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Impact of chloride (NaCl, KCl) and sulphate (Na₂SO₄, K₂SO₄) salinity on glucosinolate metabolism in *Brassica rapa*

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Abstract

Seedlings of *Brassica rapa* were exposed to increasing concentrations of NaCl, Na₂SO₄, KCl and K₂SO₄ to study the effect on glucosinolate content, composition and expression of genes of the glucosinolate biosynthetic pathway and associated transcription factors. Growth was inhibited stronger by sulphate salts and strongest by Na₂SO₄. Aliphatic, indolic and aromatic glucosinolates were differently affected by the salts in shoot and roots. A decrease in aliphatic glucosinolates in shoots of plants exposed to chloride salts corresponded to a decreased gene expression of a key enzyme for their biosynthesis. Likewise, an increased level of indolic and aromatic glucosinolates by Na₂SO₄ coincided with an increased gene expression of enzymes responsible for the biosynthesis of these glucosinolates. The results show that changes in glucosinolate content and composition under salt stress depend on the ionic composition of the salts. This has implications for the quality of *B. rapa* grown under different types of salinity.

KEYWORDS

Brassica rapa, chloride salinity, gene expression, glucosinolate metabolism, salt stress, sulphate salinity

1 | INTRODUCTION

Brassicaceae may contain high contents of glucosinolates, secondary sulphur compounds, which are responsible for their typical flavour and might have potentially anti-carcinogenic properties (Del Carmen Martinez-Ballesta, Moreno, & Carvajal, 2013; Halkier & Gershenzon, 2006; Wittstock & Halkier, 2002). Moreover, breakdown products of glucosinolates, especially isothiocyanates, have been considered to be involved in plant defence against pathogens and herbivores (Ahuja & Rohloff, 2010; Bones & Rossiter, 1996).

Glucosinolates are synthesized from amino acids or their chain elongated derivatives via the action of two cytochromes P450, a glucosyl transferase and a sulfotransferase. The basic structure of these secondary sulphur compounds consists of a *beta*-D-thioglucoside group, an N-hydroximinisulphate ester and a variable side chain (Halkier & Gershenzon, 2006). Glucosinolates can be grouped into three chemical classes: aliphatic, indolic and aromatic glucosinolate (Halkier & Gershenzon, 2006; Kliebenstein, Rowe, & Denby, 2005).

Different genes are involved in biosynthesis of the core structure of glucosinolates, viz. cytochrome P450 monooxygenase enzymes including CYP79F and CYP83A1, and in chain elongation of methionine, which is catalysed by methyl thioalkylmalate synthase enzymes, MAM1 and MAM3. In addition, some genes are involved in the biosynthesis of the core structure of indolic glucosinolates such as CYP79B2, CYP79B3 and CYP83B1 (Chen et al., 2003; Mikkelsen, Hansen, Wittstock, & Halkier, 2000; Naur et al., 2003; Reintanz et al., 2001). Several MYB transcription factors belonging to the R2R3-type MYB family have been identified to positively regulate the biosynthesis of aliphatic (MYB28, MYB29 and MYB76) and indolic (MYB34, MYB51 and MYB122) glucosinolates (Frerigmann & Gigolashvili, 2014; Gigolashvili, Yatushevich, Berger, Müller, & Flügge, 2007; Gigolashvili, Berger, et al., 2007).

The physiological function of glucosinolates in the plants is still largely unclear (Del Carmen Martinez-Ballesta et al., 2013). It has been suggested that glucosinolates might play a role in sulphur storage in plants (Bloem, Haneklaus, & Schnug, 2007; Falk, Tokuhisa, &

Gershenzon, 2007; Hirai et al., 2004, 2005). It was recently shown, however, that in sulphur-deficient *Brassica* plants, the sulphur from glucosinolates was hardly redistributed and that upon exposure of plants to excessive sulphur glucosinolates did not function as sulphur storage (Aghajanzadeh, Hawkesford, & De Kok, 2014; Aghajanzadeh, Kopriva, Hawkesford, Koprivova, & De Kok, 2015).

