Molecular Mechanisms of Salinity Tolerance in Brassicaceae

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Front Cover: Photographs of *Cochlearia pyrenaica, Thlaspi arvense, Cochlearia danica* and *Brassica oleracea* (from top to bottom), taken from "www.ukwildflowers.com".
Back Cover: *Cochlearia x hollandica* plants, growing on hydroponics at different salt concentrations in the climate chamber of the Genetics group at the Vrije University, Amsterdam The Netherlands. Photograph by author.

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To my Beloved Family

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Chapter 1

General Introduction

1.1 The salinity problem

Salinity is a major constraint to food production. The percentage of agricultural land that is affected by high salinity is continously increasing throughout the world. The extent of the problem is illustrated by the fact that one-third of the global arable land (Munns, 2002), or half of the irrigated arable land (Zhu, 2001) is significantly affected by salinity. Some soils are naturally saline, i.e, inland salt lakes and soils formed from saline parent material. This is called natural or primary salinity. Deposition of salt from the ocean carried by wind and rain also falls under this category (Munns and Tester, 2008). Secondary salinity is due to human activities such as land clearing and over-irrigation or irrigation with saline water, often in combination with poor drainage. Salinity problems in the soil and surface water occur when more water enters into the ground water system (through a process called recharge) than is discharged from the system. More incoming water causes the water table to rise. As ground water rises, it dissolves the soluble salts, which were already stored in the sub-soil and brings salty water into the reach of plant roots. Plant uptake along with evaporation of the water from soil surface, concentrates the salts more and more in the top-soil.

1.2 Classification of soils on the basis of soluble salts

Soil salinity is defined as the total concentration of salts dissolved in the soil solution. It is usually measured as electric conductivity (EC), and soils with an EC level of 4 dSm^{-1} or higher, are generally considered to be saline (Munns, 2005; Munns and Tester, 2008). Saline soils are often sodic (Zhang *et al.,* 2010). Sodicity is defined on the basis of the concentration of exchangeable sodium (Na^{+}), relative to the sum of excangeable calcium (Ca^{2+}) and magnesium (Mg^{2+}). It is measured by the 'sodium absorption ratio' (SAR), which is calculated as $[Na^{+}]/\sqrt{\{([Ca^{2+}]+[Mg^{2+}])/2\}}$. Sodic soils are typically clayey, alkaline (pH 8.5-12), and poorly structured, i.e. sticky when wet, or hard and crusty when dry, which hampers root penetration and seedling establisment (Munns, 2005). Excessive salt, mainly NaCl, in saline/sodic soils does not only destroy the soil physical structure, but also lowers the water potential, which hinders plant water uptake. As a result, plants may exhibit signs of drought even when the soil is wet or waterlogged.

1.3 Salinity effects on plant growth

Seed germination, seedling growth and vigour, vegetative growth, flowering and fruit set are adversely affected by salinity in few crops (Sairam and Tyagi, 2004). The effects imposed by salinity on plant yield can be classified as primary and secondary. Primary effects are osmotic stress and ionic stress, the latter often being mainly exerted by the Na component of NaCl (Blumwald, 2000; Hasegawa *et al.,* 2000; Munns and Tester, 2008). However, in some woody perennials, such as citrus and grapevine, chloride seems to be the more toxic component when accumulated in leaves (White and Broadley, 2001), since Na is largely retained in the woody stem (Flowers and Yeo, 1988). It is believed that the detrimental effects of salinity on plants are brought about by the combination of both ionic and osmotic effects (Kronzucker and Britto, 2010). On short-term, after a sudden rise in soil salinity, plants will initially suffer from the osmotic effect, leading to plant water deficit, stomatal closure and, consequently, cessation of carbon assimilation and growth (Munns, 2005). In extreme cases, cell division and expansion may also be more directly inhibited, through a loss of turgor pressure (Munns, 2002). On longer term, exposure to salinity will lead to the accumulation of high levels of Na and Cl within plant tissues, which ultimately causes ion toxicity. Abundance of Na^+ and Cl^- ions within the cytoplasm may disrupt enzyme activities and photosynthesis processes, in part through replacement of potassium (K) by Na (Kant *et al*., 2006). Ionic stress progresses rather slowly, manifested as accellerated senescence of older leaves, or foliar necrosis, starting at the tips and margins of the leaves. The extent of Na specific damage depends on the rate of foliar Na accumulation and on how effectively Na can be compartmentalized within tissues and cells (Tester and Davenport, 2003).

The secondary effects of salinity are those that are attributable to stress-induced generation of reactive oxygen species, which causes the oxidative damage to proteins, lipids, or nucleic acids (Hasegawa *et al.,* 2000; Zhu, 2001; Chinnusamy *et al.,* 2006). Another potential secondary effect of salinity is potassium deficiency (Silberbush and Ben-Asher, 2001). Potassium (K) is a plant macronutrient with a large number of physiological functions: it is essential for protein synthesis, photosynthesis and for the activity of glycolytic enzymes, while it also plays a role as an osmoticum in cell expansion and turgor-driven movements (Schroeder *et al.,* 1994). Because Na is similar to K and many K transporters do not discriminate sufficiently between these two cations, excessive external Na may lead to impaired K acquisition and ultimately K deficiency.

1.4 Variation for salinity tolerance

The tolerance to soil salinity varies greatly among plant species (Munns and Tester, 2008). Among cereals, rice is highly salt-sensitive whereas barley is relatively tolerant. Variation in salt tolerance is even more pronounced among dicotyledonous crops, of which legumes are relatively sensitive, even more than rice (Lauchli, 1984). Among wild plant species, saltbushes (*Atriplex halimus, A. vesicaria*) and several other members of the Chenopodeaceae (*Suaeda sp*., *Salicornia sp*.) can grow at salinity levels far in excess of that of seawater (Zhu, 2007; Munns and Tester, 2008). These species are extreme examples of so-called halophytes. Halophytes are usually defined as species that are able to grow and reproduce at 200 mM NaCl (± 20 dSm^{-1}) in the soil solution (Flowers and Colmer, 2008). Only a small minority of higher plant species, about 2%, are halophytes, the remaining 98% being termed 'glycophytes' (Dajic, 2006). Halophytes can be further classified as 'facultative halophytes', i.e. species occurring both in saline and non-saline habitats, usually exhibiting moderate degrees of salt tolerance, or 'obligate halophytes', which are confined to saline habitats, usually exhibiting high degrees of salt tolerance and, particularly in case of Chenopodiaceae, a physiological requirement of salt for optimal growth (Flowers and Colmer, 2008). There is also variation in salt tolerance among glycophytes, i.e, the genetic plant model species, *Arabidopsis thaliana*, is relatively salt-sensitive. Recently, its close relative, *Thellungiella halophila*, has been adopted as a model plant for salt tolerance research. This species has been claimed to be extremely tolerant to salt, but also to temperature extremes and drought (Taji *et al.,* 2004; Gong *et al.,* 2005; Amtmann *et al.,* 2005). The *T. halophila* genome shares 95% identity with that of *A. thaliana* (Radyukina *et al.,* 2007), which allows the use of most of the molecular tools available for *A. thaliana* (Karrenberg and Widmer, 2008).

The salt tolerance mechanisms operating in halophytes are far from understood. In general, it seems reasonable to assume that high-level salt tolerance is a complex trait, involving, at least, multiple physiological changes at the level of uptake, plant-internal transport, and compartmentalization of Na and K, the synthesis and transport of 'compatible solutes' and, at least in a number of species, functional alterations of anatomical structures, such as glands and hairs (Flowers and Colmer, 2008). Moreover, salt tolerance at halophyte level must have been independently evolved in different subclasses, orders and families of higher plants. Therefore, it is to be expected that the nature of salt tolerance mechanisms in halophytes is variable, dependent on a species' phylogenetic origin (Flowers and Colmer, 2008).

1.4.1 Morphological adaptations to salinity in halophytes

Many halophytes, particularly among the dicotyledonous ones, have succulent leaves and stems, and thick-walled, heavily cutinized epidermal cells. This has been explained by the 'physiological drought hypothesis', which states that saline environments are in fact dry, owing to the low osmotic water potential of the soil. However, in practice, at least coastal succulent halophytes appear to maintain a relatively constant water potential gradient between the leaves and the soil, and do not develop considerable tissue water deficits during the growing season, which argues against this hypothesis (Rozema and Schat, 2012). The precise role of 'electrolyte succulence' in halophytes remains to be elucidated. Salt secretion from the shoot, through 'salt glands' is another, more obvious, morphological adaptation to salinity, which occurs in some dicotyledonous or monocotyledenous halophytes. A number of halophytes, particularly among the Chenopodiaceae/Amaranthaceae, use unicellular or multicellular epidermal appendages, called 'salt hairs' or 'salt bladders', to store, and eventually remove, excessively accumulated foliar salt (Thomson *et al.,* 1988).

1.4.2 Physiological adaptations to salinity in halophytes

It is often believed that halophytes and glycophytes basically utilize the same mechanisms to cope with salt, but that the capacities or efficiencies of (at least a subset of) these mechanisms are enhanced in halophytes, in comparison with glycophytes (Volkov *et al.,* 2003; Taji *et al.,* 2004; Inan *et al.,* 2004; Kant *et al.,* 2006; Munns and Tester, 2008; Ellouzi *et al.,* 2011). However, in fact there is barely any evidence either in favor or against this hypothesis. Although the molecular physiology of salt tolerance in glycophytes, in particular *A. thaliana*, has been reasonably well explored (Munns and Tester, 2008), the regulation and expression patterns of the genes involved have, with few exceptions, not been studied in halophytes thus far. Moreover, to rigidly test any hypothesis concerning the roles for particular genes in salt tolerance in halophytes, one would need genetically accessible halophyte models, which are, apart from *T. halophila*, not available to date. However, since salt tolerance mechanisms are almost certainly subject to phylogenetic bias (see above), one would probably need a phylogenitically diverse array of accessible model halophytes in order to get a more or less comprehensive view of the phenomenon (Rozema and Schat, 2012). In the first place, there seem to be differences between the salt tolerance mechanisms in monocotyledonous halophytes and those in the majority of dicotyledenous ones. For example, dicotyledonous halophytes tend to accumulate Na in the shoot, using it as a 'cheap' osmolyte, whereas monocotyledonous halophytes tend to exclude Na from their body, using K as an osmolyte

instead (Zhu, 2007; Flowers and Colmer, 2008).

1.5 Controlling Na entry and xylem loading/un-loading

Since Na is the most toxic component of salt, control of the cellular Na concentration, particularly in the photosynthetically active tissues, is critical for salt tolerance (Tester and Davenport, 2003). Accumulation of Na to toxic concentrations can be prevented by: a) restricting Na entry into the root, b) excreting Na from root cells into the soil, c) retrieving Na from the transpirational xylem stream to recirculate it to the root (Zhu, 2002). Na uptake from the soil solution into the root symplast occurs passively in the root epidermis and cortex, energetically favored by the electrochemical gradient (Higinbothum, 1973; Tester and Davenport, 2003; Apse and Blumwald, 2007). The casparian strips in the endodermis prevent apoplastic Na influx into the root stele. Plants generally exclude about 97% of the Na back into the soil at the root surface to prevent toxic levels of Na accumulation in the shoots (Munns *et al.,* 2000).

Passive Na uptake is likely to be mediated by ion channels or uniporters. Ca^{2+}-sensitive Na uptake takes place via Non-Selective Cation Channels (NSCC's) (Demidchik and Maathuis, 2007). NSCC's are further catagorized into CNGC's (Cyclic Nucleotide-Gated Channels), GLR's (Glutamate Activated Channels; Davenport, 2002). LCT1 (Low Affinity Cation Transporters) may also be involved (Very and Sentenac, 2003) in Na uptake. Ca^{2+}-insensitive influx of Na probably occurs to some extent through NSCC's (Davenport and Tester, 2000), but several other transporters seem to be involved too, including members of the HKT family (Platten *et al.,* 2006). Several HKT family members are involved in the long-distance plant internal transport of Na (Sunarpi *et al.,* 2005; Davenport *et al.,* 2007; Møller *et al.,* 2010; Plett *et al.,* 2010), but others are high-affinity K^+/Na^+ symporters which can also mediate low-affinity Na influx into roots (Rubio *et al.,* 1995). AtHKT1 has four membrane-pore-membrane (MPM) motifs and eight transmembrane domains with two cation binding sites, one specific for Na and the other binds either Na or K (Gassmann *et al.,* 1996). On the basis of the presumed specificity of the second binding site, the HKT family has been divided into two sub-families. Transporters from sub-family 1 would preferentially conduct Na across the membrane, and have a serine residue in the first of the four pore-loop domains (motif S-G-G-G), whereas members of sub-family 2 would be non-Na-preferent, having a glycine in this position (motif G-G-G-G). Genes encoding sub-family 1 members occur in both monocots and dicots, having a long intron near the 3' end, whereas sub-family 2 is confined

to monocots, having a shorter intron near the 3'-end (Platten *et al.,* 2006). Each member was assigned a new name according to the new classification (Platten *et al.,* 2006), in which AtHKT1 has been renamed as AtHKT1;1.

1.5.1 HKT sub-family 2

Very few studies have been conducted on the roles of sub-family 2 HKT transporters in plant responses to salinity. The best studied member of this sub-family is TaHKT2;1 from wheat (Laurie *et al.,* 2002), which is the first HKT transporter identified in plants (Schachtman and Schroeder, 1994). In general, the transcription of *HKT* genes from sub-family 2 has been shown to be increased under K starvation in wheat, rice and barley (Horie *et al.,* 2001; Wang *et al.,* 1998). *TaHKT2;1* is mainly expressed in root cortical cells (Schachtman and Schroeder, 1994), and permits the conductance of both Na and K (Rubio *et al.,* 1995; Gassmann *et al.,* 1996). Silencing of *TaHKT2;1* leads to a lower root Na uptake and a lower Na concentration in the xylem sap, resulting in improved tolerance to salinity (Laurie *et al.,* 2002), consistent with the fact that wheat is a salt-excluder species. OsHKT2;1 is a unique member of sub-family 2, since it possesses a serine residue in the first putative selectivity pore-forming loop (Kato *et al.,* 2001), and exhibits preference for Na over K (Horie *et al.,* 2001). More recently, Afaq *et al.,* (2011) expressed *HvHKT2;1* (*Hordeum vulgare*) in *Xenopus* oocytes and found that HvHKT2;1 can transport both Na and K over a large range of external concentrations. Barley (*Hordeum vulgare)* has eight isoforms of HKT (Huang *et al.,* 2008) and *HvHKT2;1* is the most prominently expressed one. Barley plants in which *HvHKT2;1* was over-expressed show increased Na uptake and loading into the xylem, leading to increased Na accumulation in shoot tissues. Remarkably, the increased uptake and translocation of Na improved the salinity tolerance of the transgenic lines (Afaq *et al.,* 2011).

1.5.2 HKT sub-family 1

Transport of Na to the shoot is not properly understood. After uptake into the root symplasm, Na moves symplastically across the endodermis and is released from the xylem parenchyma cells into the xylem. To prevent Na accumulation in shoots it is crucial to maintain a low Na concentration in the xylem, which can be achieved either by minimizing the Na entry to the xylem from the root symplast, or by maximizing the retrieval of Na fom the xylem before it reaches sensitive tissues in the shoot (Apse and Blumwald, 2007). HKT members of sub-family 1 have been reported to be expressed chiefly in the xylem parenchyma cells, playing a role in the xylem loading or deloading of Na. The most studied member of the *HKT1* sub-family 1 is *AtHKT1;1*, which is the only *HKT* gene in the *A. thaliana* genome. *AtHKT1;1* was

originally identified as an *Arabidopsis* homologue of wheat *TaHKT2;1* (Schachtman and Schroeder, 1994). Rus *et al.,* (2001) found that *Athkt* mutants had lower total tissue Na concentrations than wild-type, and they supposed that AtHKT1 would be involved in Na uptake from the external medium. Later on, Maser *et al*., (2002) showed that *Athkt1* mutants ad almost the same foliar Na concentration as that of wild-type, but a lower concentration in the root. Berthomieu *et al.,* (2003), proposed a "recirculation model", according to which AtHKT1;1 would somehow allow Na retranslocation from the shoot to the root via the phloem. They suggested that such recirculation would be crucial for plant salt tolerance. Later on, another working model ("exclusion") was proposed by Sunarpi *et al.,* (2005). They suggested that AtHKT1;1 plays an important role in Na detoxification in plants through resorbing Na from the xylem vessels into xylem parenchyma cells, thus reducing salt transport to the leaf mesophyll. The model of Sunarpi *et al*., (2005) was further supported by Davenport *et al.,* (2007), who used radioactive tracer ($^{22}Na^{+}$) flux measurements and ion accumulation assays to show that AtHKT1;1 is involved in the accumulation of Na in the root via retrieval of Na from the xylem into parenchyma cells, but not in root Na uptake, nor in its recirculation via the phloem. Møller *et al.,* (2010), using an enhancer trap system, demonstrated that transgenic *AtHKT1;1* over-expression in the pericycle conferred salt tolerance, whereas non-tissue-specific over-expression under the 35S-CMV promoter did not, which again confirms that *HKT1*-mediated salt tolerance relies on resorbing Na from the xylem. More recently, Plett *et al.,* (2010) also used enhancer trap lines of rice and *A. thaliana*, and showed that over-expression of *AtHKT1;1* in the mature root cortex yielded a more efficient exlusion of Na from the shoot and enhanced salinity tolerance in both species. Similar functions have been proposed for sub-family 1 HKT's in rice (Ren *et al.,* 2005) and wheat (James *et al.,* 2006; Byrt *et al.,* 2007).

1.6 Sodium efflux from the root

Sodium efflux from root cells is a frontline defense mechanism that prevents the accumulation of toxic levels of Na in the cytosol and Na transport to the shoot. Most of the passively entered Na is actively pumped back from the root into the root-environment via the plasma-membrane Na^{+}/H^{+} antiporter SOS1 (Shabala *et al*., 2005), particularly in the meristimatic part of the root tip, of which the cells are devoid of a large central vacuole for Na sequestration (Shi *et al*., 2002).

1.6.1 SOS-signalling pathway components

The SOS signalling pathway consists of three main components (SOS1, SOS2, SOS3). SOS3 is a Ca^{2+}-binding protein (Qiu *et al.,* 2002), which is sensitive to cytosolic Ca^{2+} levels. One of the consequences of salt stress is an increase in the cytoplasmic Ca^{2+} concentration. Upon sensing increased cytoplasmic Ca^{2+}, SOS3 binds to and activates SOS2, which is a Ser/Thr protein kinase (Liu *et al.,* 2000). This SOS2-SOS3 complex ultimately phosphorylates and activates NHX1 and other transporters involved in vacuolar Na^+ transport (Qiu *et al.,* 2004), along with SOS1 (Qiu *et al.,* 2002). This interaction between SOS2 and SOS3 is also supported by *sos2sos3* double mutant analysis, which indicates that the two genes function in the same pathway (Halfter *et al.,* 2000). *AtSOS2* was found to be up-regulated under salt exposure (Liu *et al.,* 2000). *SOS2* was also isolated from *Brassica napus* and *BnSOS2* was also found to be induced upon salt exposure, both in root and shoot after 12 h (Wang *et al.*, 2004).

SOS1 has 22 introns and 23 exons. The N-terminal region of the protein is highly hydrophobic and has 12 predicted transmembrane domains. The C-terminal region of SOS1 is highly hydrophilic and supposed to be cytosolic (Mahajan *et al.,* 2008). SOS1 is an electroneutral Na^+/H^+ exchanger that is specific for Na. GUS expression under the *AtSOS1* promoter exhibited a high promoter activty in root epidermal cells (particularly at root tip), and in stelar cells throughtout the plant (Shi *et al*., 2002). *Atsos1* mutants are extremely salt-sensitive and have combined defects in Na extrusion and long distance transport of Na from root to shoot (Qiu *et al.,* 2002; Shi *et al.,* 2002). Thus, the suggested roles of SOS1 are: a) to pump Na back into the soil solution b) to decrease Na delivery to the shoot under salt exposure by retrieval from the xylem (Shi *et al*., 2002). Similar functions for SOS1 proteins have been proposed for *Populus euphratica* (Wu *et al.,* 2007), *T. halophila* (Vera-Estrella *et al.,* 2005), wheat (Mullan *et al.,* 2007) and rice (Martinez-Atienza *et al.,* 2007). *AtSOS1* transcript levels are significantly up-regulated by salt treatment, but not affected by abscisic acid or cold stress. Moreover, *AtSOS1* mRNA is more abundant in roots than in shoots (Mahajan *et al.,* 2008).

SOS1 also seems to play a role in the oxidative stress response. RCD1 is an important transcriptional regulator of oxidative stress-responsive genes and it has been shown that the C-terminal tail of SOS1 interacts with RCD1 both under salt and oxidative stress (Katiyar-Agarwal *et al.,* 2006). The RCD1 protein resides in the nucleus under non-stress conditions, but under salt/oxidative stress it can also be found in the cytoplasm near the cell periphery.

Several oxidative stress-responsive genes were found to be regulated by both RCD1 and *SOS1*, which clearly shows the involvement of SOS1 in preventing oxidative stress injury (Katiyar-Agarwal *et al.,* 2006). Recently, Oh *et al.,* (2010) reported that AtSOS1 is not only involved in Na extrusion into the root environment, but also plays some role in endocytosis, the shaping of the vacuole, and intracellular pH maintenance.

1.7 Ion compartmentation

Along with other mechanisms, control of ion movement across the tonoplast (and the plasma membrane) to maintain a low Na concentration in the cytosol is a key factor in cellular salinity tolerance. Plant cells typically maintain a high K^+/Na^+ ratio in their cytosol with K between 100 and 200 mM and Na below 10 mM (Higinbotham, 1973). This high K/Na ratio is very important for the functioning of many cytosolic enzymes. Under salt stress, in order to avoid damage to the cytosolic enzymatic machinery, plants tends to sequester excessive Na in the vacuole by means of vacuolar antiporters, e.g. NHX's.

1.7.1 Vacuolar Na^+/H^+ antiport

Na^+/H^+ exchange at the tonoplast essentially contributes to the maintenance of a high K/Na ratio in the cytosol (Apse *et al.,* 1999; Gaxiola *et al.,* 1999). This process is mainly mediated by the NHX family of Na^+/H^+ antiporters. In *Arabidopsis,* there are six members (*NHX1-6*), of which *AtNHX1* and *AtNHX2* are strongly expressed in all plant tissues except the root tip, whereas *AtNHX3* and *AtNHX4* transcripts are almost exclusively present in flowers and roots (Silva and Geros, 2009). AtNHX1, -2, -3 and -4 are localized to the tonoplast, whereas AtNHX5 and -6 are localized to endosomal compartments (Bassil *et al.,* 2011a). AtNHX1 has 12 transmembrane domains (Sato and Sakaguchi, 2005) with a hydrophobic, luminal N-terminal and a hydrophilic, cytosolic C-terminal. The transmembrane domains numbered five and six are the predicted active sites (Silva and Geros, 2009).

NHX proteins control the cytosolic Na concentrations and regulate pH, cell expansion, vesicular trafficking and protein targeting (Orlowski and Grinstein, 1997; Bassil *et al.,* 2011a; Bassil *et al.,* 2011b). Over-expression of *AtNHX1* or its orthologs from other plant species has been shown to confer salt tolerance to a wide range of host species (Apse *et al.,* 1999; Zhang and Blumwald, 2001; Xue *et al.,* 2004; He *et al.,* 2005; Yu *et al.,* 2007; Chen *et al.,* 2007; Liu *et al.,* 2008; Zhang *et al.,* 2008). *NHX* transporters have been found to be strongly induced under salt stress (Qiu *et al.,* 2004; Yokoi *et al.,* 2002; Zhang *et al.,* 2008). It has been suggested that AtNHX1 activity is regulated through interaction with the protein

kinase SOS2 (Qiu *et al.,* 2004). *Beta vulgaris BvNHX1* has been shown to be regulated by a MYB transcription factor (Adler *et al.,* 2010).

1.7.2 Proton pumps (PM-ATPase, V-ATPase, V-PP_iase)

The function of NHX proteins in pH regulation and Na sequestration is linked to the activity of proton pumps (Silva *et al.,* 2010). There are three major proton pumps in a plant cell, the plasma membrane H^+-ATPase (PM-ATPase), the vacuolar H^+-ATPase (V-ATPase) and the vacuolar proton translocating pyrophosphatase (V-PP_iase). Out of these pumps, the V-ATPase is the oldest and most complex one (Gaxiola *et al.,* 2007). It is an ATP-dependent pump, which actively translocates the H^+ ion across the tonoplast into the vacuole (Barkla *et al.,* 1995). The V-ATPase is also essential for a proper structure and functioning of the Golgi apparatus (Strompen *et al.,* 2005). V-ATPase is a multi-unit enzyme composed of the peripheral V_1-complex and the membrane-integral V_0-complex. The V_1-complex consists of eight subunits (A, B, C, D, E, F, G, and H), of which A (catalytic) and B (non-catalytic ATP-binding), each of which represented by three molecules, are responsible for ATP hydrolysis. Probably one molecule of each of the subunits C, D, E, F, G, and H, are known to assemble the stalk (Gaxiola *et al.,* 2007). The V_0-complex consists of five subunits (a, c, c", d and e), which are responsible for H^+ ion translocation (Gaxiola *et al.,* 2007). The most abundant subunit of the V_0 complex is c (Sze *et al.,* 1999), represented by five molecules, which form the proton-conducting pore. In *Arabidopsis*, these 13 subunits of V-ATPase are encoded by a total of 27 genes (Sze *et al.,* 2002).

Na^+/H^+ antiporters, such as NHX1, use the proton motive force generated by the V-ATPase and V-PP_iase to couple the downhill movement of H^+ with the uphill movement of Na (against its electrochemical potential) (Blumwald, 1987). Increased acitivity of these pumps will acidify the vacuole, and thus create a steeper electrochemical gradient for Na^+/H^+ exchange.

V-ATPase activity was found to be stimulated by NaCl exposure in *Mesembryanthemum crystallinum* (Bremberger and Luttge, 1992; Barkla *et al.,* 1995), *Salicornia bigelovii* (Ayala *et al.,* 1996), *Sueda salsa* (Wang *et al.,* 2001; Qiu *et al.,* 2007), cucumber (Kabala and Klobus, 2008), *Populus euphratic*a (Silva *et al.,* 2010), and potato (Queirós *et al.,* 2009). On the other hand, in *Daucus carrota,* V-ATPase activity remained unaffected under salt treatment (Colombo and Cerana, 1993). Measurements on tonoplast-enriched membrane vesicles, isolated from *S. salsa* leaves, demonstrated that the ATP-hydrolytic and H^+-pumping activities were more than two-fold increased under salt stress

(200 mM of NaCl), in comparison with the non-exposed controls (Qiu *et al.,* 2007). The same was observed in *M. crystallinum* (Barkla *et al.,* 1995).

It has been shown that the trancript levels of some subunits are up-regulated in response to salt stress. A salt-induced increase of subunit A transcription has been observed in salt-adapted and salt-stressed cell suspension cultures of tobacco (Narasimhan *et al.,* 1991). Transcriptional activation of subunit c has been shown in leaves and roots of six-week old halophytic *M. crystallinum* treated with 350 mM or 400 mM NaCl for 8 (Low *et al.,* 1996), or 24 h (Tsiantis *et al.,* 1996), respectively. Later on, Golldack and Dietz (2001) exposed *M. crystallinum* for 72 hr and observed that the degree of up-regulation was similar for all the subunits (A, B, E, F and c) and apparently coordinated on the longer term. Expression of subunit D in *A. thaliana* was not affected by NaCl exposure (Kluge *et al.,* 1999). In tomato leaves, induction of subunit A transcripts was found to be temporary, followed by a decrease of the transcript level after three days of salt stress (Binzel, 1995).

1.8 Synthesis of compatible solutes

Compatible solutes are low molecular weight organic compounds that reside mainly in the cytosol to balance the osmotic pressure of the inorganic ions in vacuole (Flowers *et al.,* 1977). They include linear polyols (glycerol, mannitol, sorbitol), cyclic polyols (inositol, pinitol, and other mono- and dimethylated inositol derivatives), amino acids (glutamate, proline) and betaines (glycine betaine, alanine betaine) (Zhu, 2007). Generally, they do not interfere with protein structure and functioning, but alleviate inhibitory effects of hazardous ion concentrations on enzyme activity (Bohnert and Shen, 1999). Most of them are synthesized and accumulated in response to osmotic stress. The accumulation of these solutes lower the osmotic potential of the cell, which helps to maintain the water balance under osmotic stress. Compared with synthesizing organic solutes, uptake of inorganic ions (e.g. Na^+, Ca^{2+} and K^+) is also a source of osmotic adjustment in plants (Gagneul *et al.,* 2007). To maintain an osmotic gradient for the uptake of water, many halophytic plants accumulate Na/K to a concentration equal to or greater than that of the surrounding solution (Merchant and Adams, 2005).

In some plants, inorganic ions play more important roles in osmotic adjustment than do compatible solutes (Munns and Tester, 2008). Both organic solutes and inorganic ions such as Na^+ and K^+, play crucial roles in osmotic adjustment to saline and dry conditions, and their type, content and relative contribution varies among cultivars, species, and even among

different organs of the same plant (Ashraf and Bashir, 2003). Decreasing the solute potential and osmotic adjustment within the cell may not be the only essential function of compatible solutes. Even when present at osmotically insignificant concentrations such solutes may function to scavenge reactive oxygen radicals and stabilize the tertiary structure of proteins. Shen *et al.,* (1997) showed that mannitol at concentrations of less than 100 mM in chloroplasts reduced the damage of, specifically, hydroxyl radicals. The synthesis of compatible solutes is costly and hence involves a potential growth penalty. The ATP requirement for the synthesis or accumulation of solutes has been estimated as 3.5 for Na, 34 for mannitol, 41 for proline, 50 for glycine betaine, and approximately 52 for sucrose (Raven, 1985). That is why Na is often called a "cheap osmolyte".

1.9 Salinity tolerance in halophytes: A complex trait

As follows from the above, salinity tolerance is a complex trait, involving a) the accumulation and compartmentalization of ions for osmotic adjustment, b) the synthesis of compatible solutes, c) efficient signaling pathways and efficient regulation of 'salt tolerance genes' (Volkov *et al.,* 2003; Taji *et al.,* 2004), d) the ability to accumulate essential nutrients (particularly K) in the presence of high concentrations of Na, e) the ability to limit the entry of Na into the transpiration stream either by reducing initial Na entry into roots or by controlling xylem loading/un-loading, f) the ability to regulate transpiration in the presence of high concentrations of Na and Cl (Flowers and Colmer, 2008).

1.10 Outline of this thesis

In chapter 1 the scientific background of the research questions addressed in this thesis has been presented. The main aim of the research was to compare the expression levels of salt tolerance candidate genes between halophytic and glycophytic species, and to assess the potential role of *cis*-regulatory alteration of the expression levels of these genes in the evolution of high-level salt tolerance in halophytes.

In chapter 2 four Cochlearia species, among which two halophytes (*C. anglica*, *C. x hollandica*), a relatively salt-tolerant glycophyte (*C. danica*), and a metallophyte (*C. pyrenaica*) have been compared for salt and heavy metal tolerance, the expression of four candidate salt tolerance genes (*HKT1, SOS1, NHX1*, *VATD*) and a candidate heavy metal tolerance gene (*MTP1*), as well as the Na, K, Cd, Zn concentrations in roots and shoots.

In chapter 3 six Brassicaceae (three halophytes and three glycophytes) have been

compared for their expression levels of *NHX1*, *SOS1* and the *V-ATPase* subunit-D (*VATD*), as well as the accumulation of Na and K in roots and shoots. *A. thaliana nhx1* and *sos1* mutants and wild-type were transformed, respectively, with *NHX1, SOS1* and *GUS*, both under the endogenous natural *NHX1, SOS1* and *VATD* promoters from *A. thaliana* and those from *C. x hollandica*. T_1 lines were subjected to Real-Time PCR in order to check the expression levels in the transgenic lines.

In chapter 4 the activities of the *HKT1* promoters from Thellungiella species (*T. halophila*, ecotype Shandong and *T. botschantzevii*, ecotype Saratov) have been compared with their *A. thaliana* homolog through promoter-cDNA swapping and ectopic expression in the *A.t.hkt1* mutant. T_1 lines were subjected to Real-Time PCR, to check the expression level of *HKT1*. The lines were also compared for salt tolerance, foliar water content, and Na and K accumulation.

In chapter 5 the results of the work reported in this thesis are discussed within a broader context, and directions for future research are given.

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Chapter 2

Variation in salt and heavy metal tolerance and the expression levels of candidate tolerance genes among four *Cochlearia* species with distinct habitat preferences

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Abstract

We compared four Cochlearia species for salt and zinc (Zn) and cadmium (Cd) tolerance and accumulation, and for the transcript levels of candidate tolerance genes for salt and Zn. Salt tolerance decreased in the order *C. anglica* > *C. x hollandica* > *C. danica* > *C. pyrenaica*, corresponding to the salinity levels at the sites of population origin. Only *C. anglica* and *C. x hollandica* appeared to be true halophytes, maintaining considerable growth rates and showing no visible damage when grown at 200 mM NaCl. Of the four salt tolerance candidate genes, *HKT1* was expressed more or less in proportion with the salt tolerance level of the species, at least in the roots of salt-exposed plants. The expression in the shoot was particularly high in *C. anglica*, but not in *C. x hollandica*. Also the other candidate salt tolerance genes *SOS1*, *NHX1* and a *V-ATPase* subunit-D encoding gene, *VATD*, were highly expressed in *C. anglica* or *C. x hollandica*. In particular, *SOS1* was highly expressed in *C. anglica*, though only in roots, and *C. x hollandica*, both in roots and shoots. *NHX1* was highly expressed in *C. anglica* and *C. x hollandica*, in both species only in roots, and *VATD* in *C. x hollandica*, both in roots and shoots, but not in *C. anglica*. These results suggest that *C. anglica* and *C. x hollandica* may have evolved partly different mechanisms for salt tolerance.

As expected, of all the species, only *C. pyrenaica* was hypertolerant to Zn, since the population under study originated from a former Zn mine. There was no detectable hypertolerance to Cd, however, which is unusual for metallicolous plant populations from calamine soil. The expression level of the candidate Zn tolerance gene was higher in *C. pyrenaica* than in *C. danica* and *C. anglica*, but not significantly different from that in the *C. x hollandica*.

Keywords; Salt tolerance, heavy metal tolerance, Cochlearia, *HKT1, SOS1, NHX1, VATD, MTP1*

2.1 Introduction

Only a small number of higher plant species are capable to grow and reproduce in environments with extreme soil chemistry, such as salt marshes or strongly heavy metal-enriched ('metalliferous') sites (Ernst, 1974; Flowers and Colmer, 2008). The species found on saline or metalliferous soil are called 'halophytes' and 'metallophytes', respectively. These species, or at least their salt or metal exposed populations, exhibit strongly enhanced levels of salt or metal tolerance ('hypertolerance'), in comparison with species/populations from 'normal' soils (Antonovics *et al*., 1971; Yeo and Flowers, 1980; Flowers and Colmer, 2008).

The molecular basis and physiological mechanisms underlying cases of hypertolerance to extreme soil chemistry are largely elusive. It is assumed, often more or less implicitly, that high-level salt or metal tolerance would be due to altered regulation, copy number expansion, or minor non-synonymous changes of universal homeostatic genes, rather than unique halophyte or metallophyte genes (Flowers and Colmer, 2008; Clemens, 2001; Hanikenne and Nouet, 2011). Indeed, based on recent breakthroughs in metallophyte research, it seems that the Zn and Cd hypertolerance and hyperaccumulation phenotypes in the Zn/Cd hyperaccumulating metallophyte, *Arabidopsis halleri*, are ultimately dependent on a strongly enhanced, largely constitutive ('deregulated') expression, through copy number expansion and altered *cis*-regulation, of a number of genes which had been previously shown to be involved in metal homeostasis in the congeneric non-metallophyte model species, *Arabidopsis thaliana.* These genes include those encoding the 1b P-type heavy metal transporting ATPase, *HMA4* , and the Zn^{2+}/H^{+} antiporter, *MTP1* (= *ZTP1*= *ZAT*) (Dräger *et al*., 2004; Talke *et al*., 2006; Courbot *et al*., 2007; Willems *et al*., 2007; Hanikenne *et al*., 2008), which are responsible for the loading of Zn and Cd into the xylem (Hussain *et al*., 2004), and the vacuolar sequestration of excessive cellular Zn (van der Zaal *et al*., 1999; Krämer, 2005), respectively. Whereas these genes are single-copy in the non-metallophytes *A. thaliana* or *A. lyrata*, they are triplicated (*HMA4*) or at least pentaplicated *(MTP1)* in *A. halleri*, with the *HMA4* copies all in a tandem arrangement (Hanikenne *et al*., 2008; Shahzad *et al*., 2010). It is remarkable that the same genes are similarly over-expressed in another Brassicaceae Zn/Cd hyperaccumulator, *Noccaea (=Thlaspi) caerulescens* (Assunção *et al*., 2001; Lochlainn *et al*., 2011), with four *HMA4* copies in a tandem arrangement (Lochlainn *et al*., 2011). The latter is a striking case of parallel molecular evolution, because the Zn hyperaccumulation trait must have been evolved independently in *Noccaea* and *Arabidopsis*

(Verbruggen *et al*., 2009).

As mentioned before, the molecular basis of heavy metal hypertolerance phenomena is still poorly understood, but a picture is beginning to emerge, at least for hyperaccumulating metallophytes, which is ultimately due to the availability of segregating metallophyte x non-metallophyte crosses (Willems *et al*., 2007; Courbot *et al*., 2007; Frérot *et al*., 2010), and extensive transcriptomic comparisons between (hyperaccumulator) metallophytes and related non-metallophytes (Becher *et al*., 2004, Weber *et al.,* 2004; Hammond *et al.,* 2006; van de Mortel *et al.,* 2006), which allowed for a well-considered selection of candidate genes (Talke *et al*., 2006). However, most of these candidates have not been validated yet. It has also been helpful that both hyperaccumulator metallophyte genetic models, *A. halleri* and *N. caerulescens*, share a high degree of DNA identity with the general plant genetic model, *A. thaliana*, which allows for the use of most of the sophisticated molecular tools, as well as the complete DNA sequence information and extensive gene annotations available for the latter species. Another favorable circumstance is that at least *A. halleri* has been shown to be genetically accessible, which allows the validation of candidate genes through RNAi-mediated silencing (Hanikenne *et al*., 2008).

In comparison with heavy metal hypertolerance, salt hypertolerance is less understood, because of the more complex and phylogenetically biased nature of the underlying mechanisms (Flowers and Colmer, 2008). Moreover, salt hypertolerance is a species-wide, or even genus-wide property, which strongly restricts the possibilities to make properly segregating halophyte x glycophyte crosses for QTL mapping, co-segregation analysis, or analysis of recombinant inbred line (RIL) collections. For this reason, QTL analyses of salt tolerance have thus far been confined to glycophyte crop species, like rice (Koyama *et al.,* 2001). However, there are no valid reasons to suppose, genes that control the (limited) variation in salt tolerance among glycophyte varieties would generally also be ones that control the much bigger difference in salt tolerance between halophytes and glycophytes.

The most obvious way to identify salt hypertolerance genes is through comparing the transcriptomes of halophytes and (preferably closely related) glycophytes, and validating the emerging candidate genes, preferably both through suppression in the halophyte and heterologous over-expression in the glycophyte (or the other way around, if tolerance is thought to result from suppression). To date extensive comparisons of gene expression patterns between halophytes and glycophytes are lacking, except for the case of *Thellungiella halophila/salsuginea* and *A. thaliana* (Kant *et al.,* 2006). However, although *T. halophila* has

the advantages of genetic accessibility (Fang *et al.*, 2006; Ali *et al.*, 2012) and a sufficient degree of DNA identity with *A. thaliana* to allow cross-species transcriptome comparisons using *A. thaliana* cDNA micro-arrays (Volkov *et al.,* 2003; Gong *et al.,* 2005), it may not be the ideal salt tolerance model. First, *T. halophila* is also highly tolerant to stresses other than salt, e.g. drought and cold (Bressan *et al.,* 2001; Inan *et al.,* 2004) and, although it may survive exposure to seawater-like salt concentrations for quite some time (Bressan *et al.,* 2001; Inan *et al.,* 2004), its growth is already strongly retarded at fairly low levels of salt exposure (Vera-Estrella *et al.,* 2005), which is not typical of the halophytes of sea water flooded salt marshes, for example (Flowers and Colmer, 2008). Regardless of this, out of all the transcriptional differences found between *T. halophila* and *A. thaliana*, thus far only that of *SOS1,* which encodes a plasma membrane-located Na^+ effluxing Na^+/H^+ antiporter (Shi *et al.,* 2000) has been more or less convincingly shown to be essential for the superior salt tolerance of *T. halophila*, through RNAi-mediated silencing (Oh *et al.,* 2007).

