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NECLA PEHLİVAN GEDİK

LI SUN

NEELAM MISHRA

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Defensive manoeuvres of *NHX1* and *SOS1* co/overexpression in plant salt tolerance

Necla PEHLİVAN^{1*}, Li SUN², Neelam MISHRA³

¹Department of Biology, Recep Tayyip Erdogan University, Rize, Turkey

²Department of Biology, Texas Tech University, Lubbock, TX, USA

³Department of Botany, St. Joseph's College, Bangalore, Karnataka, India

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Abstract: Excessive accumulation of salts is one of the most important factors affecting the production quality of plants. The difficulty to develop salt stress tolerant crops has prevented the security of the global food system. The classical crop breeding approach that uses random mutagenesis/recombination is time-consuming in the face of an ever-increasing human population and extreme weather pattern shifts. Today, technologies introducing foreign genetic material into plants have the best potential to assist in crop breeding improvement due to its high efficiency, accuracy, low risk of off-target effects, and minimal labour compared to classical methods. The notion of Na⁺/H⁺ antiporters in this technology has increased rapidly in recent years with numerous successful examples. In the awe of rapidly developing modern techniques, which do not yet exist at the required scale to face the aforementioned challenges, the current knowledge of the co/overexpressing Na⁺/H⁺ antiporters (*NHX1* and *SOS1*) will be explored as a potential method to produce staple crops with greater resilience to over concentrated ions and abnormally high osmotic stress.

Key words: Arabidopsis, multiple ectopic gene expression, salt stress, gene pyramiding, sodium/proton antiporter

1. Introduction

1.1. Worldwide stress and trend

Overuse of inorganic fertilizers (201.66 million tonnes total N + P₂O₅ + K₂O use by the end of 2020 (FAO, 2009) and rising sea levels (0.095 mm/year²) due to climate change continue to impact the global ecosystem. Moreover, population growth (predicted to rise 40% by 2050) is outpacing agricultural production which would need to increase by 70% all over the world and arable land area is becoming unsuitable (less than 0.20 ha per capita by 2050) for global food, fibre, and forage supply (FAO, 2009; Fischer and Connor, 2018). Crops should be grown reasonably well on limited land under various drastic environmental conditions such as electromagnetic field, water deficit, flooding, salinity, high or low temperature, UV light, and toxic contaminants to keep pace with growing demands. To achieve this goal, various different perspectives/methods have been explored and a big path has been taken during the past two decades (Mittler and Blumwald, 2010). Salt stress given overall ecological impacts, is one of the most detrimental and widespread.

1.2. Overview of salt stress

During natural process, plants in the ocean continued to maintain their ability to survive in saline conditions, while

those adapted to terrestrial life have lost their ability to tolerate high saline conditions and became glycophytic. Furthermore, major crop cultivars that feed the world population today are predominantly glycophytic land plants (natrophiles) and these plants are sensitive to high salt concentrations (Zhang and Shi, 2013). This is partially due to the long domestication period aimed at traits other than salt tolerance when most soils were low in salt on earth (mainly Na⁺) (Cheeseman, 2015). Domestication that did not target salt tolerance decreased the very low salt tolerance of these glycophytes. This domestication period has also wiped out most of the genetic diversity in our crop species thus there is little potential for plants evolving independently and adapt to saline environment (Cheeseman, 2015).

1.3. Plants' response to salt stress

The chemical compound NaCl is the most significant osmoticum in salt stress. Keeping the NaCl concentrations in the cell cytoplasm at low levels by regulating the ion flux, is therefore vital for accumulating basic monovalent ions such as K⁺ (Flowers et al., 2014). It will in turn maintain the electrostatic equilibrium.

Two interconnected strategies are being used for intra- and intercellular Na⁺ homeostasis: Na⁺ export

* Correspondence: necla.pehliwan@erdogan.edu.tr

across the plasma membrane or vacuolar/endosomal compartmentalization of Na^+ (Adem et al., 2015). Mainly H^+ electrochemical potentials (by P-type or V-type ATPases or pyrophosphatase) regulates Na^+ flow across plasma, tonoplast, or endosomal membranes (Flowers and Yeo, 1995). While Na^+ influx is controlled by either NHX family of cation/ H^+ transporters (Apse et al., 1999) or nonselective cation channels (NSCCs), or high-affinity K^+ transporters (HKTs) (Zhang et al., 2010), Na^+ efflux is controlled by Salt-Overly-Sensitive1 (SOS1) on the plasma membrane, which is a close relative of bacterial and mammalian Na^+/H^+ antiporters (Niu et al., 1995). Among them SOS1 and NHX1 together facilitate protoplasmic viability to control net Na^+ concentrations in the root xylem (Munns and Tester, 2008).