The glucosinolate content and composition may be affected by nutrient supply, especially sulphur and nitrogen (Kim, Matsuo, Watanabe, & Watanabe, 2002), but also by environmental factors such as temperature, length of the photoperiod (Charron & Sams, 2004), season (Rosa & Rodrigues, 2001), drought (Radovich, Kleinhenz, & Streeter, 2005) and salinity (López-Berenguer, Martínez-Ballesta, Moreno, Carvajal, & García-Viguera, 2009). Salinity is one of the major environmental constraints affecting plant growth, development and physiology (Munns & Tester, 2008; Zhu, 2001). Na^+ and Cl^- both are commonly considered the most important toxic ions to plants under salt stress (Hasegawa, Bressan, Zhu, & Bohnert, 2000), and studies focused on the effect of NaCl on glucosinolates (Guo, Yuan, & Wang, 2013; Keling & Zhujun, 2010; Yuan, Wang, Guo, & Wang, 2010). However, a higher toxicity of SO_4^{2-} over Cl^- and Na^+ has been observed in *Brassica rapa* in a recent comparative study exposing plants to NaCl, Na_2SO_4 , KCl and K_2SO_4 (Reich et al., 2016). The effect of these different salts on growth, mineral nutrient composition and their impact on primary sulphur metabolites and their metabolism has been studied in detail (Reich et al., 2016). In this study, the impact of NaCl, Na_2SO_4 , KCl and K_2SO_4 salinity on glucosinolate metabolism was studied in *B. rapa*. In addition to their impact on glucosinolate content and composition, also the transcript levels of the genes involved in the biosynthesis and the transcription factors involved the regulation of biosynthesis of aliphatic and indolic glucosinolates were investigated.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

Seeds of *B. rapa* cv. Komatsuna (Van der Wal, Hoogeveen, The Netherlands) were germinated in vermiculite in a climate-controlled room. Day and night temperatures were 21 and 18°C ($\pm 1^\circ\text{C}$), respectively, and relative humidity was 60%–70%. The photoperiod was 14 h at a photon fluence rate of $300 \pm 20 \mu\text{mol m}^{-2} \text{s}^{-1}$ (400–700 nm) at plant height, supplied by Philips GreenPower LED (deep red/white 120) production modules.

Ten-day-old seedlings were transferred into a 25% Hoagland nutrient solution (pH 5.9) consisting of 1.25 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 1.25 mM KNO_3 , 0.25 mM KH_2PO_4 , 0.5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 11.6 μM H_3BO_3 , 2.4 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.24 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.08 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.13 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ and 22.5 μM Fe^{3+} -EDTA in 30-L containers (20 sets of plants per container; three plants per set) and pre-grown without additional salt for 3 days, and then, salts were gradually increased during the following 3 days. For NaCl and KCl, the steps were 25, 50 and 100 mM, and for Na_2SO_4 and K_2SO_4 , the steps were 12.5, 25 and 50 mM. For half of the plants, the final concentrations were 50 mM for the chloride and 25 mM for the sulphate

salts (low salt concentrations), for the other half 100 mM for the chloride and 50 mM for the sulphate salts (high salt concentrations). Seedlings were grown in these concentrations for nine and eight more days, respectively. Then 21-day-old seedlings were harvested. Roots were separated from the shoots, and their fresh weight was determined immediately.

For determination of the dry matter content, plant tissue was dried at 80°C for 24 hr. For glucosinolate analysis, plant material was frozen immediately in liquid N_2 and stored at -80°C .