Current hypothesis on molecular salt hypertolerance mechanisms are largely based on large-scale *A. thaliana* mutant screenings, or on more specific approaches, including the functional characterization, silencing and transgenic over-expression of genes expected to be involved in plant responses to salt or drought, usually based on the transcriptional analysis of stress responses. This yielded a large number of genes that appeared to be essential for wild-type-like tolerance and responses to salt. Some of these genes encode plasma membrane-located Na transporters, such as SOS1 (see above) and HKT1. HKT1 seems to counteract excessive Na accumulation in the shoot, possibly via resorbing xylem Na into the xylem parenchyma, thus facilitating downward Na transport into the root via the phloem (Berthomieu *et al.,* 2003). Although considerable downward Na transport via the phloem has been considered unlikely (Flowers and Colmer, 2008), there can be no doubt about the importance of HKT1 for wild-type-level salt tolerance in *A. thaliana,* given the extreme Na hypersensitivity of the *hkt1* mutants (Maser *et al.,* 2002). Another transporter shown to be essential for wild-type salt tolerance level in *A. thaliana* is the vacuolar Na^+/H^+ antiporter, NHX1 (Sottosanto *et al.,* 2007). Since SOS1 and NHX1 are Na^+/H^+ antiporters, energized by transmembrane proton gradients, the proton pump of the plasma membrane, and those of the tonoplast, the vacuolar proton ATPase and the proton translocating pyrophosphatase are also considered to be essential for normal wild-type salt tolerance (Gaxiola *et al.,* 2007). Because of their obvious importance in Na homeostasis and salt acclimation in *A. thaliana* and other glycophytes, it is often more or less implicitly assumed that the superior salt tolerance in

halophytes should result from an altered expression of the same set of genes, together with genes involved in K transport/accumulation, or the synthesis of 'compatible osmolytes, such as proline or glycinebetaine (Flowers and Colmer, 2008). However, although this idea seems to be plausible at first sight, it is thus far not supported by any evidence, except for the case of *SOS1* in *T. halophila* (see above). Surprisingly, there are virtually no reports in which the expression patterns of such obvious salt hypertolerance candidate genes have been compared between halophytes and glycophytes, although it does not seem to be too difficult to do so.

When comparing gene expression patterns between population or species, there is always the possibility of phylogenetic or ecological bias unrelated with the trait of interest. To avoid such bias as much as possible, and because of the practical advantages of a high cDNA sequence identity with *A. thaliana*, the best studied metallophyte and halophyte plant models are all Brassicaceae, i.e. *A. halleri*, *N. caerulescens* and *T. halophila*. In addition to *T. halophila*, which may not be the ideal halophyte model (see above), other Brassicaeae have been also described as halophytes, o.a., *Crambe maritima*, *Cakile maritima* (Debez *et al.,* 2004), and *Lobularia maritima* (Popova *et al.,* 2008). However, none of these species seems to be able to complete its life cycle in controlled experiments at > 200 mM NaCl (De Vos *et al.,* 2010; H. Schat, unpublished), which is often considered as a criterion for being 'a true halophyte' (Flowers and Colmer, 2008). Moreover, although their distributions are mainly coastal, they grow at higher elevation above sea level, e.g. in fore dunes or on cliffs, where the ground-water is usually non-saline, or at most slightly and temporarily brackish (De Vos *et al.,* 2010). Under these conditions, these plants are probably mainly confronted with salt via the air (deposition of 'salt spray' on the shoot), rather than the ground-water (Wells and Shunk, 1938; De Vos *et al.,* 2010). The most obvious candidate halophyte among the European Brassicaceae is doubtlessly *Cochlearia anglica*, which typically occurs in the more elevated parts of coastal salt marshes (Rozema *et al.,* 1985; Pegtel, 1999).

In general, the genus Cochlearia might provide good opportunities to study plant adaptation to extreme edaphic conditions. First, based on the species' ecological preference regarding soil salinity in North-Western Europe, the genus is expected to host at least one 'true halophyte', e.g., *C. anglica*, as well as a glycophyte, *C. pyrenaica*, and two species with a rather strict preference for brackish soils, e.g. *C. x hollandica* and *C. officinalis*, as well as one which occurs both on slightly brackish soils and non-saline soils, *C. danica* (Rozema *et al.,* 1985). Second, *C. pyrenaica* is a metallophyte, capable to grow on calamine soils that are toxically enriched in Zn, Cd and Pb (Reeves, 1988), whereas the other species are strictly

non-metallicolous at species level. The species are expected to be closely related among each other. Most likely, *C. officinalis* is an autotetraploid of *C. pyrenaica*, *C. anglica* is an autooctoploid, arosen through duplication of the *C. officinalis* genome, and *C x hollandica* is the hexaploid hybrid of *C. anglica* and *C. officinalis* (Koch *et al.,* 1998; Pegtel, 1999), whereas *C. danica* probably arose as an allohexaploid hybrid of *C. pyrenaica* and *C. officinalis*, followed by chromosome complementing (Koch *et al*., 1998). In this study we compared salt tolerance along with Zn and Cd tolerance among a salt marsh population of *C. anglica*, a brackish beach plain population of *C x hollandica*, a foredune population of *C. danica* and a metallicolous population of *C. pyrenaica*. To check their putative involvement in salt or heavy metal hypertolerance we compared the expression of four genes that have often been supposed to be involved in salt tolerance, i.e., *SOS1*, *HKT1*, *NHX1,* and *VATD* (encoding the vacuolar proton ATPase, subunit-D), as well as a heavy metal hypertolerance gene that has been validated as such in *A. halleri*, *MTP1* (see above). The *Actin-2* (*Act-2*) was used as an internal reference gene.

2.2 Materials and Methods

2.2.1 Plant materials and growth conditions

Seeds of *C. anglica (C.a.)* were collected from a coastal salt marsh, called 'the Slufter', at the island of Texel in the Wadden Sea, the Netherlands. At this site *C. anglica* grows together with 'true halophytes' like *Limonium vulgare*, *Halimione portulacoides*, *Triglochin maritima*, *Juncus gerardii*, *Aster tripolium*, *Festuca rubra ssp. littoralis*, *Plantago maritima*, *Glaux maritima* and *Spergularia marina*. Seeds of *C. danica* (*C.d.*) were also collected at the island of Texel, but from a foredune at the "Mokbaai", where it grows in a vegetation consisting of glycophytes only (mainly *Festuca rubra ssp. arenaria*, *Galium verum*, *Leontodon nudicaulis*, *Erodium cicutarium*, *Geranium molle*). Seeds of *C. x hollandica* (*C.h.*) were collected from a brackish coastal beach plain at the island of Voorne, the Netherlands, where the species grows together with more or less 'halotolerant glycophytes' which are often found along the upper edge of coastal salt marshes, such as *Rumex crispus*, *Potentilla anserina*, *Phragmitis australis*, and *Agrostis stolonifera*, or species with a preference for brackish environments, such as *Scirpus maritimus*. Seeds of *C. pyrenaica* (*C.p.*) were collected from a former zinc mine near La Calamine, Belgium (see Assunção *et al*., 2003, for a site description). The seeds were sown in organic garden soil (Jongkind BV, No. 1, Aalsmeer, The Netherlands) and after

three weeks, seedlings were transferred to hydroponic culture, in 1-L polyethylene pots containing a modified half strength Hoagland's solution composed of 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 1 µM KCl, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.1 µM $CuSO_4$, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 20 µM Fe(Na)EDTA, and 2 mM 2-(N-morpholino)ethanesulphonic acid (MES), adjusted to pH 5.5 using KOH. Nutrient solutions were renewed weekly and plants were grown in a growth chamber (20/15 °C day/night; 220 µmol PAR m^{-2} s^{-1} at plant level, 14 h d^{-1}; relative humidity 75%).

2.2.2 Heavy metal tolerance test

After two weeks of growth in hydroponics, plants were exposed to Zn (2-, 50-, 150-, 450- and 1350 µM $ZnSO_4$), or Cd (0-, 20-, 40-, 80-, 160- and 320 µM $CdSO_4$), twelve plants per species per concentration, in a background solution of the same composition as the pre-culture solution. Prior to exposure, the roots were stained black by dipping them in a stirred suspension of finely powdered active carbon (Schat and Ten Bookum, 1992). After five days of exposure, the growth, i.e. the length of the unstained root segment of the longest root, was measured. After one week of exposure the plants were harvested. Prior to harvest the roots were desorbed in an ice-cold 5 mM $Pb(NO_3)_2$ solution for 30 min. For RNA isolation, parts of the roots and shoots were snap-frozen in liquid nitrogen and stored at -80 °C. The rest of the roots and the shoots were dried in a oven at 65 °C for three days

2.2.3 Salt tolerance test

After 10 days of growth in hydroponics, plants were stepwise exposed to increasing NaCl concentrations, in a background solution of the same composition, (one week per step, to allow osmotic adjustment). The concentration steps were 50-, 100-, 200-, and 400 mM. The final concentrations were 0-, 100-, 200- and 400 mM (10 plants per concentration per species). As soon as the final exposure concentration had been reached, plants were weighed, after quickly drying roots through blotting on paper tissue, and then they were kept exposed for another 25 days at the same concentration, after which they were weighed again. Then plant parts were harvested and stored for analysis, as described above. The relative growth rate was calculated as the difference between the natural logarithms of the final and initial fresh weights, divided by the time of exposure to the final NaCl concentration (3.5 weeks).

2.2.4 Measurement of Zn, Cd, Na and K

Zn and Cd in roots and shoots samples were measured in pooled samples, consisting of three plants each. About 100 mg of powdered dried plant material was digested in 2 ml of 37% (v/v) HCl; 65% (v/v) HNO_3 (1;4, v/v) in Teflon cylinders for 7 hours at 140 °C, after which the volume was adjusted to 10 ml with demineralised water. Zn and Cd concentrations were determined on an atomic absorption spectrophotometer (Perkin Elmer AAS100). For Na and K analysis the materials of three plants were pooled and powdered. Twenty mg of plant material was extracted (90 ºC) in 2 ml of demineralized H_2O in 2 ml eppendorfs for 1 hour. After cooling, the extracts were filtered through Spin-X® Centrifuge tube Filters (Costar, 0.22 µM Nylon). Proper dilutions were made in demineralised water. Na and K concentrations were determined, using flame emission, on an atomic absorption spectrophotometer (Perkin Elmer AAS100).

2.2.5 RNA extraction and cDNA preparation

RNA was extracted from all *Cochlearia* frozen roots and leaves using the Trizol™ (Invitrogen) method, following the manufacturer's instructions. Single-stranded cDNA was synthesized from total RNA (2.5 µg, boiled for 1 minute), using 100 Units of M-MLV Reverse Transcriptase (Invitrogen), 2 mM dNTPs, 100 mM DTT, 10X RT buffer and 10 µM oligo dT primer, at 42 ºC for 1hr.

2.2.6 Amplification of *HKT1, NHX1, SOS1, VATD*, *MTP1* and *Act-2*

PCR's were performed on cDNA/gDNA to amplify the orthologues of *HKT1, NHX1, SOS1*, *VATD, MTP1* and *Act-2* from all the *Cochlearia* species. Degenerate primers were designed (Table S2.1: Supplementary Information) based on the regions that appeared to be most conserved among the plant species represented in GenBank. The first PCR was performed on 2 µl of cDNA/gDNA. PCR reactions were performed as follows; a hot start for 3 min at 96 °C, followed by 26 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min, and a final extension step of 10 min at 72 °C. Nested PCR was done on 2 µl of the first PCR product, using the nested primers sets (Table S2.1: Supplementary Information), following the same programme as for the first PCR. The amplified DNA fragments were gel-purified using the Agarose Gel DNA extraction Kit (Roche, Applied Science) and cloned into pGEM-T Easy (Technical Manual, pGEM®-T Easy Vectors, Promega). All the fragments were sequenced using the Big Dye Terminator kit

(Applied Biosystems) and samples were run on ABI PRISM 3100 DNA Sequencer. Data base searches were conducted with BLAST service at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and TAIR (www.arabidopsis.org).

2.2.7 Primer designing and Real-Time PCR

Gene-specific Real-Time PCR primers, with a G/C ratio between 50 and 60% and a melting point between 58 and 60 ºC for the *HKT1, NHX1, SOS1*, *VATD, MTP1* and *Act-2,* were designed on the basis of their obtained partial sequences (Table S2.2: Supplementary Information). All the primer pairs were intron spanning (except for *VATD*, which does not have introns), to avoid gDNA amplification. The quantitative assessment of mRNA levels was performed with SensiMix™ SYBR No-ROX kit (Bioline), including the SYBR® Green I dye, dNTPs, stabilisers and enhancers, and using the Bio-Rad MJ Research Opticon™ Real-Time PCR detection system (Applied Biosystems Inc., IJssel, The Netherlands). A dilution series (5-, 10-, 20-, 40- and 80 times) of the cDNA samples in water was tested to identify the cDNA concentrations that produced cycle threshold values between 18 and 30, and PCR efficiencies of > 1.98. The final reaction conditions were 10 µl SensiMix™ SYBR No-ROX matser mix, 0.75 µl forward primer (final concentration of 250 nM), 0.75 µl of reverse primer (final concentration of 250 nM) and cDNA in a total reaction volume of 20 µl. An initial step of 95 ºC for 10 min was used to activate the polymerase. Cycling conditions were; melting step at 95 ºC for 10 s and annealing-extension at 60 ºC for 20 s, with 40 cycles, at the end melting curve from 60 ºC to 90 ºC, read every 0.5 ºC, for 10 s. All Real-Time PCR reactions were performed in experimental triplicates, and a maximum difference of one cycle between the C_T's of the triplicate samples was considered acceptable. Negative controls were included for each primer pair to check for significant levels of any contaminants. Expression values were calculated using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). Shoots and roots of individual plants were used for RNA extraction and for Real-Time PCR analysis. All the primers used for Real-Time PCR are given in Table S2.2 (Supplementary Information).

2.2.8 Statistics

Statistic analysis was performed using one way and two-way ANOVA. The MSR statistic was used for a posteriori comparisons of individual means (Rohlf and Sokal, 1981). When necessary, data was subjected to logarithmic transformation prior to analysis.

2.3 Results

2.3.1 Salt tolerance

The effect of NaCl on the relative growth rate (RGR) differed strongly between the species. While all the species grew comparably fast in the control solution, there were pronounced inter-specific differences at all the NaCl exposure levels tested (Fig. 1). As expected, *C. pyrenaica* was clearly the most salt-sensitive species; it died at 400 mM and it showed heavy chlorosis and spotted necrosis at 200 mM NaCl. *C. danica* survived, but lost fresh weight at 400 mM, and showed heavy chlorosis of the older leaves at 200- and 400 mM. *C. x hollandica* and *C. anglica* showed slight chlorosis of the very oldest leaves at 400 mM, but

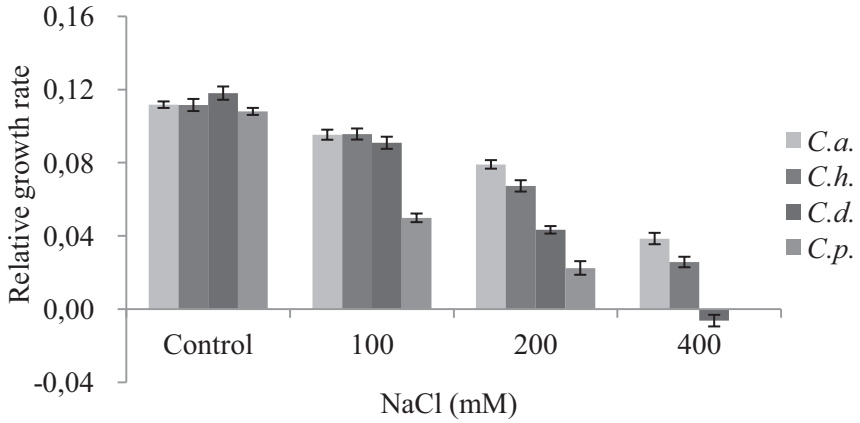

Fig. 1 Effect of salt treatment on Relative growth rate (RGR) of *Cochlearia anglica* (*C.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Cochlearia pyrenaica* (*C.p.*). Error bars are ±SE.

not at all at 200 mM. Both of these species maintained considerable fresh biomass increments even at 400 mM. At 200 mM the RGR of each of the species was significantly different from each of the others. In summary, their salt tolerance unambiguously decreased in the order *C. anglica* > *C. x hollandica* > *C. danica* > *C. pyrenaica*.

2.3.2 Na and K accumulation

There were no big differences between the species' root and shoot Na concentrations after exposure to 200 mM NaCl, except that *C. x hollandica* had relative low Na accumulation in the shoot (Fig. 2). In control conditions there were no considerable inter-specific differences in the root K concentrations (Fig. 3). The shoot K concentrations were significantly higher in *C. anglica* and *C. pyrenaica* than in *C. x hollandica* and *C. pyrenaica*, both in the control and the 200 mM NaCl (Fig. 3). The 200 mM NaCl exposure strongly and significantly decreased the shoot K concentrations, to a comparable degree in all the species. The same was found for

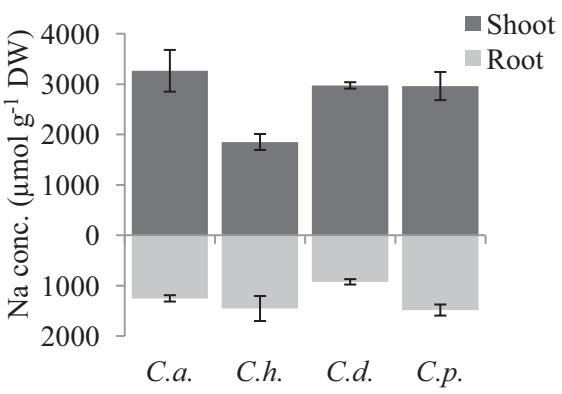

Fig. 2 Shoot and root sodium concentrations of *Cochlearia anglica* (*C.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Cochlearia pyrenaica* (*C.p.*) after three weeks of exposure to 200 mM NaCl. Error bars are ±SE. Black bars, shoot Na concentration: grey bars, root Na concentration

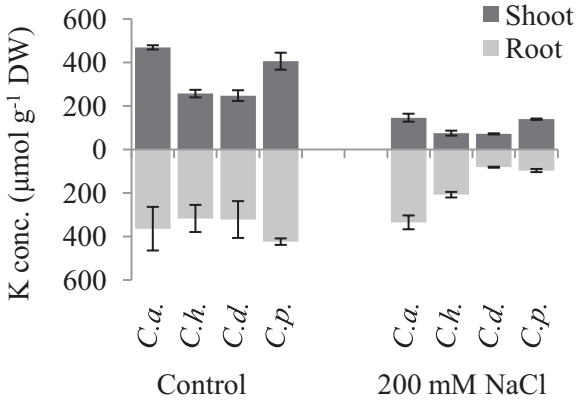

Fig. 3 Shoot and root potassium concentrations of *Cochlearia anglica* (*C.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Cochlearia pyrenaica* (*C.p.*) after three weeks of exposure to 200 mM NaCl. Error bars are ±SE. Black bars, shoot K concentration: grey bars, root K concentration.

the root K concentrations in *C. danica* and *C. pyrenaica*. On the other hand, *C. anglica* and *C. x hollandica* maintained root K concentrations under NaCl exposure that were not significantly different, or only slightly lower than those in the control treatment, respectively (Fig. 3).

2.3.3 Zinc and cadmium tolerance

As estimated from the root growth test, *C. pyrenaica* appeared to be significantly more Zn tolerant than any of the other species (Fig. 4). All of the other species, were

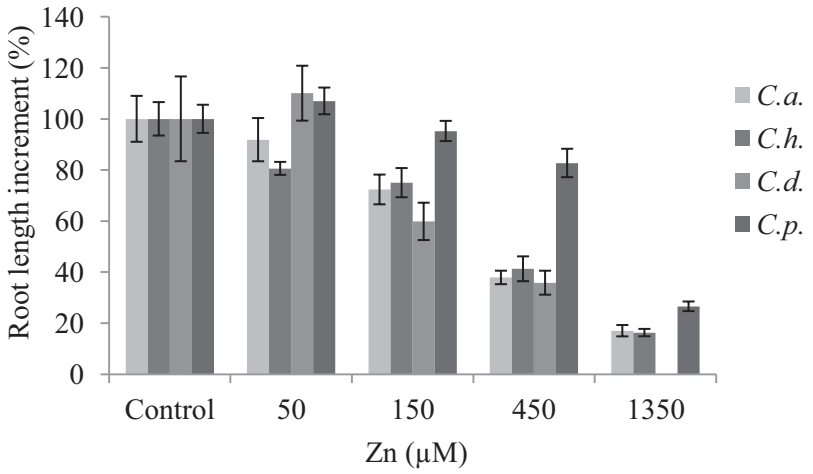

Fig. 4 Relative effect of Zn on root growth in *Cochlearia anglica* (*C.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Cochlearia pyrenaica* (*C.p.*) (*C.d.* did not show any root growth at 1350 µM). Means are given as % of controls. Error bars are ±SE.

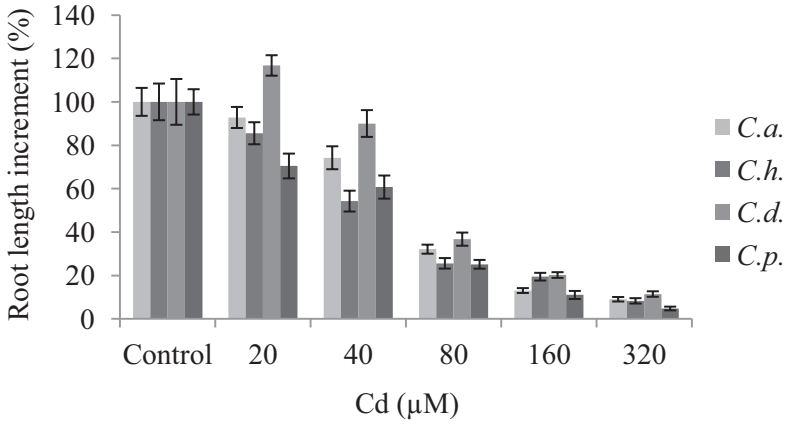

Fig. 5 Relative effect of Cd treatments on root growth of *Cochlearia anglica* (*C.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Cochlearia pyrenaica* (*C.p.*). Means are given as % of controls. Error bars are ±SE.

not considerably different among each other (Fig. 4). Regarding Cd tolerance, *C. danica* was significantly, albeit only slightly more tolerant than all of the other species, including *C. pyrenaica* (Fig. 5).

2.3.4 Zn and Cd accumulation

The root and shoot Zn concentrations after one week of exposure varied significantly between species (Fig. 6), and also the species x Zn concentration interaction was

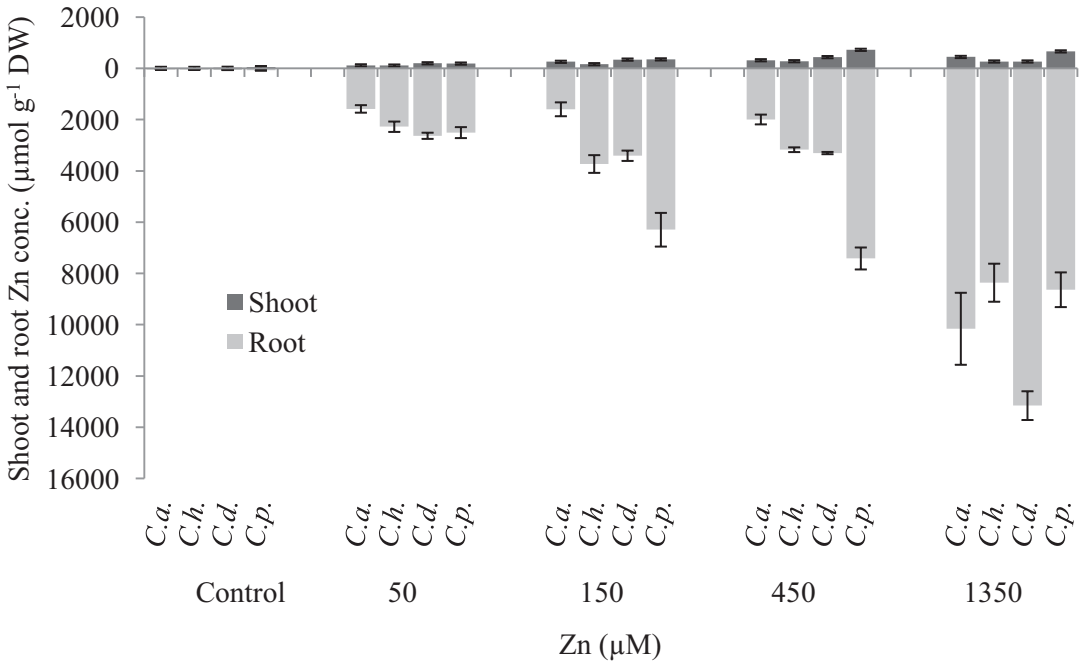

Fig. 6 Effect of Zn treatments on shoot and root Zn accumulation in *Cochlearia anglica* (*C.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Cochlearia pyrenaica* (*C.p.*). Error bars are ±SE. Black bars, shoot Zn concentration: grey bar, root Zn concentration.

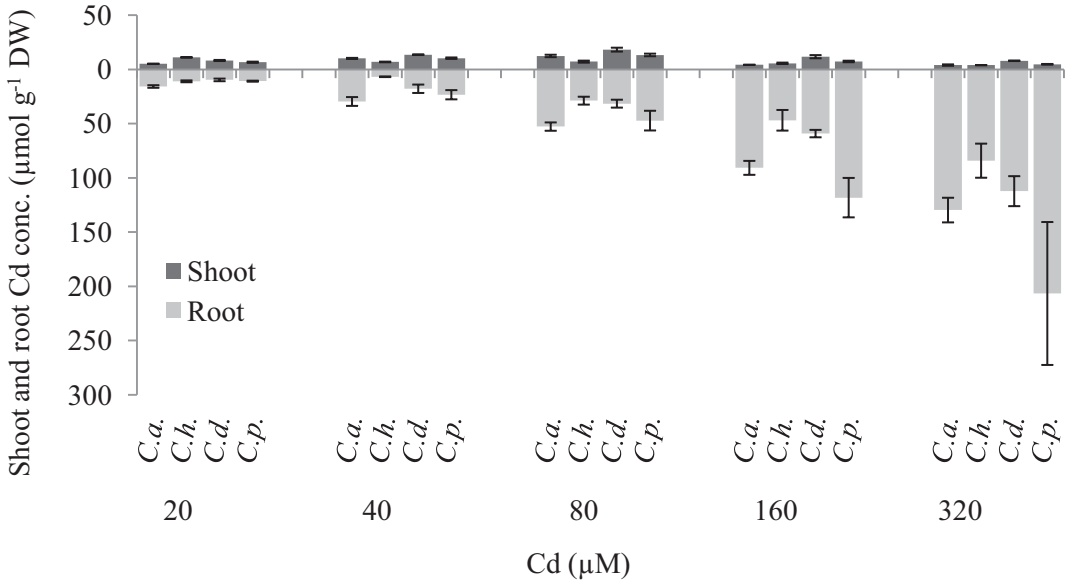

Fig. 7 Effect of Cd treatments on shoot and root Cd accumulation in *Cochlearia anglica* (*C.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Cochlearia pyrenaica* (*C.p.*). Error bars are ±SE. Black bars, shoot Cd concentration: grey bar, root Cd concentration.

significant ($P < 0.01$). All the species accumulated Zn primarily in the root. Of all the species, at the 150- and 450 µM Zn treatments *C. pyrenaica* showed the highest, and *C. anglica* the lowest root Zn concentrations, respectively (Fig. 6). At 450- and 1350 µM Zn, *C. pyrenaica* showed shoot Zn concentrations that were significantly higher than those of all the others. The Cd concentrations in root and shoot also varied significantly between species, the pattern being different from that for Zn. All the species accumulated Cd primarily in the root (Fig. 7).

2.3.5 Expression of salt and Zn/Cd tolerance candidate genes

2.3.5.1 *HKT1* expression

The *HKT1* cDNA sequences obtained for *C. anglica*, *C. x hollandica* and *C. danica* were 98-99% identical among each other, and 81-82% identical with *AtHKT1*. The corresponding predicted protein sequences were 97-100% identical among each other, and 74-76% identical with AtHKT1.

The *HKT1* cDNA sequence obtained for *C. pyrenaica* was notably different, sharing 76-77% nucleotide identity with the other Cochlearia species, and 82% with *AtHKT1* (63-65% and 74% at the protein level, respectively) (Table S2.4: Sequence alignment; S2.1: Supplementary Information).

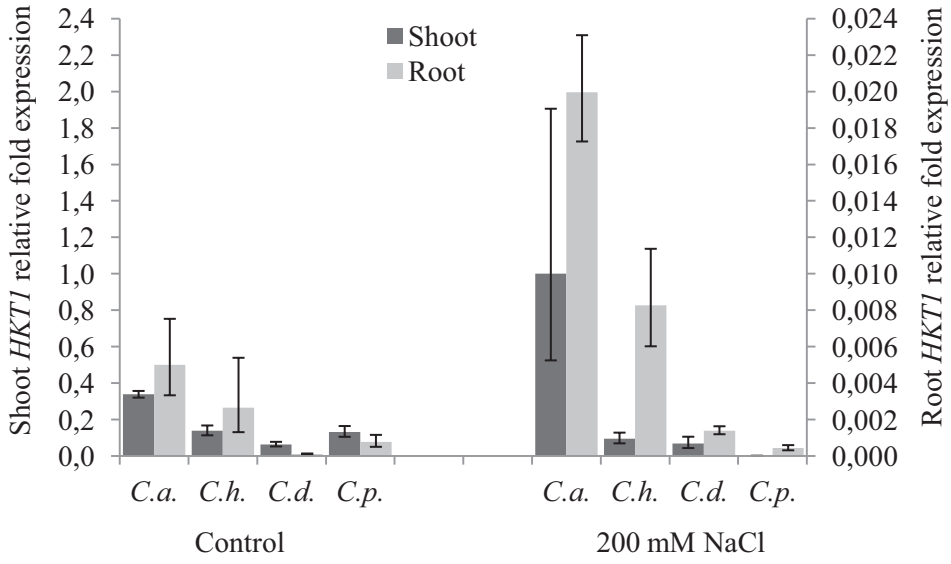

Fig. 8 Expression of *HKT1* in shoot and root of *Cochlearia anglica* (*C.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Cochlearia pyrenaica* (*C.p.*). The gene expression was normalized to the highest expression, which was assigned a value of 1. Each value is average of three independent biological replicates. Error bars are ±SE. Black bars, shoot *HKT1* expression: grey bars, root *HKT1* expession.

In all the species *HKT1* expression was much higher in the shoots than in the root (Fig. 8). In the shoot *C. anglica* showed significantly higher expression levels than did the other species, both in the control and the 200 mM NaCl. There was no significant effect of salt exposure, except for *C. pyrenaica*, where the gene expression was strongly down-regulated by salt exposure (Fig. 8). Also in the roots, under control conditions, the *HKT1* transcript concentration was significantly higher in *C. anglica* than in either of the other species, and significantly affected by salt exposure (Fig. 8). However, there was significant up-regulation in *C. x hollandica* and *C. danica.* In the 200 mM NaCl treatment the expression levels in the two most salt-tolerant species, *C. anglica* and *C. x hollandica*, were similar among each other, and significantly higher than in *C. danica* and *C. pyrenaica*.

2.3.5.2 *SOS1* expression

The Cochlearia *SOS1* cDNA sequences obtained were 98-99% identical among each other, and 88-90% identical with *AtSOS1*. The corresponding predicted protein sequences were 95-99% identical among each other, and 88-91% identical with AtSOS1 (Table S2.5: Sequence alignment; S2.2: Supplementary Information).

In the absense of salt, *SOS1* was particularly expressed in *C. x hollandica*, but barely in *C. anglica* and *C. danica* (Fig. 9). In all the species, the expression in the

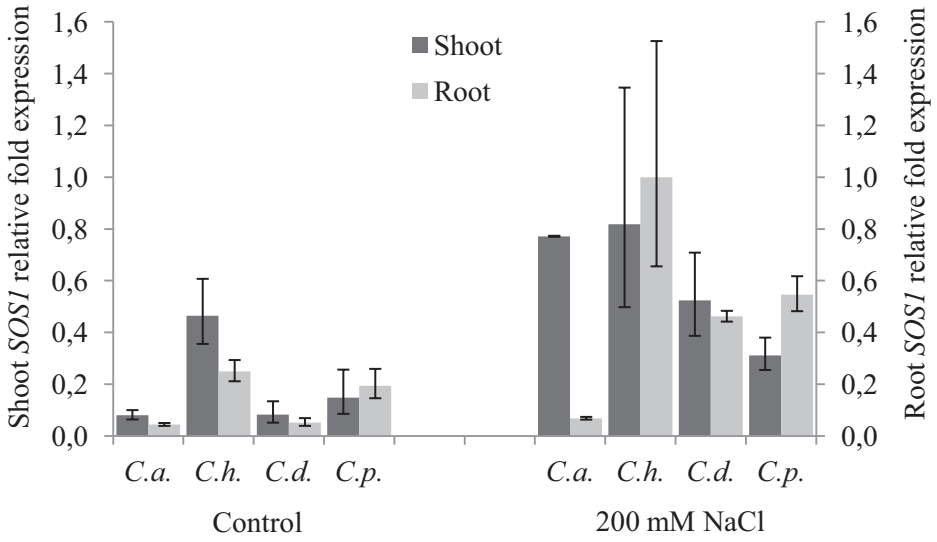

Fig. 9 Expression of *SOS1* in shoot and root of *Cochlearia anglica* (*C.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Cochlearia pyrenaica* (*C.p.*). The gene expression was normalized to the highest expression, which was assigned a value of 1. Each value is the average of three independent biological replicates. Error bars are ±SE. Black bars, shoot *SOS1* expression: grey bars, root *SOS1* expession.

shoot was up-regulated under salt exposure, though only strongly and significantly so in *C. anglica* and *C. danica*. *SOS1* expression in the root was up-regulated by salt treatment in all species, particularly in *C. x hollandica* and *C. danica*, but not at all in *C. anglica* (Fig. 9).

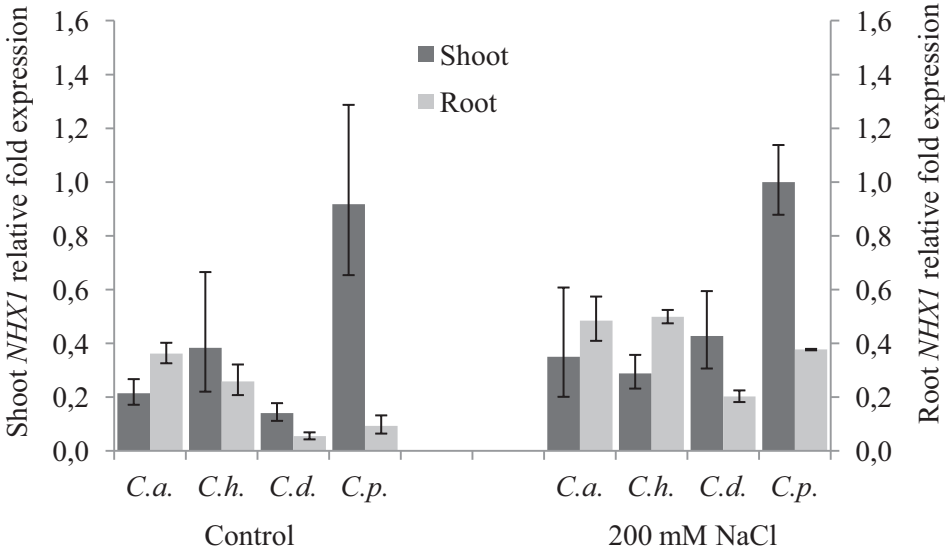

Fig. 10 Expression of *NHX1* in shoots and roots of *Cochlearia anglica* (*C.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Cochlearia pyrenaica* (*C.p.*). The gene expression was normalized to the highest expression, which was assigned a value of 1. Each value is the average of three independent biological replicates. Error bars are ±SE. Black bars, shoot *NHX1* expression: grey bar, root *NHX1* expession.

2.3.5.3 *NHX1* expression

The Cochlearia *NHX1* cDNA sequences obtained were 92-99% identical among each other, and 90-91% identical with *AtNHX1*. The corresponding predicted protein sequences were 96-99% identical among each other, and 94-96% identical with AtNHX1 (Table S2.6: Sequence alignment; S2.3: Supplementary Information).

In the absence of salt, *NHX1* expression in the shoot was highest in *C. pyrenaica* and lowest in *C. danica* (Fig. 10). Salt treatment did not significantly increase the shoot expression level, except in *C. danica*. In the roots, in the absence of salt, *NHX1* expression was significantly higher in *C. anglica* and *C. x hollandica* than in *C. danica* and *C. pyrenaica*. Salt treatment enhanced root *NHX1* expression significantly and strongly in *C. pyrenaica* and *C. danica*, but much less in *C. x hollandica* and *C. anglica*, although the latter two species maintained a significantly higher expression level than the former two also under salt treatment (Fig. 10).

2.3.5.4 *VATD* expression

The Cochlearia *VATD* cDNA sequences obtained were 98-99% identical among each other, and 86-87% identical with *AtVATD*. The corresponding protein sequences were 99% identical among each other, and 96-98% identical with AtVATD (Table S2.7: Sequence alignment; S2.4: Supplementary Information).

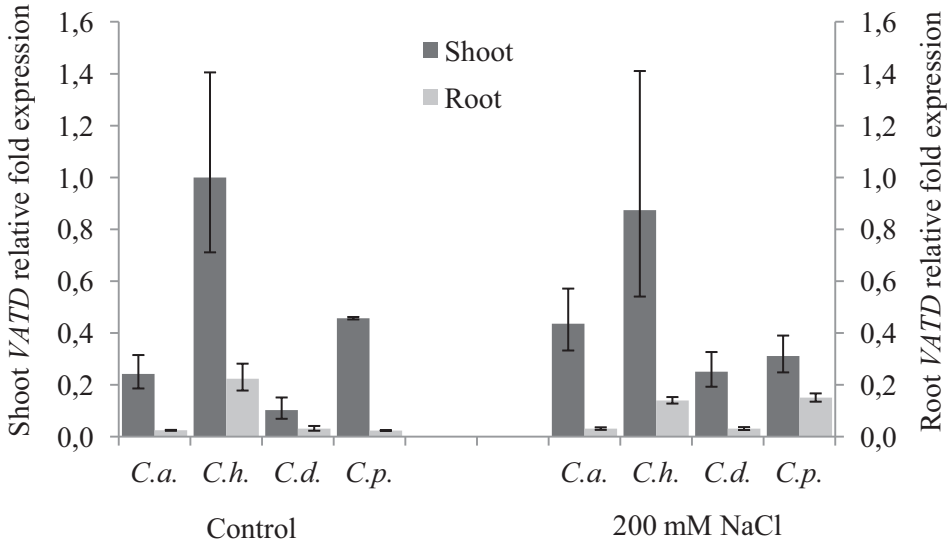

Fig. 11 Expression of *VATD* in shoots and roots of *Cochlearia anglica* (*C.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Cochlearia pyrenaica* (*C.p.*). The gene expression was normalized to the highest expression, which was assigned a value of 1. Each value is the average of three independent biological replicates. Error bars are ±SE. Black bars, shoot *VATD* expression: grey bar, root *VATD* expession.

In all the species *VATD* was more strongly expressed in the shoot than in the root. In the absence of salt, the shoot expression was significantly higher in *C. x hollandica* and *C. pyrenaica* than in *C. anglica* and *C. danica* (Fig. 11). In the salt treatment the shoot expression level was about two-fold up-regulated in *C. anglica* and *C. danica*, but slightly and insignificantly down-regulated in *C. x hollandica* and *C. pyrenaica*. Also in the root, in the absence of salt, *VATD* was significantly higher expressed in *C. x hollandica* than in any of the other species. Salt treatment did not significantly affect the root expression levels, except for *C. pyrenaica*, where the gene was up-regulated to the level found in *C. x hollandica*.

2.3.5.5 *MTP1* expression

The Cochlearia *MTP1* cDNA sequences obtained were 95-99% identical among each other, and 85% identical with *AtMTP1*. The corresponding protein sequences were 96-100% identical among each other, and 85-90% identical with AtMTP1 (Table S2.8: Sequence alignment; S2.5: Supplementary Information).

