1974) and wheat (Hampson and Simpson 1990). Comparative studies within *Brassica* species are still very scarce. Additionally, the results of a study by Paek et al. (1988) on calli of *B. campestris* revealed that  $\text{Na}_2\text{SO}_4$  had a stronger negative impact on biomass. The authors of the study also noted that sulfate accumulated much less under  $\text{Na}_2\text{SO}_4$  than chloride under  $\text{NaCl}$  salinity. This unequal uptake of  $\text{Na}^+$  and its anion under sulfate salinity could explain the increased inhibitory effect on growth (also concluded by Meiri et al. 1971; Navarro et al. 2003). Another observation from older studies is that excess sulfate inhibits calcium uptake (Hayward and Wadleigh 1949) but this also holds true for  $\text{NaCl}$  and the application of additional calcium usually leads to an amelioration of salt stress (Cramer 2002; Kaya et al. 2002; Shabala et al. 2006). However, Johansen and Loneragan (1975) observed that  $\text{Na}_2\text{SO}_4$  reduced 75 % of  $\text{K}^+$  uptake compared to the absence of  $\text{Na}^+$  while the same concentration of  $\text{NaCl}$  reduced it only by 50 %. A link to cation homeostasis therefore seems likely. Interestingly,  $\text{Na}^+$  accumulated mainly in shoots when jack pines were exposed to  $\text{NaCl}$  whereas it mainly accumulated in the roots under  $\text{Na}_2\text{SO}_4$  salinity (Apostol et al. 2002). This is another hint that the translocation to the shoot and its control might be a crucial process under salt stress. Besides promoting the toxicity of  $\text{Na}^+$ , sulfate could also have direct toxic effects (Visscher et al. 2010).

Salt tolerance is known to be a physiological complex and trait, which challenges attempts at improvement. As *Brassica* is a diverse genus with high agricultural importance, many efforts have been made to identify and develop salt tolerant cultivars. Salt tolerance in *B. napus*, for example, could be increased tremendously in transgenic plants with an enhanced  $\text{Na}^+$  accumulation in the vacuole (Zhang et al. 2001). In another transgenic approach, plants of *B. juncea* with an introduced bacterial pathway for the synthesis of glycine-betaine showed increased germination rates and seedling growth (Prasad et al. 2000) but the occurrence of sulfate salinity and its increased toxicity compared with chloride salinity also suggests correlations of salt tolerance with sulfate uptake, assimilation and whole plant distribution. More research needs to be carried out on the toxicity of excessive sulfate and its possible role in exacerbating  $\text{Na}^+$  toxicity.

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