2.2 | Glucosinolate content and composition

The glucosinolates contents were analysed with RP-HPLC and a UV detection method, as described by Mugford et al. (2009) and Mugford, Matthewman, Hill, & Kopriva (2010). The glucosinolates were extracted from 50 mg freeze-dried plant material in 1.5 ml 70% methanol (v/v) containing the internal standard 4-hydroxybenzylglucosinolate (Roth, Germany) at 70°C for 45 min. The extract was centrifuged for 5 min at 13,000 g. After centrifugation, 1 ml of the supernatant was loaded on a DEAE Sephadex A-25 column. The column was washed with 2×0.5 ml water and 20 mM sodium acetate. After washing, the column was treated overnight with sulfatase to convert the glucosinolates to their desulphated derivatives. The desulphated glucosinolates were eluted off the column with 2×0.5 ml water and were separated by reverse-phase HPLC and quantified by UV absorption at 229 nm relative to the internal standard using response factors recalculated from Brown, Tokuhisa, Reichelt, and Gershenzon (2003) (response factor of sinalbin is 0.4; that is, the sinigrin-derived factors were multiplied by 2.5). The detection limit of the method is 3 nmol/g for aliphatic and aromatic glucosinolates and ca. 0.75 nmol/g for indolic glucosinolates, the recovery is >90%, and the variation (as RSD percentage) is <10%.

2.3 | RNA extraction

Total RNA was isolated by a modified hot phenol method (Verwoerd, Dekker, & Hoekema, 1989). Frozen ground plant material was extracted in hot (80°C) phenol/extraction buffer (1:1, v/v), 1 g/ml. The extraction buffer contained 0.1 M Tris-HCl, 0.1 M LiCl, 1% SDS (w/v), 10 mM EDTA, pH 8.0. After mixing, 0.5 ml of chloroform-isoamyl alcohol (24:1, v/v) was added. After centrifugation (13,400 g) for 5 min at 4°C, the aqueous phases were transferred to new tube. After adding an equal volume of chloroform and isoamyl alcohol, the total RNA was precipitated by 4 M LiCl overnight at 4°C. Total RNA was collected and washed with 70% ethanol. Possible genomic DNA contamination was removed with a DNAase treatment step (Promega, USA). Phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol were used for further purification, and total RNA was precipitated by ethanol and dissolved in diethylpyrocarbonate-treated water. The quantity and quality of RNA were checked using ThermoNanoDrop 2000, and RNA in each was adjusted to the same concentration. The integrity of RNA was checked by electrophoresis by loading 1 μg RNA on a 1% TAE-agarose gel.

2.4 | Real-time quantitative PCR of the enzymes involved in biosynthesis of glucosinolates and their relative MYB transcriptional factors

DNA-free intact RNA (1 µg) was reverse-transcribed into cDNA with oligo-dT primers using a first-strand cDNA synthesis kit (Promega) according to the manufacture-supplied instructions. Subsequently, the cDNA was used as a template in real-time PCR experiments with gene-specific primers.

To design primers for genes involved in the biosynthesis of glucosinolates as well as their relative MYB transcriptional factors, the CDS of *Arabidopsis thaliana* genes were used to query homologous *B. rapa* sequences which are available in the *B. rapa* genome sequence portal <http://www.brassica-rapa.org>. The full-length sequences of these genes can be found under following accession numbers: MAM1/MAM3 (Bra029355, Bra018524, Bra029356, Bra021947, Bra013009 and Bra013011), CYP79F1 (AT1G16410 and Bra026058), CYP83A1 (AT4G13770 and Bra032734), CYP79B2 (AT4G39950 and Bra011821), CYP79B3 (AT2G22330 and Bra030246), CYP83B1 (AT4G31500 and Bra034941), MYB 28 (AT5G61420 and Bra029311), MYB 29 (AT5G07690 and Bra005949), MYB 34 (AT5G60890 and Bra035954, Bra029350, Bra029349 and Bra013000) and MYB 51 (AT1G18570 and Bra016553 and Bra031035). Relative transcript levels were normalized based on expression of *B. rapa* actin as a reference gene. To design primers, *Arabidopsis* actin 2 gene (NM112764.3) was used to query homologous *B. rapa* (JN120480.1) sequences. Gene-specific primer sets are listed in Table 1.

RT-PCR was performed on an Applied Bio Systems' 7300 real-time PCR system using the SYBR Green master mix kit (Thermo Scientific) based on manufacturer's instructions. The transcript level of the target gene and actin was measured using the comparative Ct method. Analysis of qPCR data was performed using three independent RNA preparations from separate plant tissues.