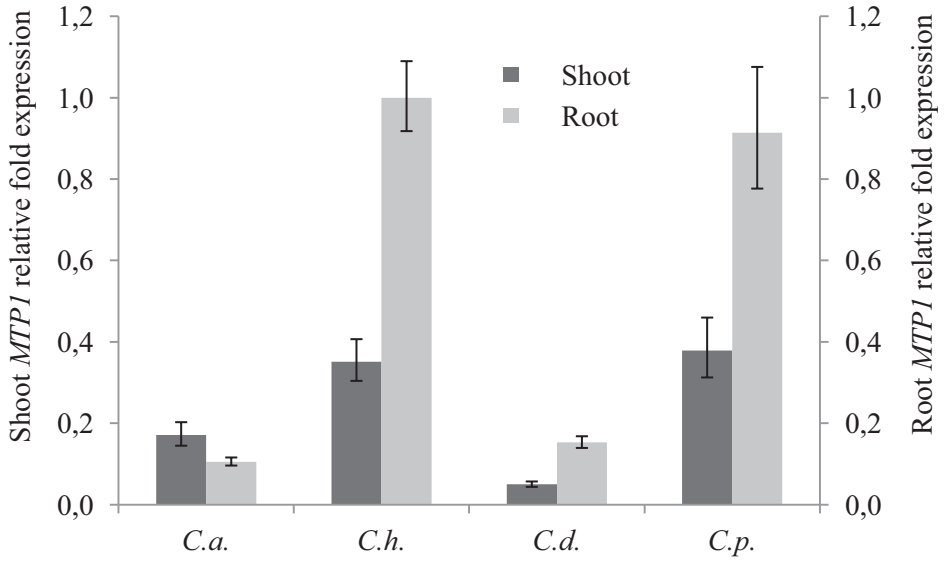

Fig. 12 Expression of *MTP1* in shoot and root of *Cochlearia anglica* (*C.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Cochlearia pyrenaica* (*C.p.*). The gene expression was normalized to the highest expression, which was assigned a value of 1. Each value is the average of three independent biological replicates. Error bars are ±SE. Black bars, shoot *MTP1* expression: grey bars, root *MTP1* expession.

MTP1 was much more expressed in *C. x hollandica* and *C. pyrenaica* than in *C. anglica* and *C. danica*, both in the roots and in the shoots (Fig. 12). In all the species, except *C. anglica*, *MTP1* expression was significantly higher in roots than in shoots.

2.4 Discussion

Our study clearly demonstrated differential salt tolerance among the Cochlearia species under study, decreasing in the order *C. anglica* > *C. x hollandica* > *C. danica* > *C. pyrenaica*, which is precisely the order to be expected on the basis of the species' ecological preferences regarding the soil salinity level in their natural habitat. The superior salt tolerance in *C. anglica* and, though to a lower degree, *C. x hollandica* seems to be associated with a high capacity to prevent a salt-imposed decrease of the K concentration in the root, though not in the shoot (Fig. 3). In addition, in *C. x hollandica*, but not in *C. anglica*, it is associated with a relatively low rate of Na accumulation in the shoot (Fig. 2).

Regarding the expression of the candidate salt tolerance genes under study here, there are clearly species-specific patterns, of which the relationships with the inter-specific variation in salt tolerance are not immediately evident. Only for *HKT1* expression in roots in the salt treatment, there is a clear-cut correlation with salt tolerance, although the level of *HKT1* expression in the roots, in comparison with that in the shoots, is overall low (Fig. 8). In addition, the root *HKT1* expression is strongly induced by the salt treatment in *C. anglica*, *C. x hollandica* and *C. danica*, but not in *C. pyrenaica*, which is by far the most salt-sensitive species (Fig. 8). Moreover, the shoot *HKT1* expression is highest, both in the control and the salt treatment, in the most salt-tolerant species, *C. anglica*. These results suggest that variation in *HKT1* expression may contribute to the variation in salt tolerance among Cochlearia species, indeed. However, the high *HKT1* expression, particularly in the shoot, in *C. anglica* is not associated with low Na accumulation in the shoot, in comparison with the more salt-sensitive species, which is at violence with the proposed function of the gene product, i.e. retranslocating Na from the shoot to the root (Sunarpi *et al.,* 2005; Davenport *et al.,* 2007). On the other hand, *C. x hollandica*, in which *HKT1* is only salt-induced in the root (Fig. 8), clearly shows a lower rate of shoot Na accumulation in the salt treatment, suggesting that in this species HKT1 might function to resorb Na from the xylem (Davenport *et al.,* 2007; Møller *et al.,* 2010).

The expression levels of the other candidate salt tolerance genes are not straight-forwardly reconcilable with the variation in salt tolerance among the species. However, there are some remarkable patterns. For all the genes, in the majority of

cases, the highest expression levels are found in either *C. anglica* or *C. x hollandica*, or both, in comparison with *C. danica* and *C. pyrenaica*, e.g., *HKT1* in root and shoot (Fig. 8), *SOS1* in root and shoot (Fig. 9), *NHX1* in roots (Fig. 10), *VATD* in shoot (Fig. 11), most often both in the controls and the salt treatment. However, notable exceptions are *NHX1* in shoot and *VATD* in root, the former both in the control and the salt treatment, the latter only in the salt treatment, which show the highest expression levels in the most salt-sensitive species, *C. pyrenaica* (Figs. 10, 11). These results leave open the possibility that enhanced expression of *SOS1*, *NHX1*, or *VATD* may also contribute the superior salt tolerance of *C. anglica* or *C. x hollandica*. If so, then these contributions should be expected to be strongly species-specific in case of *SOS1* and *VATD* in roots, which are much less expressed in *C. anglica* than in *C. x hollandica* under salt treatment (Figs. 9, 11). On the other hand the *HKT1* expression level in shoot in the salt treatment seems to be much higher in *C. anglica* than in *C. x hollandica* (Fig. 8). These results may be taken to suggest that *C. anglica* and *C. x hollandica* possess different salt tolerance mechanisms, at least in part, in agreement with their very different shoot Na and root K concentrations in the salt treatment (Figs. 2, 3). This is remarkable, because *C. x hollandica* is an allohexaploid with *C. anglica* being most probably the most salt tolerant parent species. Unfortunately, we were not able to include the other parent species, *C. officinalis*, in this study. *C. officinalis* is expected to exhibit considerable salt tolerance too, in view of its clear-cut ecological preference for brackish environments. It might well be that *C. x hollandica* expresses the salt tolerance mechanisms of *C. officinalis*, or a combination of component traits of the salt tolerance mechanisms in both parent species, rather than the *C. anglica* mechanism, in which *HKT1* over-expression seems to be a key trait. To resolve this issue, future comparisons of the gene expression patterns and the Na and K allocation patterns between *C. anglica*, *C. x hollandica* and *C. officinalis* are required.

As expected *C. pyrenaica*, or at least the population under study here, shows considerable Zn hypertolerance, in comparison with the other species. The population does not show any Cd hypertolerance, in contrast to other metallophytes growing at the same site (Assunção *et al*., 2003). This is remarkable, because metallophytes from calamine soils have thus far invariably been reported to be hypertolerant to both Zn and Cd (Macnair, 1993; Schat *et al.,* 1996; Assunção *et al*., 2003).

This multiple hypertolerance is supposed to be due to the frequent co-occurrence of toxic enrichments of Zn and Cd in calamine soils, rather than to common genetic determinants (Schat and Vooijs, 1997; Jack *et al*., 2007), although a partial pleiotropic genetic control of Zn and Cd tolerance has been established in *A. halleri* (Willems *et al*., 2007; Courbot *et al*., 2007). The apparent absence of any Cd hypertolerance in the *C. pyrenaica* population under study is enigmatic, but it confirms the genetic independence of Cd and Zn hypertolerance, such as previously found in another non-hyperaccumulator metallophyte, *Silene vulgaris* (Jack *et al.,* 2007).

The expression patterns of the vacuolar Zn transporter gene, *MTP1*, seem to be neither correlated with Zn tolerance, nor with Zn or Cd accumulation in roots and shoots. Although the gene is highly expressed in *C. pyrenaica*, it is also expressed high in *C. x hollandica*, which is clearly non-hypertolerant to Zn and Cd.

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Supplementary Information

Variation in salt and heavy metal tolerance and the expression levels of candidate tolerance genes among four *Cochlearia* species with distinct habitat preferences

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Supplementary Tables; S2.1-S2.8

Supplementary sequence alignments; S2.1-S2.5

Supplementary Tables

Pair	Primer	Sequence
Outer	HKT1 Deg.Fwd1	CHTTYTCNRTBTTYWCMRYNGTBTCNAC
	HKT1 Deg.Rev1	GYNSWRAAMCCVACRTTBCCRTAYGC
Inner	HKT1 Deg.Fwd2	YTGYGGNTTYRTSCCBAMVAAYGA
	HKT1 Deg.Rev2	TVARNAYRYTGAARTTKAKBGGRTC
Outer	NHX1 Deg.Fwd1	GTKCTKAATCARGAYGAKACACC
	NHX1 Deg.Rev1	TCRATRTCCAAKGCATCCATWCCRAC
Inner	NHX1 Deg.Fwd2	GTATTYGGRGARGGTGTYGTRAATGATGC
	NHX1 Deg.Rev2	AAYGACAWWGTTGCAAARGYATGC
Outer	SOS1 Deg.Fwd1	CTCRTYVTBGGVATTGCYCTYGGATC
	SOS1 Deg.Rev1	GTKGANCCATTMACWATMAGHGTYAG
Inner	SOS1 Deg.Fwd2	CTKCCKGCBCTTCTTTTYGAGAGTKC
	SOS1 Deg.Rev2	CRATTCCRCCHGTGAAGAAAABAAAC
Outer	VATD Deg.Fwd1	GTVGTKCCSACKGTKACDATGCTYG
	VATD Deg.Rev1	CCTCYTCTTGTADCCCTGDATCTTC
Inner	VATD Deg.Fwd2	GCTCGBCTYGTYGGYGCKACMMGMGG
	VATD Deg.Rev2	CTGAAGAARTCCTCBCKYTCRAGCTC
Outer	MTP1 Deg.Fwd1	CTGYGGAGARGCVCCNTGYGG
	MTP1 Deg.Rev1	CCTTGTTRAGCACCATRTCHGCATC
Inner	MTP1 Deg.Fwd2	GYTTCHGGDGAYGCHMANGAACG
	MTP1 Deg.Rev2	CYTTYCCCACYGTGMTAGCCCAWATG

Supplementary Table S2.1 Degenerate primer pairs for 1st and nested PCR.

Name	Sequence
(C.h.)Act-2F	ATGTCGCTATCCAAGCTGTTC
(C.h.)Act-2R	CACCATCACCAGAATCCAACA
(C.a.,C.d.,C.p.)Act-2F	ATGTCGCTATTCAAGCTGTTC
(C.a.,C.d.,C.p.,)Act-2R	CACCATCACCAGAATCCAGCA
(C.h.,C.a.,C.d.,)HKT1F	CGATTTCTCAACACTCTCACCAGC
(C.p.)HKT1F	CAAGACACTCGGGAGAAACC
(C.p.)HKT1R	GTCTTTTGTTCGGTCAACGGC
(C.h.,C.d.)HKT1R	GGATTCATTCCCTCTCTCTTTG
(C.a.)HKT1R	CGGTTTCTTTCCCTCTCTCTTTG
(C.h.,C.d.)NHX1F	GCCACCCATTATATTCAATGCAG
(C.h.,C.d.)NHX1R	GCATAATAGTCACGAAATTGCGG
(C.a.,C.p.)NHX1F	GCCACCCATAATATTCAACGCAG
(C.a.,C.p.)NHX1R	CACCTAGAGTTATGACAGTGCAAG
(C.h.,C.a.,C.d.,C.p.)SOS1F	CTGAAGGCATTCTCGACAG
(C.h.,C.a.,C.d.,C.p.)SOS1R	AGAACTCCAACAACAACACAACG
(C.h.,C.a.,C.d.,C.p.)VATDF	GAGGACAGAGAACATAGCTGG
(C.h.,C.a.,C.d.,C.p.)VATDR	CTTGTACTTGTTGACCACCTCTAG
(C.h.,C.a.,C.d.,C.p.)MTP1F	GTGGAAGATAGTGGATCTGATATG
(C.h.,C.a.,C.d.,C.p.,)MTP1R	GCAATCCCTTTTCTAGTTTCGTAG

Supplementary Table S2.2 Primers used for RT-PCR

	C.a.	*C.h.*	*C.d.*	*C.p.*
HKT1	JQ435901	JQ435890	JQ435889	JQ435888
NHX1	JQ435894	JQ350805	JQ435893	JQ435895
SOS1	JQ435899	JQ350806	JQ435898	JQ435900
VATD	JQ638709	JQ350807	JQ638708	JQ638710
MTP1	JQ435877	JQ435878	JQ435876	JQ435875
Act-2	JQ435882	JQ435881	JQ935965	JQ435883

Supplementary Table S2.3 Accession number from GenBank.

	C.a.	*C.h.*	*C.d.*	*C.p.*	*A.t.*
		Protein basis			
C.a.		97	97	65	76
C.h.	98		100	65	63
C.d.	98	99		63	74
C.p.	77	76	76		74
A.t.	82	81	81	82	
		Nucleotides basis			

Supplementary Table S2.4 Identity Percentage for *HKT1*

	C.a.	*C.h.*	*C.d.*	*C.p.*	*A.t.*
		Protein basis			
C.a.		99	95	95	90
C.h.	99		95	97	91
C.d.	98	98		99	88
C.p.	98	99	99		90
A.t.	89	90	88	90	
		Nucleotides basis			

Supplementary Table S2.5 Identity Percentage for *SOS1*

	C.a.	*C.h.*	*C.d.*	*C.p.*	*A.t.*
		On Protein basis			
C.a.		96	97	98	96
C.h.	92		99	96	94
C.d.	92	99		97	95
C.p.	99	92	92		96
A.t.	91	90	90	91	
		Nucleotides basis			

Supplementary Table S2.6 Identity Percentage for *NHX1*

	C.a.	*C.h.*	*C.d.*	*C.p.*	*A.t.*
		Protein basis			
C.a.		99	99	99	97
C.h.	98		99	99	97
C.d.	99	99		99	98
C.p.	99	99	99		96
A.t.	87	87	87	86	
		Nucleotides basis			

Supplementary Table S2.7 Identity Percentage for *VATD*

	C.a.	*C.h.*	*C.d.*	*C.p.*	*A.t.*
		Protein basis			
C.a.		100	97	98	88
C.h.	99		98	99	90
C.d.	96	95		96	85
C.p.	99	99	96		87
A.t.	85	85	85	85	
		Nucleotides basis			

Supplementary Table S2.8 Identity Percentage for *MTP1*

Supplementary sequence alignments

```
C.a.HKT1     AACATGGTCATATTTCGCAAGAACTCGGGTCTCCTTTGGATTTTAATACCACAAGTTCTGATGGGAAACACTT
C.h.HKT1     ---------------CGCAAGAACTCGGGTCCCCTTTGGATTTTAATACCTCAAGTTTTGATGGGAAACACTT
C.d.HKT1     ------------------------------------------------------------------------
C.p.HKT1     AACATGATCATATTTAGCAAGAACTCGGGTCTACTCTTGCTTTTAATCCCTCAAGTGTTTATGGGCAACACTC
A.t.HKT1     AACATGATCATCTTTCGCAAGAACTCTGGTCTCATCTGGCTCCTAATCCCTCAAGTACTGATGGGAAACACTT

C.a.HKT1     TGTTTCCTTGCTTCTTGAGGTTGCTATTATGGGGACTTGATAAAATCACAAAGCGTGAAGAGTATGGTTATAT
C.h.HKT1     TGTTTCCGTGCTTCTTGAGGTTGCTATTATGGGGACTTGATAAAATCACAAAGCGTGAAGAGTATGGTTATAT
C.d.HKT1     ----------------------------TGGGGACTTGATAAAATCACAAAGCGTGAAGAGTATGGTTATAT
C.p.HKT1     TTTTCCCTTGCTTCTTGGTTTTGACCATATGGGTACTCTCTAAAACAACAAAACGTGAAGAGTTTGGTTACAT
A.t.HKT1     TGTTCCCTTGCTTCTTGGTTTTGCTCATATGGGGACTTTATAAGATCACAAAGCGTGACGAGTATGGTTACAT

C.a.HKT1     TCTCAAGAACCACAAGAAGATGAGATACTCTCATCTACTCTCCGTGCGTCTTTGTGTTAGTCTTGGCTTGACG
C.h.HKT1     TCTCAAGAACCACAAGAAGATGAGATACTCTCGTCTACTCTCCGTGCGTCTTTGTGTTAGTCTTGGCTTGACG
C.d.HKT1     TCTCAAGAACCACAAGAAGATGAGATACTCACGTCTACTCTCCGTGCGTCTTTGTGTTAGTCTTGGCTTGACG
C.p.HKT1     TCTCAAGAACCACAAGAATATGGGTTACACACATCTACTCTCGGTTCGTCTTTGTGTTCTTGTTGGTGTAACG
A.t.HKT1     TCTCAAGAACCACAATAAGATGGGATACTCTCATCTACTCTCGGTTCGTCTATGTGTTCTTCTTGGAGTGACG

C.a.HKT1     GTGTTAGGGTTTTTGATAATACACCTTATTTTGTTATGTGTCTTTGAGTGGAGATTGGAGTCTCTTCAAGGAA
C.h.HKT1     GTGTTAGGGTTTTTGATGATACACCTTGTTTTGTTATGTGTCTTTGAGTGGAGATTGGAGTCTCTTCAAGGAA
C.d.HKT1     GTGTTAGGGTTTTTGATGATACACCTTGTTTTGTTATGTGTCTTTGAGTGGAGATTGGAGTCTCTTCAGGGAA
C.p.HKT1     GTTTTAGGGTTTGTAATGATAGAGCTTTTGCTCTTTTGCACATTTGAATGGAACTCAAAGTCTCTAGAAGGTT
A.t.HKT1     GTGCTAGGGTTTCTGATAATACAGCTTCTTTTCTTCTGCGCCTTTGAATGGACCTCTGAGTCTCTAGAAGGAA
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C.a.HKT1     TGAATTGGTACGAGAAGATTGTTGGTTCTTTGTTTCTAGTGGTTAACACAAGACATGCCGGTGAAACAATAGT
C.h.HKT1     TGAATTGGTACGAGAAGATTGTTGGTTCTTTGTTTCTAGTGGTTAACACAAGACATGCCGGTGAAACAATAGT
C.d.HKT1     TGAATTGGTACGAGAAGATTGTTGGTTCTTTGTTTCTAGTGGTTAACACAAGACATGCCGGTGAAACAATAGT
C.p.HKT1     TGAGTTGGTACGAGAAATTAATTGGATCGTTGTTTCAAGTGACCAATACAAGACACTCGGGAGAAACCATTGT
A.t.HKT1     TGAGTTCGTACGAGAAGTTGGTTGGATCGTTGTTTCAAGTGGTGAATTCGCGACACACCGGAGAAACTATAGT

C.a.HKT1     CGATTTCTCAACACTCTCACCAGCTATATTGATACTATTCACCTTCATAATGTATCTTCCACCATACACATTA
C.h.HKT1     CGATTTCTCAACACTCTCACCAGCTATATTGATACTATTCACCTTCATAATGTATCTTCCACCATACACATTA
C.d.HKT1     CGATTTCTCAACACTCTCACCAGCTATATTGATACTATTCACCTTCATAATGTATCTTCCACCATACACATTA
C.p.HKT1     TGATCTATCTACACTTTCACCGGCTATCTTGATACTCTTCCTCGTCATGATGTATCTTCCTCCATACACATTC
A.t.HKT1     AGACCTCTCTACACTTTCCCCAGCTATCTTGGTACTCTTTATTCTTATGATGTATCTTCCTCCATACACTTTA

C.a.HKT1     TTTATGACGTTGACTAAGAAAAATAAGAATAACAAAGAGAGAGGG---AAAGAAA---CCGAAAATGAAAAAG
C.h.HKT1     TTTATGACGTTGACTAAGAAAAAGAAGAATAACAAAGAGAGAGGG---AATGAAT---CCGAAAATGAAAAAG
C.d.HKT1     TTTATGACGTTGACTAAGAAAAAGAAGAATAACAAAGAGAGAGGG---AATGAAT---CCGAAAATGAAAAAG
C.p.HKT1     TTTATGCCGTTGACCGAACAAAAGACTAAGAAAGAAGGAGAAGACAATTATGATTATCCTGAAAATGGATATA
A.t.HKT1     TTTATGCCGTTGACGGAACAAAAGACGATAGAGAAAGAAGGAGGAGATGATGATT---CCGAAAATGGAAAGA

C.a.HKT1     AAGCAAAGAAGAGTGGATTCTTTGTGTCGCAACTTTCCTTTTTGGCGATATGCATCTTTCTTGTTTGCACTAC
C.h.HKT1     AAGCAAAGAAGAGTGGATTCTTTGTGTCGCAACTTTCCTTTTTAGCGATATGCATCTTTCTTGTTTGCACTAC
C.d.HKT1     AAGCAAAGAAGAGTGGATTCTTTGTGTCGCAACTTTCCTTTTTAGCGATATGCATCTTTCTTGTTTGCACTAC
C.p.HKT1     AAAGAACAAAGAATGTTCTCTTCATGTCACAACTTACCTTTTTGGCTATGTGCGTTTTTCTAATTTCCATCAC
A.t.HKT1     AAGTTAAAAAGAGTGGACTCATCGTGTCACAACTTTCCTTTTTGACGATATGTATCTTTCTCATTTCAATCAC

C.a.HKT1     CGAAAGACAGAAATTACAACGAGATCCACTCAATTTCAACGTCTTTAACATCACTCTAGAAGTTATCAGTGCG
C.h.HKT1     CGAAAGACAGAAATTACAACGAGATCCACTCAATTTCAACGTCTTTAACATCACTCTAGAAGT----------
C.d.HKT1     CGAAAGACAGAAATTACAACGAGATCCACTCAATTTCAACGTCTTTAACATCACTCTAGAAGTTATCAGTGCG
C.p.HKT1     CGAAAGGGAAAAACTTCGACA------------------------------------------------------
A.t.HKT1     CGAAAGGCAAAATCTACAACGTGATCCGATAAATTTCAACGTCCTTAACATCACTCTCGAAGTTATCAGTGCA

C.a.HKT1     TATGGAAACGTGGGATT
C.h.HKT1     -----------------
C.d.HKT1     TATGGCAACGTCGGCTT
C.p.HKT1     -----------------
A.t.HKT1     TATGGAAACGTTGGTTT
```
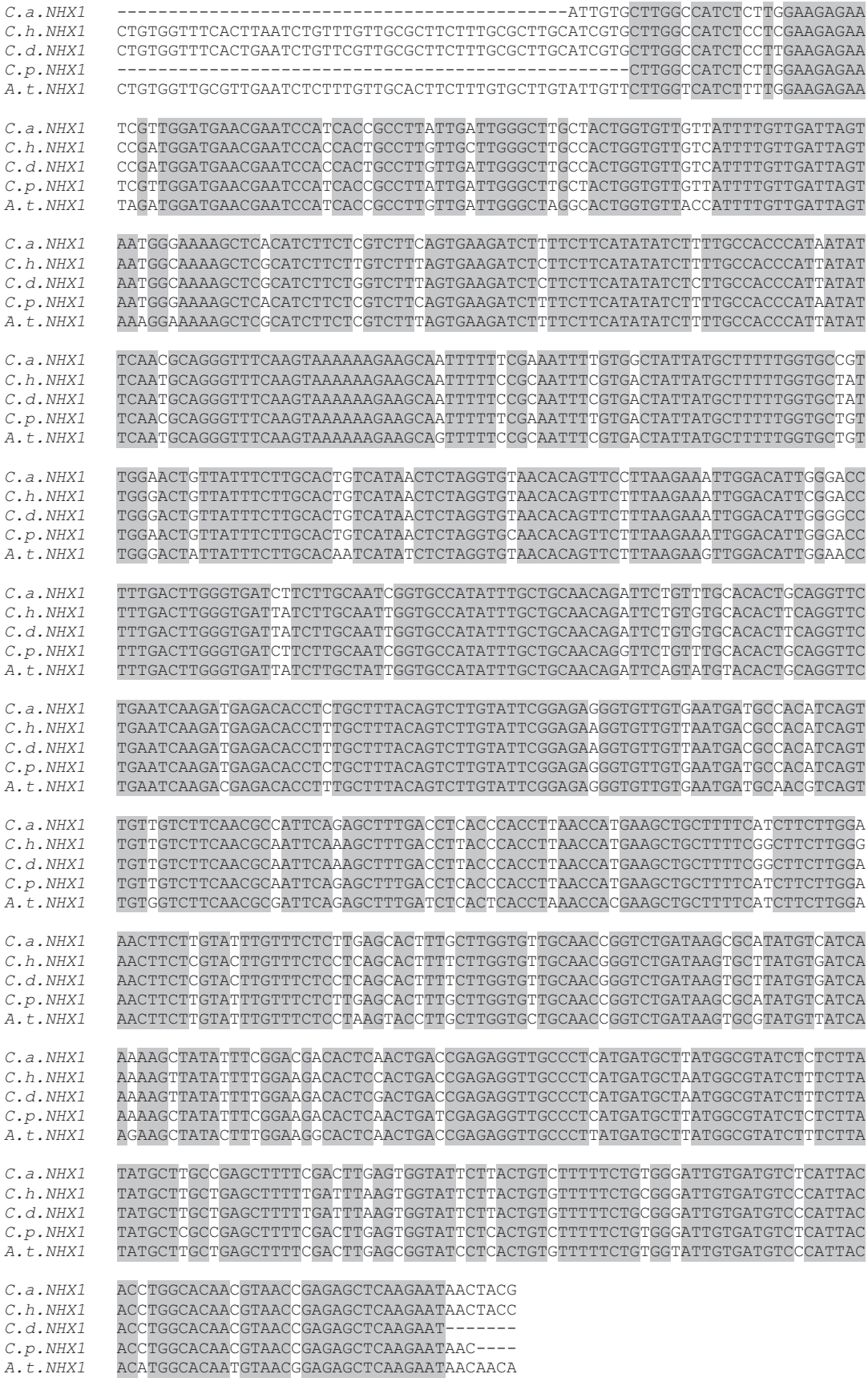

Supplementary sequence alignment S2.1. Sequence alignments of *C.a.HKT1*, *C.h.HKT1*, *C.d.HKT1* and *C.p.HKT1* with *A.t.HKT1* on nucleotides basis.

```
C.a.SOS1     ----------------------------------------AAAGGTGACAGCCAAAAAAGTTTGCATCACT
C.h.SOS1     ACGTTGGGCATGTTTTATGCTGCCCTTGCAAGGACAGCATTTAAAGGTGACAGCCAAAAAAGTTTGCATCACT
C.d.SOS1     ------------TTTTATGCTGCCCTTGCAAGGACAGCATTTAAAGGTGACAGCCAAAAAAGTTTGCATCACT
C.p.SOS1     ------------------------------------------------------------TTTGCATCACT
A.t.SOS1     ACTTTGGGCATGTTTTATGCTGCATTTGCAAGGACAGCCTTTAAAGGTGACAGTCAAAAAAGCTTGCATCACT

C.a.SOS1     TCTGGGAAATGGTCGCCTATATTGCCAATACTTTGATTTTTATCCTCGGTGGTGTTGTCATAGCTGAAGGCAT
C.h.SOS1     TCTGGGAAATGGTCGCCTATATTGCCAATACTTTGATTTTTATCCTCAGTGGTGTTGTCATAGCTGAAGGCAT
C.d.SOS1     TCTGGGAAATGGTCGCCTATATTGCCAATACTTTGATTTTTATCCTCAGTGGTGTTGCCATAGCTGAAGGCAT
C.p.SOS1     TCTGGGAAATGGTCGCCTATATTGCCAATACTTTGATTTTTATCCTCAGTGGTGTTGTCATAGCTGAAGGCAT
A.t.SOS1     TCTGGGAAATGGTTGCATATATTGCAAACACTTTGATATTTATCCTCAGTGGTGTTGTCATTGCTGAAGGCAT

C.a.SOS1     TCTCGACAGCGATAAGATTGCCTACCAAGGGAATTCATGGGCATTTCTCTTTCTACTATATCTTTATATTCAA
C.h.SOS1     TCTCGACAGCGATAAGATTGCCTACCAAGGGAATTCATGGGCATTTCTCTTTCTACTATATCTTTATATTCAA
C.d.SOS1     TCTCGACAGCGATAGGATTGCCTACCAAGGGAGTTCATGGGGATTTCTCTTTCTACTATATCTTTATATTCAA
C.p.SOS1     TCTCGACAGCGATAGGATTGCCTACCAAGGGAGTTCATGGGGATTTCTCTTTCTACTATATCTTTATATTCAA
A.t.SOS1     TCTCGACAGTGATAAGATTGCCTACCAAGGGAATTCATGGCGATTTCTTTTTCTGCTATACGTTTACATCCAA

C.a.SOS1     CTGTCACGTTGTGTTGTTGTTGGAGTTCTATATCCATTTTTATGCCGTGTTGGCTATGGTTTGGATTGGAGAG
C.h.SOS1     CTGTCACGTTGTGTTGTTGTTGGAGTTCTATATCCATTTTTATGCCGTGTTGGCTATGGTTTGGATTGGAGAG
C.d.SOS1     CTGTCACGTTGTGTTGTTGTCGGAGTTCTATATCCATTTTTATGCCGTGTTGGCTATGGTTTGGATTGGAGAG
C.p.SOS1     CTGTCACGTTGTGTTGTTGTTGGAGTTCTATATCCATTTTTATGCCGTGTTGGCTATGGTTTGGATTGGAGAG
A.t.SOS1     CTATCGCGTGTTGTTGTTGTTGGAGTTCTATATCCACTTTTATGTCGTTTTGGCTATGGTTTGGATTGGAAAG

C.a.SOS1     AAGCCATTATACTTGTATGGTCTGGTTTGAGGGGTGCAGTGGCGCTCTCGCTTTCTTTATCTGTGAAGCAATC
C.h.SOS1     AAGCCATTATACTTGTATGGTCTGGTTTGAGGGGTGCAGTGGCGCTCTCGCTTTCTTTATCTGTGAAGCAATC
C.d.SOS1     AAGCCATTATACTTGTATGGTCTGGTTTGAGGGGTGCAGTGGCGTTCTCGCTTTCTTTATCTGTGAAGCAATC
C.p.SOS1     AAGCCATTATACTTGTATGGTCTGGTTTGAG----------------------------------------
A.t.SOS1     AATCCATTATACTCGTATGGTCTGGTTTGAGGGGCGCAGTGGCTCTTGCACTTTCTTTATCCGTGAAGCAATC
```

Supplementary sequence alignment S2.2. Sequence alignments of *C.a.SOS1*, *C.h.SOS1*, *C.d.SOS1* and *C.p.SOS1* with *A.t.SOS1* on nucleotides basis.



Supplementary sequence alignment S2.3. Sequence alignments of *C.a.NHX1*, *C.h.NHX1*, *C.d.NHX1* and *C.p.NHX1* with *A.t.NHX1* on nucleotides basis.

```
C.a.VATD   ------------AAGAAGAAGAGTGATGCTTTAACAGTTCAGTTCAGGGCTCTTCTCAAGAAGATCGTTGTAG
C.h.VATD   ------------AAGAAGAAGAGTGATGCTTTAACAGTTCAGTTCAGGGCTCTTCTCAAGAAGATCGTTGTAG
C.d.VATD   CATGCTCTGCTCAAGAAGAAGAGTGATGCTTTAACAGTTCAATTCAGGGCTCTTCTCAAGAAGATCGTTGTAG
C.p.VATD   CATGCTCTGCTCAAGAAGAAGAGTGGTGCTTTAACAGTTCAGTTCAGGGCTCTTCTCAAGAAGATCGTTGTAG
A.t.VATD   CATGCTCTCCTCAAGAAAAAGAGTGATGCTTTAACTGTTCAGTTTAGGGCACTTCTCAAGAAAATCGTTACAG

C.a.VATD   CGAAAGAGTCCATGGGAGATATGATGAAGACATCGTCTTTCGCTCTTACGGAAGTCAAGTATGTAGCTGGCGA
C.h.VATD   CGAAAGAGTCCATGGGAGATATGATGAAGACATCGTCTTTCGCTCTTACGGAAGTCAAGTACGTAGCTGGCGA
C.d.VATD   CGAAAGAGTCCATGGGAGATATGATGAAGACATCGTCTTTCGCTCTTACGGAAGTCAAGTACGTAGCTGGCGA
C.p.VATD   CGAAAGAGTCCATGGGAGATATGATGAAGACATCGTCTTTCGCTCTTACGGAAGTCAAGTACGTAGCTGGCGA
A.t.VATD   CTAAGGAGTCTATGGGAGATATGATGAAGACATCGTCTTTTGCTCTTACCGAAGTAAAGTATGTTGCTGGTGA

C.a.VATD   TAGCGTCAAGCACGTCGTGCTGGAGAACGTTAAAGAAGCTACTCTGAAAGTTCGTTCGAGGACAGAGAACATA
C.h.VATD   TAGCGTCAAGCACGTCGTGCTGGAGAACGTTAAAGAAGCTACTCTGAAAGTTCGTTCGAGGACAGAGAACATA
C.d.VATD   TAACGTCAAGCACGTCGTGCTGGAGAACGTTAAAGAAGCTACTCTGAAAGTTCGTTCGAGGACAGAGAACATA
C.p.VATD   TAGCGTCAAGCACGTCGTGCTGGAGAACGTTAAAGAAGCTACTCTGAAAGTTCGTTCGAGGACAGAGAACATA
A.t.VATD   CAATGTCAAACATGTTGTCCTCGAGAACGTTAAAGAAGCTACTTTGAAGGTTCGTTCTCGGACAGAGAATATC

C.a.VATD   GCTGGTGTGAAGCTACCAAAGTTTGATCATTTCTGTGAAGGCGAGACCAAGAACGATTTGACGGGTTTAGCTA
C.h.VATD   GCTGGTGTGAAGCTACCAAAGTTTGATCATTTCTCTGAAGGCGAGACCAAGAACGATTTGACGGGTTTAGCTA
C.d.VATD   GCTGGTGTGAAGCTACCAAAGTTTGATCATTTCTCTGAAGGCGAGACCAAGAACGATTTGACCGGTTTAGCTA
C.p.VATD   GCTGGTGTGAAGCTACCAAAGTTTGATCATTTCTCTGAAGGCGAGACCAAGAACGATTTGACGGGTTTAGCTA
A.t.VATD   GCTGGAGTGAAGCTGCCTAAGTTTGATCACTTCTCTGAAGGTGAGACCAAGAATGACTTGACCGGTTTAGCTA

C.a.VATD   GAGGTGGTCAACAAGTACAAGCTTGCCGTGTTGCTTATGTGAAAGTTATCGAAGTTTTAGTCGAGCTTGCTTC
C.h.VATD   GAGGTGGTCAACAAGTACAAGCTTGCCGTGTTGCTTATGTGAAAGTTATCGAAGTTTTAGTCGAGCTTGCTTC
C.d.VATD   GAGGTGGTCAACAAGTACAAGCTTGCCGTGTTGCTTATGTGAAAGTTATCGAAGTTTTAGTCGAGCTTGCTTC
C.p.VATD   GAGGTGGTCAACAAGTACAAGCTTGCCGTGTTGCTTATGTGAAAGTTATCGAAGTTTTAGTCGAGCTTGCTTC
A.t.VATD   GAGGTGGTCAACAGGTCCGAGCTTGCCGTGTTGCTTATGTGAAAGCCATTGAAGTTCTAGTTGAGCTTGCTTC

C.a.VATD   TCTTCAGACATCCTTCTTGACGCTCGACGAAGCTATCAAGACGACTAATCGCAGGGTCAACGCTTTGGAGAAT
C.h.VATD   TCTTCAGACATCCTTCTTGACGCTCGACGAAGCTATAAAGACGACTAATCGCAGGGTCAACGCTTTGGAGAAT
C.d.VATD   TCTTCAGACATCCTTCTTGACGCTCGACGAAGCTATCAAGACGACTAATCGCAGGGTCAACGCTTTGGAGAAT
C.p.VATD   TCTTCAGACATCCTTCTTGACGCTCGACGAAGCTATAAAGACGACTAATCGCAGGGTCAACGCTTTGGAGAAT
A.t.VATD   TCTCCAGACTTCTTTCTTGACCCTTGATGAAGCAATCAAGACGACTAACCGTAGGGTCAACGCTCTGGAGAAT

C.a.VATD   GTTGTGAAACCGAAGATTGAGAACACGATTAGTTACATCAAGGGAGAGCTTGACGAGCTTGAACGAGAGGACT
C.h.VATD   GTTGTGAAACCGAAGATTGAGAACACGATTAGTTACATCAAGGGAGAGCTTGATGAGCTTGAGAGAGAAGACT
C.d.VATD   GTTGTGAAACCGAAGATTGAGAACACGATTAGTTACATCAAGGGAGAGCTTGATG------------------
C.p.VATD   GTTGTGAAACCGAAGATTGAGAACACGATTAGTTACATCAAGGGAG--------------------------
A.t.VATD   GTGGTGAAACCAAAGCTGGAGAATACAATCAGTTACATCAAGGGAGAGCTTGATGAGCTTGAGAGAGAGGATT

C.a.VATD   TCTTCAGGCTTAAGAAGATTCAGGGTTACAAGA
C.h.VATD   TCTTCAGGCTTAAGAAGATTCAAGGATACAAGA
C.d.VATD   -----------------------------------
C.p.VATD   -----------------------------------
A.t.VATD   TCTTCAGGTTGAAGAAGATTCAGGGATACAAGA
```