TABLE 1 List of primer sequences for qPCR analysis

Gene	Primer sequences (5'-3')	
	Forward	Reverse
MAM1/MAM3 ^a	TCAAAGCMAACACTCCTG ^a	CCACTTCTTTCRCCTATTCC ^a
CYP79F1	CTCCTGGACCACCAGGAT	CTCGAGCGATCTCGTCAG
CYP83A1	GGATGGGCCAAAAAATACGG	TGCGTCTTGAGAAGCTCTTT
CYP79B2	GGCTCCACAGCATCATGAA	GAGCGTCTTGTTGCTTGAGT
CYP79B3	ATGCTTACGGGATTGGATCTAAAC	GGTTTGATTTCATCAGCGGTAA
CYP83B1	CGAACCAGTCATCCCAATTCTT	CGGCTGTGTCACGAGAAA
MYB28	GCATCTAGTTCGACAARCR	RGKGTGAAACCGGAGG
MYB29	GACTCAAACCCGAGTAACC	GGAGTTAAAGGAACCATAGTTTCT
MYB34	GATCCAACCACTCACAACCC	GCGATGWTTGTGGAGTTTC
MYB51	CAAGTGTCAACGTTGACTC	GAG RCGACGTAGCGTTA
Actin	AGCAGCATGAAGATCAAGGT	GCTGAGGGATGCAAGGATAG

^aThe same primers are applied to amplify both MAM1 and MAM3 genes.

2.5 | Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). A one-way analysis of variance (ANOVA) was performed, and the treatment means were compared using Tukey's HSD all-pairwise comparisons at the $p = .01$ level as a post hoc test (see figures).

3 | RESULTS

3.1 | Impact of chloride and sulphate salinity on growth

Plant biomass was slightly reduced by 50 mM NaCl, 25 mM Na₂SO₄ and K₂SO₄ but hardly affected by 50 mM KCl (Figure 1). In addition, the exposure of plants to 100 mM NaCl and KCl resulted in significant decrease in plant biomass by 30% and 16%, respectively. However, the effect of 50 mM Na₂SO₄ and K₂SO₄ was stronger and resulted in a reduction in 74% and 50% in plant biomass, respectively (Figure 1). Furthermore, the shoot-to-root ratio was not affected by chloride and sulphate salinity (Figure 1). Dry matter content was hardly changed by any salts in roots (Figure 1). Shoot dry matter content was slightly increased by 100 mM KCl and NaCl and approximately doubled by 50 mM K₂SO₄ and Na₂SO₄ (Figure 1).

3.2 | Impact of chloride and sulphate salinity on glucosinolate content and composition

The shoot and root of *B. rapa* contained a variety of aliphatic (glucanapin, progoitrin and glucobrassicinapin), indolic (glucobrassicin, neoglucobrassicin and 4-hydroxyglucobrassicin) and aromatic (glucanasturtiin) glucosinolates (Table 2). The root contained an almost 1.5-fold higher content of glucosinolates (mainly indolic glucosinolates) than the shoot, whereas the aliphatic glucosinolates were the predominant glucosinolates in the shoot and followed by the indolic and aromatic glucosinolates (Figure 2).

FIGURE 1 Impact of chloride (NaCl and KCl) and sulphate salinity (Na₂SO₄ and K₂SO₄) on plant biomass, shoot-to-root ratio and dry matter content of shoot and roots of *Brassica rapa*. Grey bars: 50 mM NaCl and KCl, 25 mM Na₂SO₄ and K₂SO₄; black bars: 100 mM NaCl and KCl and 50 mM Na₂SO₄ and K₂SO₄. Data represent the mean of three measurements with nine plants in each ($\pm$ SD). Different letters indicate significant difference ($p < .01$; one-way ANOVA, Tukey's HSD all-pairwise comparisons as a post hoc test)

TABLE 2 Nomenclature of the individual glucosinolates identified in shoots and roots of *Brassica rapa*

GSL type	Trivial name	Chemical name
Aliphatic	Gluconapin (4C) ^a	3-Butenyl GSL
	Progoitrin (4C) ^a	2-Hydroxy-3-butenyl GSL
	Gluco brassicanapin (5C) ^a	4-Pentenyl GSL
Indolic	Gluco brassicinin	Indol-3-ylmethyl GSL
	Neoglucobrassicinin	1-Methoxy-indol-3-ylmethyl GSL
	4-Hydroxyglucobrassicinin	4-Hydroxy-indol-3-ylmethyl GSL
Aromatic	Gluconasturtiin	2-Phenylethyl GSL

^aCarbon side chain.

Exposure of plants to 50 and 100 mM NaCl and KCl did not affect the total glucosinolate content and the glucosinolate composition of the root, whereas it resulted in an up to 60% decrease in total glucosinolate content of the shoot, which could be attributed to a decrease in the aliphatic glucosinolate content (Figure 2).

Exposure of plants to 25 mM Na₂SO₄ and 25 mM K₂SO₄ did not significantly affect the total glucosinolate content of both root and shoot and the glucosinolate composition of the root (Figure 2). However, the total glucosinolate content and composition of both root and shoot were substantially affected upon exposure to 50 mM Na₂SO₄ and resulted in an enhanced total glucosinolate content (2.2-fold) in the root due to an increase in aliphatic (3.2-fold), indolic (twofold) as well as aromatic glucosinolate content (3.8-fold). Also in the shoot, exposure to 50 mM Na₂SO₄ resulted in an increase in total glucosinolate content (1.8-fold), which could be ascribed to an increase in indolic glucosinolates (4.5-fold), aromatic glucosinolates (3.3-fold) as well as aliphatic glucosinolate content (1.4-fold; Figure 2). However, exposure of plants to 50 mM K₂SO₄ did not affect the total glucosinolate content of both root and shoot. Moreover, the glucosinolate composition of the root remained unaffected, though in the shoot, the indolic glucosinolate and aromatic glucosinolate contents were significantly increased (threefold and twofold, respectively; Figure 2).

3.3 | Impact of chloride and sulphate salinity on transcript levels of genes involved in glucosinolate biosynthesis

In the shoot, transcript level of the CYP79F1, the gene for enzyme catalysing the biosynthesis of the core structure of aliphatic glucosinolates, was decreased at 50 mM NaCl and KCl. No significant changes were observed in the transcript level of MAM1/MAM3, which is involved in the side-chain biosynthesis, and that of CYP83A1, which is involved in biosynthesis of the core structure of aliphatic glucosinolates (Figure 3). NaCl and KCl salinity did not affect the transcripts levels of MAM1/MAM3, CYP79F1 and CYP83A1 in the root (Figure 3). Similarly, Na₂SO₄ and K₂SO₄ salinity did not significantly affect the transcript levels of MAM1/MAM3, CYP79F1 and CYP83A1 in root nor shoot (Figure 3). The transcript levels of CYP79B2, CYP79B3 and CYP83B1, encoding enzymes catalysing the biosynthesis of indolic glucosinolates, were hardly affected in root and shoot of plants exposed to 100 mM NaCl and KCl, and only the expression of CYP79B3 was decreased in the shoot (Figure 4). Exposure of plants to 50 mM Na₂SO₄ and K₂SO₄ did not affect the transcript levels of CYP79B2 and CYP79B3 of the root, although the transcript level of CYP83B1 was substantially increased (5.7-fold) at 50 mM Na₂SO₄ (Figure 4). In the shoot, the transcript levels of CYP79B2, CYP79B3 and CYP83B1 were substantially increased (15-, 2.7- and 3.5-fold, respectively) upon exposure to 50 mM Na₂SO₄ (Figure 4). In the shoot of plants exposed to 50 mM K₂SO₄, only the transcript level of CYP83B1 was substantially increased (2.3-fold), whereas that of CYP79B3 was slightly decreased and that of CYP79B2 not significantly affected (Figure 4).