Supplementary sequence alignment S2.4. Sequence alignments of *C.a.VATD*, *C.h.VATD*, *C.d.VATD* and *C.p.VATD* with *A.t.VATD* on nucleotides basis.

```
C.a.MTP1   GCTTCTATTCGGAAGCTTTGTATCGCTGTAGTCTTGTGTCTTTTGTTCATGAGCGTCGAAGTTGTTGGTGGCA
C.h.MTP1   ------------------------------------------------------------------------
C.d.MTP1   ---------------CTTTGTATCGCTGTAGTCCTGTGTCTTTTGTTTATGAGCGTAGAAGTTGTTGGTGGCA
C.p.MTP1   GCTTCTATTCGGAAGCTTTGTATCGCTGTAGTCTTGTGTCTTTTGTTCATGAGCGTCGAAGTTGTTGGTGGCA
A.t.MTP1   GCTTCTATGCGGAAGCTTTGTATCGCCGTCGTGCTGTGTCTAGTGTTCATGAGTGTTGAAGTTGTTGGTGGGA

C.a.MTP1   TCAAAGCCAATAGTCTGGCTATACTAACCGATGCTGCCCATTTGCTCACTGACGTCGCTGCCTTTGCTATCTC
C.h.MTP1   ------------------------------------------------------------------------
C.d.MTP1   TCAAAGCCAATAGTCTGGCTATACTAACCGATGCTGCCCATTTGCTCACTGACGTTGCTGCCTTTGCTATCTC
C.p.MTP1   TCAAAGCCAATAGTCTGGCTATACTAACCGATGCTGCCCGTTTGCTCACTGACGTCGCTGCCTTTGCTGTCTC
A.t.MTP1   TTAAAGCCAATAGTTTAGCTATATTAACCGATGCAGCTCATTTGCTCTCTGACGTTGCTGCCTTTGCTATCTC

C.a.MTP1   TTTGTTCTCCTTGTGGGCTGCTGGCTGGGAAGCAACTCCGAGGCAGACTTATGGGTTTTTCAGGATTGAGATT
C.h.MTP1   ------------------------------------------------------------------------
C.d.MTP1   TTTGTTCTCCTTGTGGGCTGCTGGCTGGGAAGCTACTCCAAGGCAGACTTACGGGTTTTTCAGGATTGAGATT
C.p.MTP1   TTTGTTCTCCTTGTGGGCTGCTGGCTGGGAAGCAACTCCGAGGCAGACTTATGGGTTTTTCAGGATTGAGATT
A.t.MTP1   CCTCTTCTCATTGTGGGCTGCTGGCTGGGAAGCGACTCCTAGGCAGACTTACGGGTTCTTCAGGATTGAGATT

C.a.MTP1   TTGGGAGCTCTTGTATCTATCCAGCTCATTTGGCTGCTCACCGGTATTTTGGTTTATGAAGCCATTATCAGAC
C.h.MTP1   ------------------------------------------------------------------------
C.d.MTP1   TTGGGTGCTCTTGTATCTATCCAGCTCATTTGGTTGCTCACTGGTATTTTGGTTTATGAAGCCATTATCAGAC
C.p.MTP1   TTGGGAGCTCTTGTATCTATCCAGCTCATTTGGCTGCTCACCGGTATTTTGGTTTATGAAGCCATTATCAGAC
A.t.MTP1   TTGGGTGCTCTTGTATCTATCCAGCTCATTTGGTTGCTCACGGGTATTCTGGTTTATGAAGCGATTATCAGAA
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C.a.MTP1    TTATTACCGAGACCAGTGAGGTTAATGGATTCCTCATGTTTCTTGTTGCTGCCTTTGGCCTTGCGGTGAATAT
C.h.MTP1    ------------------------------------------------------------------------
C.d.MTP1    TTATTACCGAGACCAGTGAGGTTAATGGATTTCTCATGTTTCTTGTTGCTGCCTTTGGCCTTGCGGTGAATAT
C.p.MTP1    TTATTACTGAGACCAGTGAGGTTAATGGATTCCTCATGTTTCTTGTTGCTGCCTTTGGCCTTGCGGTGAATAT
A.t.MTP1    TTGTTACAGAGACCAGTGAGGTTAATGGATTCCTCATGTTTCTGGTTGCTGCCTTTGGTCTAGTGGTGAACAT

C.a.MTP1    AGTAATGGCTGTTCTGCTTGGACATGATCATGGTCATAGTCATGGGCATGGACATGGTCATGACCACAGTCAC
C.h.MTP1    ------------------------------------------------------------------------
C.d.MTP1    AGTAATGGCTGTTCTGCTTGGACATGATCATGGTCATAGTCATGGGCATGGACATGGTCATGACCACAGTCAC
C.p.MTP1    AGTAATGGCTGTTCTGCTTGGACATGATCATGGTCATAGTCATGGGCGTGGACATGGTCATGAACACAGTCAC
A.t.MTP1    CATAATGGCTGTTCTGCTAGGGCATGATCATGGTCACAGTCATGGACATGGGCATGGCCACGGCCATG---AC

C.a.MTP1    GGTGGGAACCATAGCCATGGGGTGACAGTAACCACCCATCACCATCACGG---CCACGGCCACGATCACGATC
C.h.MTP1    ----------------------------------------------CACGG---CCACGATCACGATCATGGTC
C.d.MTP1    GGTGGGAACCATAGCCATGGGGTGACAGTAACCACACATCTCCATCACGGCCACCACGGTCACGATCATGGTC
C.p.MTP1    GGTGGGAACCATAGCCATGGGGTGACAGTAACCACCCATCACCATCACGG---CCACGATCACGATCATGGTC
A.t.MTP1    CATCACAATCATAGCCATGGGGTGACTGTTACCACTCATCACCATCATCACGATCATGAACATGGCCATAGTC

C.a.MTP1    ATAGTCACGGAGAGGACAAACATCATGCTCATGAGGATGATGTAACAGAGTCATTGCTGGACAAATCAAACCC
C.h.MTP1    ATAGTCACGGAGAGGACAAACATCATGCTCATGAGGATGATGTAACAGAGTCATTGCTGGACAAATCAAACCC
C.d.MTP1    ATAGTCACGGAGAGGACAAACATCATGCTCACGAGGATGATGTAACAGAGTCATTGCTGGAGAAATCAAACCC
C.p.MTP1    ATAGTCACGGAGAGGACAAACATCATGCTCATGAGGATGATGTAACAGAGTCATTGCTGGACAAATCAAACCC
A.t.MTP1    ATGGTCATGGAGAGGACAAGCATCATGCTCATGGG---GATGTTACTGAGCAATTGTTGGACAAATCGAAGAC

C.a.MTP1    TCGAGCCGCGGAGAAAGAGAAAAAAAAGAGAAACATCAATGTCCAAGGAGCTTATCTCCATGTCCTTGGTGAT
C.h.MTP1    TCGAGCCGCGGAGAAAGAGAAAAAAAAGAGAAACATCAATGTCCAAGGAGCTTATCTCCATGTCCTGGGTGAT
C.d.MTP1    TCCGGCCGCAGAGAAAGAGAAAAAAAAGAGAAACATCAATGTACAAGGAGCTTATCTCCATGTACTTGGTGAT
C.p.MTP1    TCGAGCCGCGGAGAAAGAGAAAAAAAAGAGAAACATCAATGTCCAAGGAGCTTATCTCCATGTCCTTGGTGAT
A.t.MTP1    TCAAGTCGCAGCAAAAGAGAAAAGAAAGAGAAACATCAATCTCCAAGGAGCTTATCTGCATGTCCTTGGGGAT

C.a.MTP1    TCAATCCAGAGCGTTGGTGTGATGATCGGAGGGGCACTCATATGGTACAACCCCAAGTGGAAGATAGTGGATC
C.h.MTP1    TCAATCCAGAGCGTTGGTGTGATGATCGGAGGGGCACTCATATGGTACAACCCCAAGTGGAAGATAGTGGATC
C.d.MTP1    TCAATCCAGAGCGTTGGTGTGATGATTGGAGGGGCTATCATATGGTACAACCCAAAGTGGAAGATAGTGGATC
C.p.MTP1    TCAATCCAGAGCGTTGGTGTGATGATCGGAGGGGCACTCATATGGTACAACCCCAAGTGGAAGATAGTGGATC
A.t.MTP1    TCCATCCAGAGTGTTGGTGTTATGATTGGAGGAGCTATCATTTGGTACAATCCGGAATGGAAGATAGTGGATC

C.a.MTP1    TGATATGCACGCTTGCCTTTTCGGTTATTGTATTGGGAACAACCATCAACATGATAAGAAACATTCTAGAAGT
C.h.MTP1    TGATATGCACGCTTGCCTTTTCGGTTATTGTATTGGGAACAACCATCAACATGATAAGAAACATTCTAGAAGT
C.d.MTP1    TGATATGCACGCTAGCCTTTTCGGTTATTGTGTTGGGAACAACAATCAACATGATAAGAAACATTCTAGAAGT
C.p.MTP1    TGATATGCACGCTTGCCTTTTCGGTTATTGTATTGGGAACAACCATCAACATGATAAGAAACATTCTAGAAGT
A.t.MTP1    TGATCTGCACACTTGCCTTTTCGGTTATTGTCCTAGGAACAACCATCAACATGATTCGCAACATTCTAGAAGT

C.a.MTP1    ATTGATGGAGAGCACACCGAGAGAGATCGATGCTACGAAACTAGAAAAGGGATTGCTGGAGATGGAAGAAGTG
C.h.MTP1    ATTGATGGAGAGCACACCGAGAGAGATCGATGCTACGAAACTAGAAAAGGGATTGCTGGAGATGGAAGAAGTG
C.d.MTP1    GTTGATGGAGAGCACACCGAGGGAGATTGATGCTACGAAACTAGAAAAGGGATTGCTGGAGATGGAAGAAGTG
C.p.MTP1    ATTGATGGAGAGCACACCGAGAGAGATCGATGCTACGAAACTAGAAAAGGGATTGCTGGAGATGGAAGAAGTG
A.t.MTP1    ATTGATGGAGAGTACACCCAGAGAGATTGACGCCACAAAGCTCGAAAAGGGTTTGCTCGAAATGGAAGAAGTG

C.a.MTP1    GTGGCAGTGCATGAGCT---
C.h.MTP1    GTGGCAGTGCATGAGCTT--
C.d.MTP1    GTGGCAGTGCATGAGCTTCA
C.p.MTP1    GTGGCAGTGCATGAGCTTC-
A.t.MTP1    GTGGCTGTTCATGAGCTCCA
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Supplementary sequence alignment S2.5. Sequence alignments of *C.a.MTP1*, *C.h.MTP1*, *C.d.MTP1* and *C.p.MTP1* with *A.t.MTP1* on nucleotides basis.

Chapter 3

Salt tolerance and candidate salt tolerance gene expression levels in Brassicaceae

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Abstract

We compared six Brassicaceae glycophytes and halophytes for salt tolerance and the expression levels in roots and shoots of the candidate salt tolerance genes, *NHX1*, *SOS1*, and *VATD*, encoding vacuolar Na^+/H^+ antiporter, the plasmamembrane Na^+/H^+ antiporter, and subunit-D of vacuolar proton ATPase, respectively. Salt tolerance decreased in the order of *Cochlearia x hollandica* >> *Cochlearia danica*/*Thellungiella botschantzevii* > *Brassica oleracea* > *Thlaspi arvense* > *Arabidopsis thaliana.* The highest expression levels of *NHX1, SOS1,* and *VATD* were consistently found in *C. x hollandica*, both in shoots and roots, and both in control plants and salt-treated ones. Salt-imposed induction of *NHX1* was observed in *C. danica* (shoot and root) and *B. oleracea* (shoot). *SOS1* was up-regulated by salt treatment in the shoots of *C. x hollandica* and *C. danica*, and *VATD* in the shoot of *T. arvense*.

Expression of *NHX1* gDNA under the *C. x hollandica NHX1* promoter in the *A.t.nhx1* mutant background yielded, irrespective of the gDNA source, 30-fold and two-fold enhanced expression levels, in comparison with those in wild-type *A. thaliana* and *C. x hollandica*, respectively. This suggests that high expression level in *C. x hollandica* is completely explained by altered *cis*-regulation of this gene. Promoter swap experiments showed that the *C. x hollandica SOS1* and *VATD* promoters were five-fold and two-fold more active than corresponding *A. thaliana* promoters, respectively. However, particularly in the case of *VATD*, this is not sufficient to explain the difference in the wild-type expression levels between *C. x hollandica* and *A. thaliana*.

Keywords; Brassicaecea, *NHX1,* promoter swapping, Salt tolerance, *SOS1*, *VATD*

3.1 Introduction

On the basis of their level of salt tolerance, plants can be classified as a halophyte or a

glycophyte. Halophytes are defined as plants that can still complete their life cycle with at least 200 mM NaCl (Flowers and Colmer, 2008). Halophytes are widely but unevenly spread over higher plant families and orders (Flowers *et al.,* 1977). They exhibit, in a phylogenetically biased way, a broad variety of adaptations to salt, including specific morphological structures, such as salt glands or bladder cells. The physiological determinants of the superior salt tolerance in halophytes are poorly known and, most probably, also subject to phylogenetic biasness (Flowers and Colmer, 2008). There is strong evidence that salt tolerance in halophytes within the Poales order is associated with enhanced levels of selectivity for K over Na (Flowers and Colmer, 2008), leading to Na exclusion and the maintenance of high cellular K levels under salt exposure (Colmer *et al.,* 2006). Dicotyledonous halophytes exhibit much lower degrees of K/Na selectivity and accumulate Na often to much higher levels in their tissues, using it as a 'cheap' osmolyte (Flowers *et al.,* 1977; Flowers and Colmer, 2008). Since cytoplasmic Na tolerance does not seem to exist in halophytes, it is therefore often believed that at least salt accumulating halophytes must have evolved enhanced capacities for Na compartmentalization at the levels of organs, tissues, cells and subcellularly (Flowers and Colmer, 2008). All halophytes must also be capable to synthesize and accumulate 'compatible solutes', to achieve osmotic adjustment of the cytoplasm and organelles other than the vacuole. There is a huge variation, even within plant families, in the types of compatible solutes used by halophytes. These include linear polyols (glycerol, mannitol, sorbitol), cyclic polyols (inositol, pinetol or other mono- and dimethylated inositol derivatives), amino acids (proline, glutamate), betaines (glycine betaine, alanine betaine), and a variety of sugars (Dajic, 2006). It has often been suggested that halophytes should exhibit enhanced capacities for compatible solute accumulation, but there is no hard evidence either in favor or against this hypothesis. In general, glycophytes also tend to accumulate such compounds when under exposure to a broad variety of stresses, including salt, drought, frost, or heavy metal toxicity (Munns and Tester, 2008).

The molecular mechanisms of salt tolerance have been investigated almost exclusively in glycophytes thus far, in particular the plant genetics model, *Arabidopsis thaliana*, except for the recently proposed halophyte model species, *Thellungiella halophila* (Inan *et al.,* 2004; Gong *et al.,* 2005). These studies have revealed a number of genes that appeared to be essential for wild-type-level salt tolerance in *A. thaliana*, including those encoding the Na transporters SOS1, which is a Na effluxing plasmamembrane-located Na^+/H^+ antiporter (Shi *et al.,* 2000), *NHX1*, a vacuolar Na^+/H^+ antiporter (Pardo *et al.,* 2006),

and *HKT1*, a plasmamembrane-located Na influxer, supposed to be a $Na^+(K^+)/H^+$ symporter (Rubio *et al.,* 1995), or a $Na^+(K^+)$ channel (Horie *et al.,* 2009; Kronzucker and Britto, 2011), supposed to resorb Na from the xylem and to promote Na retranslocation to the root via the phloem (Berthomieu *et al.,* 2003). Other genes supposed to be essential for normal salt tolerance in glycophytes are those encoding the plasmamembrane H^+-ATPase, the vacuolar H^+-ATPase, *VAT*, and the vacuolar H^+ translocating pyrophosphatase (*V-PPA*), because their gene products are responsible for the maintenance of the electric potential or pH gradient required for passive or secondary active Na transport by HKT1, or SOS1 and NHX1, respectively (Vera-Estrella *et al.,* 2005; Martinez-Atienza *et al.,* 2007; Silva and Geros, 2009).

Many authors have assumed, often more or less implicitly, that the high level of salt tolerance in halophytes would rely, at least in part, on enhanced expression of one or more of these genes, as appears from the high number of transgenic over-expression studies that have been performed with at least *SOS1*, *NHX1*, *PPA* and, more recently, *HKT1* (Ashraf and Akram, 2009; Zhang *et al.,* 2008; Oh *et al.,* 2007; Baisakh *et al.,* 2012). In virtually all of these studies, it has been claimed that over-expression of either of these genes, usually under the 35S-CMV promoter, resulted in improved salt tolerance in the glycophytic host (Ashraf and Akram, 2009). Evidently, apart from the question of whether these claims are valid (Flowers and Colmer, 2008), such transgenic experiments can never prove that (enhanced expression levels of) these genes are also responsible for the superior salt tolerance in halophytes, in comparison with glycophytes. To resolve this issue, one should compare the expression patterns of these genes in halophytes and glycophytes and, in case of a difference, prove that this difference is responsible for at least some part of the difference in salt tolerance between the halophyte and the glycophyte under study, preferably through silencing the gene in the halophyte down to the level prevailing in the glycophyte reference species. To date this has only been done for *SOS1* in *T. halophila*, in a study which used *A. thaliana* as a glycophyte reference (Oh *et al.,* 2007). This study strongly suggested that altered expression of *SOS1* in the root is a major determinant of the superior salt tolerance of *T. halophila*, indeed.

There is no common opinion on the type of molecular changes, which underly high-level salt tolerance in halophytes to date. In attempts to genetically engineer improved salt tolerance in glycophyte crops, many investigators have used cDNA's of halophytic origin (Ashraf and Akram, 2009, and references therein), which apparently reflects the belief that

structural changes at the protein level could be responsible, at least in part, for the superior salt tolerance in halophytes. The effects of transgenes from halophytic and glycophytic sources have only seldomly been compared in a single experiment, but the few studies available to date unequivocally suggest that the transgene source is irrelevant for its effect in the host (Chang-Quing *et al.,* 2008; Li *et al.,* 2008). This suggests that non-synonymous mutations in the coding regions of particular genes may not be primarily responsible for the superior salt tolerance in halophytes. Indeed, it is more likely that halophytes and glycophytes basically use the same set of genes to cope with salt, but express them in a different way, most likely through altered *cis*-regulation (Wittkopp *et al.,* 2004) or, such as established for heavy metal tolerance in metallophytes, a combination of altered *cis*-regulation and gene copy number expansion (Hanikenne *et al.,* 2008).

Reports on comparisons of gene expression patterns between halophytes and glycophytes are remarkably scarce to date, which hampers a deeper understanding of the salt tolerance mechanisms in halophytes. Extensive transcriptome comparisons are only available for *T. halophila* and *A. thaliana*, which share sufficient DNA identity to allow the use of *A. thaliana*-based cDNA micro-arrays (Taji *et al.,* 2004; Gong *et al.,* 2005). However, there are reasons to believe that *T. halophila* might not be the ultimate halophyte model. First, it has the slow maximum growth rate typical of a "stress tolerator" (Grime, 1979), which is not apparent in coastal halophytes (Flowers and Colmer, 2008). Second, although it seems to survive seawater salinity level for a fairly long period, its growth rate is already severely inhibited at relatively low salinity (Inan *et al.,* 2004), which is also different from coastal halophytes (Flowers and Colmer, 2008). Moreover, *T. halophila* is also tolerant for several other stresses, like temperature extremes, or drought, which is, again, often not the case in coastal halophytes (Bressan *et al.,* 2001; Inan *et al.,* 2004), and may lead to difficulties in distinguishing specific 'salt tolerance genes' from (other) 'stress tolerance genes'.

In view of the above, it would be interesting to gain much more information on the expression patterns of common sense-based candidate salt tolerance genes in halophytes other than *T. halophila,* and in glycophytes other than *A. thaliana*. In this study we address the question whether enhanced expression of *SOS1*, *NHX1*, or the genes encoding the vacuolar proton ATPase (we measured *VATD,* encoding subunit-D), could contribute to the superior salt tolerance in halophytes in comparison with various glycophytes. A second aim was to establish correlations, if any, between Na allocation patterns and gene expression patterns. To facilitate gene identification we confined our selection of halophytes and

glycophytes to the Brassicaceae family. We selected a coastal halophyte, *Cochlearia x hollandica* (Pegtel, 1999), which is the allohexaploid hybrid of *C. anglica* and *C. officinalis* (Koch *et al.,* 1998), a continental inland halophyte, *Thellungiella botschantzevii* (German, 2008), the glycophytes *Thlaspi arvense* and *A. thaliana*, as well as *Cochlearia danica* and *Brassica oleracea*, which could be expected to be relatively salt-tolerant glycophytes, in view of their more or less coastal distribution patterns. We checked the supposed halophyte/glycophyte status of these species by growing them with and without NaCl in the nutrient solution. Finally, to assess the potential role of *cis*-regulatory alterations in the evolution of differential candidate salt tolerance gene expression between halophytes and glycophytes, we isolated and cloned the upstream (partial) promoter sequences of *SOS1*, *NHX1*, and *VATD* from the most salt tolerant species, *C. x hollandica*. We expressed the gDNA coding regions or cDNA from *C. x hollandica* and *A. thaliana*, in the corresponding *A. thaliana* mutant background or wild-type, both under the *A. thaliana* and the *C. x hollandica* supposed promoter sequences and compared the expression levels of the transgenes.

3.2 Materials and Methods

3.2.1 Plant materials and growth conditions

Seeds of *C. x hollandica* were collected from a 'green beach' at the island of Voorne, The Netherlands. Seeds of *C. danica* were collected from a foredune at the island of Texel, The Netherlands. Those of *T. botschantzevii* originated from solonchak-type soil at Saratov, Russia, and those of *T. arvense* from a roadside population near the campus of the Vrije Unversiteit, Amsterdam, The Netherlands. Seeds of *Brassica oleracea* were collected from a coastal limestone cliff near Étretat, France.

Seeds were sown in garden peat soil (Jongkind BV, No. 1, Aalsmeer, The Netherlands) and three weeks after germination, seedlings were transferred to hydroponics, in 1-L polyethylene pots (three plants per pot) containing a modified half strength Hoagland's solution composed of 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 1 µM KCl, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.1 µM $CuSO_4$, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 20 µM Fe(Na)EDTA, in demineralised water buffered with 2 mM 2-(N-morpholin)ethanesulphonic acid, pH 5.5, adjusted with KOH. Nutrient solutions were renewed weekly and plants were grown in a growth chamber (20/15 °C day/night; 200 µmol m^{-2} s^{-1}, 14 h d^{-1}; relative humidity 75%). After ten days of growth in hydroponics, half of the

plants were exposed to NaCl, in a background solution of the same composition. The NaCl concentration was stepwise increased (4 days per step), to allow osmotic adjustment. The concentration steps were 50-, 100-, and 200 mM. After 3 weeks of exposure to 200 mM NaCl the plants were harvested, dried and stored for analysis. Parts of the roots and shoots were snap-frozen in liquid nitrogen and stored at -80 °C until RNA extraction.

3.2.2 Measurement of Na and K

For Na and K analysis the materials of three plants were pooled and powdered. Twenty mg of plant material was extracted (90 ºC) in 2 ml of demineralized H_2O in 2 ml eppendorfs for 1 hour. After cooling, the extracts were filtered through Spin-X® centrifuge tube filters (Costar, 0.22 µM Nylon). Proper dilutions were made in demineralized H_2O. Na and K concentrations were determined, using flame emission, on an atomic absorption spectrophotometer (Perkin Elmer AAS100).

3.2.3 Cloning of *NHX1, SOS1* and *VATD*

To amplify *NHX1, SOS1* and *VATD*, first and nested PCR's were performed with primer pairs designed on the basis of conserved regions of the sequences available for *NHX1, SOS1* and *VATD* in GenBank (Table S3.1: Supplementary Information). The reactions were performed as follows: initiation with denaturation for 3 min at 96 °C, followed by 26 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min, and a final extension step of 10 min at 72 °C. The first and nested PCR were performed on 2 µL cDNA and 2 µL of the first PCR product, respectively. The amplified fragments were purified using the Agarose Gel DNA extraction Kit (Roche, Applied Science), following the manufacturer's instructions, and cloned into pGEM-T Easy (Technical Manual, pGEM®-T Easy Vectors, Promega) for sequencing. Sequences of cloned genes fragments were determined using the Big-Dye Terminator protocol and ABI PRISM 3100 DNA Sequencer. Data base searches were conducted with BLAST service at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and TAIR (www.arabidopsis.org). The accession number from GenBank are given in table S3.2 (Supplemetary Information).

3.2.4 RNA isolation and first strand cDNA synthesis

Plant tissue harvested from salt-treated or control plants were homogenized in liquid nitrogen. The homogenates were suspended in 1.5 ml Trizol (38% phenol, 0.8 M guanidine

thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M NaAc (pH5) and 5% glycerol was added, after which the samples were extracted with 0.3 ml chloroform. The nuclei were precipated with an equal volume of iso-propanol and washed with 75% ethanol. The pellet was dissolved in 500 µl RNase free H_2O. A DNase treatment was performed by adding 50 µl DNase buffer (0.5 M Tris/HCl pH 7.6, 0.1 M $MgCl_2$) and 3 µl RNase free DNase I (Roche) to remove the possible DNA contamination, followed by an incubation step for 30 minutes at 37 ºC. Next a phenol:chloroform extraction was performed and RNA was precipitated again using 2-propanol. The pellet was washed with 70% EtOH and dissolved in RNase free H_2O. Total RNA was quantified spectrophotometrically (Nanodrop, ND100). RNA was only used if the ratio between spectrophotometer readings (260 nm : 280 nm) were between 1.8 and 2.0, denoting minimal contamination from cellular proteins. The integrity of the total RNA was checked by Formaldehyde Agarose (FA) Gel Electrophoresis. FA gel preparation and electrophoresis was done as described in RNeasy® Mini Handbook, 4th edition (QIAGEN). cDNA was synthesized from total RNA (2.5 µg, boiled for 1 minute) using 100 Units M-MLV Reverse Transcriptase (Invitrogen), 2 mM dNTPs, 100 mM DTT, 10X RT buffer and 10 µM oligo dT primer at 42ºC for 1 hour. The first strand cDNA was checked using the housekeeping gene *Actin-2* (*Act-2*) to assess the quality of the reverse transcription. PCR products were run on a 1.5% agrose gel.

3.2.5 Real-Time PCR

Primers were designed with a G/C ratio between 50 and 60% and a melting point between 58 and 60 °C (Table S3.3: Supplementary Information). Gene-specific primers for *NHX1, SOS1*, *VATD* and *Act-2* were separately designed, on the basis of obtained partial sequences of *B.o., T.a., C.h., C.d.,* and *T.b.* The quantitative assessment of mRNA levels was performed with SensiMix™ SYBR No-ROX kit (Bioline) using the Bio-Rad MJ Research Opticon™ Real Time PCR System detection system (Applied Biosystems Inc., IJssel, The Netherlands) using *Act-2* as an internal control. SensiMix™ SYBR No-ROX kit includes the SYBR® Green I dye, dNTPs, stabilisers and enhancers. As a ready to-use premix, only primers and template needed to be added. A dilution series (5-, 10-, 20-, 40- and 80 times) of the cDNA samples in water was tested to identify the cDNA concentrations that produced cycle threshold values between 18 and 30, and PCR efficiencies of > 1.98. The final reaction conditions were, 10 µL SensiMix™ SYBR No-ROX matser mix, 0,75 µL forward primer (final concentration of 250 nM), 0,75 µL of reverse primer (final concentration of 250 nM) and cDNA in a total

reaction volume of 20 µL. An initial step of 95 °C for 10 min was used to activate the polymerase. Cycling conditions were: melting step at 95 °C for 15 s and annealing/extension at 60 °C for 20 s, with 40 cycles, at the end melting curve from 60 ºC to 90 ºC, read every 0.5 ºC, for 10 s. All Real-Time PCR reactions were performed in triplicate, and a maximum difference of one cycle between the C_T values of the replicates was considered acceptable. Negative controls were included for each primer pair to check for significant levels of any contaminants. Expression values were calculated using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). Shoots and roots of individual plants were used for RNA extraction and for Real-Time PCR analysis.

3.2.6 *SOS1, NHX1* and *VATD* promoter sequencing, transgene constructs, and transformation of *A. thaliana*

SOS1, NHX1 and *VATD* promoters from *C. x hollandica (C.h.)* were sequenced by chromosome walking on gDNA with gene-specific reverse primers (Table S3.4: Supplementary Information), using the Clontech (PT3042-2) Universal Genome walker kit. This yielded 1857 bp of the *C.h.SOS1* promoter, 2122 bp of the *C.h.NHX1* promoter and 1003 bp of the *C.h.VATD* promoter, counted from the start codon (ATG) of *C.h.SOS1*, *C.h.NHX1* and *C.h.VATD*, respectively. Constructs were made with the following promoter/coding region combinations: *p35S::C.h.SOS1, A.t.SOS1prom::A.t.SOS1, A.t.SOS1 prom::C.h.SOS1, C.h.SOS1prom::A.t.SOS1, C.h.SOS1prom:: C.h.SOS1, p35S::C.h.NHX1, C.h.NHX1prom(2kb)::A.t.NHX1, C.h.NHX1prom (1.5kb)::C.h.NHX1, C.h.NHX1prom(2kb):: C.h.NHX1* (*A.t.NHX1prom::A.t.NHX1* is missing as we did not succeed in amplifying the *A.t.NHX1* promoter), *A.t.VATD prom::GUS, C.h.VATDprom::GUS*. For *SOS1* and *NHX1* we used gDNA, while for *GUS* we used cDNA. All PCR's were done with specific sense and antisense primers (Tables S3.5, S3.6 and S3.7 for *NHX1, SOS1* and *VATD*, respectively: Supplementary Information), using the "Phusion® High Fidelity DNA Polymerase (Finnzymes)" on gDNA/cDNA to amplify the coding regions and on gDNA to amplify the promoters. The sense and anti-sense primers of all the constructs described above have attB1 and attB2 sites. DNA recombinant technique was performed according to the GATEWAY® Cloning System. BP recombination reactions were done between attB-flanked DNA fragments and appropriate attP-containing pDONR221 P1, P2 entry vector, using BP Clonase® II enzyme mix, to generate an entry clone, and the LR recombination reaction between the entry clone and a Gateway® destination vector, using LR Clonase® II, to

generate an expression clone. We used pH7WG2(-p35S) {constructed from pH7WG2} as a destination vector for promoter::gene constructs, pH7WG2 for only the gene, and pHGWFS7 for promoter::GUS construct. These binary vector contains a hygromycin phosphotransferase (hpt) gene, which confers resistance to hygromycin in transformed cells. Later, these binary vectors were introduced through electroporation into the *Agrobacterium tumefaciens* strain C58 (pMP90).

3.2.7 Screening of transformant lines

Seeds of homozygous *A.t.sos1* and *A.t.nhx1* mutants (Col) and wild-type were sown on soil. *A.t.sos1* and *A.t.nhx1* mutants and *A. thaliana* (wild-type) were transformed with the constructs by the flower dip method (Clough and Bent, 1998). T_0 seeds were surface sterilised and sown on 0.8% (w/v) gelrite plates containing 0.5% Murashige and Skoog (MS) salts at pH 5.7-5.9 with 50 µg ml^{-1} hygromycin. The plates were kept vertically to allow recording of the root growth. After two weeks, there was a clear difference between transformed and un-transformed plants. The transgenic plants were transferred to hydroponics, containing a modified half-strength Hoagland's nutrient solution (see above). Selected T_1 progeny was used to check expression of *SOS1*, *NHX1* and *GUS*. The relative transcript levels were measured by Real-Time PCR taking *Act-2* as a positive internal control.

3.2.8 Statistics

Statistical analysis was carried out using one-way and two-way ANOVA. Individual means were compared using Tukey's test.

3.3 Results

3.3.1 Salt tolerance

There were obvious differences in salt tolerance among the species tested. *A. thaliana* was clearly the most salt-sensitive, of the species under study: all the plants died within three weeks upon exposure to 200 mM NaCl. The next most sensitive species was *T. arvense*, which showed some mortality and heavy damage: almost 90% of the leaves of the surviving plants were dead. The other species did not exhibit mortality, but their responses to the salt treatment were clearly different. In *B. oleracea* about 80% of the leaves were still alive, but chlorotic with spotted necrosis at the end of the experiment, except for youngest ones, which

remained glaucous green. Also *C. danica* and *T. botschantzevii* showed chlorosis/necrosis and enhanced senescence, though only of the older leaves (40-60%), whereas *C. x hollandica* remained completely green and healthy. None of the species showed foliar chlorosis, necrosis, or senescence in the control treatment, except for the cotyledons and the oldest leaves. In summary, the salt tolerance decreased in the order *C. x hollandica* >> *C. danica*/*T. botschantzevii* > *B. oleracea* > *T. arvense* > *A. thaliana.*

3.3.2 Na and K concentrations in root and shoot

The shoot Na concentrations in green leaves in salt-exposed plants were not significantly different between the species, except for *B. oleracea*, which exhibited higher foliar Na concentrations than any of the other species (Fig. 1). The root Na concentrations were

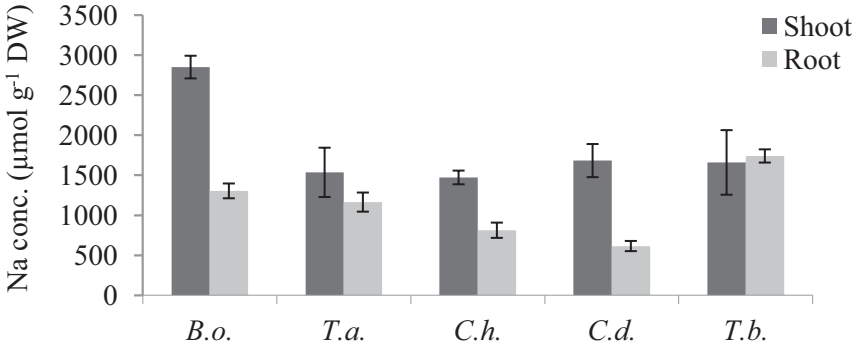

Fig. 1 Shoot and root sodium concentrations of *Brassica oleracea* (*B.o.*), *Thlaspi arvense* (*T.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Thellungiella botschantzevii* (*T.b.*), after three weeks of exposure to 200 mM NaCl in the nutrient solution. Values are means ±SE of five samples. Black bars, shoot Na concentration: grey bars, root Na concentration.

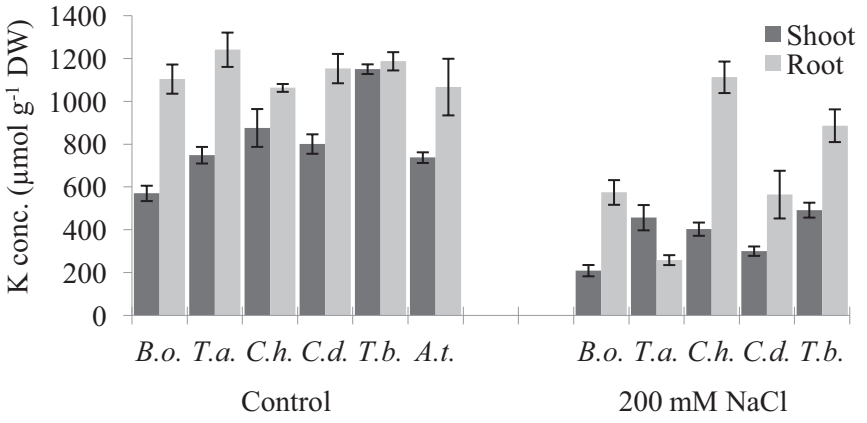

Fig. 2 Shoot and root potassium concentrations in *Brassica oleracea* (*B.o.*), *Thlaspi arvense* (*T.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Thellungiella botschantzevii* (*T.b.*), and *Arabidopsis thaliana* (*A.t.*) in control solution (0 mM NaCl) and after three weeks of exposure to 200 mM NaCl. Values are means ±SE of five samples. Black bars, shoot K concentration: grey bars, root K concentration.

significantly different between species, decreasing in the order *T. botschantzevii* > *B. oleracea* > *T. arvense* > *C. x hollandica* > *C. danica* (Fig. 1).

The shoot K concentrations in the control plants varied significantly between species, being highest in *T. botschantzevii* and lowest in *B. oleracea* (Fig. 2). The same pattern was maintained in the salt treated ones, but in all the species the K concentrations were 40-60% lower than in the control plants. The root K concentrations were not significantly different between species under control conditions, but the salt treatment decreased the root K concentrations strongly and significantly in *T. arvense* (75%), *B. oleracea* and *C. danica* (50%), but barely and insignificantly in *T. botschantzevii*, or not at all in *C. x hollandica*, in comparison with the control plants (Fig. 2).

3.3.3 Expression of salt tolerance candidate genes

3.3.3.1 *NHX1* expression

The *NHX1* cDNA and predicted protein sequences obtained from the different species were 87-98% and 91-98% identical among each other, respectively (Table S3.8: Sequence alignment S3.1: Supplementary Information).

In all the species *NHX1* was more strongly expressed in shoots than in roots (Fig. 3). Of all the species, *C. x hollandica* showed the highest *NHX1* expression levels, both in shoots and

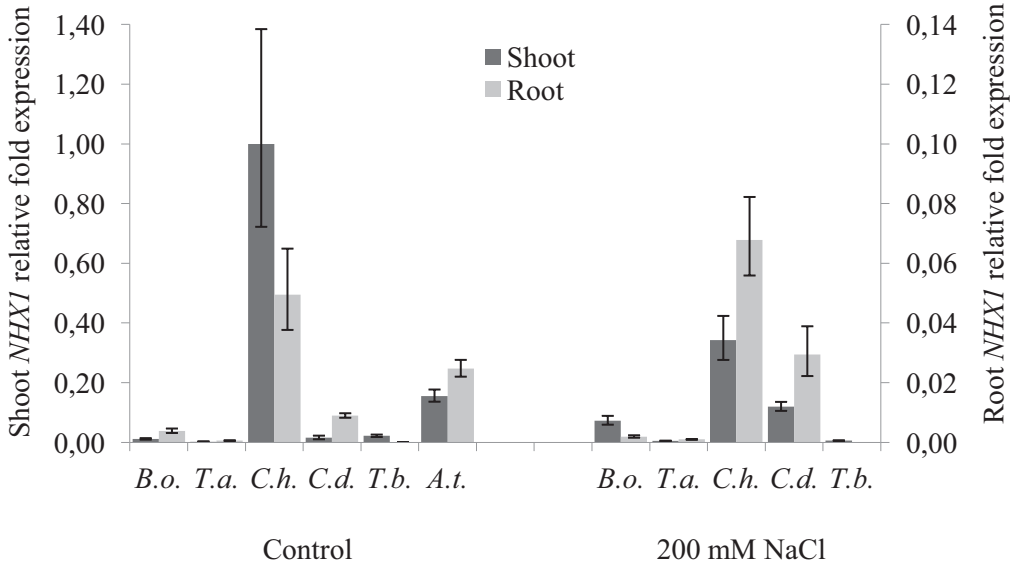

Fig. 3 Expression of *NHX1* in shoots and in roots of *Brassica oleracea* (*B.o.*), *Thlaspi arvense* (*T.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Thellungiella botschantzevii* (*T.b.*), and *Arabidopsis thaliana* (*A.t.*). The gene expression was normalized to the highest expression, which was assigned a value of 1. Each value is the average of three independent biological replicates. Error bars are ±SE. Black bars, shoot *NHX1* expression: grey bars, root *NHX1* expression.

roots, and both in control plants and NaCl-treated ones. There was no significant effect of the salt treatment in this species. There was significant salt-induced *NHX1* expression in *C. danica*, both in shoot and root, as well as in *B. oleracea*, though only in the shoot ($P < 0.01$). *T. arvense* and *T. botschantzevii* showed extremely low *NHX1* expression levels, both in shoots and roots, unaffected by the salt treatment. On the other hand, *A. thaliana* showed showed relatively high *NHX1* expression levels, at least under control conditions (Fig. 3).

3.3.3.2 *SOS1* expression

The *SOS1* cDNA and predicted protein sequences obtained for the different species were 88-99% and 84-98% identical among each other, respectively (Table, S3.9: Sequence alignment S3.2: Supplementary Information).

Of all the species, *C. x hollandica* exhibited by far the highest *SOS1* expression levels in roots, both in the control and the salt treatment (Fig. 4). The shoot expression levels were also highest among all the species, but not significantly higher than in *B. oleracea* and *C. danica* in the control treatment, and not significantly higher than *C. danica* in the salt treated plants. Significant induction of *SOS1* expression by the salt treatment was found in *C. x hollandica*, *C. danica*, and *T. botschantzevii,* albeit only in their shoots. In the salt treatment the root and shoot *SOS1* expression levels were significantly lower in *T. botschantzevii* than in any of the other species. This was also the case in the control treatment, except for *A. thaliana*, which showed even lower expression levels ($P < 0.05$).

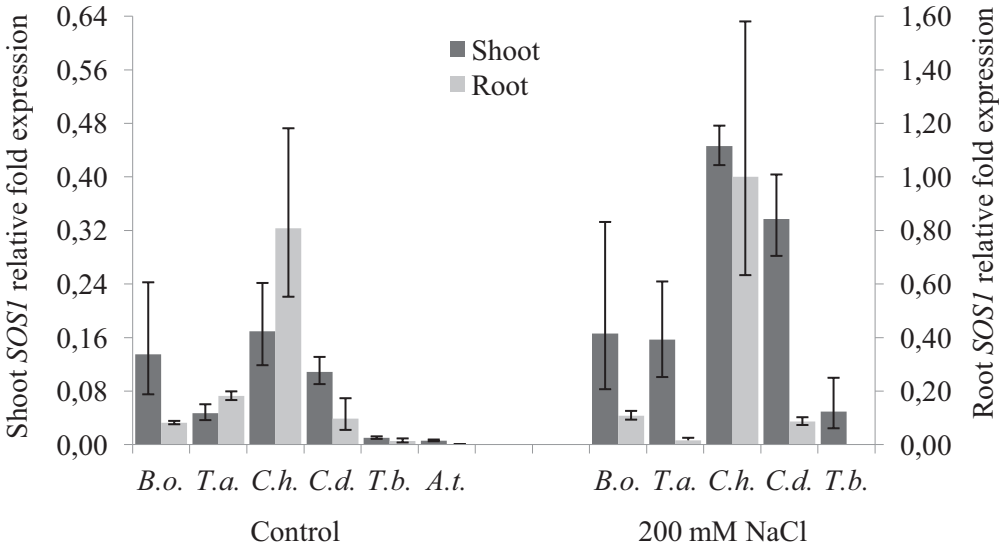

Fig. 4 Expression of *SOS1* in shoots and roots of *Brassica oleracea* (*B.o.*), *Thlaspi arvense* (*T.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Thellungiella botschantzevii* (*T.b.*), and *Arabidopsis thaliana* (*A.t.*). The gene expression was normalized to the highest expression, which was assigned a value of 1. Each value is the average of three independent biological replicates. Error bars are ±SE. Black bars, shoot *SOS1* expression; grey bars, root *SOS1* expression.

3.3.3.3 *VATD* expression

The *VATD* cDNA and predicted protein sequences obtained for the different species were 85-99% and 92-97% identical among each other, respectively (Table S3.10: Sequence alignment S3.3: Supplementary Information).

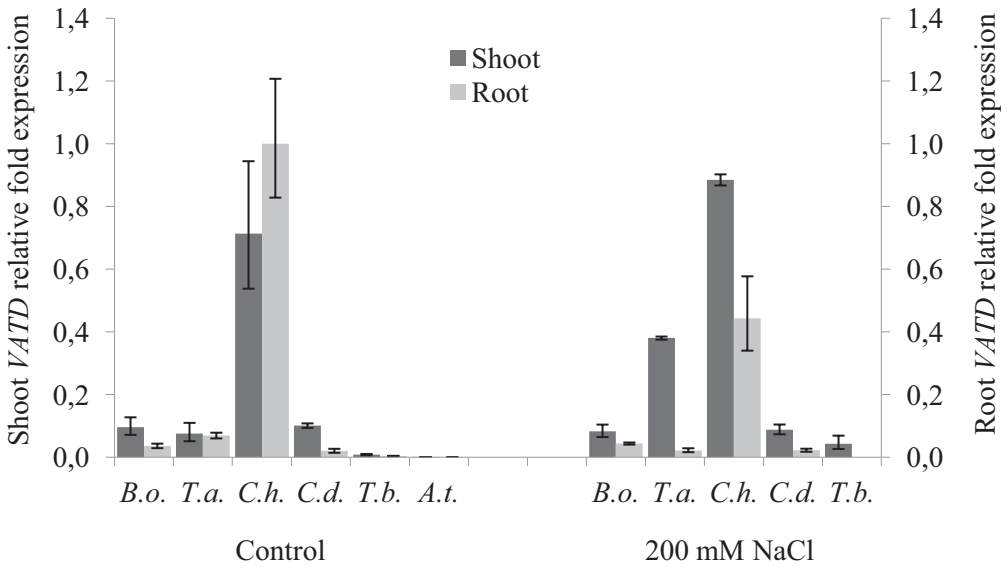

Fig. 5 Expression of *VATD* in shoots and roots of *Brassica oleracea* (*B.o.*), *Thlaspi arvense* (*T.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Thellungiella botschantzevii* (*T.b.*), and *Arabidopsis thaliana* (*A.t.*). The gene expression was normalized to the highest expression, which was assigned a value of 1. Each value is the average of three independent biological replicates. Error bars are ±SE. Black bars, shoot *VATD* expression: grey bars, root *VATD* expression.

Of all the species, *C. x hollandica* also showed the highest expression of *VATD*, both in root and shoot and both in the control and the salt treatment (Fig. 5). In all cases, *VATD* expression was significantly higher in *C. x hollandica* than in all of the other species, of which *B. oleracea*, *T. arvense*, and *C. danica* in turn exhibited a significantly higher expression than *T. botschantzevii* and, in the control, *A. thaliana*. There was no induction by salt in any of the species.