3.4 | Impact of chloride and sulphate salinity on transcript levels of transcription factors involved in the regulation of glucosinolate synthesis

The transcript levels of MYB28 and MYB29, which code for transcription factors in the regulation of the genes involved in the

FIGURE 2 Impact of chloride (NaCl and KCl) and sulphate salinity (Na₂SO₄ and K₂SO₄) on aliphatic, indolic, aromatic and total glucosinolate content in root and shoot of *Brassica rapa*. Grey bars: 50 mM NaCl and KCl, 25 mM Na₂SO₄ and K₂SO₄; Black bars: 100 mM NaCl and KCl and 50 mM Na₂SO₄ and K₂SO₄. Data represent the mean of three measurements with nine plants in each ($\pm$ SD). Different letters indicate significant difference ($p < .01$; one-way ANOVA, Tukey's HSD all-pairwise comparisons as a post hoc test)

biosynthesis of aliphatic glucosinolates, were unaffected in both root and shoot of plants exposed to NaCl, KCl, Na₂SO₄ and K₂SO₄ salinity (Figure 5). Similarly, the transcript levels of MYB34 and MYB51, for transcription factors involved in the regulation of the biosynthesis of indolic glucosinolates, were hardly affected in both root and shoot of plants exposed to NaCl and KCl salinity (Figure 6). In contrast, transcript levels of MYB34 and MYB51 were substantially affected in both root and shoot of plants exposed to Na₂SO₄ (Figure 6). The transcript levels of MYB34 and MYB51 in the root were fourfold and 18-fold, and the levels in the shoot were 4.5-fold and 12.5-fold increased, respectively (Figure 6). Similarly, the transcript level of MYB34 in the shoot was increased by twofold upon exposure of plants to K₂SO₄, while it remained unaffected in the root (Figure 6). The transcript level of MYB51 was hardly affected in the root or shoot of plants exposed to K₂SO₄ (Figure 6).

4 | DISCUSSION

This study reconfirmed that sulphate salinity has a greater negative impact on plant biomass production in *Brassica* than chloride salinity at a comparable osmolality, irrespective whether the cation was Na⁺ or K⁺ (Reich et al., 2016). The content of chloride hardly changed by

sulphate salts, while the sulphate content was reduced in the root of plants exposed to chloride salts. Exposure of plants to NaCl caused a slightly higher increase in sodium content than exposure to Na₂SO₄. In addition, both sodium salts caused an increase in potassium in both shoot and roots to a similar extent. However, the exposure of plants to KCl led to a slightly higher increase in potassium content than exposure to K₂SO₄ (Reich et al., 2016).

The results furthermore show that chloride and sulphate salinity differently affect glucosinolate metabolism, which in part may be due to a direct interference of sulphate salinity with the regulation of sulphur metabolism in *Brassica* (Reich, Aghajanzadeh, Stuver, Koralewska, & De Kok, 2015; Reich et al., 2016). Likewise, different impact of chloride and sulphate salts on mineral nutrient contents (Reich et al., 2016) support the idea that they may contribute differently to the accumulation of glucosinolates.

The glucosinolate content in the shoot was decreased upon exposure to NaCl and KCl, independently of the concentration in which the salts were applied. A similar decrease in glucosinolate content has been observed in broccoli sprouts upon exposure to KCl salinity (Aires, Rosa, & Carvalho, 2006). Evidently, the decrease in glucosinolate content under chloride salinity was not cation (Na⁺ and K⁺) specific because NaCl and KCl had apparently the same effect on the biosynthesis of glucosinolates.

FIGURE 3 Impact of chloride (NaCl and KCl) and sulphate salinity (Na₂SO₄ and K₂SO₄) on the transcript levels of MAM1/MAM3, CYP79F1 and CYP83A1 in shoot and root of *Brassica rapa*. Relative gene expression of these genes was determined by qRT-PCR, and the mRNA levels were compared to actin. Data on relative expression in each treatment represent the mean of three measurements with nine plants in each ($\pm$ SD). Different letters indicate significant difference ($p < .01$; one-way ANOVA, Tukey's HSD all-pairwise comparisons as a post hoc test)

There are two possible scenarios for the reduction in glucosinolates in the shoot in response to chloride salinity. First, NaCl and KCl exposure might reduce de novo synthesis of glucosinolates in the shoot or increase the degradation and turnover of glucosinolates by an enhanced activity of myrosinases (Halkier & Gershenzon, 2006; Yan & Chen, 2007). Second, the decrease in sulphate content in plants exposed to chloride salts (Reich et al., 2016) could be considered as a reason for the reduction in glucosinolates. As chloride competes with sulphate at the level of uptake (due to their common negative charge), a decrease in glucosinolates upon exposure to NaCl and KCl could be caused by a reduced sulphur availability due to decreased sulphate uptake. The Group 1 sulphate transporters (Sultr1; 1 and Sultr1; 2) are responsible for the primary uptake of sulphate at the root plasma membrane (Smith et al. 1995). It has been observed that the expression of both Sultr1; 1 and Sultr1; 2 in the roots was reduced upon exposure of the plants to chloride salts (Reich et al., 2016).

Moreover, the observed downregulation of CYP79F1 by salt stress indicated that glucosinolates might be synthesized at a lower rate upon exposure to NaCl and KCl salinity. Alternatively, because salt stress induces sulphate reduction and glutathione synthesis (Koprivova, North, & Kopriva, 2008), during chloride stress sulphur might be partitioned towards the primary assimilation and limit so the synthesis of glucosinolates.

The impact of sulphate salinity on glucosinolate metabolism was highly dependent on the cation. While K₂SO₄ had no significant effect and Na₂SO₄ substantially increased the content of glucosinolates (Figure 2). This suggests a negative effect of Cl⁻ (as the common anion in NaCl and KCl) on glucosinolate biosynthesis, rather than of Na⁺ or K⁺. An increased level of indolic and aliphatic glucosinolates has been observed in K-deficient plants, which corresponded to the transcriptional upregulation of CYP79B2 and CYP79B3 (catalysing the first step in the biosynthesis of tryptophan-derived indole glucosinolate) as well as CYP79F1 and CYP79F2 (which encode enzymes that catalyse the synthesis of short-chain and long-chain elongated methionine-derived aliphatic glucosinolates; Troufflard et al., 2010). The transcript levels of CYP83B1, SUR1 (C-S lyase) and sulfotransferase (SOT) did not show significant changes (Troufflard et al., 2010). In addition, glucosinolates in the shoot were more affected than those in the root in plants upon exposure to both chloride salts (Figure 2). However, the signal linking Na⁺ salt stress to the biosynthesis of glucosinolates and its enzymatic targets remains to be identified.

It has been shown that total sulphur and sulphate contents were substantially increased in both shoot and roots of plants exposed to 50 mM K₂SO₄ compared to control plants (Reich et al., 2016); however, it did not result in an increase in total glucosinolate content. Glucosinolate content was significantly increased in plants exposed

FIGURE 4 Impact of chloride (NaCl and KCl) and sulphate salinity (Na₂SO₄ and K₂SO₄) on the transcript levels of CYP79B2, CYP79B3 and CYP83B1 in shoot and root of *Brassica rapa*. Relative gene expression of these genes was determined by qRT-PCR, and the mRNA levels were compared to actin. Data on relative expression in each treatment represent the mean of three measurements with nine plants in each ($\pm$ SD). Different letters indicate significant difference ($p < .01$; one-way ANOVA, Tukey's HSD all-pairwise comparisons as a post hoc test)

FIGURE 5 Impact of chloride (NaCl and KCl) and sulphate salinity (Na₂SO₄ and K₂SO₄) on the transcript levels of MYB28 and MYB29 in shoot and root of *Brassica rapa*. Relative gene expression of these genes was determined by qRT-PCR, and the mRNA levels were compared to actin. Data on relative expression in each treatment represent the mean of three measurements with nine plants in each ($\pm$ SD). Different letters indicate significant difference ($p < .01$; one-way ANOVA, Tukey's HSD all-pairwise comparisons as a post hoc test)

to 50 mM Na₂SO₄; however, this increase was absent upon exposure of plants to equivalent concentrations of K₂SO₄. This difference between the effect of Na₂SO₄ and K₂SO₄ could be due to a negative effect of K⁺ on glucosinolate content, counteracting a

potential increase by an excess sulphate on glucosinolate biosynthesis. This absence of an effect upon exposure to K₂SO₄ is presumably caused by a decrease in aliphatic glucosinolates. However, indolic and aromatic glucosinolate were significantly