3.3.4 Gene expression in *A. thaliana* under the *C. x hollandica NHX1*, *SOS1* and *VATD* promoters

Chromosome walking in *C. x hollandica* yielded upstream sequences, starting from the ATG translation start, of 2167, 1989, and 1003 bp for *NHX1*, *SOS1*, and *VATD*, respectively. Alignments with the corresponding *A. thaliana* sequences produced low identity percentages (Sequence alignments S3.4, S3.5 and S3.6 respectively: Supplementary Information).

Unfortunately, our attempts to clone the *A. thaliana NHX1* promoter were unsuccessful, which precluded a direct comparison. However, when expressed in the *A.*

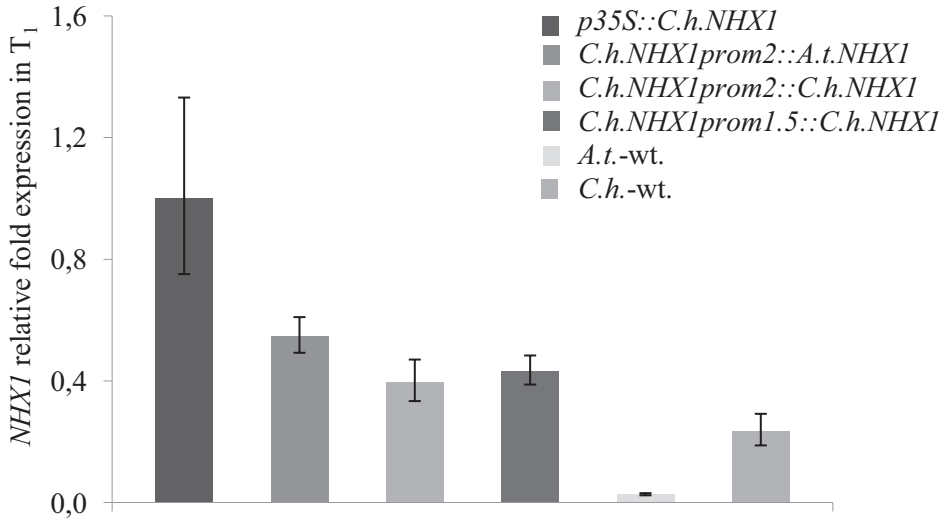

Fig. 6 *A.t.nhx1* transformed with *p35S::C.h.NHX1, C.h.NHX1prom2::A.t.NHX1, C.h.NHX1prom2:: C.h.NHX1, C.h.NHX1prom1.5::C.h.NHX1.* Expression of *NHX1* was measured in shoot of T_1 progeny by Real-Time PCR, using *A.t.*-wt. and *C.h.*-wt. as positive controls. Given are the means ±SE of five independent transgenic lines or wild-type plants.

thaliana nhx1 mutant, under the upstream sequence from *C. x hollandica*, *NHX1* transcript concentations were extremely high, i.e., even two times higher than in *C. x hollandica* itself, and more than 30-fold higher than in wild-type *A. thaliana*, irrespective of the origin of the

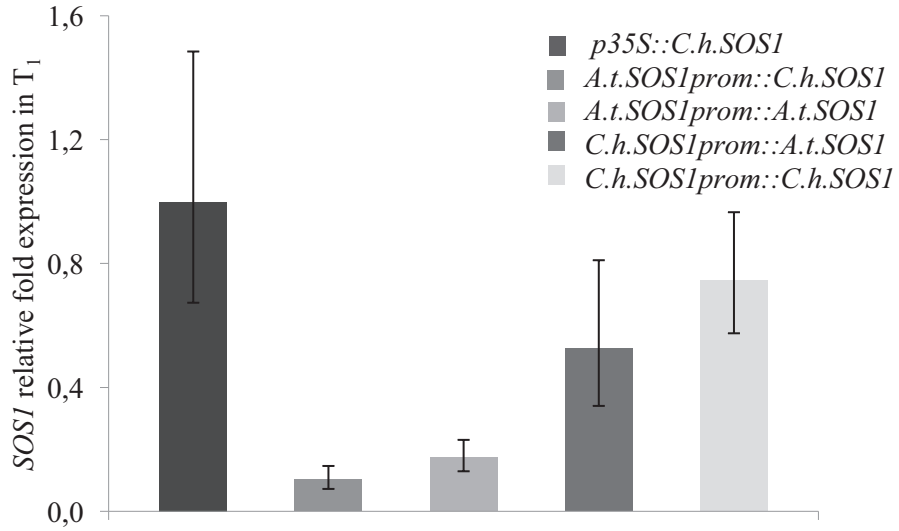

Fig. 7 *A.t.sos1* transformed with *p35S::C.h.SOS1, A.t.SOS1prom::C.h.SOS1, A.t.SOS1prom::A.t.SOS1, C.h.SOS1prom::A.t.SOS1, C.h.SOS1prom::C.h.SOS1.* Expression of *SOS1* was measured in shoot of T_1 progeny by Real-Time PCR. Given are the means ±SE of five independent transgenic lines.

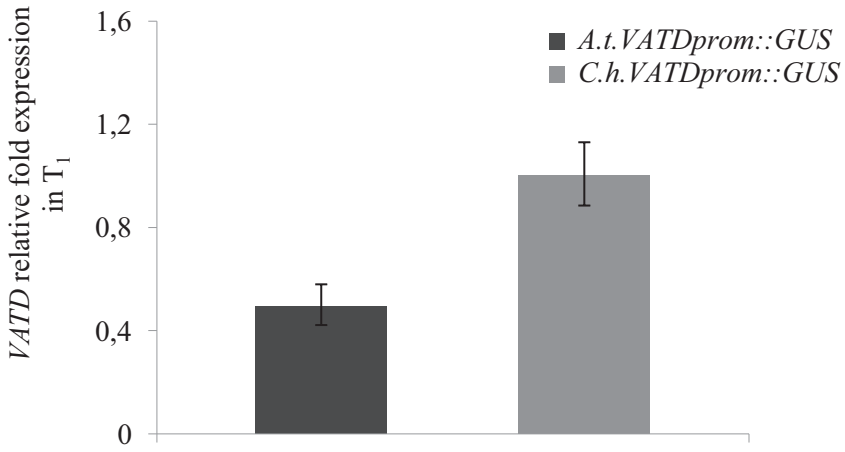

Fig. 8 *A.t.*-wild-type transformed with *A.t.VATDprom::GUS, C.h.VATDprom:: GUS.* Expression of *GUS* was measured in root of T_1 progeny by Real-Time PCR. Given are the means ±SE of five independent transgenic lines.

gDNA coding region (Fig. 6). The same results was obtained with a shorter upstream sequence from *C. x hollandica*.

Also *SOS1* was more strongly expressed ($P < 0.001$) under the *C. x hollandica* (partial) *SOS1* promoter sequence in the *A. thaliana sos1* mutant (about 5-fold) than it was under the *A. thaliana* promoter, again irrespective of the origin of the gDNA coding region (Fig. 7).

When expressed in wild-type *A. thaliana* under the *C. x hollandica VATD* promoter, the *GUS* transcript concentration was about 2-fold higher ($P < 0.05$) than it was under the *A. thaliana* promoter (Fig. 8). The GUS staining patterns were not visibly different, however. Under both promoters, particularly intense staining was observed in the root tip and the root stele (data not given).

3.4 Discussion

Our results showed that *C. x hollandica* is by far the most salt tolerant among the species under study, because it did not visibly suffer from the 200 mM salt treatment, in agreement with the results of a previous experiment (chapter 2). *C. danica* and *T. botschantzevii* were also capable to maintain some growth at 200 mM NaCl, but showed a strongly enhanced rate of leaf senescence. These species were in turn considerably more salt tolerant than *B. oleracea*, which became almost complete chlorotic, or *T. arvense* and *A. thaliana*, which showed an almost complete or complete die-back, respectively. These clear-cut inter-specific differences in salt tolerance were neither associated with differences in the Na concentration

in (green) leaves at the end of the experiment, nor with Na root-to-shoot translocation (Fig. 1), but seemingly with the capacity to maintain normal K concentrations in the root (Fig. 2). The ranking of the species according to their salt tolerance, i.e., *C. x hollandica* >> *C. danica*/*T. botschantzevii* > *B. oleracea* > *T. arvense* > *A. thaliana* seems to be more or less in agreement with the salinity levels in their natural habitat, i.e., permanently brackish soil for *C. x hollandica*, slightly and temporarily brackish or non-saline soil for *C. danica*, non-saline soil, but with deposition of air-borne salt spray for *B. oleracea*, and permanently non-saline soil for *T. arvense* and *A. thaliana*. We do not know the soil salinity level at the site of origin of the *T. botschantzevii* population under study here. In any case, in terms of its capacity to maintain growth and health at 200 mM NaCl (Flowers and Colmer, 2008), this population can not be classified as a true halophyte, in the sense that it is able to maintain high growth rates at 200 mM NaCl, but rather as a relatively salt-tolerant glycophyte. Of course there is a possibility that *T. botschantzevii* , as a species, displays intra-specific variation in salt tolerance. However, we have meanwhile checked salt tolerance in about 10 Asian continental *T. botschantzevii/salsuginea/halophila* populations, and none of them performed significantly better at 200 mM NaCl than did the Saratov population, included in this study. Overall, in our hands, *T. botschantzevii* behaves as a 'salt endurer', surviving long periods of high salinity in a more or less quiescent state, rather than a true halophyte.

The expression differences found between *C. x hollandica* and *C. danica* agree very well with those found in a previous study, in which four Cochlearia species, among which two halophytes, *C. anglica* and *C. x hollandica*, and two glycophytes, *C. danica* and *C. pyrenaica*, were compared among each other. In the latter study *SOS1*, *NHX1* and *VATD* were all higher expressed in *C. x hollandica* than in *C. danica*, except for *NHX1* in the shoot, due to a strong salt-imposed induction of *NHX1* expression in *C. danica*, which is also apparent in the present study (Fig. 3). However, the expression levels of *NHX1* in the shoot, both in the control and under salt exposure, and *VATD* in the root, albeit only under salt exposure, were higher in the salt-sensitive metallophyte, *C. pyrenaica*, than they were in *C. x hollandica*. In the present study *C. x hollandica* consistently exhibits the highest expression levels for *NHX1*, *SOS1* and *VATD*, suggesting that a high level of expression of these genes may be required for the high level of salt tolerance in this species, indeed. On the other hand, it is also clear that a strongly enhanced expression of *NHX1* in the shoot and of *VATD* in the root, such as observed in *C. pyrenaica*, is not sufficient to produce any considerable salt tolerance (chapter 2). One might argue that the high *VATD* expression in *C. pyrenaica* could

be related with its zinc tolerance, which is, in non-hyperaccumulator metallophytes, supposed to be based on an enhanced sequestration of zinc in root cell vacuoles, mediated by an enhanced expression of Zn^{2+}/H^{+} antiporters of the *MTP* family (Krämer, 2005). However, *VATD* was highly expressed in *C. pyrenaica* exclusively under salt exposure (chapter 2), whereas Zn tolerance in metallicolous populations is known to be a constitutive trait (Hanikenne *et al.,* 2008). In addition, it is very difficult to conceive a role for *NHX1* in metal sequestration. As yet we do not have any valid explanation for the high expression of these genes in *C. pyrenaica*. Anyway, the results of the present study may be taken to suggest that strongly enhanced expression levels of *NHX1* or *VATD* are exceptional among glycophytes.

Finally, although we can not tell whether the isolated *C. x hollandica* promoter fragments of *NHX1*, and *SOS1* contain all the relevant response elements, it is remarkable that they both confer significantly higher transcript levels than their *A. thaliana* analogues. This is a strong indication that *C. x hollandica* has acquired the high expression levels of these genes through altered *cis*-regulation, at least in part. In the case of *NHX1*, heterologous expression under the *C. x hollandica* promoter is apparently enough to produce the high expression level found in *C. x hollandica*. In the case of *SOS1*, however, the *C. x hollandica* promoter produces about five times more expression than the *A. thaliana* one, whereas the 'wild-type' expression levels are ± 15-fold different. The *C. x hollandica VATD* promoter that we used however, contains the complete intergenic region between *VATD* and the upstream gene. Nevertheless, it is only two times stronger than the *A. thaliana* one, whereas the difference in wild-type *VATD* expression levels is about 50-fold. This clearly suggests that the strongly enhanced expression levels of *SOS1* and, particularly, *VATD* are not exclusively due to altered *cis*-regulation, but also to altered *trans*-regulation or copy number expansion.

In conclusion, high-level salt tolerance in *C. x hollandica* is associated with a strongly enhanced expression, in comparison with most glycophytes, of the Na transporters, *NHX1* and *SOS1*, and the vacuolar proton ATPase, the latter producing the proton motive force required for the Na^{+}/H^{+} antiport activity of NHX1. The high expression levels of these genes in *C. x hollandica* seem to rely on altered *cis*-regulation, albeit only partly so for *SOS1* and *VATD*. Overall, these results suggest that salt tolerance in *C. x hollandica* is based on efficient efflux of Na from the cytoplasm, and possibly on the ability to maintain a normal K concentration in its roots under Na exposure.

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Chapter 3

Supplementary Information

Salt Tolerance and Candidate Salt Tolerance Gene Expression Levels in Brassicaceae

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Supplementary Tables; S3.1-S3.10

Supplementary sequence alignments; S3.1-S3.6

Supplementary Tables

Pair	Primer	Sequence
Outer	NHX1Deg.Fwd1	GTKCTKAATCARGAYGAKACACC
	NHX1Deg.Rev1	TCRATRTCCAAKGCATCCATWCCRAC
Inner	NHX1Deg.Fwd2	GTATTYGGRGARGGTGTYGTRAATGATGC
	NHX1Deg.Rev2	AAYGACAWWGTTGCAAARGYATGC
Outer	SOS1Deg.Fwd1	CTCRTYVT.B.GGVATTGCYCTYGGATC
	SOS1Deg.Rev1	GTKGANCCATTMACWATMAGHGTYAG
Inner	SOS1Deg.Fwd2	CTKCCKGCBCTTCTTTTYGAGAGTKC
	SOS1Deg.Rev2	CRATTCCRCC.H.GTGAAGAAAABAAAC
Outer	VATDDeg.Fwd1	GTVGTKCCSACKGTKAC.D.ATGCTYG
	VATDDeg.Rev1	CCTCYTCTTGTADCCCTGDATCTTC
Inner	VATDDeg.Fwd2	GCTCGBCTYGTYGGYGCKACMMGMGG
	VATDDeg.Rev2	CTGAAGAARTCCTCBCKYTCRAGCTC

Supplementary Table S3.1 Degenerate primer pairs used to amplify *NHX1*, *SOS1*, *VATD* in 1st and nested PCR.

	NHX1	*SOS1*	*VATD*	*Act-2*
B.o.	JQ435891	JQ435896	JQ638707	JQ435879
T.a.	JQ435892	JQ435897	JQ638706	JQ435880
C.h.	JQ350805	JQ350806	JQ350807	JQ435881
C.d.	JQ435893	JQ435898	JQ638708	JQ935965
T.b.	DQ995339	EF207775	JQ638705	-

Supplementary Table 3.2 Accession numbers, obtained from GenBank, of *Brassica oleracea* (*B.o.*), *Thlaspi arvense* (*T.a.*), *Cochlearia x hollandica* (*C.h.*), *Cochlearia danica* (*C.d.*) and *Thellungiella botschantzevii* (*T.b.*).

Primers	Sequence
(B.o.,C.d.)Act-2F	ATGTCGCTATTCAAGCTGTTC
(B.o.,T.a.,C.d.,T.b.,A.t.)Act-2R	CACCATCACCAGAATCCAGCA
(T.a.,T.b.,A.t.)Act-2F	ATGTCGCCATCCAAGCTGTTC
(C.h.)Act-2F	ATGTCGCTATCCAAGCTGTTC
(C.h.)Act-2R	CACCATCACCAGAATCCAACA
(B.o.,T.a.,T.b.)NHX1F	TCTTCTGGTCTTCAGTGAAGATC
(B.o.)NHX1R	GCATAATAGTCACAAAGTTGCGG
(T.a.)NHX1R	CAGTTCCAATAGCACCAAAAAGC
(C.h.,C.d.,T.b.,A.t.)NHX1R	GCATAATAGTCACGAAATTGCGG
(C.h.,C.d.,A.t.)NHX1F	GCCACCCATTATATTCAATGCAG
(B.o.,T.a.)SOS1F	GGTGACAGCCAAAGAAGTTTG
(B.o.,T.a.)SOS1R	GAGAATGCCTTCAGCTATGG
(C.h.,C.d.,T.b.,A.t.)SOS1F	CTGAAGGCATTCTCGACAG
(C.h.,C.d.,T.b.,A.t.)SOS1R	AGAACTCCAACAACAACACAACG
(B.o.,T.b.)VATDF	GACATCGTCTTTTGCTCTTACCG
(B.o.)VATDR	CACCAGCGATGTTCTCTTGC
(T.a.,A.t.)VATDF	CCTCGAGAACGTTAAAGAAGCTAC
(T.a.,A.t.)VATDR	GACCTGTTGACCACCTCTAG
(C.h.,C.d.,)VATDF	GAGGACAGAGAACATAGCTGG
(C.h.,C.d.)VATDR	CTTGTACTTGTTGACCACCTCTAG
(T.b.)VATDR	CTCCTGCGATGTTCTCTTGC

Supplementary Table S3.3 Primers used in real-time qPCR. Abbreviations; *B. oleracea* (*B.o.*), *T. arvense* (*T.a.*), *C. x hollandica* (*C.h.*), *C. danica* (*C.d.*), *T. botschantzevii* (*T.b.*), *A. thaliana* (*A.t.*), forward primer (F) and reverse primer (R).

Chromosome walk	Primer Name	Primer Sequence
C. x hollandica NHX1		
1st Chromosome walk*	Outer R1	CAAGGCAGTGGTGGATTCGTTC
	Inner R2	GTTCATCCATCGGTTCTCTTCAAGG
2nd Chromosome walk	Outer R3	GCGAGAAAACGAAGGAATCGCTAG
	Inner R4	CGAGAGAGACGTGTACGGTCC
3rd Chromosome walk	Outer R5	GACTGGGTAAAATATCCGTTAGATTCG
	Inner R6	GATCCGATCCGTGATTCGTCTCG
C. x hollandica SOS1		
1st Chromosome walk *	Outer R1	GTGAACTTCCATTGAAAAGGCACTCTC
	Inner R2	GAAGTTCAGGGTCAATATCATTCC
C. x hollandica VATD		
1st Chromosome walk *	Outer R1	CTTGTACTTGTTGACCACCTCTAG
	Inner R2	CGATGTCTTCATCATATCTCCCATG
2nd Chromosome walk	Outer R3	GAACACACTATGTGGGCTCCATG
	Inner R4	GTCCGTTTTACATAAACAAGAAACAAC

Supplementary Table S3.4 Primers used for chromosome walking on *C. x hollandica* gDNA to amplify the *NHX1* (2122 bp), *SOS1* (1857 bp) and V-ATPase subunit-D (*VATD*, 1003 bp) promoter sequences upstream of ATG. "R" Stands for reverse primer. *****1st Chromosome walking primers set is specific to the coding sequences of the respective gene, while 2nd and 3rd pairs, if any, are specific to the respective promoter sequences.

Construct		Primer name	Primer sequence
p35S::*C.h.NHX1*	*C.h.NHX1*	C.h.NHX1F	GGGGACAAGTTTGTACAAAAAAGCAGGCT-ATGGCAATGCTGGCATCACAT
		C.h.NHX1R	GGGGACCACTTTGTACAAGAAAGCTGGGT-ATATCGTTGAAAGTGTCCATG
C.h.NHX1prom2:: A.t.NHX1	*C.h.NHX1prom*	C.h.NHX1promF	GGGGACAAGTTTGTACAAAAAAGCAGGCT-GTCGGTTTAACTAAGTCGGTC
		C.h.NHX1promR	AGAGAATCCAACATCTTCTATACCCACTGG-TTTCC
	A.t.NHX1	A.t.NHX1F	GTGGGTATAGAAGATGTTGGATTCTCTAGT-GTCG
		A.t.NHX1R	GGGGACCACTTTGTACAAGAAAGCTGGGTG-CTCTCAAAACGTTAGGACAG
C.h.NHX1prom2:: C.h.NHX1	*C.h.NHX1prom*	C.h.NHX1promF	GGGGACAAGTTTGTACAAAAAAGCAGGCTG-TCGGTTTAACTAAGTCGGTC
		C.h.NHX1promR	GACACGAGAGAGCCTAAATGTG
	C.h.NHX1	C.h.NHX1F	GCTGGCATCACATTTAGGCTC
		C.h.NHX1R	GGGGACCACTTTGTACAAGAAAGCTGGGTAT-ATCGTTGAAAGTGTCCATG
C.h.NHX1prom1.5:: C.h.NHX1	*C.h.NHX1prom*	C.h.NHX1promF	GGGGACAAGTTTGTACAAAAAAGCAGGCTGT-CGGTTTAACTAAGTCGGTC
		C.h.NHX1promR	AGAGAATCCAACATCTTCTATACCCACTGGTT-TCC
	C.h.NHX1	C.h.NHX1F	GTGGGTATAGAAGATGTTGGATTCTCTAGTGT-CG
		C.h.NHX1R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCT-CTCAAAACGTTAGGACAG

Supplementary Table S3.5 Primers used to make *NHX1* constructs.

Construct		Primer name	Primer sequence
p35S::*C.h.SOS1*	*C.h.SOS1*	C.h.SOS1F	GGGGACAAGTTTGTACAAAAAAGCAGGCT-ATGTACTCCGTTTCAGCTTTT
		C.h.SOS1R	GGGGACCACTTTGTACAAGAAAGCTGGGT-GCAAACATTCATCGAAATAGC
A.t.SOS1prom::C.h.SOS1	*A.t.SOS1prom*	A.t.SOS1promF	GGGGACAAGTTTGTACAAAAAAGCAGGCT-GCATTTCATTAGGATCGACGG
		A.t.SOS1promR	AAAGCTGAAACGGAGTACATTTTATTTGAA-TATATCTAAGAAGCAACAAC
	C.h.SOS1	C.h.SOS1F	CTTAGATATATTCA AATAAAATGTACTCCGT-TTCAGCTTTT
		C.h.SOS1R	GGGGACCACTTTGTACAAGAAAGCTGGGTGC-AAACATTCATCGAAATAGC
A.t.SOS1prom::A.t.SOS1	*A.t.SOSprom*	A.t.SOS1promF	GGGGACAAGTTTGTACAAAAAAGCAGGCTGC-ATTTCATTAGGATCGACGG
	A.t.SOS1	A.t.SOS1R	GGGGACCACTTTGTACAAGAAAGCTGGGTCA-ACATACACTAGCTTATTCT
C.h.SOS1prom::A.t.SOS1	*C.h.SOS1prom*	C.h.SOS1promF	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC-TTGACATACCCTGACACAC
		C.h.SOS1promR	CGTCGATTACAGTCGTCATAAAGTATACAGCT-ATTTATAAACAACTAT
	A.t.SOS1	A.t.SOS1F	TAAATAGCTGTATACTTTATGACGACTGTAAT-CGACGCG
		A.t.SOS1R	GGGGACCACTTTGTACAAGAAAGCTGGGTCA-ACATACACTAGCTTATTCT
C.h.SOS1prom::C.h.SOS1	*C.h.SOS1prom*	C.h.SOS1promF	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC-TTGACATACCCTGACACAC
	C.h.SOS1	C.h.SOS1R	GGGGACCACTTTGTACAAGAAAGCTGGGT GCAAACATTCATCGAAATAGC

Supplementary Table S3.6 Primers used to make *SOS1* constructs.

Construct		Primer name	Primer sequence
C.h.VATDprom::GUS	*C.h.VATDprom*	C.h.VATDpromFwd	GGGGACAAGTTTGTACAAAAAAGCAGGCT - ATACTGGAACGTGGCTAAGGC
		C.h.VATDpromRev	GGGGACCACTTTGTACAAGAAAGCTGGGT - CATTGTAACCGTTGGAACCAC
A.t.VATDprom::GUS	*A.t.VATDprom*	A.t.VATDpromFwd	GGGGACAAGTTTGTACAAAAAAGCAGGCT-ATACTGGAGCGGGGATAAGGC
		A.t.VATDpromRev	GGGGACCACTTTGTACAAGAAAGCTGGGT-GGGAACCACATTCAAACGCGC

Supplementary Table S3.7 Primers used to make *VATD* constructs.

	A.t.NHX1	B.o.NHX1	T.a.NHX1	C.d.NHX1	C.h.NHX1	T.b.NHX1
	Coding sequence					
A.t.NHX1		88	91	89	89	91
B.o.NHX1	91		91	87	88	91
T.a.NHX1	96	95		91	91	94
C.d.NHX1	94	92	95		98	92
C.h.NHX1	93	93	95	98		92
T.b.NHX1	97	97	98	96	97	
	Amino acid sequence					

Supplementary Table S3.8 Identity percentages for *NHX1*. Abbreviations; *B. oleracea* (*B.o.*), *T. arvense* (*T.a.*), *C. x hollandica* (*C.h.*), *C. danica* (*C.d.*), *T. botschantzevii* (*T.b.*), *A. thaliana* (*A.t.*).

	A.t.SOS1	B.o.SOS1	T.a.SOS1	C.d.SOS1	C.h.SOS1	T.b.SOS1
	Coding sequence					
A.t.SOS1		88	88	91	88	91
B.o.SOS1	85		99	99	98	91
T.a.SOS1	85	97		98	98	91
C.d.SOS1	86	94	96		98	92
C.h.SOS1	86	96	96	95		91
T.b.SOS1	91	91	91	88	93	
	Amino acid sequence					

Supplementary Table S3.9 Identity percentages for *SOS1*. Abbreviations; *B. oleracea* (*B.o.*), *T. arvense* (*T.a.*), *C. x hollandica* (*C.h.*), *C. danica* (*C.d.*), *T. botschantzevii* (*T.b.*), *A. thaliana* (*A.t.*).

	A.t.VATD	B.o.VATD	T.a.VATD	C.d.VATD	C.h.VATD	T.b.VATD
	Coding sequence					
A.t.VATD		88	90	87	86	92
B.o.VATD	94		89	87	86	90
T.a.VATD	96	94		86	86	91
C.d.VATD	97	94	97		99	86
C.h.VATD	97	92	95	96		85
T.b.VATD	96	96	96	95	92	
	Amino acid sequence					

Supplementary Table S3.10 Identity percentages for *VATD*.
Abbreviations; *B. oleracea* (*B.o.*), *T. arvense* (*T.a.*), *C. x hollandica* (*C.h.*), *C. danica* (*C.d.*), *T. botschantzevii* (*T.b.*), *A. thaliana* (*A.t.*).

Supplementary Sequence Alignments

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B.o.NHX1        ------------------------------------------------------------
T.a.NHX1        ------------------------------------------------------------
C.h.NHX1        TTGTTGTCGACATCTGGTCACGCCTCTGTGGTTTCACTTAATCTGTTTGTTGCGCTTCTT
C.d.NHX1        TTGTTGTCGACATCTGATCACGCCTCTGTGGTTTCACTGAATCTGTTCGTTGCGCTTCTT
T.b.NHX1        ------------------------------------------------------------
A.t.NHX1        ------------------------------------------------------------

B.o.NHX1        ------TGTATTGTCCTTGGCCATCTTCTGGAAGAGAACCGATGGATGAACGAATCCATC
T.a.NHX1        ---------ATTGTGCTTGGCCATCTTTTGGAGGAGAACCGATGGATGAACGAATCCATC
C.h.NHX1        TGCGCTTGCATCGTGCTTGGCCATCTCCTCGAAGAGAACCGATGGATGAACGAATCCACC
C.d.NHX1        TGCGCTTGCATCGTGCTTGGCCATCTCCTTGAAGAGAACCGATGGATGAACGAATCCACC
T.b.NHX1        ------TGTATTGTGCTTGGCCATCTTTTGGAGGAGAACCGATGGATGAACGAATCCATC
A.t.NHX1        ------TGTATTGTTCTTGGTCATCTTTTGGAAGAGAATAGATGGATGAACGAATCCATC

B.o.NHX1        ACCGCCTTATTGATTGGGCTGGCTACTGGTGTTGTCATGTTGTTGATTAGTAATGGCAAA
T.a.NHX1        ACCGCCTTAATGATTGGGCTGGCCACTGGTGTTGTCATTTTGTTGATTAGTAAAGGAAAA
C.h.NHX1        ACTGCCTTGTTGCTTGGGCTTGCCACTGGTGTTGTCATTTTGTTGATTAGTAATGGCAAA
C.d.NHX1        ACTGCCTTGTTGATTGGGCTTGCCACTGGTGTTGTCATTTTGTTGATTAGTAATGGCAAA
T.b.NHX1        ACAGCGTTGTTGATTGGGCTTGCCACTGGTGTTGTCATTTTGTTGATTAGTAAAGGAAAA
A.t.NHX1        ACCGCCTTGTTGATTGGGCTAGGCACTGGTGTTACCATTTTGTTGATTAGTAAAGGAAAA

B.o.NHX1        AGCTCACATCTTCTGGTCTTCAGTGAAGATCTTTTCTTCATATATCTTTTGCCACCCATT
T.a.NHX1        AGCTCACATCTTCTGGTCTTCAGTGAAGATCTTTTCTTCATATATCTTTTGCCGCCCATA
C.h.NHX1        AGCTCGCATCTTCTTGTCTTTAGTGAAGATCTCTTCTTCATATATCTTTTGCCACCCATT
C.d.NHX1        AGCTCGCATCTTCTGGTCTTTAGTGAAGATCTCTTCTTCATATATCTCTTGCCACCCATT
T.b.NHX1        AGCTCACATCTTCTGGTCTTCAGTGAAGATCTTTTCTTCATATATCTCTTGCCACCCATA
A.t.NHX1        AGCTCGCATCTTCTCGTCTTTAGTGAAGATCTTTTCTTCATATATCTTTTGCCACCCATT

B.o.NHX1        ATATTCAATGCTGGATTTCAAGTGAAAAAGAAACAGTTTTTCCGCAACTTTGTGACTATT
T.a.NHX1        ATATTCAATGCAGGGTTTCAAGTTAAAAAGAAGCAATTTTTCCGGAATTTCATAACTATC
C.h.NHX1        ATATTCAATGCAGGGTTTCAAGTAAAAAAGAAGCAATTTTTCCGCAATTTCGTGACTATT
C.d.NHX1        ATATTCAATGCAGGGTTTCAAGTAAAAAAGAAGCAATTTTTCCGCAATTTCGTGACTATT
T.b.NHX1        ATATTCAATGCAGGGTTTCAAGTAAAAAAGAAGCAATTTTTCCGCAATTTCGTGACTATT
A.t.NHX1        ATATTCAATGCAGGGTTTCAAGTAAAAAAGAAGCAGTTTTTCCGCAATTTCGTGACTATT

B.o.NHX1        ATGCTCTTTGGTGCTATTGGAACTGTTGTCTCTTGCACTGTCATAACACTAGGTGTAACA
T.a.NHX1        ATGCTTTTTGGTGCTATTGGAACTGTTATTTCTTGCACTGTAATAACTCTAGGTGTAACG
C.h.NHX1        ATGCTTTTTGGTGCTATTGGGACTGTTATTTCTTGCACTGTCATAACTCTAGGTGTAACA
C.d.NHX1        ATGCTTTTTGGTGCTATTGGGACTGTTATTTCTTGCACTGTCATAACTCTAGGTGTAACA
T.b.NHX1        ATGCTTTTTGGTGCTATTGGAACTGTTATCTCTTGCACTGTCATAACTTTAGGTGTAACG
A.t.NHX1        ATGCTTTTTGGTGCTGTTGGGACTATTATTTCTTGCACAATCATATCTCTAGGTGTAACA
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B.o.NHX1          CAGTTCTTTAAGAAACTGGACATTGGGACCTTTGACTTGGGTGATTATCTTGCAATCGGT
T.a.NHX1          CAGTTCTTTAAGAAATTGGACATTGGGACCTTTGACTTGGGTGATTATCTTGCAATCGGT
C.h.NHX1          CAGTTCTTTAAGAAATTGGACATTCGGACCTTTGACTTGGGTGATTATCTTGCAATTGGT
C.d.NHX1          CAGTTCTTTAAGAAATTGGACATTGGGGCCTTTGACTTGGGTGATTATCTTGCAATTGGT
T.b.NHX1          CAGTTCTTTAAGAAACTGGATATTGGGACCTTTGACTTGGGTGATTATCTTGCAATTGGT
A.t.NHX1          CAGTTCTTTAAGAAGTTGGACATTGGAACCTTTGACTTGGGTGATTATCTTGCTATTGGT

B.o.NHX1          GCTATATTTGCTGCAACAGATTCCGTTTGCACACTGCAGGTTCTGAACCGAGATGAGACA
T.a.NHX1          GCCATATTTGCTGCAACAGATTCTGTGTGCACACTGCAGGTTCTGAATCAAGATGAGACA
C.h.NHX1          GCCATATTTGCTGCAACAGATTCTGTGTGCACACTTCAGGTTCTGAATCAAGATGAGACA
C.d.NHX1          GCCATATTTGCTGCAACAGATTCTGTGTGCACACTTCAGGTTCTGAATCAAGATGAGACA
T.b.NHX1          GCTATATTTGCTGCAACAGATTCTGTGTGCACTCTGCAGGTTCTGAATCAAGATGAGACA
A.t.NHX1          GCCATATTTGCTGCAACAGATTCAGTATGTACACTGCAGGTTCTGAATCAAGACGAGACA

B.o.NHX1          CCTCTGCTTTACAGTCTTGTATTCGGAGAAGGTGTTGTGAATGACGCCACATCAGTTGTT
T.a.NHX1          CCTTTGCTTTACAGTCTTGTATTCGGAGAGGGTGTTGTGAATGATGCCACATCAGTTGTA
C.h.NHX1          CCTTTGCTTTACAGTCTTGTATTCGGAGAAGGTGTTGTTAATGACGCCACATCAGTTGTT
C.d.NHX1          CCTTTGCTTTACAGTCTTGTATTCGGAGAAGGTGTTGTTAATGACGCCACATCAGTTGTT
T.b.NHX1          CCTTTGCTATACAGTCTTGTATTCGGAGAGGGTGTTGTGAATGATGCCACATCGGTTGTT
A.t.NHX1          CCTTTGCTTTACAGTCTTGTATTCGGAGAGGGTGTTGTGAATGATGCAACGTCAGTTGTG

B.o.NHX1          GTCTTCAACGCGATTCAGAGCTTTGACCTCACCCACCTTAACCATGAAGCTGCTTTTCGA
T.a.NHX1          ATCTTCAATGCAATTCAGAGCTTTGACCTCACCCACCTTAACCATGAAGCTGCTTTCCAT
C.h.NHX1          GTCTTCAACGCAATTCAAAGCTTTGACCTTACCCACCTTAACCATGAAGCTGCTTTTCGG
C.d.NHX1          GTCTTCAACGCAATTCAAAGCTTTGACCTTACCCACCTTAACCATGAAGCTGCTTTTCGG
T.b.NHX1          GTCTTCAACGCAATTCAGAGCTTTGACCTCACCCACCTTAACCATGAAGCTGCTTTTCAT
A.t.NHX1          GTCTTCAACGCGATTCAGAGCTTTGATCTCACTCACCTAAACCACGAAGCTGCTTTTCAT

B.o.NHX1          CTTCTTGGGAACTTTTTCTATCTGTTTCTCCTCAGCACCTTGCTTGGTGTTGCGACTGGT
T.a.NHX1          CTTCTTGGAAACTTCTTGTATTTGTTTCTCCTGAGCACTTTGCTTGGTGCAGCAACCGGT
C.h.NHX1          CTTCTTGGGAACTTCTCGTACTTGTTTCTCCTCAGCACTTTTCTTGGTGTTGCAACGGGT
C.d.NHX1          CTTCTTGGAAACTTCTCGTACTTGTTTCTCCTCAGCACTTTTCTTGGTGTTGCAACGGGT
T.b.NHX1          CTTCTTGGAAACTTCTTGTATTTGTTTCTTCTGAGCACATTGCTTGGTGTTGCAACCGGT
A.t.NHX1          CTTCTTGGAAACTTCTTGTATTTGTTTCTCCTAAGTACCTTGCTTGGTGCTGCAACCGGT

B.o.NHX1          CTGATAAGTGCATATGTCATCAAAAAGCTATACTTCGGAAGACACTCCACTGACCGAGAG
T.a.NHX1          CTGATAAGTGCATATGTCATCAAAAAGCTATATTTTGGAAGACACTCAACCGACCGAGAG
C.h.NHX1          CTGATAAGTGCTTATGTGATCAAAAAGTTATATTTTGGAAGACACTCCACTGACCGAGAG
C.d.NHX1          CTGATAAGTGCTTATGTGATCAAAAAGTTATATTTTGGAAGACACTCGACTGACCGAGAG
T.b.NHX1          CTGATAAGTGCCTATGTCATCAAAAAACTATATTTTGGAAGACACTCAACTGATCGAGAG
A.t.NHX1          CTGATAAGTGCGTATGTTATCAAGAAGCTATACTTTGGAAGGCACTCAACTGACCGAGAG

B.o.NHX1          GTTGCTCTCATGATGCTTATGGCGTATCTTTCTTACATGCTTGCTGAGCTTTCCGACTTG
T.a.NHX1          GTTGCACTCATGATGCTTATGGCGTATCTTTCTTATATGCTTGCTGAGCTTTTCGACTTG
C.h.NHX1          GTTGCCCTCATGATGCTAATGGCGTATCTTTCTTATATGCTTGCTGAGCTTTTTGATTTA
C.d.NHX1          GTTGCCCTCATGATGCTAATGGCGTATCTTTCTTATATGCTTGCTGAGCTTTTTGATTTA
T.b.NHX1          GTTGCCCTCATGATGCTTATGGCGTATCTGTCTTATATGCTTGCTGAGCTTTTCGACTTG
A.t.NHX1          GTTGCCCTTATGATGCTTATGGCGTATCTTTCTTATATGCTTGCTGAGCTTTTCGACTTG

B.o.NHX1          AGTGGTATTCTCACTGTGTTTTTCTGCGGGATTGTCATGTCTCATTACACCTGGCACAAC
T.a.NHX1          AGTGGTATCCTCACAGTGTTTTTCTGTGGGATTGTGATGTCACATTACACCTGGCACAAC
C.h.NHX1          AGTGGTATTCTTACTGTGTTTTTCTGCGGGATTGTGATGTCCCATTACACCTGGCACAAC
C.d.NHX1          AGTGGTATTCTTACTGTGTTTTTCTGCGGGATTGTGATGTCCCATTACACCTGGCACAAC
T.b.NHX1          AGTGGTATTCTCACCGTGTTTTTCTGTGGGATTGTGATGTCCCATTACACCTGGCACAAC
A.t.NHX1          AGCGGTATCCTCACTGTGTTTTTCTGTGGTATTGTGATGTCCCATTACACATGGCACAAT

B.o.NHX1          GTCACCGAGAGCTCAAGAATCACTACCAAGCACGCCTTCGCAACGTTGTCGTTTCT
T.a.NHX1          GTAACCGAGAGCTCAAGAATAACTA--------------------------------
C.h.NHX1          GTAACCGAGAGCTCAAGAATAACTACC-------------------------------
C.d.NHX1          GTAACCGAGAGCTCAAGAAT---------------------------------------
T.b.NHX1          GTAACCGAGAGCTCGAGAATAACTACA-------------------------------
A.t.NHX1          GTAACGGAGAGCTCAAGAATAACAACA-------------------------------
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Supplementary Sequence Alignment S3.1 Sequence alignments of *B.o.NHX1, T.a.NHX1, C.h.NHX1, C.d.NHX1, T.b.NHX1* and *A.t.NHX1* on nucleotides basis.

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B.o.SOS1          ---CAAATGGTGCTACTTGCTGGTCCTGGAGTTCTTATTTCGACGTTTTGTCTCGCAACG
T.a.SOS1          GGACAAATGGTGCTACTTGCTGGTCCTGGAGTTCTTATTTCGACGTTTTGTCTCGCAACG
C.h.SOS1          -GACAAATGGTGCTACTTGCTGGTCCTGGAGTTCTTATTTCGACGTTTTGTCTCGCAACG
C.d.SOS1          ------------------------------------------------------------
T.b.SOS1          GGACAAATGGTGCTACTTGCTGGGCCTGGAGTTCTCATTTCAACCTTTTGTCTGGCATCG
A.t.SOS1          -GACAAATGGTGTTACTTGCTGTCCCTGGAGTTCTTATTTCAACAGCTTGTCTTGGATCG
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B.o.SOS1        CTTGTTAAGCTCACGTTTCCATATGACTGGGACTGGAAAACGTCGTTGTTGCTTGGGGGA
T.a.SOS1        CTTGTTAAGCTCACGCTTCCATATGACTGGGACTGGAAAACGTCGTTGTTGCTTGGGGGA
C.h.SOS1        CTTGTTAAGCTCACGTTTCCATATGACTGGGACTGGAAAACGTCGTTGTTGCTTGGGGGA
C.d.SOS1        ------------------------------------------------------------.
T.b.SOS1        CTTGTTAAGCTCACGTTTCCGTATGACTGGGACTGGAAAACGTCGTTGTTGCTTGGGGGA
A.t.SOS1        CTTGTGAAGGTCACGTTTCCGTATGAATGGGACTGGAAAACGTCCTTGTTGCTTGGGGGA

B.o.SOS1        CTTTTAAGTGCTACAGATCCTGTTGCTGTTGTTGCTTTGCTAAAGGAGCTTGGTGCTAGT
T.a.SOS1        CTTTTAAGTGCTACAGATCCTGTTGCTGTTGTTGCTTTGCTAAAGGAGCTTGGTGCTAGT
C.h.SOS1        CTTTTAAGTGCTACAGATCCTGTTGCTGTTGTTGCTTTGCTAAAGGAGCTTGGTGCTAGT
C.d.SOS1        ------------------------------------------------------------.
T.b.SOS1        CTTTTAAGTGCTACTGATCCTGTTGCTGTTGTTGCTTTGCTTAAAGAGCTTGGTGCTAGT
A.t.SOS1        CTTTTAAGTGCTACTGATCCGGTTGCTGTTGTTGCTTTGCTAAAGGAGCTTGGTGCTAGT

B.o.SOS1        AAGAAGATAAGCACCGTGATTGAAGGGGAATCTTTGATGAACGACGGGACGGCAATTGTG
T.a.SOS1        AAGAAGATAAGCACCGTGATTGAAGGGGAATCTTTGATGAACGACGGGACGGCAATTGTG
C.h.SOS1        AAGAAGATAAGCACCGTGATTGAAGGGGAATCTTTGATGAACGACGGGACGGCAATTGTG
C.d.SOS1        ------------------------------------------------------------.
T.b.SOS1        AAGAAGCTAAGCACAGTCATTGAAGGGGAATCCCTGATGAATGATGGGACGGCTATTGTG
A.t.SOS1        AAGAAGCTAAGCACCATAATTGAAGGGGAATCCCTGATGAATGATGGGACGGCGATTGTT

B.o.SOS1        GTTTTCCGGCTATTTTTAAAGATGGTTTTAGGACACAGTTTTGGCTGGGGTTCTATAATC
T.a.SOS1        GTTTTCCAGCTATTTTTAAAGATGGTTTTAGGACACAGTTTTGGCTGGGGTTCTATAATC
C.h.SOS1        GTTTTCCAGCTATTTTTAAAGATGGTTTTAGGACACAGTTTTGGCTGGGGTTCTATAATC
C.d.SOS1        ------------------------------------------------------------.
T.b.SOS1        GTTTTCCAGTTATTCTTAAAGATGGTTATGGGTCATAGTTCTGGCTGGTCTTCTATAATC
A.t.SOS1        GTTTTCCAGTTATTCTTAAAGATGGCTATGGGGCAAAACTCTGACTGGAGTTCTATAATC

B.o.SOS1        ATATTTCTTGTTAGAGTCGCACTTGGAGCTGTAGGCATCGGTATGGCTTTTGGCATTGTC
T.a.SOS1        ATATTTCTTGTTAGAGTCGCACTTGGAGCTGTAGGCATCGGTATGGCTTTTGGCATTGTC
C.h.SOS1        ATATTTCTTGTTAGAGTCGCACTTGGAGCTGTAGGCATCGGTCTGGCTTTTGGCATTGTC
C.d.SOS1        ------------------------------------------------------------.
T.b.SOS1        ACATTTCTGATTAGAGTCGCACTTGGAGCTGTTGGCATTGGTATCGCTTTTGGCATTGCC
A.t.SOS1        AAATTTCTGCTTAAAGTCGCACTTGGAGCTGTAGGCATTGGTCTGGCGTTTGGCATTGCA

B.o.SOS1        TCAGTTCTTTGGCTCAGGTTCATACTTAATGACACAGTGATAGAGATTACTCTTACAATT
T.a.SOS1        TCAGTTCTTTGGCTCAGGTTCATACTTAATGACCCAGTGATAGAGATTACTCTTACAATT
C.h.SOS1        TCAGTTCTTTGGCTCAGGTTCATATTTAATGACACAGTGATAGAGATTACCCTTACAATT
C.d.SOS1        ------------------------------------------------------------.
T.b.SOS1        TCGGTTCTTTGGCTCAAGTTCATATTCAACGACACAGTAATTGAGATTACTCTTACGATT
A.t.SOS1        TCAGTTATTTGGCTCAAGTTCATATTCAATGACACTGTAATAGAGATTACTCTTACAATT

B.o.SOS1        GCAGTGAGCTACTTCGCATATTACACTGCTCAAGAGTGGGCTGAGGCTTCTGGTGTTTTA
T.a.SOS1        GCAGTGAGCTACTTCGCATATTACACTGCTCAAGAGTGGGCTGAGGCTTCTGGTGTTTTA
C.h.SOS1        GCAGTGAGCTACTTCGCATATTACACTGCTCAAGAGTGGGCTGAGGCTTCTGGTGTTTTA
C.d.SOS1        ------------------------------------------------------------.
T.b.SOS1        GCAGTGAGCTACTTCGCATATTACACTGCTCAAGAGTGGGCTGGGGCTTCTGGTGTTTTG
A.t.SOS1        GCAGTGAGCTATTTCGCATACTACACTGCTCAAGAGTGGGCTGGGGCTTCTGGTGTTTTG

B.o.SOS1        ACAGTGATGACTTTGGGCATGTTTTATGCTGCCCTTGCAAGGACAGCATTTAAAGGTGAC
T.a.SOS1        ACAGTGATGACTTTGGGCATGTTTTATGCTGCCCTTGCAAGGACAGCATTTAAAGGTGAC
C.h.SOS1        ACAGTGATGACGTTGGGCATGTTTTATGCTGCCCTTGCAAGGACAGCATTTAAAGGTGAC
C.d.SOS1        ----------------------TTTTATGCTGCCCTTGCAAGGACAGCATTTAAAGGTGAC
T.b.SOS1        ACGGTGATGACTTTGGGCATGTTTTATGCTGCATTTGCAAGAACAGCATTTAAAGGGGAC
A.t.SOS1        ACGGTCATGACTTTGGGCATGTTTTATGCTGCATTTGCAAGGACAGCCTTTAAAGGTGAC

B.o.SOS1        AGCCAAAGAAGTTTGCATCACTTCTGGGAAATGGTCGCCTATATTGCCAATACTTTGATT
T.a.SOS1        AGCCAAAGAAGTTTGCATCACTTCTGGGAAATGGTCGCCTATATTGCCAACACTTTGATT
C.h.SOS1        AGCCAAAAAAGTTTGCATCACTTCTGGGAAATGGTCGCCTATATTGCCAATACTTTGATT
C.d.SOS1        AGCCAAAAAAGTTTGCATCACTTCTGGGAAATGGTCGCCTATATTGCCAATACTTTGATT
T.b.SOS1        AGTCAAAGAAGTTTGCATCACTTCTGGGAAATGGTCGCATATATTGCAAATACTTTGATT
A.t.SOS1        AGTCAAAAAAGCTTGCATCACTTCTGGGAAATGGTTGCATATATTGCAAACACTTTGATA

B.o.SOS1        TTTATCCTCAGTGGTGTTGCCATAGCTGAAGGCATTCTCGACAGCGATAGGATTGCCTAC
T.a.SOS1        TTTATCCTCAGTGGTGTTGCCATAGCTGAAGGCATTCTCGACAGCGATAGGATTGCCTAC
C.h.SOS1        TTTATCCTCAGTGGTGTTGTCATAGCTGAAGGCATTCTCGACAGCGATAAGATTGCCTAC
C.d.SOS1        TTTATCCTCAGTGGTGTTGCCATAGCTGAAGGCATTCTCGACAGCGATAGGATTGCCTAC
T.b.SOS1        TTTATCCTCAGTGGTGTTGTCATTGCTGAAGGCATTCTCGACAGCGATAAGATTGCGTAC
A.t.SOS1        TTTATCCTCAGTGGTGTTGTCATTGCTGAAGGCATTCTCGACAGTGATAAGATTGCCTAC

B.o.SOS1        CAA-------------------------------------------------------
T.a.SOS1        CAAGGGAGTTCAT---------------------------------------------
C.h.SOS1        CAAGGGAATTCATGGGCATTTCTCTTTCTACTATATCTTTATATTCAACTGTCACGTTGT
C.d.SOS1        CAAGGGAGTTCATGGGGATTTCTCTTTCTACTATATCTTTATATTCAACTGTCACGTTGT
T.b.SOS1        CAAGG-----------------------------------------------------
A.t.SOS1        CAAGGGAATTCATGGCGATTTCTTTTTCTGCTATACGTTTACATCCAACTATCGCGTGTT
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B.o.SOS1        ------------------------------------------------------------
T.a.SOS1        ------------------------------------------------------------
C.h.SOS1        GTTGTTGTTGGAGTTCTATATCCATTTTTATGCCGTGTTGGCTATGGTTTGGATTGGAGA
C.d.SOS1        GTTGTTGTCGGAGTTCTATATCCATTTTTATGCCGTGTTGGCTATGGTTTGGATTGGAGA
T.b.SOS1        ------------------------------------------------------------
A.t.SOS1        GTTGTTGTTGGAGTTCTATATCCACTTTTATGTCGTTTTGGCTATGGTTTGGATTGGAAA

B.o.SOS1        ------------------------------------------------------------
T.a.SOS1        ------------------------------------------------------------
C.h.SOS1        GAAGCCATTATACTTGTATGGTCTGGTTTGAGGGGTGCAGTGGCGCTCTCGCTTTCTTTA
C.d.SOS1        GAAGCCATTATACTTGTATGGTCTGGTTTGAGGGGTGCAGTGGCGTTCTCGCTTTCTTTA
T.b.SOS1        ------------------------------------------------------------
A.t.SOS1        GAATCCATTATACTCGTATGGTCTGGTTTGAGGGGCGCAGTGGCTCTTGCACTTTCTTTA

B.o.SOS1        ------------------------------------------------------------
T.a.SOS1        ------------------------------------------------------------
C.h.SOS1        TCTGTGAAGCAATCAAGCGGAAATTCATTTCTCAGCACTGAGACTGGAACATTGTTTATT
C.d.SOS1        TCTGTGAAGCAATCAAGCGGAAATTCATTTCTCAGCACTGAGACGG--------------
T.b.SOS1        ------------------------------------------------------------
A.t.SOS1        TCCGTGAAGCAATCAAGCGGAAATTCACATATCAGCAAGGAGACTGGAACATTGTTTCTT
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Supplementary Sequence Alignment S3.2 Sequence alignments of *B.o.SOS1, T.a.SOS1, C.h.SOS1, C.d.SOS1, T.b.SOS1* and *A.t.SOS1* on nucleotides basis.

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B.o.VATD        ------GCTCTCCTCAAGAAGAAGAGCGATGCCTTAACTGTTCAGTTCAGAGCCCTTCTC
T.a.VATD        ---------CTCCTCAAGAAGAAGAGTGATGCGTTAACTGTTCAGTTCAGGGCACTTCTC
C.h.VATD        ---CATGCTCTGCTCAAGAAGAAGAGTGATGCTTTAACAGTTCAGTTCAGGGCTCTTCTC
C.d.VATD        ---CATGCTCTGCTCAAGAAGAAGAGTGATGCTTTAACAGTTCAATTCAGGGCTCTTCTC
T.b.VATD        GGCCATGCTCTTCTCAAGAAGAAGAGTGATGCATTAACTGTTCAGTTCAGGGCACTTCTC
A.t.VATD        ---CATGCTCTCCTCAAGAAAAAGAGTGATGCTTTAACTGTTCAGTTTAGGGCACTTCTC

B.o.VATD        AAGGAGTTCGTTACGGCCAAGGAATCAATGGGAGACATGATGAAGACATCGTCTTTTGCT
T.a.VATD        AAGAAAATCGTTGAAGCTAAGGAATCCATGGGAGACATGATGAAGACTTCGTCTTTTGCT
C.h.VATD        AAGAAGATCGTTGTAGCGAAAGAGTCCATGGGAGATATGATGAAGACATCGTCTTTCGCT
C.d.VATD        AAGAAGATCGTTGTAGCGAAAGAGTCCATGGGAGATATGATGAAGACATCGTCTTTCGCT
T.b.VATD        AAGAAAATCGTTACCGCTAAGGAATCCATGGGAGATATGATGGAGACATCGTCTTTTGCT
A.t.VATD        AAGAAAATCGTTACAGCTAAGGAGTCTATGGGAGATATGATGAAGACATCGTCTTTTGCT

B.o.VATD        CTTACCGAAGTCAAGTACGTGGCTGGTGAGAATGTTAAGCATGTAGTTCTCGAGAACGTT
T.a.VATD        CTCACCGAAGTCAAGTACGTTGCTGGTGAGAATGTCAAACACGTTGTCCTCGAGAACGTT
C.h.VATD        CTTACGGAAGTCAAGTACGTAGCTGGCGATAGCGTCAAGCACGTCGTGCTGGAGAACGTT
C.d.VATD        CTTACGGAAGTCAAGTACGTAGCTGGCGATAACGTCAAGCACGTCGTGCTGGAGAACGTT
T.b.VATD        CTTACCGAAGTAAAGTATGTCGCCGGTGAGAATGTCAAACATGTTGTCCTCGAGAACGTT
A.t.VATD        CTTACCGAAGTAAAGTATGTTGCTGGTGACAATGTCAAACATGTTGTCCTCGAGAACGTT

B.o.VATD        GAAGAAGCTACGCTGAAAGTTCGTTCAAGGCAAGAGAACATCGCTGGTGTGAAGCTTCCA
T.a.VATD        AAAGAAGCTACGCTGAAGGTTCGTTCCAGGACAGAGAACATTGCCGGTGTGAAGCTTCCC
C.h.VATD        AAAGAAGCTACTCTGAAAGTTCGTTCGAGGACAGAGAACATAGCTGGTGTGAAGCTACCA
C.d.VATD        AAAGAAGCTACTCTGAAAGTTCGTTCGAGGACAGAGAACATAGCTGGTGTGAAGCTACCA
T.b.VATD        AAAGAAGCTACACTGAAAGTTCGTTCCAGGCAAGAGAACATCGCAGGAGTGAAGCTTCCC
A.t.VATD        AAAGAAGCTACTTTGAAGGTTCGTTCTCGGACAGAGAATATCGCTGGAGTGAAGCTGCCT

B.o.VATD        AAGTTTGATCATTTCTCTGAAGGCGAGACCAAGAACGACTTAACCGGTTTAGCTAGAGGT
T.a.VATD        AAGTTTGATCACTTCTCTGAAGGCGAGACCAAGAATGACTTAACCGGTTTGGCTAGAGGT
C.h.VATD        AAGTTTGATCATTTCTCTGAAGGCGAGACCAAGAACGATTTGACGGGTTTAGCTAGAGGT
C.d.VATD        AAGTTTGATCATTTCTCTGAAGGCGAGACCAAGAACGATTTGACCGGTTTAGCTAGAGGT
T.b.VATD        AAGTTTGATCACTTCTCTGAAGGCGAGACCAAGAATGACTTAACCGGTTTAGCTAGAGGT
A.t.VATD        AAGTTTGATCACTTCTCTGAAGGTGAGACCAAGAATGACTTGACCGGTTTAGCTAGAGGT

B.o.VATD        GGGCAACAGGTCCAAGCTTGCCGTGTGGCTTATGTGAAAGCCATTGAGGTTCTGGTTGAG
T.a.VATD        GGTCAACAGGTCCAAGCTTGCCGTGTGGCTTATGTGAAAGCCATTCAAGTCCTGGTGGAG
C.h.VATD        GGTCAACAAGTACAAGCTTGCCGTGTTGCTTATGTGAAAGTTATCGAAGTTTTAGTCGAG
C.d.VATD        GGTCAACAAGTACAAGCTTGCCGTGTTGCTTATGTGAAAGTTATCGAAGTTTTAGTCGAG
T.b.VATD        GGTCAACAGGTCCAAGCTTGCCGTGTGGCTTATGTGAAAGCCATTGAAGTCCTAGTTGAG
A.t.VATD        GGTCAACAGGTCCGAGCTTGCCGTGTTGCTTATGTGAAAGCCATTGAAGTTCTAGTTGAG

B.o.VATD        CTTGCTTCTCTCCAGACTTCGTTCTTGACGCTTGACGAAGCAGTCAAGACGACTAACCGC
T.a.VATD        CTTGCTTCCCTCCAGACCTCGTTCTTGACGCTTGATGAAGCAATCAAGACAACCAATCGC
C.h.VATD        CTTGCTTCTCTTCAGACATCCTTCTTGACGCTCGACGAAGCTATAAAGACGACTAATCGC
C.d.VATD        CTTGCTTCTCTTCAGACATCCTTCTTGACGCTCGACGAAGCTATCAAGACGACTAATCGC
T.b.VATD        CTTGCTTCTCTCCAGACTTCGTTCTTGACGCTTGATGAAGCAATCAAGACGACCAATCGG
A.t.VATD        CTTGCTTCTCTCCAGACTTCTTTCTTGACCCTTGATGAAGCAATCAAGACGACTAACCGT
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B.o.VATD          AGGGTCAACGCTCTGGAGAATGTGGTGAAACCCAAGATTGAGAATACGATCAGTTAC---
T.a.VATD          AGGGTCAACGCTCTGGAGAACGTGGTGAAACCAAAGATTGAGAACACAATCAGCTACATC
C.h.VATD          AGGGTCAACGCTTTGGAGAATGTTGTGAAACCGAAGATTGAGAACACGATTAGTTACATC
C.d.VATD          AGGGTCAACGCTTTGGAGAATGTTGTGAAACCGAAGATTGAGAACACGATTAGTTACATC
T.b.VATD          AGGGTAAACGCTCTGGAGAATGTTGTGAAACCGAAGCTGGAGAATACTATCAGTTACATC
A.t.VATD          AGGGTCAACGCTCTGGAGAATGTGGTGAAACCAAAGCTGGAGAATACAATCAGTTACATC

B.o.VATD          ----------------
T.a.VATD          AAAGGAGAGCTT----
C.h.VATD          AAGGGAGAGCTTGATG
C.d.VATD          AAGGGAGAGCTTGATG
T.b.VATD          AAGGGAGAGCT-----
A.t.VATD          AAGGGAGAGCTTGATG
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Supplementary Sequence Alignment S3.3 Sequence alignment of *B.o.VATD, T.a.VATD, C.h.VATD, C.d.VATD, T.b.VATD* and *A.t.VATD* on nucleotides basis.

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A.t.NHX1prom      ------TAATAATAAAATAACAACATTACAAAATACCAAAATATGTAGGGTAATAGTTTT
C.h.NHX1prom      GTCGGTTTAACTAAGTCGGTCAAGGTAAATATCTACCGTATCCGCTCCGTGACTAGACGG

A.t.NHX1prom      TGCTAATATAGTTATTATATTATTACTAAAATATAAATTCACATGTTAATATTTGTTGTT
C.h.NHX1prom      ATATCCGTTAAAATCCAAATATTCGCCGGATATCCGCTCCGCCCCGTAAT-TTAAAAAAA

A.t.NHX1prom      GACAAAAACAAAAACAATGATATTACAAAAAAACAAATAGGGTGATTAAACGCTAGAAGA
C.h.NHX1prom      AATTGTAAAAATTTCAAACTTAATCTAAAATATCAATATTTACAAAAACTTTATAAATTA

A.t.NHX1prom      TCCGTAACCATTTTGATGACAAAAAGATATGGTACTAAGATGAACACGTTTTTGAGAATA
C.h.NHX1prom      TTGTTGAATAAAAATAAATAAAATCTATAGGAGATTAACTAAATAAATTATTTAATTGAA

A.t.NHX1prom      TTCAAAACAAATTCATTTCCGAAAGATTATCAATTTTCAAGCATACAGTATG-ATCTGGT
C.h.NHX1prom      TTTGAAAGAAATGCAAAACTATACCTAAGTTGTTGTGTTATCCAATTGTTTAGTTCATAT

A.t.NHX1prom      AAACTATAAATGGTAGAACCACGATAATTAACTAGTCGATTCTATATGTTATGACATAGA
C.h.NHX1prom      TTCTTTTAAATTATTTATTAAATGTTGGTATTATTTAATTTTATAATATTTT--CTTTTA

A.t.NHX1prom      CTTAGCTAGGAGCATATCCGGCAGACCGGCACTAATCTAATGATTTCATGAGTTGTTATT
C.h.NHX1prom      ATTGTTTTG--GTAAAATCATTTAAATAAAAAGATTATTTTACTTCATAATACTTATATA

A.t.NHX1prom      ATCTAAACTAATTAACAATTAATAGAATGAAATATAGTTTTATATTTAAGTTTTTTTTTT
C.h.NHX1prom      TTGTTTATTTGTTTGTGATTTATG-AATTCTTTCTAATTTACTCATTTAATAATTATTTT

A.t.NHX1prom      TTTTTTTTTTTTTTTCGGAATATCAGGAGGTTCTGGGCCGAAACCCGTAATCCCTTCAGG
C.h.NHX1prom      CTACAAATAGATATAAGAATTATTAGATTTATATACGTAATTACCAATATAAAATAAACA

A.t.NHX1prom      CCTGAAACCACAACATG--TAATATTAGTTTCGTCCCAAACAT-CACTCGAACTAACGAC
C.h.NHX1prom      TATTTATATGTAACAGAGCGGATATCCGTTTTTTAGAATTTTAGTATTTGTTATTTGCTC

A.t.NHX1prom      CTCTAAGTTTCGCCTAGGATCTTTACCAATTGAGGTAACAACGCTTGGTCAAGTTTAAAT
C.h.NHX1prom      CGCCTTTAACGGATATTGATTTTTAATATTTGTT-TTGCTTTGTAAGATAACGGTTATCC

A.t.NHX1prom      CATTGTTGCT-TTCTGTGAGATTTTGATCCCTATAACCTAATTTATCTTATGAGAAATTA
C.h.NHX1prom      GGATTTTTCAGATCAAATCGAGACGAATCACGG-ATCGGATCGAATCTAACGGATATTTT

A.t.NHX1prom      ACCTTTTTTGATAAAGGATAAGGAAAATAGTAACCTTGTATAAAAAAATTCATTGCCATT
C.h.NHX1prom      GCCCAG--CCCTAATAGAAATGTTCAAAATGAACCAATTTTCTAAACTTTAAAAGCAGGA

A.t.NHX1prom      TTCATATATATATATATATATATATATATCCTAAAATGTATATCTCTAATAAAATTAG
C.h.NHX1prom      ATAAAATAAAAG------------TATAAAACGGATGAAAAAATAATTAAACAAAAAAAA

A.t.NHX1prom      AAGAAATGATACCAAAAATAAAGGTGAAATCATGATAAAATTTTGTTTTTTTTTGTTTTG
C.h.NHX1prom      ACTAATATAAACCCCAAAACACGTTG--ACCAAAAAAAAAGTGAGAATTATAAAACTATA

A.t.NHX1prom      GTTCATTGAGTTTGCCATTAACGCTTTTTTTTATAAATAATTTCGTCTACACAAATTCA
C.h.NHX1prom      CT--ATTTGAATTACATTTCACAAAACTACATTATTTCCAAAATACTTCACAAAACTACA

A.t.NHX1prom      TCCATATAATATTTTCTTTTACTGCATCAGAGTTTCACTATGTATTTAGGTTTTTTATGA
C.h.NHX1prom      TCTAAATACTCAAAATTAAATCCAAATAAACCCTATTCAAACAATCTTATCCTCTTAATA

A.t.NHX1prom      ATTTTAAAAAAGTTCAGCATCAAGGTGAAGAAAATTCCAAAAAAATAAATAAAACGGTGA
C.h.NHX1prom      CTATACCCTAAATACTAATTAGCG--AACTCAAATGAAAAGATTTTAATTTTGGTAATTT

A.t.NHX1prom      CCCAAAAAAAACTTGTTGTCTTCAAAACCAAAAGTACTCGACAAATCAAATAGATAAATC
C.h.NHX1prom      TTAAAAGGATAAATCAAGCTTTTAAAATTTTAAAAGGGTGTAAACTTGTGAAAATATTGA
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A.t.NHX1prom      ATGAATCTTAGTACAGTCAGGTTTTATCGTCTACGATAAGTTCCACAATAAATCCAAACA
C.h.NHX1prom      ATTAAATGTAGTTTTGTGAAGGAAGGTCG-------AAAGGTTGTGAATTTCTCAAAAAA

A.t.NHX1prom      CTAAACTGTTCAGATCTCAGATAGTAACTTTAAATCAACGGCTATAAATTCGCCATTTGT
C.h.NHX1prom      AAAAACAAACCAAAACACAGCCG-----CTTTGATCAGAGAAATTAATTTAAATGGACG-

A.t.NHX1prom      ACTTTCATTTACACAACAGAGAATAATACATACACTCTATCTCTCTCCTATTCGTAACTT
C.h.NHX1prom      GCTGAGATTTGGCCAACAGAGAATAATACT----CTCTCTCTACTTTCTATTT-TGGCTC

A.t.NHX1prom      ATATTTCTCTCTCTACTGTGTTT--------AGAAATTGGAATCTTCTCTCTTCTCTATC
C.h.NHX1prom      TTTTCTCTCTCTCTACTGTGTAAGAATTACAAATAACTAAATAAGAATCATATTCCTCTC

A.t.NHX1prom      TCCCTCTCTCTTAAAAAGGGACCGTACACGTCTCTCTCTATTTCCAGTAAAAAATCGAAA
C.h.NHX1prom      TTCCTCTCCTATATAAAGG-ACCGTACACGTCTCTCTCGCTTAATTTTCACTTCTGTGAT

A.t.NHX1prom      TTTCGTATAATTTCCTCAGTCCCGTAATTTTCTCCTTTTTTTTCTTCCCCAATTCCTTCA
C.h.NHX1prom      TTTATTTTCTAG----CGATTCCTTCGTTTTCTCGCTCTCTCTCTCTCTC---TCTCTCT

A.t.NHX1prom      ATTTTCGAATTCGCCTCTCTGTTTCGTTCCTCGTAGA-CGAAGAAGAAGAAGAATCTCAG
C.h.NHX1prom      CGTTGTGAACTTCCACCTCCGTTCTGGTCTTCGAATCGCGTCTATGTTCCATTGTTCCTC

A.t.NHX1prom      GTTTTAGCTTTCGAAGCTTCCAAAATTTTGAATTTTGATCTTCTGGGCTCTTTTGTAAAT
C.h.NHX1prom      CTCTT--CTTTC-ACGAATCGCGTTCGTTGAAGAT--ATCTCCTGCGTGCGTTCAGCTTC

A.t.NHX1prom      CAGACTGAAGATATTTAGATTACCCAGAAGTTGTTCAAGGTAAAGCAATTCAGATCAGAT
C.h.NHX1prom      TAAGCT-TCGAAAATTTGAATACACGAACTCTGTATTGATATATGTTGTGGTGATCTTCG

A.t.NHX1prom      CCACCAATTTTTTTTTTTT--TTTCATTTGAAGTTTTTGTGTTTGTTGTTTGTTGTTGG
C.h.NHX1prom      CCTTTTTTGTTCATCATATAGAATCCGCTTCATGTAAGCGATTCAGAT-CTCACCGTTTT

A.t.NHX1prom      GAGATATTTGAAATCTGGGTTATTAAAGTTCCGACCTAATTTCATATTGTGCTATTACCA
C.h.NHX1prom      TGTAGCTTCGTTTGATTTTTTTTTTTTTTACTGTCATGCTT--AAACTGGGATATTACCA

A.t.NHX1prom      AAAATAGGAAATTTTGAGAATTTG-GTTTTATGTTGTCATCATTTAATAATATTAG----
C.h.NHX1prom      AAAATGGTGAATCTAGAGAATTTTTGTTGGATATTGT-ATCATTTTAGAATATATGGAAG

A.t.NHX1prom      -CCTTTTGTGGGATT---TGTGCTTTTTGTTGTTTGTATCAAAAGTCTCAGATTTGGATT
C.h.NHX1prom      ACATAATATATTTTTGATTAGCCTTTTTGGTTTTTGAGTTTTATTATTCTACTTTAGTAT

A.t.NHX1prom      CACTTTTTGTGTGTTTTTTATAGTCAAAG--CTGATTCATGCTAT--ACATTACACAGCT
C.h.NHX1prom      AGAGTTTCAAG-GTTTATTAGATTCATTTTTCTAAAACCCTTTTTGAGTTTTTAATAGCT

A.t.NHX1prom      TCACTGTTTAGTCTCAGCTCTGTT-TTCTTGAAAACAGGAATGGTTTCAGTGGACAGCAC
C.h.NHX1prom      ACTTGAAGCTGATTCATAAATGTTGCTCTTAGTCTCAGGAACAGTTTCATTGGACAGCAC

A.t.NHX1prom      GGAAAGATAAAAGAGACTTTTTTTTCCAGATTTTGCTGATCCAAAATCTG-AATAGTTGT
C.h.NHX1prom      CGAAGGATAAGAGGTTCCCCCCCGAGCTGCTGATCCAAATCTTGATGTTGTAGTTGTTGT

A.t.NHX1prom      TCATGTTCTTGGATCAAATCTG-GAAAGAG-GAAGTTTGTTG-------------GATCT
C.h.NHX1prom      TGATGTTTCGGTAGCTTTTTTTCGTGTGTGTGTACTTTGATATCGGTTTGAAAGTAATCT

A.t.NHX1prom      AGAAGAAGATAACA-------------
C.h.NHX1prom      GAAAGGGGAAACCAGGGGGTATAGAAG
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Supplementary Sequence Alignment S3.4 Sequence alignment of *A.t.NHX1* promoter (2122 bp) and *C.h.NHX1* promoter (2122 bp) upstream from ATG. Both promoter have 42 percent identity among themself.

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A.t.SOS1prom      GCATTTCATTAGGATCGACGGTTGGTCGATTAATCAATACTAACCCAAACAGTCTTTTAA
C.h.SOS1prom      -------CTTAATAAAATATATATGCAACTTAATAATTAATCTGCAAGGTTCAAAATCAA

A.t.SOS1prom      CATGCACACTTGATACCTTGTAGGTGCTTCTGGGTAGCATGAAAATGATAATTGGTGTTA
C.h.SOS1prom      ATCTCAAATATATGAAATGGAAGTGAACTCA----AACCCTAAATCGAACCATATATCGA

A.t.SOS1prom      TTCTGATATGCGAAATTGAAAATGTTGTTGTGCATCAGCTAAGCATAATTGAAAGTTTAA
C.h.SOS1prom      ACCCTATATCAAACCATAAATTTGCAGACCAAAAAAGACGAATCGAAAATAAATCAAAGA

A.t.SOS1prom      AAAATATTATAAATTTAACGTGAAGAATTGAAAGGTATCAGATTAATGTTTTGGATCAGC
C.h.SOS1prom      GAAATCGAAGACTATTTGTAGAGAAAATGGAGGGATTACCAAAGAGAAATCGAAGACGAC

A.t.SOS1prom      CGAATAGAGTCAGGCTAATCCAAATTCTCAAATATTTTCGAAACCTTAAACACATCAAAC
C.h.SOS1prom      GACGTCGAGGAAGAGAAGACGACGGCGACCCGAGGAAGAGAAGACGAAGGCGAGTTGAGG
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A.t.SOS1prom      TCCACGAAGTAAACACACTCACACACATATCAAAATCCACATTATAAATGTATTTTTGGT
C.h.SOS1prom      -AAGAGAAGATGACGGCCACCCGAGAAAGAAAAGACGACGACGAGACGAGGAAGAAAAGA

A.t.SOS1prom      AGGCTAGTCGTCTAATTCTAAAATAG--CCAATTTACATT-TGCAATTGTTTAT--TCAA
C.h.SOS1prom      CGACGACGAGACGAGGAAGAGAAGTCGACGAAGAGACGAGGTAGAGATGACGATGGCCAC

A.t.SOS1prom      AAAAGTACAACAGTAAGAGGTCTTATCAATTAAATCATAAGAAAAATATGTCCCAAAATG
C.h.SOS1prom      CCGAGGAAGAGATGACGACGGCGAGCCGAGGAAGAGATGACGACGGCGAG-CCGAGGAAG

A.t.SOS1prom      TCAAAAAACCAACTACGAATATTTCTTTTCCTTTTAAG-GCCAAACCAGGTTTTGGAATA
C.h.SOS1prom      AGAAGACGACGGCGGAAGTTAAATCGTCGGGAGAGAAGAGAGAAAGTGGGGCTAGGGTTT

A.t.SOS1prom      CTTATTACAAATAAAC---TCAATTTCAATAAATTCTTGTACAC-----AAATTAAGCAC
C.h.SOS1prom      GTTTGTGTAAATATAAGGGTTAGGTTTGTAAAGTTTTTGTAAACTTTGCACAATTGGGGA

A.t.SOS1prom      TAATTTCATTGTGAAGATACCATAGTCACATTCACATCTATATGACATCAATCTATAAAA
C.h.SOS1prom      AAATTTCAAATATCAATTTCATGAATAACTTGTTTGAAAAAAAATCATGGTGGCATTTTC

A.t.SOS1prom      TCCAAAACAAACTATATATTATATTTATATTATTTTTTTGTCATCATTATATTTCCATTA
C.h.SOS1prom      AAAAATAGTAACTCCAAATTGGGTTAAAAGGATTTTTCCTTTAAATGTTGGTCACTTTAG

A.t.SOS1prom      ATTTATCT-AATTCT--GGTTCTAATATACTCTTGGTCAGAAAAATATAAACATTGAAG
C.h.SOS1prom      TAAGCTCTTCATTCTTGGGTTATGTTAGTCAATATGACCTGATTATGGGTCACTTTAGTG

A.t.SOS1prom      AATTGGTCGGCTGAAAATTGTGAAAAATATATAGCAGAAAA-ATATGATAATGT-TATCA
C.h.SOS1prom      AATT--TTTACTATAAAATAAGACTTGTTTCTAACCAATTGGACTTAGTCTTGTGACTAA

A.t.SOS1prom      TAAACAAAATTAATAGTAAAATTTAATTTTAATTTAACACTACAGTACTATACACGTGTG
C.h.SOS1prom      TTTTCCTATTTAGAGATGAATAACCAAAAATAAAAATCCCTACATGATCATAATCATCAT

A.t.SOS1prom      TATGTATAGCTCTATAAGTATTTACTCTCTTTCAGCTATTTATTTTTTCAGTGAACGAGC
C.h.SOS1prom      CTCACAACGATTGGGATCCAATGATGATATTATAATAAATACATATCTTTTTGTTACATA

A.t.SOS1prom      ATTCTTCTTCTTCCTCTGTGTTGTTGCTTCTTAGATATATTCAAATAAA
C.h.SOS1prom      ATTATTATATTATAATATAGTTGTTTATAAATAGCTGTATACTTT----
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Supplementary Sequence Alignment S3.5 Sequence alignment of *A.t.SOS1* promoter (990 bp) and *C.h.SOS1* promoter (990 bp) upstream from ATG. Both promoter have 35 percent identity among themself.

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A.t.VATDprom      -------------------GTCATACAATATAGTGTTGAATGAGAGATTATTTTCTCCTC
C.h.VATDprom      ATACTGGAACGTGGCTAAGGCACTACGAGACAGTGGAGGAA-AGGGATAAGTTAGGCCAA

A.t.VATDprom      AACCATTTGGAAA-CTCTATACAACAAGATTTCATCCCTGAAACTTGGAAATGAAAAAAT
C.h.VATDprom      TTCAATATTGATTGCATCATGAAGGCTTTAGACAAATCTGCACTTTAGTTTCAAATCCTA

A.t.VATDprom      GTATTTGGCCGAGTTTTGTTTTTGTCCTAAGCACTAAATAAGAACGTGAGATATATAGTT
C.h.VATDprom      CTAATTATATGATGTAATATATACATATATATGTTATATTATAATGTTTTTTTTAAACTT

A.t.VATDprom      CACCGCGTCTAAAGACGAAAGTGATGGGTCTCAGATTTAGGACACGAAAGTAGGCCCCAC
C.h.VATDprom      CAACATATATTTATGTATATGCGTTATATTCAAGTTTTT----TCTTTTGTTGTTTATGT

A.t.VATDprom      CATACGTGATGAGCTGTATACGTATTTATTTTATTTGTAAAAA---ATAAGCCCTTTATA
C.h.VATDprom      AATATAT-ATATAAGTTATGCTTTTGTGAGTAACAAATAAAAATCTTTGAACCATATCAT

A.t.VATDprom      TTTACGGTTAATTACACAA-TTAGCCCATTTCTTTCTTCTCCACGATCACAGCGACGAGG
C.h.VATDprom      ATGGCATATGTTGTTTCTTGTTTATGTAAAACGGACTAAAACAAGAACATGGAGCCCACA

A.t.VATDprom      TCGAAT--ACGATCTTCTT-ACCCCTGTCGACAGTTCTGAATTAATATCAATTTTCATTG
C.h.VATDprom      TAGTGTGTTCACACAAAGTGGACCCTACCATAAACGATGAG---CTGTCCCTATTTTGTA

A.t.VATDprom      TCTTCCTATCTCTCTTTCTCTCTTTGGCTTCTTCCAATCTAATTTCAGTGATTTACTTGT
C.h.VATDprom      GCAAACCAAGTC-CTTTATGTTTATGGTTAATTACAAAACAACCCCTTTTCTTTCCTCCA

A.t.VATDprom      TAAGAATTCACCTTTTGAGAGGC-CCAGGTATGTCAATCCCAAATCTCACTCTTTTCTTC
C.h.VATDprom      CAATCAACGACAAGTTCGGATACGATCAAAATCTCAAATTTATTTCTCCAAGGTCTCTTC

A.t.VATDprom      TCTTAGTTTGGTTTGAAACAATTGAATC----TGGTGGGAAATTTAGGCGTGTATCCATA
C.h.VATDprom      TCTCTGTTTCTATTACAGAGATTCACTCGCAGAGAAGTAACCTCTTATTGTCTCTCGATA

A.t.VATDprom      TAACCTG-------ACTGCTCAATTCTGAGTCCAATTGATTAGGTGTGTTCTG-----TG
C.h.VATDprom      ATCCCCAGGTAAATTCGTCTCAGATCTTACTCTTACCGTCTCGATTCTCTCTGGTAAACA

A.t.VATDprom      ATCTAGATTTCATCTTCTGGGAAACGATCTATCCTAAAGTCGTAGACTTTGATTCAATTA
C.h.VATDprom      ATCGAATCTGAGAAATTTACCGATCTCGCTGTTCTACGATCTTGATTTCGTCTTCTGTTA
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A.t.VATDprom      G--AAAATTCGTAGACTTTGATTCAATCTGGGAACAAAAAATGGATAATCTGATGAATCT
C.h.VATDprom      ATCTAAAGTCGCAAGCTTT-ATTCAATTTAGTAATCGATAATGTAAGATCTGATGAATCT

A.t.VATDprom      CTGTATGGATCGTTCGAGATAATGATTGGG---TGCTTGGTTCATTAGCCTAAGATTTTC
C.h.VATDprom      CTGTATAGATCGTGTAAGATTATGATATGGGTTTGTTTGGTTGATTAGTCTATAAG----

A.t.VATDprom      CTAGTGAAACTTAAGAATCTTTGCGTGTTTGTTCAATTATAATTAGTAAACCAGAATTTA
C.h.VATDprom      CTTTTGG---TAATGAAACTTTGTTTGATTATTGATTAGTAAACAG------AGTAATTA

A.t.VATDprom      CGAGATTTCCATGTTGATCTCTTTCCTCAATGATGCAAGTATTGTTTCGCATCATCGAAA
C.h.VATDprom      GTAGATTT-----TTTGAAAGTTTAATCTTTGTCGAAA--AACGATTC-AATTATCGTTG

A.t.VATDprom      AATCATCTTCTGTTGTTGGCTTCTGTCAATAGAGTAAGTCTGTTTGTGTCCTTGTTTAAT
C.h.VATDprom      TTGTGTGTTGTTTTAATGGAAGAAAGCAATC---AAAATCTCTTTAT-------------

A.t.VATDprom      CCCATCTCTTTCATTTGCTGTAGTTTTGAAAT-
C.h.VATDprom      --CATTATTATTATGCAGTATTGTTTCTGAATC
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Supplementary Sequence Alignment S3.6 Sequence alignment of *A.t.VATD* promoter (1003 bp) and *C.h.VATD* promoter (1003 bp) upstream from ATG. Both promoter have 48 percent identity among themself.

Chapter 4

Expression of *HKT1* from *Arabidopsis thaliana*, or *HKT1;2* from *Thellugiella halophila* or *T. botschantzevii*, complements the *A. thaliana hkt1* mutant when they are expressed under the endogenous *A. thaliana HKT1* promoter, but not when expressed under the *T. halophila/botschantzevii HKT1;2* promoters

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Abstract

Based on a former study in which we found a higher expression level of *HKT1* in *Thellungiella halophila/botschantzevii*, in comparison with *Arabidopsis thaliana*, we compared the activities of the *HKT1;2* promoters from *T. salsuginea* (=*halophila*, ecotype Shandong; 1822 bp) and *T. botschantzevii* (ecotype Saratov; 1811 bp) with the *HKT1* promoter from *Arabidopsis thaliana* (846 bp), by comparing *HKT1*/*HKT1;2* transcript concentrations in the *A. thaliana hkt1* mutant background. We also assessed NaCl tolerance in the transgenic lines, using *A. thaliana* wild-type and *A.t.hkt1* as controls. Expressing either *HKT1* or *T.s.HKT1;2* under the *A.t.HKT1* promoter more or less completely reversed the salt hypersensitivity of the mutant, whereas expressing either of the genes under the *T.s.HKT1;2* promoter did not. Expressing the genes under the 35S-CMV promoter yielded incomplete complementation. Complementation of the mutant was not consistently associated with significant changes of the Na or K shoot concentrations under salt exposure. When expressed under either of the *Thellungiella* promoters, the levels of gene expression were very low, in fact below detection limit, suggesting that we missed important upstream response elements.

Keywords: Gene expression, HKT1, promoter swapping, Thellungiella

4.1 Introduction

The detrimental effects of high salinity levels on plants are the consequence of

osmotic stress and the toxicity of excess sodium ions (Munns and Tester, 2008). Halophytes are defined as plants that can grow and complete their life cycle at high salinity (> 200 mM NaCl) while the others are known as glycophytes (Flowers and Colmer, 2008). The mechanisms underlying high-level salt tolerance in halophytes are poorly understood, but the trait is often, more or less implicitly, supposed to depend on enhanced capacities for cellular Na and K compartmentalization and homeostasis, or compatible organic osmolyte synthesis, through alterations of the expression patterns of genes encoding Na^+/K^+ transporters or genes involved in the synthesis or breakdown of compatible solutes (Flowers and Colmer, 2008). The Na transporters, SOS1, NHX1 and HKT1 have often been considered to play key roles in salt tolerance in halophytes (Ashraf and Akram, 2009), however, comparisons between their expression patterns in halophytes and glycophytes are, with few exceptions (Kant *et al.,* 2006), not available to date.

In a recent study (chapter 2) we compared *HKT1* expression among halophytic and glycophytic species of Cochleria, and found much higher expression levels in the halophytic species than in the glycophytic, supporting the hypothesis that enhanced *HKT1* expression may be crucial for high-level salt tolerance, indeed. We also found a high expression level of an *HKT1*-like gene in *Thellungiella botschantzevii*, a close relative of the *Arabidopsis thaliana* related salt cress, *T. halophila*, which is thought to be a suitable halophyte model species (Inan *et al*., 2004).

HKT is a gene family which is increasingly studied for its role in long distance Na transport. In 2003 Berthomieu *et al.,* (2003)*,* proposed a model for A.t.HKT1 functioning. According to this model, A.t.HKT1 is involved in the recirculation of Na from the shoot towards the root through loading Na from the phloem companion cells into phloem, thus eliminating Na from the shoot. Another working model was proposed by Sunarpi *et al*., (2005). They found the protein to be localized at the plasma membrane of xylem parenchyma cells and suggested that A.t.HKT1 plays an important role in Na detoxification in plant aerial parts via resorbing Na from xylem vessels into xylem parenchyma cells (Sunarpi *et al.,* 2005). This was further supported by Davenport *et al.,* (2007), they use radioactive traces ($^{22}Na^+$) flux measurements and ion accumulation assays and showed that *A.t.HKT1* is involved in root accumulation of Na via retrieval of Na from the xylem into parenchyma cells but is not involved in root influx or recirculation in the phloem. More recently Plett *et al*.,

(2010) used enhancer trap lines for transformation in rice and *A. thaliana* and showed that both species over-expressing *A.t.HKT1* in mature root cortex had greater shoot Na exclusion and thus increased salinity tolerance. However, Sunarpi *et al.,* (2005) have not completely excluded a role for A.t.HKT1 in phloem Na loading. They found weak signals of GUS in aerial parts (leaves) and up-regulation of *A.t.HKT1* in the shoot in response to a mild increase of NaCl, with an associated reduction of the Na concentration of the phloem sap. Jacoby (1979) showed that Na translocation to phloem is an important process to maintain low Na contents in shoot of bean, suggesting that Na recirculation could be an important mechanism against salinity tolerance in plant (Hauser and Horie, 2010).

Recently, it became clear that *T. salsuginea* (= *halophila*) has at least two *HKT1* genes, *HKT1;1* and *HKT1;2*. T.s.HKT1;2, however, is a K transporter, whereas T.s.HKT1;1 is a Na transporter, like A.t.HKT1 (Ali *et al*., 2012). Since *T.s.HKT1;1* is barely expressed, in comparison with *T.s.HKT1;2*, it seems that the relatively high expression of *T.s.HKT1*, in comparison with *A.t.HKT1* (see above) is due to *T.s.HKT1;2*, and that the contribution of HKT1 to salt tolerance in *Thellungiella* is associated with the maintenance of a high degree of K selectivity under NaCl exposure, rather than Na resorption from the xylem.

In this study, we compared the activity of four promoters: the *A.t.HKT1prom* (846 bp), the *Thellungiella halophila (=salsuginea)* ecotype Shandong promoter (*T.s.HKT1;2prom*, 1822 bp), the *Thellungiella botschantzevii* ecotype Saratov promoter (*T.b.HKT1;2prom*, 1811 bp) and the 35S-CMV promoter through examining *A.t.HKT1* and *T.s.HKT1;2* gene expression in the *A. thaliana hkt1* mutant background. We also compared the potential of the constructs to reverse the Na hypersensitivity phenotype of the *A.t.hkt1* mutant.

4.2 Materials and Methods

4.2.1 Plant material and experimental conditions

T. salsuginea and *T. botschantzevii* seeds, originating from a coastal area near Shandong, China, and a solontchak soil in Saratov, Russia, were sown in garden soil (Jongkind BV, number 7, Aalsmeer, the Netherlands). Three-weeks old seedlings were transfered to hydroponics (see below) and plants were harvested, snap-frozen

and stored at -80 ºC until DNA and RNA extraction (see below). Seeds of *A. thaliana* (Col) wild-type, *A.t.hkt1* mutants and transgenic lines were surface sterilized in 96% ethanol then in 10% bleach, washed three times with sterilised water, dissolved in 0.1% agarose and sown on 0.8% (w/v) gelrite plates containing 0.5% Murashige and Skoog (MS) salts at pH 5.7-5.9 with 25 µg ml^{-1} hygromycin for transgenic lines, 25 µg ml^{-1} kanamycin for the *A.t.hkt1* mutants and no antibiotic for wild-type on square petri plates and put them vertically. Seeds were germinated at 22 ºC under 10 hr-light/14 hr-dark photoperiod. After two weeks, seedlings were transferred to hydroponics culture in 1-L polyethylene pots (three plants per pot, each plant belonging to different transgenic lines/mutant/wild-type) containing a modified half-strength Hoagland's solution (Schat and Ten Bookum, 1992). Plants were grown in a climate room at a light intensity of 220 µmol m^{-2} s^{-1} at plant level, for 10 h d^{-1}, 20/15 ºC day/night, 75% RH. Nutrient solutions were renewed twice a week. After two weeks in hydroponics, plants were exposed to NaCl (0 and 50 mM), ten plants per treatment. After two weeks of exposure, plants were harvested, shoots and roots fresh weight (grams) were measured.

4.2.2 Tolerance index (T.I.) and water content

The tolerance index (T.I.) was calculated over ten biological replicates of each transgenic line using the formula:

$$\text{T.I.} = \frac{\text{Average fresh weight at 50 mM}}{\text{Average fresh weight at control}}$$

The percentage of water in fresh weight was calculated as:

$$= \frac{(\text{fresh weight} - \text{dry weight})}{\text{fresh weight}} \times 100$$

4.2.3 Determination of Na and K concentrations

Na/K concentrations were determined in roots and shoots (ten plants per population per concentration, two plants were pooled together) by taking 20 mg of dry material in 2 ml eppendorfs. 2 ml water was added in each eppendorf then boiled for one hour at 90 ^{o}C. After cooling they were filtered through a Spin-X® Centrifuge tube Filter

(Coaster, 0.22 µM Nylon). After proper dilution, Na/K were determined on a Flame Atomic Absorption Spectrophotometer (Perkin Elmer AAS100) by flame emission.

4.2.4 RNA and DNA extraction and 1st strand cDNA synthesis

RNA was extracted from frozen shoot tissues using Trizol™ (Invitrogen) following the manufacturer's instructions and as described in Jack *et al.,* (2007). Single-stranded cDNA was synthesized from total RNA (2.5 µg, boiled for 1 min) using 100 Units M-MLV Reverse Transcriptase (Invitrogen), 2 mM dNTP's, 100 mM DTT, 10X RT buffer and 10 µM oligo dT primer. DNA was isolated according to Karp *et al.,* (1999).

4.2.5 *T.s./T.b. HKT1;2* promoters sequencing, constructs making, transformation of the *A.t.hkt1* mutant

The *HKT1;2* promoters from *T. salsuginea* ecotype Shandong and *T. botschantzevii* ecotype Saratov were sequenced by chromosome walking on gDNA using gene specific reverse primers, using the Clontech (PT3042-2) Universal Genome walker kit. 1822 bp from *T. salsuginea*, 1811 bp from *T. botschantzevii* and 846 bp from *A. thaliana* (Maser *et al.,* 2002) upstream from start codon (ATG) of *HKT1,* were used as promoters. Following constructs were prepared: *35S-CMVprom::T.s.HKT1;2, A.t.HKT1prom::T.s.HKT1;2, A.t.HKT1prom::A.t.HKT1, T.s.HKT1;2prom::A.t.HKT1, T.b.HKT1;2prom::A.t.HKT1*. PCR's were done using specific sense and antisense primers using the "Phusion® High Fidelity DNA Polymerase" (Finnzymes), on cDNA to amplify coding region and on gDNA to amplify promoters. Sense primers of *A.t.HKT1prom, T.b.HKT1;2prom, T.s.HKT1;2prom* and *T.s.HKT1;2prom::A.t.HKT1* contain "CACC" 5' overhang which is necessary for directional cloning in pENTR/D Topo, while sense and antisense primers of *A.t.HKT1prom::A.t.HKT1*, *A.t.HKT1prom*::*T.s.HKT1;2* and *T.b.HKT1;2prom*::*A.t.HKT1* have attB1 and attB2 sites (Table S4.1: Supplementary Information). All DNA recombinant techniques were performed according to the GATEWAY Cloning System. BP recombination reaction was done between attB-flanked DNA fragment and appropriate attP-containing donor vector using BP Clonase® II enzyme mix, to generate an entry clone then LR recombination reaction between the entry clone and a Gateway® destination vector, using LR Clonase® II to generate an expression clone. We used pH7WG2,

pHGWFS7 (Karimi *et al.,* 2002) and pH7WG2(-35Sprom) {constructed from pH7WG2} as destination vectors. For *T.s.HKT1;2*, under the control of the cauliflower mosaic virus CMV-35S promoter, we used pH7WG2. For all the promoters analysis we used pHGWFS7 and for all other constructs (which have promoter with them) pH7WG2(-35Sprom) was used. These binary vector contains a hygromycin phosphotransferase (hpt) gene, which confers resistance to hygromycin in transformed cells. Later, these binary vectors were introduced into the *Agrobacterium tumefaciens* strain C58 (pMP90) by electroporation.

4.2.6 Screening of transformant lines

Seeds of homozygous *A.t.hkt1* mutants (Col) were obtained from NASC stock center (N6531), and sown on soil along with wild-type. *A.t.hkt1* mutants were transformed with the constructs (described above) by the flower dipping method (Clough and Bent, 1998). Transgenic T_0 seeds were surface sterilised and sown on 0.8% (w/v) gelrite plates containing 0.5% Murashige and Skoog (MS) salts at pH 5.7-5.9 with 50 $\mu g\ ml^{-1}$ hygromycin for screening. The plates were kept vertically to see the root growth. After two weeks, there was a clear difference in transformed and un-transformed plants. The transgenic plants were transferred to hydroponics solution containing a modified half-strength Hoagland's nutrient solution (Schat and Ten Bookum, 1992). After two weeks in hydroponics, samples were taken from roots and leaves to extract RNA. RNA extraction and cDNA synthesis was performed as described above (Chapter 2). Then the relative transcript levels were measured by Real-Time PCR taking *Actin-2* (*Act-2*) as an internal control.

4.2.7 Statistics

Statistic analysis was performed using one way and two-way ANOVA. MSR statistic was used for a posteriori comparisons of individual means (Rohlf and Sokal, 1981). When necessary, data were subjected to logarithmic transformation prior to analysis.

4.3 Results

4.3.1 Selection and molecular analysis of T_0/T_1 transgenic plants

The amplified *T. salsuginea* cDNA sequence appeared to be *T.s.HKT1;2*. It shared

83% identity, on a nucleotide basis, with *A. thaliana HKT1* (Sequence alignment S4.1: Supplementary Information). On a protein basis, T.s.HKT1;2 and A.t.HKT1 were 79% identical (Sequence alignment S4.2: Supplementary Information). The promoter alignment of *T. botschantzevii* and *T. salsuginea* with *A. thaliana* showed that both the *T.b.HKT1;2* and *T.s.HKT1;2* promoter sequences shared 38% identity with the corresponding *A.t.HKT1* promoter (Sequence alignment S4.3: Supplementary Information). The *T.b.HKT1;2* (1811 bp) and *T.s.HKT1;2* (1822 bp) promoters were cloned for this experiment. We successively performed two experiments with two sets of independent transgenic lines.

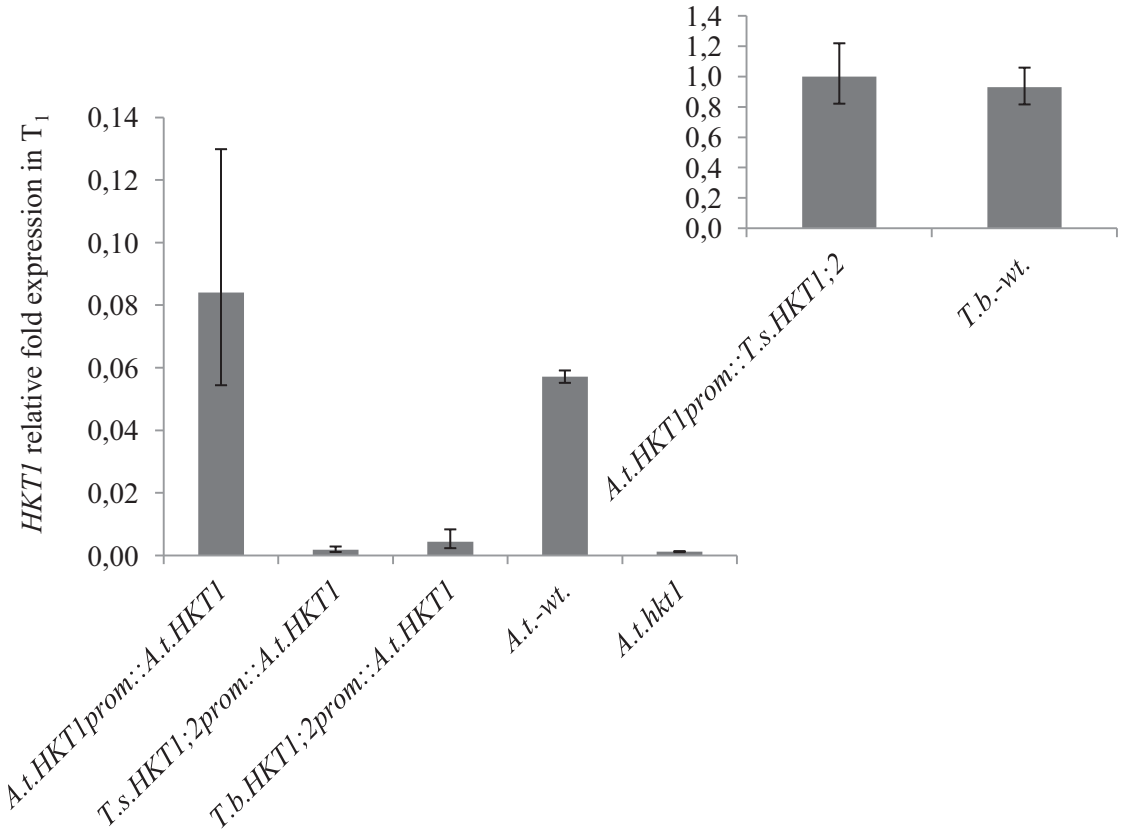

Fig. 1 *A.t.hkt1* transformed with *A.t.HKT1prom::T.s.HKT1;2, A.t.HKT1prom::A.t.HKT1, T.s.HKT1;2prom::A.t.HKT1, T.b.HKT1;2prom::A.t.HKT1.* Expression of *HKT1* was measured in T_1 progeny by Real-Time PCR. Using *A. thaliana* wt. and *A.t.hkt1* as positive and negative controls, respectively. Error bars are ±SE.

PCR and Real-Time PCR analyses were performed on all the T_0 plants and 3-4 randomly selected plants from the T_1 progeny (the 1st generation of transgenic plants) to determine the expression levels (Fig. 1) of the transgenes. *T.s.HKT1;2* was expressed strongly under *A.t.HKT1prom*, in comparison with any other promoter (Fig. 1). Also *A.t.HKT1* was well expressed, approximately at the level of wild-type *A.*

thaliana, when under its native promoter. The *T.b.HKT1;2prom::A.t.HKT1* and *T.s.HKT1;2prom::A.t.HKT1* constructs were not detectably expressed.

4.3.2 Tolerance index (T.I.)

Under 50 mM NaCl exposure both of the constructs with the *A.t.* promoter, *A.t.HKT1prom::A.t.HKT1* and *A.t.HKT1prom::T.s.HKT1;2*, complemented the *A.t.hkt1* mutant, approximately to wild-type level (Fig. 2). As expected, the constructs that were not detectably expressed did not complement the *A.t.hkt1* mutant to any extent. The *35S-CMVprom::T.s.HKT1;2* construct complemented the mutant in one of the experiments, but not in the other, in spite of the fact that in both experiments the *T.s.HKT1;2* expression level was at least one order of magnitude higher under the 35S-CMV promoter than under any of the others. Overall, the results of the two experiments were consistent (Fig. 2).

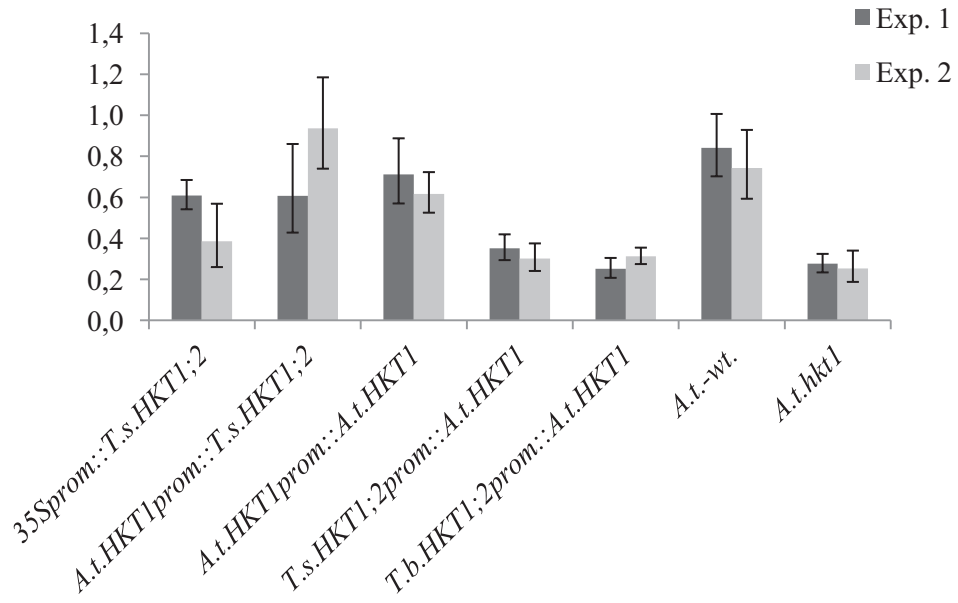

Fig. 2 Tolerance Index on the basis of fresh weight measured in T_1 progeny of *35Sprom::T.s.HKT1;2, A.t.HKT1prom::T.s.HKT1;2, A.t.HKT1prom::A.t.HKT1, T.s.HKT1;2prom::A.t.HKT1, T.b.HKT1;2 prom::A.t.HKT1,* wt. and *A.t.hkt1*. Using *A. thaliana* wt. and *A.t.hkt1* as positive and negative controls, respectively. Error bars are ±SE. Black bars represent values from the first experiment: grey bars represent values from the second experiment.

4.3.3 Water content of fresh leaves

Consistent with their tolerance index, lines harboring the *A.t.HKT1prom::T.s.HKT1;2* and *A.t.HKT1prom::A.t.HKT1* constructs maintained wild-type-like water percentages in their leaves (> 90%) under salinity stress, whereas the plants with the *Thellungiella*

promoters desiccated to a degree comparable with the *A.t.hkt1* mutant (Fig. 3). The construct with the 35S-CMV promoter complemented the mutant in the first experiment, but incompletely in the second one. Overall, the results of the two experiments were consistent.

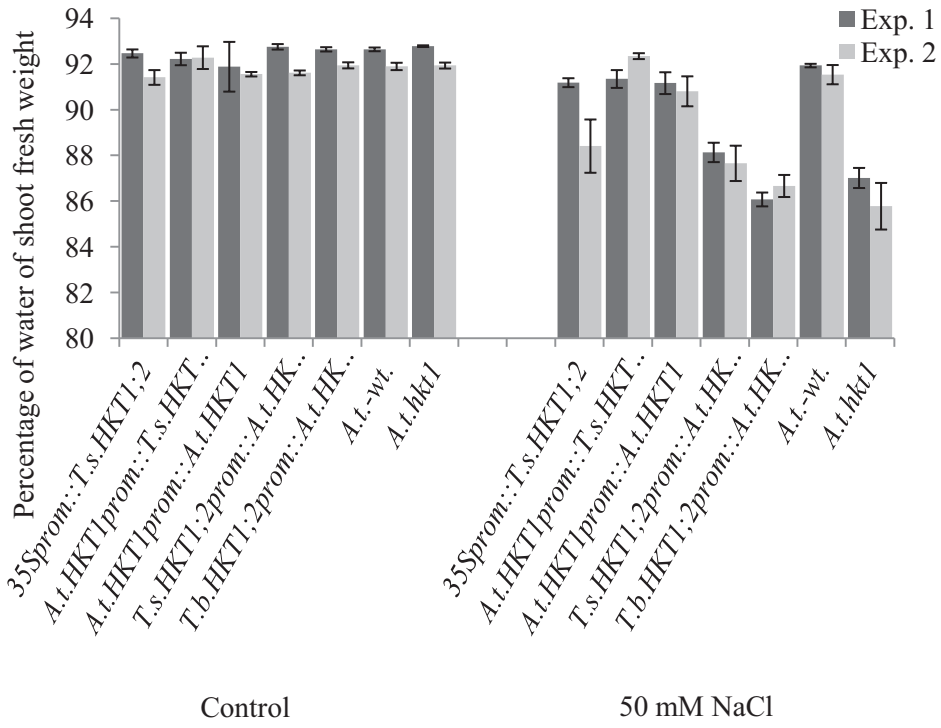

Fig. 3 Water content (percentage of shoot fresh weight) in T_1 progeny of *35Sprom::T.s.HKT1;2, A.t.HKT1prom::T.s.HKT1;2, A.t.HKT1prom::A.t.HKT1, T.s.HKT1;2prom::A.t.HKT1, T.b.HKT1;2 prom::A.t.HKT1*.Using *A. thaliana* wt. and *A.t.hkt1* as positive and negative controls, respectively. Error bars are ±SE. Black bars represent values from the first experiment: grey bars represents values from the second experiment.

4.3.4 Na and K analysis in transgenic T_1 plants

We compared Na and K accumulation in shoots and roots of wild-type and transgenic lines. In both experiments the plants with the *35S-CMVprom::T.s.HKT1;2* construct exhibited a lower Na concentration in their shoots than any of the other lines. In experiment 1, the foliar Na concentration in the *A.t.HKT1prom::A.t.HKT1* line was significantly higher than in the *35S-CMVprom::T.s.HKT1;2* line, but significantly lower than in the other lines, while in experiment 2, the *A.t.HKT1prom::T.s.HKT1;2* line showed a significantly foliar Na concentration than any of the other lines. Overall, the results of both experiments exhibited the same trend (Fig. 4). The root Na concentrations were highly erratic and there was no

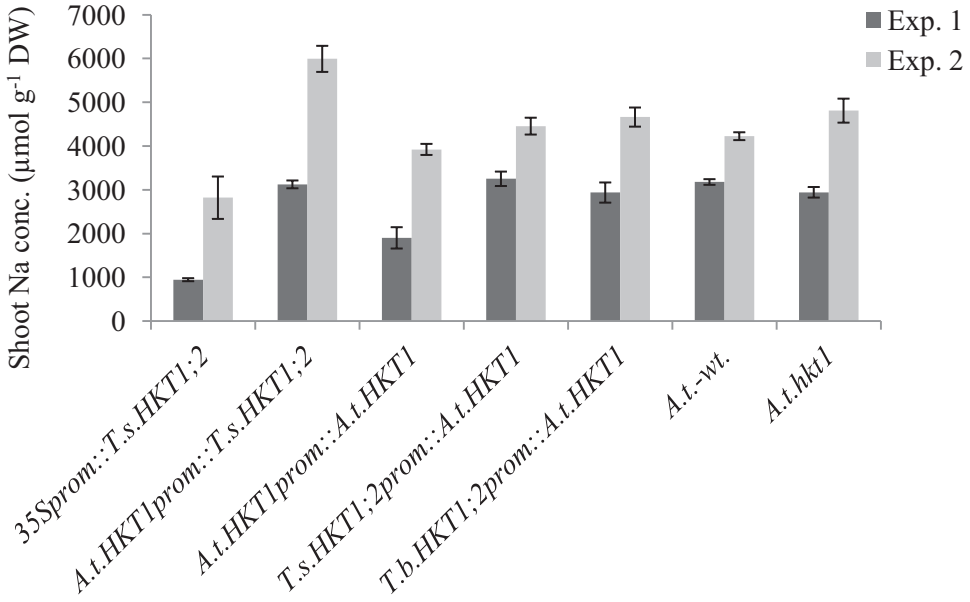

Fig. 4 Na concentrations in shoots of T_1 progeny from *35Sprom::T.s.HKT1;2, A.t.HKT1prom::T.s.HKT1;2, A.t.HKT1prom::A.t.HKT1, T.s.HKT1;2prom::A.t.HKT1, T.b.HKT1;2 prom::A.t.HKT1*. Using *A. thaliana* wt. and *A.t.hkt1* as positive and negative controls, respectively. Error bars are ±SE. Black bars represent values from the first experiment: grey bars represent values from the second experiment.

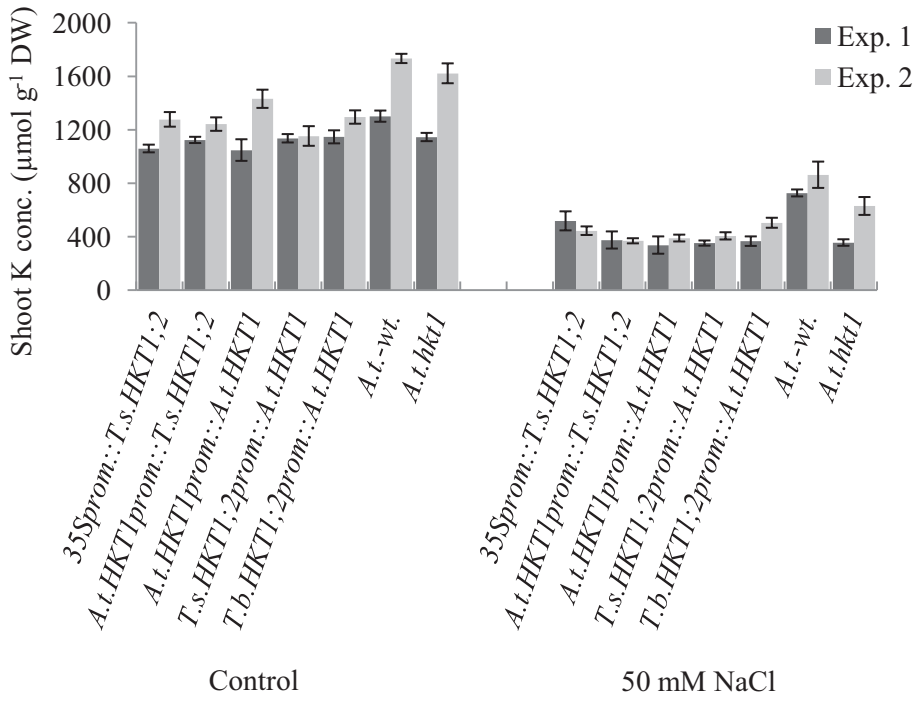

Fig. 5 K concentration in the shoots of T_1 progeny from *35Sprom::T.s.HKT1;2, A.t.HKT1prom::T.s.HKT1;2, A.t.HKT1prom::A.t.HKT1, T.s.HKT1;2prom::A.t.HKT1, T.b.HKT1;2 prom::A.t.HKT1*. Using *A. thaliana* wt. and *A.t.hkt1* as positive and negative controls, respectively. Error bars are ±SE. Black bars represent values from the first experiment: grey bars represent values from the second experiment.

consistency between the results of the two experiments (data not shown).

The shoot K concentrations were not significantly different between lines, except for wild-type, which showed a significantly higher foliar K concentration than all the other lines, apart from the *A.t.hkt1* mutant line, though in experiment 1 exclusively under NaCl exposure (Fig. 5). The root K concentrations were erratic and inconsistent (data not shown).

4.4 Discussion

As clearly shown by the tolerance index, only the *A.t.HKT1prom::A.t.HKT1* and *A.t.HKT1prom::T.s.HKT1;2* constructs yielded a more or less completely complemented the *A.t.hkt1* mutant regarding its salt hypersensitivity phenotype. The *T.s.HKT1;2prom::A.t.HKT1* and *T.b.HKT1;2prom::A.t.HKT1* constructs did not yield any detectable complementation at all, while the *35S-CMVprom::T.s.HKT1;2* construct only incompletely complemented the mutant. The same conclusion can be drawn on the basis of the foliar water contents in the salt treatment. The complete lack of complementation obtained with the *T.s.HKT1;2prom::A.t.HKT1* and *T.b.HKT1;2 prom::A.t.HKT1* constructs is doubtlessly owing to the lack of detectable *A.t.HKT1* expression in the transgenic lines transformed with these constructs. The latter could be due to the absence from the *A. thaliana* genome of an essential transcriptional activator or, more likely, the lacking of an essential response element located upstream of the sequences that we used. The incomplete complementation provided by the *35S-CMVprom::T.s.HKT1;2* construct is most probably owing to a non-tissue-specific expression of *T.s.HKT1;2* (Møller *et al*., 2010).

Our observation that *A.t.HKT1* and *T.s.HKT1;2* are both able to complement the *A.t.hkt1* mutant is not self-evident, since A.t.HKT1 is a Na-selective transporter (Uozumi *et al*., 2000), whereas T.s.HKT1;2 is K-specific, even in the presence of NaCl (Ali *et al.,* 2012). A.t.HKT1 is supposed to provide salinity tolerance through resorbing Na from the xylem, thus preventing its accumulation in the shoot (Berthomieu *et al.,* 2003; Sunarpi *et al.,* 2005), while T.s.HKT1;2 is supposed to do the same through maintaining a sufficient K uptake under salinity stress (Ali *et al.,* 2012). However, although both genes doubtlessly complemented the *A.t.hkt1* mutant under our experimental conditions, we did not find significant differences in the foliar Na or K contentrations between the corresponding transformant lines. We not even found consistent and significant differences in the foliar Na or K concentrations

between wild-type *A. thaliana* and the *A.t.hkt1* mutant, although the difference in salinity tolerance was beyond doubt. As yet we do not have any explanation for these phenomena. It may be that initial differences in Na and K concentration, in so far existent at all, may have been obscured by toxicity or long-lasting stress.

In conclusion, when expressed under the *A. thaliana HKT1* promoter, both *HKT1* from *A. thaliana* and *HKT1;2* from *T. salsuginea* restore a wild-type level of salinity tolerance in the *A.t.hkt* mutant, although the precise underlying mechanisms remain elusive.

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Chapter 4

Supplementary Information

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Supplementary Tables; S4.1
Supplementary sequence alignments; S4.1-S4.3

Construct		Primer name	Primer Sequence
35Sprom:: T.s.HKT1;2	*T.s.HKT1;2*	T.s.HKT1;2F	GGGGACAAGTTTGTACAAAAAAGCA-GGCTATGGAGAGAGTTGTGGACAAG
		T.s.HKT1;2R	GGGGACCACTTTGTACAAGAAAGCT-GGGTAGATTTACGAAGATGAAGGAT
A.t.HKT1prom ::T.s.HKT1;2	*A.t.HKT1prom*	A.t.HKT1promF	GGGGACAAGTTTGTACAAAAAAGCAGGCT-CATCCCATGTTACTCCATGTG
		A.t.HKT1promR	CCACAACTCTCTCCATTTTAGTTCTCGAGTC
	T.s.HKT1;2	T.s.HKT1;2F	GACTCGAGAACTAAAATGGAGAGAG-TTGTGGACAAG
		T.s.HKT1;2R	GGGGACCACTTTGTACAAGAAAGCT-GGGTAGATTTACGAAGATGAAGGAT
A.t.HKT1prom ::A.t.HKT1	*A.t.HKT1prom*	A.t.HKT1promF	GGGGACAAGTTTGTACAAAAAAGCA-GGCTCATCCCATGTTACTCCATGTG
		A.t.HKT1promR	CGAACGGATTTTTGCTATTTT TGCC
	A.t.HKT1	A.t.HKT1F	ATGGACAGAGTGGTGGCAAAAATAGC
		A.t.HKT1R	GGGGACCACTTTGTACAAGAAAGCT-GGGTATTAACGATGATGCAAACTAC
T.s.HKT1;2prom ::A.t.HKT1	*T.s.HKT1;2prom*	T.s.HKT1;2promF	CACCGTGCAATTTGAAAACTACTCC
		T.s.HKT1;2promR	CACTCTGTCCATTTTGTATATATCTTACC
	A.t.HKT1	A.t.HKT1F	TAAGATATATACAAAATGGACAGAGTGGTGG
		A.t.HKT1R	ATTAACGATGATGCAAACTAC
T.b.HKT1;2prom ::A.t.HKT1	*T.b.HKT1;2prom*	T.b.HKT1;2promF	GGGGACAAGTTTGTACAAAAAAGC-AGGCTGTGCAATTTGAAAACTACTCC
		T.b.HKT1;2promR	GTCCATTTGTATATATCTTACCAGAG
	A.t.HKT1	A.t.HKT1F	GATATATACAAATGGACAGAGTGGTGG
		A.t.HKT1R	GGGGACCACTTTGTACAAGAAAGCT-GGGTATTAACGATGATGCAAACTAC

Supplementary Sequence S4.1. Primers used for GATWAY cloning

Supplementary sequence alignments