FIGURE 6 Impact of chloride (NaCl and KCl) and sulphate salinity (Na₂SO₄ and K₂SO₄) on the transcript levels of MYB34 and MYB51 in shoot and root of *Brassica rapa*. Relative gene expression of these genes was determined by qRT-PCR, and the mRNA levels were compared to actin. Data on relative expression in each treatment represent the mean of three measurements with nine plants in each ($\pm$ SD). Different letters indicate significant difference ($p < .01$; one-way ANOVA, Tukey's HSD all-pairwise comparisons as a post hoc test)

enhanced upon exposure to K₂SO₄. An increased transcript level of CYP83B1 and MYB34 indicates a specific induction of indolic and aromatic glucosinolate biosynthesis by K₂SO₄, as these genes are involved in biosynthesis and the regulation of biosynthesis of indolic glucosinolates.

There are a number of possible explanations for the substantial increase in glucosinolate content in both shoot and root of plants exposed to Na₂SO₄. The first could be an increase in the overall organic sulphur content (derived from subtracting the sulphate content from the total sulphur content), which has been already observed in a previous study in *B. rapa* exposed to 50 mM Na₂SO₄ (Reich et al., 2016). It revealed that sulphur and organic sulphur content did not accumulate more upon exposure to Na₂SO₄ than upon exposure to K₂SO₄. Likewise, sulphur and organic sulphur contents in plants exposed to 100 mM KCl were the same as those in plants exposed to NaCl (Reich et al., 2016). Therefore, it seems that Na⁺ had the same effect as K⁺ on sulphate uptake and accumulation of organic sulphur content. The second explanation would be a synergistic effect of Na⁺ and sulphate on a promotion of glucosinolate biosynthesis, mediated by the increased transcript levels of the genes involved in biosynthesis of aliphatic (CYP79F1) and indolic (CYP79B2, CYP79B3 and CYP83B1) glucosinolates or inert effect of Na⁺ on glucosinolate metabolism.

The severe reduction in growth in plants exposed to 50 mM Na₂SO₄ could also be considered as an explanation for the increase in glucosinolate content. However, there was no overall correlation found between biomass and glucosinolate content, as K₂SO₄ did also decrease biomass but hardly affected total glucosinolates, while chloride salts decreased biomass which was accompanied with reduction in glucosinolates content. Thus, it can be concluded that alterations in glucosinolate content and composition under salt stresses might not be solely attributed to changes in growth.

The physiological role of glucosinolates in the response of plants to salinity stress is not completely understood. However, the induction of glucosinolate accumulation under salinity has been evidenced when external salt concentrations rise above the tolerance level (López-Berenguer, Martínez-Ballesta, García-Viguera, & Carvajal, 2008; Pang et al., 2012; Yuan et al., 2010). In addition, some studies suggest that glucosinolates might be involved in osmotic adjustment (López-Berenguer et al., 2009; López-Pérez, Martínez Ballesta, Maurer, & Carvajal, 2009). Therefore, the increased level of glucosinolates in plants exposed to Na₂SO₄ observed in this study could be considered as part of the plant defence response to salt stress.

5 | CONCLUSIONS

It is evident that chloride and sulphate salinity differently effect on content and composition of glucosinolates. The impact of sulphate salinity on glucosinolate metabolism was cation dependent, whereas the decrease in glucosinolate content under chloride salinity was not cation (Na⁺ and K⁺) specific. It was demonstrated that the changes in glucosinolates content coincided with an alteration of gene expression of key enzymes for their biosynthesis.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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