```
T.s.HKT1;2        ATGGAGAGAGTTGTGGACAAGTTAGCTAAAATCTTTTCGCAACATGCTAAATCTCTCCCC
A.t.HKT1          ATGGACAGAGTGGTGGCAAAAATAGCAAAAATCCGTTCGCAGCTTACTAAATTACGTTCA

T.s.HKT1;2        CTTTTCTTCCTTTACTTCTTCTACTTCTTGTTCTTCTCCTTCTTGGGGTTCTTGGCACTC
A.t.HKT1          CTATTCTTCCTTTACTTCATCTACTTCTTGTTCTTCTCCTTTTTAGGGTTTTTGGCACTC

T.s.HKT1;2        AAGATCTCAAAGCCAAGAACCACTTCACGTCCTCATGACTTGGATCTGTTCTTCACTTCT
A.t.HKT1          AAGATCACAAAGCCAAGAACCACTTCACGTCCTCATGACTTTGACCTTTTCTTCACTTCT

T.s.HKT1;2        GTCTCCGCCATCACTGTCTCCTCCATGTCAACCATCGACATGGAAGTCTTCTCAAACACC
A.t.HKT1          GTCTCTGCCATCACCGTCTCTTCCATGTCTACCGTCGACATGGAAGTCTTCTCCAACACC

T.s.HKT1;2        CAACTTATCATCATTACTATCCTCATGTTTCTAGGCGGCGAGATCTTCACTTCTTTCGTG
A.t.HKT1          CAACTTATCTTCCTCACTATCCTCATGTTCCTCGGTGGCGAAATCTTCACCTCCTTTCTC

T.s.HKT1;2        AATCTCTACTTCTCTCATTTCATTAACTTC---------------AAAATCAAACATCTT
A.t.HKT1          AACCTCTACGTCTCCTATTTCACCAAGTTCGTCTTCCCTCATAACAAGATTAGACATATT

T.s.HKT1;2        GTGGGCTCTTTCAACTTCGACCGTCCTATCAATGATCCGGGTAGTGATCTTGAGAATGTT
A.t.HKT1          TTGGGATCTTATAATTCGGACAGTTCCATCGAGGATCG---CTGTGACGTTGAGACTGTT

T.s.HKT1;2        ACTAATCATGTCAAGCTTTCTAGTCAGATCAATGAAAGGGCCTCTAAGTGTTTGTACTCG
A.t.HKT1          ACTGATTATCGCGAGGGTCTTATCAAGATCGATGAAAGGGCATCTAAGTGCTTGTACTCG

T.s.HKT1;2        GTGGTTCTTGGTTACCTTTTTGTAACCAACATAGCTGGTTCCACGTTGCTTCTTCTGTAC
A.t.HKT1          GTGGTTCTTAGTTACCATCTTGTTACTAACCTAGTTGGCTCTGTGTTGCTTCTTGTGTAC

T.s.HKT1;2        GTAAATTTTGTTAAAACGGCGAGAGATGTTCTTAGTTCCAAAAAAATCTCACCTCTCACT
A.t.HKT1          GTAAATTTTGTTAAAACGGCGAGAGATGTTCTTAGTTCCAAAGAAATCTCACCTCTCACT

T.s.HKT1;2        TTCTCGGTCTTCACAGCTGTCTCTACGTTATCAGACTGTGGATTTGTCCCCACGAATGAG
A.t.HKT1          TTCTCCGTCTTCACAACTGTTTCCACGTTTGCAAACTGCGGATTTGTCCCCACGAATGAG

T.s.HKT1;2        AACATGATCATCTTCCGAAAGAACTCTGGCCTCCTCTGGCTCTTAATCCCTCAAGTATTC
A.t.HKT1          AACATGATCATCTTTCGCAAGAACTCTGGTCTCATCTGGCTCCTAATCCCTCAAGTACTG
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T.s.HKT1;2        ATGGGAGACACTTTGTTTCCTTGCTTCTTGGTTTTGGCCATATGGGGACTTCATAAGATC
A.t.HKT1          ATGGGAAACACTTTGTTCCCTTGCTTCTTGGTTTTGCTCATATGGGGACTTTATAAGATC

T.s.HKT1;2        ACAAATCGAGAAGAATTGGGTTACATTCTCAAGAATCACAAGAAGATGGGATACTCTCAT
A.t.HKT1          ACAAAGCGTGACGAGTATGGTTACATTCTCAAGAACCACAATAAGATGGGATACTCTCAT

T.s.HKT1;2        TTACTCTCCGTTCGTCTTTGTGTTCTTCTTGCTTTGACGGTGTTAGGGCTTGTGATGATA
A.t.HKT1          CTACTCTCGGTTCGTCTATGTGTTCTTCTTGGAGTGACGGTGCTAGGGTTTCTGATAATA

T.s.HKT1;2        CAGTTTCTTCTATTCTGCACCTTTGAATGGAACTCTGAGTCTCTTGAAGGAATGAATTCC
A.t.HKT1          CAGCTTCTTTTCTTCTGCGCCTTTGAATGGACCTCTGAGTCTCTAGAAGGAATGAGTTCG

T.s.HKT1;2        TACGAGAAGTTGGTTGGATCGTTGTTTCAAGTTGTCAACTCGAGACACACTGGAGAAACC
A.t.HKT1          TACGAGAAGTTGGTTGGATCGTTGTTTCAAGTGGTGAATTCGCGACACACCGGAGAAACT

T.s.HKT1;2        GTTGTCGACCTCTCTACACTTTCTCCAGCAATCTTGGTACTCTTCATCCTCATGATGTAT
A.t.HKT1          ATAGTAGACCTCTCTACACTTTCCCCAGCTATCTTGGTACTCTTTATTCTTATGATGTAT

T.s.HKT1;2        CTTCCTCCCTACACACTATTCATGCCGTTGACCGTAGAAAAG---AATAAGAAAGAGGGT
A.t.HKT1          CTTCCTCCATACACTTTATTTATGCCGTTGACGGAACAAAAGACGATAGAGAAAGAAGGA

T.s.HKT1;2        G---AACACGATTCCGGAGATGAAATTAAAGGAAAGAAGAATGGGTTCTACGTGTCACAA
A.t.HKT1          GGAGATGATGATTCCGAAAATGGAAAGAAAGTTAAAAAGAGTGGACTCATCGTGTCACAA

T.s.HKT1;2        CTCACCTTTCTAGCGATATGTATCTTTCTCATTTCCACCACCGAAAGTCAAAAACTAAGA
A.t.HKT1          CTTTCCTTTTTGACGATATGTATCTTTCTCATTTCAATCACCGAAAGGCAAAATCTACAA

T.s.HKT1;2        CGAGATCCACTCAATTTCAACATCCTCAACATCACTTTCGAAGTTATCAGTGCATATGGA
A.t.HKT1          CGTGATCCGATAAATTTCAACGTCCTTAACATCACTCTCGAAGTTATCAGTGCATATGGA

T.s.HKT1;2        AACGTTGGGTTCACGACCGGTTACAGCTGCGAGCGGCGCCTAGACATCAGCGATGGTAGC
A.t.HKT1          AACGTTGGTTTCACTACCGGGTACAGCTGTGAACGGCGTGTGGACATCAGCGATGGTGGC

T.s.HKT1;2        TGTAAAGACGCAAGTTATGGGTTTGCAGGACGATGGAGTCCCGTTGGAAAATTCATACTT
A.t.HKT1          TGCAAAGACGCGAGTTATGGGTTTGCAGGACGATGGAGTCCAATGGGAAAATTCGTACTA

T.s.HKT1;2        ATAATAGTAATGTTTTATGGTAAATTTAAGCAATTCTCAGCTAAATCTGGCAGAGCGTGG
A.t.HKT1          ATAATAGTAATGTTTTATGGTAGGTTTAAGCAGTTCACAGCCAAATCTGGCCGCGCATGG

T.s.HKT1;2        ATACTTTATCCTTCATCTTCGTAA
A.t.HKT1          ATTCTTTACCCCTCGTCTTCCTAA
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Supplementary sequence alignment S4.1. Sequence alignments of *T.s.HKT1;2* and *A.t.HKT1* for nucleotide, both have 83% identity among themselves on nucleotides basis.

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T.s.HKT1;2        MERVVDKLAKIFSQHAKSLPLFFLYFFYFLFFSFLGFLALKISKPRTTSRPHDLDLFFTS
A.t.HKT1          MDRVVAKIAKIRSQLTKLRSLFFLYFIYFLFFSFLGFLALKITKPRTTSRPHDFDLFFTS

T.s.HKT1;2        VSAITVSSMSTIDMEVFSNTQLIIITILMFLGGEIFTSFVNLYFSHFINF-----KIKHL
A.t.HKT1          VSAITVSSMSTVDMEVFSNTQLIFLTILMFLGGEIFTSFLNLYVSYFTKFVFPHNKIRHI

T.s.HKT1;2        VGSFNFDRPINDPGSDLENVTNHVKLSSQINERASKCLYSVVLGYLFVTNIAGSTLLLLY
A.t.HKT1          LGSYNSDSSIEDR-CDVETVTDYREGLIKIDERASKCLYSVVLSYHLVTNLVGSVLLLVY

T.s.HKT1;2        VNFVKTARDVLSSKKISPLTFSVFTAVSTLSDCGFVPTNENMIIFRKNSGLLWLLIPQVF
A.t.HKT1          VNFVKTARDVLSSKEISPLTFSVFTTVSTFANCGFVPTNENMIIFRKNSGLIWLLIPQVL

T.s.HKT1;2        MGDTLFPCFLVLAIWGLHKITNREELGYILKNHKKMGYSHLLSVRLCVLLALTVLGLVMI
A.t.HKT1          MGNTLFPCFLVLLIWGLYKITKRDEYGYILKNHNKMGYSHLLSVRLCVLLGVTVLGFLII

T.s.HKT1;2        QFLLFCTFEWNSESLEGMNSYEKLVGSLFQVVNSRHTGETVVDLSTLSPAILVLFILMMY
A.t.HKT1          QLLFFCAFEWTSESLEGMSSYEKLVGSLFQVVNSRHTGETIVDLSTLSPAILVLFILMMY

T.s.HKT1;2        LPPYTLFMPLTVEK--NKKEGEHDSGDEIKGKKNGFYVSQLTFLAICIFLISTTESQKLR
A.t.HKT1          LPPYTLFMPLTEQKTIEKEGGDDDSENGKKVKKSGLIVSQLSFLTICIFLISITERQNLQ

T.s.HKT1;2        RDPLNFNILNITFEVISAYGNVGFTTGYSCERRLDISDGSCKDASYGFAGRWSPVGKFIL
A.t.HKT1          RDPINFNVLNITLEVISAYGNVGFTTGYSCERRVDISDGGCKDASYGFAGRWSPMGKFVL

T.s.HKT1;2        IIVMFYGKFKQFSAKSGRAWILYPSSS
A.t.HKT1          IIVMFYGRFKQFTAKSGRAWILYPSSS
```

Supplementary sequence alignment S4.2. Sequence alignments of *T.s.HKT1;2* and *A.t.HKT1* for protein, both have 79% identity among themselves on protein basis.

```
T.s.HKT1;2prom.     -----------AAACTACTCCAATTTAGATGAAACGTATTGTTGTGGAAACGCCTCTTGC
T.b.HKT1;2prom.     GTGCAATTTGAAAACTACTCCAATTTAGATGAAACGTATTGTTGTGGAAACGCCTCTTGC
A.t.HKT1prom.       -----------CAACTTG-CAAGCTCTAATGATTCACAAG-TTGATAACAC-CATTTTGC

T.s.HKT1;2prom.     ATTTTCTCCTTGCCTATTTACAAAGAATATTTGGTATAGATCCACTCATCACTCTACATT
T.b.HKT1;2prom.     ATTTTCTCCTTGCCTATTTACGAAGAATATTTGGTATAGATCCACTCATCAATCTACATT
A.t.HKT1prom.       AAGCTCTAATAATG-ATTCATTAAACAAATTGGCAATTTTCAAATACCAACACCACCCTT

T.s.HKT1;2prom.     TGTATTGGATTCCTCAGTAGACAGCTTAATCTTGGCTTCTATGTTTGCAATGGTGTCATA
T.b.HKT1;2prom.     TATATTGGATTCCTGAGTAGACAGCTTAATCTTGGCTTCTATGTTTGCAATGGTGTCATA
A.t.HKT1prom.       TTTCTTGTTAAAATTACTATTGTTTATTTTATTTTATTTAATGTTA--AATTATATTTAA

T.s.HKT1;2prom.     CATTTGACTTAACAT-ACTCATGAGAGTATCGCTCCTCTATGAACATTTTTTAA-AAAAG
T.b.HKT1;2prom.     CATTTGACTTAACAT-ACTCATGAGAGTATCGCTCCTCTATGAACATTTTTCAAGAAAAG
A.t.HKT1prom.       AAGAAAAATAACCTTGGTACATATGAAATGCTAACTTTTTCAAGAGTTATTTTA--AAAA

T.s.HKT1;2prom.     TTCTAGATTTCTCAAGCATTAAACTGATCATGAATAGTCAAAGTTTGTAGATAATCATTT
T.b.HKT1;2prom.     TTCTAGATTTCTCAAGCATTAAATTGATCATGAATAGTCAAAGTTTGTAGATAATCATTT
A.t.HKT1prom.       AACAGAATTTCTAATATATCTATTG--TCTTGATTCAAACCAAATT--TGGATGCCATTT

T.s.HKT1;2prom.     TTGAAA--CAAACATCCCTAACACGGTTCTATACTTCTTAAAATATCTAGACTTTGAGT-
T.b.HKT1;2prom.     TTGAAA--CAAACATCCCTAACACGGTTCTCTACTTCTTAAAATATCTAGACTTTGAGT-
A.t.HKT1prom.       TTGAACTTCAATCCTCCACCACCTTGAATTGTGCT-CAAACGGTTTCTAATATTCTTGTC

T.s.HKT1;2prom.     TATACGTATTAAATGTAATAACAATCATCAATGTGAGAGTATGCAAACTAGAAGCTACAT
T.b.HKT1;2prom.     TATACATATTAAATGTAATAACAATCATCAATGTGAGAGTATGCAAACTAGAAGCTACAT
A.t.HKT1prom.       TATGATCATCACAAGTATTTCCGTTG------GTGATGATTGCTCCCCATGCCTCCTCCT

T.s.HKT1;2prom.     CTTCAATGTAAAAAGATGTCACTATCCTCAAGATGAATTTTAAAGGTT--GTCATATATA
T.b.HKT1;2prom.     CTTCAATGTAAAAACATGTCACTATCCTCGAGATGAATTTCAAAGGTTAAGTCATATATA
A.t.HKT1prom.       ATTCTGT-TCATTCCATATTGAGTGTATAGAGTTCTGTAGTGCATACCGAAGTATGTAGC

T.s.HKT1;2prom.     CTTTCTAGTTGACATGGAGGAATCTAAAGTAGTAGTTCTTCAAGTGCATGAGTTCATCCA
T.b.HKT1;2prom.     CTTTCTAG-TGACATGGAGGAATCTAAATTAGTAGTTCTTCAAGTGCATGATTTAATCCA
A.t.HKT1prom.       TCTTCGTTTTGTCCACAAGGTTTTCAGATAAAATCTTCATAACCTCTGTCCAACTCACCG

T.s.HKT1;2prom.     TGGTGTGTGTTCAAACCGAATCATAACCTCTAG---GGTCACTCCTTGCCTGTGTTGGAT
T.b.HKT1;2prom.     TGGTGTGTGTTCAAACCGAATCATAACCTCTAG---GGTCACTCCTTGCCTGTGTTGGAT
A.t.HKT1prom.       AG------AATCTGTCCGAGAAAATTCCCCTTGTTAGGTCTTCCCATATCTTTTTGAGTA

T.s.HKT1;2prom.     TTTTTTTTTTTTTG-----GTAAGGCTCTTCCAACTCCACTTTTTTTTTGTTTCTGGTGT
T.b.HKT1;2prom.     TTTTTTTTTTTTTTTTTTTTGTAAGGCTCTTACAACTCCACTTTTTGTTTGTTTCTGGTGT
A.t.HKT1prom.       TAGGCAGTGGAAAAAT----AAGTGTTCCCTCGTCTCTACTCGTTCATTGCAAAAAATGC

T.s.HKT1;2prom.     G--AACATTCTCTCATCAAATGGCAGTTGCTCAT---ATGACTTATCTTGAATATATTGG
T.b.HKT1;2prom.     G--AACATTCTCTCATCAAATGGCAGTTGCTCAT---ATGACTTATCTTGAATATATTGG
A.t.HKT1prom.       AGCTCGAATCTACATTACAATTCCACTTCCTCATTCTCTCCCCTGTTGCGACTCTGTTTT

T.s.HKT1;2prom.     TCAAATTTGTCTTCCTACTCTTGTGCCCAATCATTCTTGTGCCCAATCATTGTAAACCAT
T.b.HKT1;2prom.     TCAAATTTGTCTTCCTACTCTTGT----------------GCCCAATCATTGTAAACCAT
A.t.HKT1prom.       TTACAACCGTATTGTTATACTTTGGAGTAG-CGTATGGGAACCATATCTCATTTTGTCCC

T.s.HKT1;2prom.     AGCCATTAACTACTTTACTTTTTTTTTTT--GTATTTCTACAAGCAAAAAT--ATGAAA
T.b.HKT1;2prom.     AGCCATTAACTACTTTACTTTTTTTTTTTTTGTATTTCTACAAGCAAAAAT--ATGAAA
A.t.HKT1prom.       T-CCATCGGCGGCTTTGCTAACCGCAGCTG-TGACCAAGAACATTTATTTTTCCCTTTTA

T.s.HKT1;2prom.     GAAATACAATTGGCAGATTATGCATTGACATCAAGAATCTTATTTTCTTTCTTTTATTAT
T.b.HKT1;2prom.     GAAATACAATTGGCAGATTATGCAATGACATCAATAATCTTATTTTCTTT-TTTTATTAT
A.t.HKT1prom.       ACAGAACTATATCGTGATCGTTCAGT-TCTCCATTCAGCTTCTGTTTTCTTATTTCTTCT

T.s.HKT1;2prom.     TTTATCTAATTTGAAAAAAGCTTTTATACGTCAAAGTATAATCGACTGACTAACACGTGT
T.b.HKT1;2prom.     TTTATCTAATTTGAAAAAAGCTTTTAAACGTCAAAGTATAATCGACTGACTAACACGTGT
A.t.HKT1prom.       TCAACACGGTTTAAGATACCCTTTCGATGGTTTCTCCTTCGTGTCATGGACATCACCTCT

T.s.HKT1;2prom.     GGCCATTTATGTCAAATATAAAGTTTGAGATAACAAACTTAATATAATAAAATATATCTG
T.b.HKT1;2prom.     GGCGATTTATGTCAAATATAAAGTTTGAAATAACAAACTTAATATAATAAAATATATCTG
A.t.HKT1prom.       TTCATTGTGCTTGAACTAGGAATCCCTAGATCAATG-CATCCTCTCTAAGACATCCCATG

T.s.HKT1;2prom.     TATGTATAT--------CAAGCATACACATAAAGTC--AGTGTACTTGTATCTATTTTTA
T.b.HKT1;2prom.     TATGTATAAGAAGATATCAAGCATACCCATAAAGTC--AGTGTACTTGTATCTATTTTTA
A.t.HKT1prom.       TTACTCCATG--------TGTCAATACCAAAAAGACGTAGTTTCGCCGCTTTTAACCACT

T.s.HKT1;2prom.     TTAAAAGGTAAAGTTTTCTTTATTTTCTCTATTTTAATTTTACGACTTATTTATTTTTTC
T.b.HKT1;2prom.     TTAAAAGGTAAAG-TTTCTTTATTTTCTCTATTTTAATTTTACGACTTATTTATTTTTTC
A.t.HKT1prom.       ACCATGACTGTTGATCTCTTTAAATAATTAAAAAGTGGTGTTCCCCATGAAGACCG-TTC
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T.s.HKT1;2prom.     TTTTTTATTATTTTAAATTTTTTTTAGACTATTGGAAG---TTTTTAATTTTTTGGAGGT
T.b.HKT1;2prom.     CTTTTTATTATTTTAAAATTTATTTAGACTATTGGAAGTTTTTTTTTTATTTTTGGGAAGT
A.t.HKT1prom.       ACTTCCATCAATTTATGGTCTTCTTGGACCAATTTCGTT--TATCCAAACTATTACCCCT

T.s.HKT1;2prom.     TGTTTGAATTTTTTTATTAGTTTT-CCATTTATATTTCTATTTATTAATGATTTATATCA
T.b.HKT1;2prom.     TGTTAGAATTTTTTTATTAGTTTTCCCATTTATATTTCTATTTATTAATGATTTATATCA
A.t.HKT1prom.       ACAACATCGTTTATAAATTGTCTTTACATTTTGATCTAGAATTTGATCT-ATTTTGCACG

T.s.HKT1;2prom.     TTTGTTTATCTGTTATTTTTATCATCTTTAAGAAATAAATAACAAAAAACAGAAAATT--
T.b.HKT1;2prom.     TTTGTTTATTTGTTATTTCTATCATCTATAAGAAATAAATAACAAACAACAGAAAATT--
A.t.HKT1prom.       AATCCTCTTCCACCTTTTCAGGCATGTACGAGCAATATAACATGAGTTTGATCAAATTTT

T.s.HKT1;2prom.     ----GATTATAATGAAATTGACATCTAAAATATCTTTGATGAAAATATTTATTTATCTTT
T.b.HKT1;2prom.     ----GATTATAATGAAATTGACATCTAAGATCTCTTTGATGAAAATATTTATTTATCTTT
A.t.HKT1prom.       GCATGGTCTCTACGAAGTTGAGTCATGCAATTTTTACCTATAATTTTTGTTCTCGGCATT

T.s.HKT1;2prom.     TCTATAACTGAAATATTATTATAATATTGTGCTTAATATAAATGTGACACGTAAGATAAT
T.b.HKT1;2prom.     TCTACTACTTGAATATTATTATAATATTGTGCTTAATATAAATGTGACACGTAAGATAAT
A.t.HKT1prom.       TTTATAGCTCTCTACTTAGGATATAAGTAACATGGAGTTGGATGAATCCTTTTCTTTATA

T.s.HKT1;2prom.     TCGTAGGCTAATTTT---AGAGAG--AAAAAACTCGTTTTAAAATTTTTAATATTTTAAT
T.b.HKT1;2prom.     TCGTAGGCTAATTTT---AGAGAGAAAAAAAACTCGTTTTTAAAATTTTAATATTTTAAT
A.t.HKT1prom.       TTGAAATCGAATCTTCAATGCTAAAAATAAAATCCACTCAATCAGTGAGAGAATTATTAT

T.s.HKT1;2prom.     GAAATATTTTG-------GTTGTTTCCAATAATTTGTAGGCTAATTAAGGAAATCTCTCT
T.b.HKT1;2prom.     TAAATATTTTG-------GTTGTTTCCAATAATTTGTAGGCTAATTAAGGAAATCTCTCT
A.t.HKT1prom.       GGAACAAGCTATAAAGACGAAAGAATCACTCAATCATACAATAAAGAAGGATGTTTTTTT

T.s.HKT1;2prom.     ATTTCTAGAGTCGAGAAAGGAAAATATTCGGGGAAAACAAAAGTTCAT----ATAGAAAA
T.b.HKT1;2prom.     ATTTTTAGAGTCGAGAAAGGAAAATATTCGGGGAAAACAAAAGTTCAT----ATAGAAAA
A.t.HKT1prom.       CATTTAAACTAAGAAAGAGAACTTTTCCACGTGAATTAAAATAGACATCTCAATAATAAA

T.s.HKT1;2prom.     TACTTTCATGTTGGTCCTCATACACTTATGTGGCTGAGAATGTTCTTATGCGTATATATA
T.b.HKT1;2prom.     TACTTTCATGTTGGTCCTCATACACCTATGTGGCTGAGAATGTTCTTATGCGTATATATA
A.t.HKT1prom.       GACGATTTTTTTTCTTCTCTTTTTCCTCTCACTTTGTATTGTATTGTATTTCTATATATT

T.s.HKT1;2prom.     AACACAAGGGCCAAT-----AATATAATTCGTAGACCGACCCAAGAAATAAAATGG--AG
T.b.HKT1;2prom.     AACACAAGGGCCAAT-----AATATAATTCGTAGACCGACCCAAGAAATAAAATGG--AG
A.t.HKT1prom.       TCTTCCTCTTACAAATACCCTTTTAAATTGGAAAGAAAAAACAGGAATCGCTATCATCAG

T.s.HKT1;2prom.     AGTTTTTCTTTTTTCTTGAAATCCTAGAAATTTCGTATTCCACAATCTCCT----CGGAT
T.b.HKT1;2prom.     AGTTTTTCTTTTTTCTTGAAATCCTAGAAAATTCGTATTCCACAATCTCCT----CGGAT
A.t.HKT1prom.       TAATAGTCATCATTAATCAATTTATATGTAATATGTGGCTGACAATTTCCATGTACGTGT

T.s.HKT1;2prom.     AAGAGCAGGTTTAAAACCCCTACCCGTTTCCCGCACTCGAACCTTCGACCTCTGTCTCTC
T.b.HKT1;2prom.     AAGAGCAGGTTTAAAACCCCTACCCGTTTCCCGCACTCGAACCTTCGACCTCTGTCTCTC
A.t.HKT1prom.       AATATGTAATATATAAACACAACTTATGGCCAG---TATAATATTAATGCTTAAACCGAC

T.s.HKT1;2prom.     CGGTCAAAGGGTTCTCTGGTAAGATATATACAA
T.b.HKT1;2prom.     TG----------------GTAAGATATATACAA
A.t.HKT1prom.       TCG-----------------AGAACTAAA---
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Supplementary sequence alignment S4.3. Sequence alignments of *T.s.HKT1;2* promoter, *T.b.HKT1;2* promoter and *A.t.HKT1* promoter. *T.s.HKT1;2* promoter and *T.b.HKT1;2* promoter have 95% identity among themselves while having 38%, 38% identities with *A.t.HKT1* promoter, respectively on nucleotides basis.

Chapter 5

General Discussion

As outlined in chapter 1, high-level salt tolerance in halophytes is a poorly understood phenomenon. It is commonly believed to be a complex trait, involving alterations of Na/K homeostasis and compatible solute accumulation (Flowers and Colmer, 2008). Its genetic architecture can thus be assumed to be complex too (Rozema and Schat, 2012). The target loci for natural selection under the pressure of high salinity are completely unknown. Many authors implicitly assume that salt tolerance relies on the over-expression of (a subset of) the genes that have been shown to be essential for wild-type-level salt tolerance in *A. thaliana*, such as *HKT1*, the SOS pathway genes, *NHX1*, or the genes encoding the vacuolar and plasma membrane proton pumps, which create the electrochemical gradient for secondary active or passive transmembrane Na transport (Ashraf and Akram, 2009). The results described in chapter 2 and, particularly, chapter 3 of this thesis are clearly in support of this idea.

As suggested by the promoter swap experiments described in chapter 3, at least the high expression of *NHX1* in the halophyte, *C. x hollandica*, can be completely explained by altered *cis*-regulation, in agreement with the hypothesis that (micro-) evolution proceeds chiefly via *cis*-regulatory change (Wittkopp *et al.,* 2004). The *C.h.SOS1* promoter was about 5-fold more active than the corresponding one from *A. thaliana*, again in conformity with this hypothesis, although the difference in wild-type transcript levels is more than 5-fold, that is, about 15-fold. The *C.h.VATD* promoter, on the other hand, was only 2-fold more active than the *A.t.VATD* one, whereas the wild-type *C.h.VATD* transcript level was about 50-fold higher than that of *A.t.VATD*, suggesting that evolutionary mechanisms other than alteration of *cis*-regulatory sequences must have played a dominant role here. A plausible candidate mechanism would be copy number expansion, such as shown for, e.g., the heavy metal tolerance gene *HMA4* in the heavy metal hyperaccumulators *Noccaea caerulescens* (Lochlainn *et al.,* 2011) and *A. halleri* (Hanikenne *et al.,* 2008), and for *MTP1* in *A. halleri* (Shahzad *et al.,* 2010). It would be very interesting to check the copy numbers of *VATD* and other salt tolerance candidate genes in the halophytes and glycophytes under study here in the near future. In general, both gene copy number

expansion, either through tandem replication due to unequal cross-over, or otherwise, and altered *cis*-regulation play major roles in the (micro-) evolution of high-level heavy metal tolerance in metallophytes, the latter having been suggested to serve as a general model for plant evolutionary genomics (Hanikenne and Nouet, 2011). However, most metallophytes are, from the evolutionary viewpoint, relatively young, in comparison with halophytes (Ernst, 1974; Rozema and Schat, 2012). It is also conceivable, therefore, that structural changes at the protein level might have contributed to the evolution of salt tolerance in halophytes or, in other words, that halophytes might possess 'unique salt tolerance genes'. A possible example of such a unique gene might be *T.s.HKT1;2*. There are two *HKT1* isomorphs in *T. salsuginea*, *T.s.HKT1;1* and *T.s.HKT1;2*, whereas *A. thaliana* has only one, *A.t.HKT1*. As shown by Ali *et al.,* (2012), *T.s.HKT1;1* is a Na-specific transporter, like *A.t.HKT1*, whereas *T.s.HKT1;2* showes K specificity even in the presence of NaCl, which appeared to be due to two acid substitutions in the protein. The same authors provided strong arguments that *T.s.HKT1;2* does contribute to the high salt tolerance level in *T. salsuginea*, in comparison with *A. thaliana*, implying that *T.s.HKT1;2* might indeed represent a unique salt tolerance gene, since it is not present in the glycophyte reference species, *A. thaliana*. On the other hand, many other glycophytes possess *HKT1*-like transporters, of which at least some with a considerable K preference, and it can not be excluded that one or more of them might have been 'lost from *A. thaliana*', rather than 'acquired by *T. halophila*'. Moreover, there is no evidence that *T.s.HKT1;2* has been evolved under the pressure of high salinity. In any case, many investigators use transgenes of halophyte origin in their attempts to improve salt tolerance in glycophytic hosts, which reflects the belief that halophytes could have, in terms of salt tolerance, structurally better proteins than glycophytes (Rozema and Schat, 2012). However, there is no experimental evidence in favor of this idea. On the contrary, in so far orthologous transgenes from halophytic and glycophytic origin have been compared at all, their effects on the host were not significantly different (Chang-Qing *et al.,* 2008; Li *et al.,* 2008).

In chapter 4 it has been shown that *T.b.HKT1;2*, which is orthologous with *T.s.HKT1;2*, complemented the salt-hypersensitive *A.t.hkt1* mutant when expressed under the *A.t.HKT1* promoter. This finding is highly remarkable, since the *A.t.HKT1* promoter is expected to be active in the xylem parenchyma (Davenport *et al.,* 2007)

and, therefore, that expressing *T.b.HKT1;2* under the *A.t.HKT1* promoter could lead to K resorbtion from the xylem, which is unlikely to promote salt tolerance. In any case, there was definitely complementation of the mutant, as shown by the restoration of wild-type-level foliar water contents under salinity exposure. The mechanism behind this complementation remains elusive, and deserves further study.

As outlined above, it seems that halophytes and glycophytes basically use the same genes to cope with salinity, but express them, or at least a subset of them, in a different way. Although we did obtain indications that salt tolerance at halophyte level requires enhanced expression levels of genes involved in Na and K transport and homeostasis, we still lack the direct evidence. Throughout the period of this PhD project there was no time left to establish the salt tolerance of the T_1 lines with the strong *C. x hollandica NHX1* and *SOS1* promoters. This should be one of the first things to do in a follow-up study. In general, the literature barely provides direct evidence of a role for Na^+/K^+ transporters or other candidate salt tolerance genes in halophytes. Admittedly, a lot of investigators case over-expressed canditate salt tolerance genes, either from halophytic or glycophytic origin, in glycophytic hosts, and claimed improved salt tolerance in the transgenic lines (Ashraf and Akram, 2009; Rozema and Schat, 2012; for a survey). However, regardless of the question after the validity of the phenotyping methodology (Flowers and Colmer, 2008), these results as such do not prove that the superior levels of salt tolerance in halophytes, in comparison with glycophytes, would rely on naturally enhanced expression levels of these genes. First, apart from this thesis, comparisons of the expression patterns of these genes between halophytes and glycophytes are barely available. Second, in virtually all of the transgenic experiments, the transgenes were expressed under the constitutive, non-tissue-specific 35S-CMV promoter, which may strongly hamper gene functioning, in extreme cases even leading to a decrease in salt tolerance of the host, owing to incorrect cell or tissue specificity (Møller *et al.,* 2009). Third, even when correct over-expression, e.g. under a natural halophyte promoter, of a transgene would not improve salt tolerance in a glycophytic host, then it is still possible that it does contribute to the superior salt tolerance in a halophyte, because gene functioning can strongly depend on the presence or absence of other factors in the genetic background. Therefore, direct evidence of the role of candidate salt tolerance genes in halophytes requires their silencing in a halophyte genetic background. Thus far this

has exclusively been done for *SOS1* in *T. halophila*, which is presently the only genetically accessible halophyte model (Oh *et al.,* 2007). It would be desirable to have an array of genetically accessible halophytes of different families, in the first place because the mechanisms and molecular determinants of salt tolerance are probably subject to phylogenetic bias (Flowers and Colmer, 2008). Second, *T. halophila/salsuginea* might not represent an ideal halophyte model (chapter 3, Rozema and Schat, 2012).

In conclusion, the physiological and genetic determinants of high-level salt tolerance in halophytes are largely unknown. In part, this is owing to the fact that high-level salt tolerance is usually a species-wide, or even genus-wide trait, which precludes (candidate) gene identification via intra-specific comparison, co-segregation analysis, or QTL mapping (Rozema and Schat, 2012). Furthermore, inter-specific comparisons of gene expression patterns between related halophytes and glycophytes are almost completely lacking, which is a major omission of salt tolerance research thus far. Full transcriptome comparisons are thus far only possible between *T. halophila* and *A. thaliana*. It would be desirable to have more possibilities for full transcriptome comparisons, which should be made possible through modern 'deep sequencing' techniques. Of course, full genome comparisons will be useful too. Finally, to characterize differentially expressed candidate genes, it would be desirable to have an array of genetically accessible halophytes. The development of suitable protocols to genetically transform halophytes is urgently required.

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Chapter 6

Summary

Salt tolerance in halophytes is a poorly understood and a complex trait, involving alterations of the uptake and plant-internal transport and compartmentalization of Na and K at the levels of organs, tissues, cells and organelles, as well as compatible solute synthesis and, occasionally, morphological/anatomical adaptations. Studies in glycophytes, mainly *Arabidopsis thaliana*, have identified a number of genes that are essential for a wild-type salt tolerance level, such as *SOS1*, *SOS2*, *SOS3*, *HKT1*, *NHX1*, and the vacuolar proton pumps, *VAT* and *PPA*. It is often assumed that halophytes and glycophytes basically use same set of genes to cope with salinity, but express at least a subset of them in a different way. However, the latter genes, i.e. those that make the difference between salt tolerance at halophyte and at glycophyte level, have not been identified thus far, apart from *SOS1* in *Thellungiella halophyla*. This is mainly owing to an overall lack of comparative studies on gene expression patterns between halophytes and glycophytes.

In chapter 2 we compared salt tolerance and expression of *SOS1*, *HKT1*, *NHX1* and *VATD* (subunit-D of the vacuolar proton ATPase) among four Cochlearia species, of which two halophytes (*C. anglica* and *C. x hollandica*), a more or less salt tolerant glycophyte (*C. danica*), and a metal-tolerant glycophyte (*C. pyrenaica*). In agreement with the mean soil salinity levels in their natural habitats, their salt tolerance, estimated from the relative growth rate over a series of salt concentrations in the nutrient solution, decreased in the order *C. anglica* > *C. x hollandica* > *C. danica* > *C. pyrenaica*. Only *C. anglica* and *C. x hollandica* remained green and vital at 200 mM NaCl, which is often used as a criterion for being a halophyte. *HKT1* expression in the root correlated well with the species' salt tolerance levels, decreasing in the same order. In case of the other genes, the highest expression levels were found either in *C. anglica* or *C. x hollandica*, except for *NHX1* in shoots. Overall, our results are in agreement with the hypothesis that salt tolerance in halophytes relies, at least in part, on enhanced expression of a subset of the genes that are responsible for wild-type-level salt tolerance in *Arabidopsis thaliana* or other glycophytes.

To corroborate the role of the Na^+/H^+ antiporters, *SOS1* and *NHX1* and the vacuolar proton ATPase, *VAT*, in salt tolerance in other Brassicaceae family members, we also compared salt tolerance and these genes expression levels in *C. x hollandica*, *C. danica, Thellungiella botschantzevii* (ecotype Saratov), *Brassica oleracea*, *Thlaspi arvense* and *Arabidopsis thaliana* in an additional experiment, described in chapter 3. All the species were exposed to 200 mM of NaCl for three weeks, and their salt tolerance levels were inferred from the degrees of salt-induced visible damage, i.e. chlorosis, necrosis, enhanced senescence, and mortality. Based on these criteria salt tolerance decreased in the order *C. x hollandica* > *C. danica*/*T. botschantzevii* > *B. oleracea* > *T. arvense* > *A. thaliana*. The highest expression levels of *NHX1, SOS1* as well as *VATD* were consistently found in the most salt tolerant species, *C. x hollandica*, both in shoots and roots, and both in control plants and salt-treated ones. Salt-imposed induction of *NHX1* was observed in *C. danica* (shoot and root) and *B. oleracea* (shoot). *SOS1* was up-regulated by salt treatment in the shoots of *C. x hollandica* and *C. danica*, and *VATD* in the shoot of *T. arvense*. To assess the contribution of altered *cis*-regulation in the strongly enhanced expression levels of these genes in *C. x hollandica, NHX1* and *SOS1* from *A. thaliana* and *C. x hollandica*, or *GUS*, were expressed in *A. thaliana*, under the natural *NHX1, SOS1* and *VATD* promoters from *C. x hollandica* and *A. thaliana*, respectively. It appeared that the *C. x hollandica NHX1* and *SOS1* promoters were much more active than the corresponding ones from *A. thaliana*. The *C.h.VATD* promoter, however, was only two-fold more active than the *A.t.VATD* promoter, suggesting that the superior expression levels of *NHX1* and *SOS1*, but not that of *VATD*, in *C. x hollandica* may be largely explained by altered *cis*-regulation.

In chapter 4, *HKT1* from *A. thaliana* and *HKT1;2* from *T. botschantzevii* and *T. salsuginea* were expressed under the *A.t.HKT1* promoter, the 35S-CMV promoter and the *T.s.*- and *T.b.HKT1;2* promoters, in the *A. thaliana hkt1* mutant, which is strongly compromised in salt tolerance. Expression under the *T.s.* and *T.b.* promoters did not yield any significant expression, possibly because of the lacking of an essential upstream response element, and thus failed to complement the mutant. When expressed under the *A.t.HKT1* promoter, both *A.t.HKT1* and *T.b.HKT1;2* fully complemented the mutant, in that their expression restored a wild-type-like salt tolerance level. This is remarkable, because A.t.HKT1 is a Na-specific transporter,

responsible for Na retrieval from the xylem, whereas T.b./T.s.HKT1;2 is a K-transporter, involved in K uptake.

Samenvatting

Zouttolerantie bij halofyten is een complex en slecht begrepen fenomeen. Halofyten en glycofyten worden verondersteld verschillend te zijn met betrekking tot de opname, het transport, de allocatie en de sub-cellulaire compartimentering van Na en K, maar ook wat betreft het vermogen om 'compatibele' organische osmotica te synthetiseren. Sommige halofyten vertonen ook morfologisch-anatomische aanpassingen, zoals zoutharen of zoutklieren. Onderzoek aan glycofyten, vooral *Arabidopsis thaliana*, heeft een aantal genen aan het licht gebracht die essentieel zijn voor een wild-type zouttolerantie niveau, zoals *SOS1*, *SOS2*, *SOS3*, *HKT1*, *NHX1*, en de vacuolaire protonpompen, *VAT* en *PPA*. Over het algemeen wordt aangenomen dat halofyten en glycofyten dezelfde genen gebruiken om zich aan te passen aan hoge zoutgehalten, maar dat halofyten ten minste een deel van deze genen sterker tot expressie brengen. Echter, met uitzondering van *SOS1* bij *Thellungiella halophyla,* zijn de genen die bijdragen aan het verschil in zouttolerantie tussen halofyten en glycofyten nog niet geïdentificeerd. Dat is vooral te wijten aan een gebrek aan studies waarin de genexpressiepatronen van halofyten en glycofyten op directe wijze vergeleken zijn.

In hoofdstuk 2 werden de zouttolerantie- en de expressieniveaus van *SOS1*, *HKT1*, *NHX1* en *VATD* (subunit-D van het vacuolaire proton *ATPase*) onderling vergeleken tussen vier Cochlearia soorten, waarvan twee halofyten (*C. anglica* and *C. x hollandica*), een min of meer zouttolerante glycofyt (*C. danica*) en een metaal-tolerante glycofyt (*C. pyrenaica*). In overeenstemming met het gemiddelde zoutgehalte van de bodem in hun natuurlijke omgeving, nam de zouttolerantie, afgemeten aan het effect van zout op de relatieve groeisnelheid, af in de volgorde: *C. anglica* > *C. x hollandica* > *C. danica* > *C. pyrenaica.* Alleen *C. anglica* en *C. x hollandica* bleven groen en vitaal bij 200 mM NaCl, het algemeen aanvaarde criterium voor een halofyt. De expressie van *HKTI* nam in dezelfde volgorde af. De hoogste expressieniveaus van de overige genen werden gemeten bij ofwel *C. anglica*, ofwel *C. x hollandica*, behalve de expressie van *NHXI* in het blad. Deze resultaten zijn grotendeels in overeenstemming met de hypothese dat zouttolerantie bij halofyten (deels) afhankelijk is van een verhoogde expressie van een subset van de genen die essentieel zijn voor het wildtype-niveau van zouttolerantie bij glycophyten.

Om meer aanwijzingen te verkrijgen betreffende de rol van de Na^+/H^+ antiporters, *SOS1* en *NHX1*, en het vacuolaire proton *ATPase*, *VAT*, bij de zouttolerantie van andere leden van de familie der Kruisbloemigen (Brassicaceae), werden in een aanvullend experiment, beschreven in hoofdstuk 3, de zouttolerantie en de genexpressieniveaus vergeleken tussen *C. x hollandica*, *C. danica, Thellungiella botschantzevii* (ecotype Saratov), *Brassica oleracea*, *Thlaspi arvense* en *Arabidopsis thaliana.* Gedurende drie werden alle soorten blootgesteld aan 200 mM NaCl. De zouttolerantie werd geschat op basis van de mate waarin deze behandeling zichtbare schade veroorzaakte, zoals chlorose, necrose, of versnelde veroudering van het blad, of sterfte van de plant. Op grond van die criteria nam de zouttolerantie af in de volgorde: *C. x hollandica* > *C. danica*/*T. botschantzevii* > *B. oleracea* > *T. arvense* > *A. thaliana*. De hoogste expressieniveaus van *NHX1, SOS1,* en *VATD* werden gevonden bij de meest zouttolerante soort, *C. x hollandica*, zowel in de bladeren als in de wortels, en zowel onder controlecondities, als onder blootstelling aan zout. Een door zout geïnduceerde verhoging van de expressie van *NHXI* werd waargenomen bij *C. danica* (in bladeren en wortels) en *B. oleracea* (in bladeren). Inductie door zout van *SOSI* werd gemeten in de bladeren van *C. x hollandica* en *C. danica*, en van *VATD* in de bladeren van *T. arvense*. Om de bijdrage van eventuele veranderingen in de genpromotors van *HHX1*, *SOS1* en *VATD* aan de sterk verhoogde expressieniveaus van deze genen in *C. x hollandica* vast te stellen, werden *NHX1* en *SOS1* van *A. thaliana* en *C. x hollandica*, of *GUS*, tot expressie gebracht in *A. thaliana*, zowel onder de natuurlijke *NHX1, SOS1* en *VATD* promotors van *C. x hollandica*, als die van *A. thaliana*. Het bleek dat de *C. x hollandica NHX1* en *SOS1* promotors vele malen actiever waren dan die van *A. thaliana*. Echter, de *C.h.VATD* promotor was slechts tweemaal zo actief als de *A.t.VATD* promotor. Deze resultaten suggereren dat de sterk verhoogde expressieniveaus van *C.h.NHX1* en *C.h.SOS1*, maar niet dat van *C.h.VATD*, geheel of grotendeels verklaard kunnen worden door veranderingen in de *cis*-regulerende sequenties van die genen.

In een ander experiment (hoofdstuk 4) werden *HKT1* van *A. thaliana* en *HKT1;2* van *T. botschantzevii* en *T. salsuginea* tot expressie gebracht in de extreem zoutgevoelige *A. thaliana hkt1* mutant, onder de *A.t.HKT1* promotor, de 35S-CMV promotor, zowel als de *T.s.*- en *T.b.HKT1;2*-promotors. De *T.s.*- en *T.b.HKT1;2* promotors vertoonden geen detecteerbare activiteit, vermoedelijk vanwege het

ontbreken van een essentieel upstream respons element, en de constructen met deze promotors complementeerden, in overeenstemming hiermee, de mutant niet. Expressie onder de *A.t.HKT1* promotor, zowel van *A.t.HKT1* als *T.b.HKT1;2,* complementeerde de mutant volledig. Dit is uiterst merkwaardig, omdat A.t.HKT1 een Na-specifieke transporter is, verantwoordelijk voor de resorptie van Na vanuit het xyleem, terwijl de T.b./T.s.HKT1;2 een K-specifieke transporter is, betrokken bij de opname van K in de wortel.

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