2. Breeding strategies to improve salt stress tolerance: classic and modern (trans-gene delivery)

Unlike conventional breeding, chemical priming, or methods of char-application that enriches the soil, genetic engineering techniques' pros outweighs the cons of salt stress tolerance. Though the real sensor(s) has/have been claimed not found for overconcentrated cellular Na^+ for a long time (Ismail, 2014), there is a brand-new evidence harmonizing Na caused ionic/osmotic stress sensing and Ca^{2+} signaling in monocation-induced [Ca^{2+}] increases 1 (moca1) Arabidopsis mutants (Jiang et al., 2019). A joint group from US and China proposed a new model valid for salt cation sensing system which has not been identified before and/or similar to any known previous systems reported in any kingdom: Na^+ ions bind to a specific member of sphingolipid class (glycosyl inositol phosphorylceramides) depolarize the membrane potential to allow activation of Ca^{2+} channels, subsequently Ca^{2+} influx. Current developments to elucidate the response to salt at biochemical and molecular levels have made it possible to produce salt tolerant crops along with the help of well-documented genetic transformation technologies (e.g., Agrobacterium mediated gene transfer, particle bombardment with gene gun, or DNA uptake into protoplasts) (Rao et al., 2017). Production of salt tolerant transgenic lines has been successfully pursued by genes encoding either transcription factors, proton pumps (Li et al., 2018), ion transporters (Pehlivan et al., 2016), etc. Despite these innovations, the engineering of a salt tolerant plant takes at least 5 years (Flowers and Muscolo, 2015), while large-scale roll out could only be possible within a decade at least.

3. Basic characteristics of Na^+/H^+ antiporters in cell

Stable ion homeostasis in cytoplasm is largely done through the entire ion transport web in plant cells including Na^+/H^+ antiporters on lipid membranes (Figure 1). Phylogenetic

analysis showed that these electroneutral or electrogenic membrane proteins exist in all the kingdoms from the bacteria (CPA2 family of NapA, NhaA), yeast (SOD2, Nha1) (Apse et al., 1999) human (NHE1) (Okazaki et al., 2019) and to the plants responsible for pH, osmoregulation-intracellular ion balance, exchanging of sodium and protons across the plasma membrane by 1:1 or 2:1 stoichiometry (cation/proton antiporters 1 and 2 family-CPA, CPA2). These pumps in the NHX family (>200 Na^+/H^+ in the databases) undergoing conformational changes with different pH profiles are important determinants of Na^+ ions' movement in the cell during salt tolerance (Zhang and Shi, 2013). They also control the cell cycle regulation and proliferation, cell bioenergetics, transport of important biochemical signal molecules from the place where they are synthesized and packed (golgi) to specific locations (vesicle traffic), the removal of target cellular material from the cell. In addition to that, they trigger the active accumulation of K^+ ions in the vacuoles (K^+ balance) to regulate the continuity of the turgor and the sustainability of cell growth. The Arabidopsis thaliana SOS1 (AtSOS1) is found to be evolutionarily related to the bacterial NhaP (Okazaki et al., 2019). More than 38 Na^+/H^+ pumps were further reported in the Arabidopsis genome with the vacuolar AtNHX1 and membrane SOS1 proteins being the most studied pump systems (Waditee et al., 2006) (Figure 1).

3.1. Milestone studies of AtNHX1 in plants

The AtNHX1 (*Arabidopsis thaliana* sodium/proton Na^+/H^+ antiporter) is a secondary ion transporter that has been cloned by Gaxiola and his team 20 years ago. It is the most abundant and the most studied vacuolar Na^+/H^+ antiporter (Gaxiola et al., 1999). Yeast (*Saccharomyces cerevisiae*) vacuolar membrane transporter ScNHX1 was found to be identical in function to Arabidopsis AtNHX1 which was reported by the same group afterwards. The AtNHX1 and AtNHX2 are listed as dominant isoforms of NHX gene cluster. They were shown to be highly expressed in vascular tissue, meristems, and guard cells. The AtNHX1 triggers the uptake of water into the cells by sequestering sodium ions into the vacuole (alkalization of the vacuole). Therefore, it reverses the Na^+ toxicity in the cytoplasm, and maintains osmotic balance (Glenn et al., 1999). The ABA and osmotic stress induction of AtNHX1 expression prove that it indeed is responsible for osmotic regulation.

Additionally, AtNHX1 triggers another transporter and catalyses the compartmentalization of K^+ ions (by transporting K^+ ions from the cell to the vacuole, which acts as a pool for the cytosolic K^+). Thus, it regulates mineral intake as well as keeping the intracellular K^+ concentration high. The transporter also affects the transpiration rate by controlling stomata movements (Leidi et al., 2010) and vesicle trafficking in cell (Hamaji et al., 2009). These

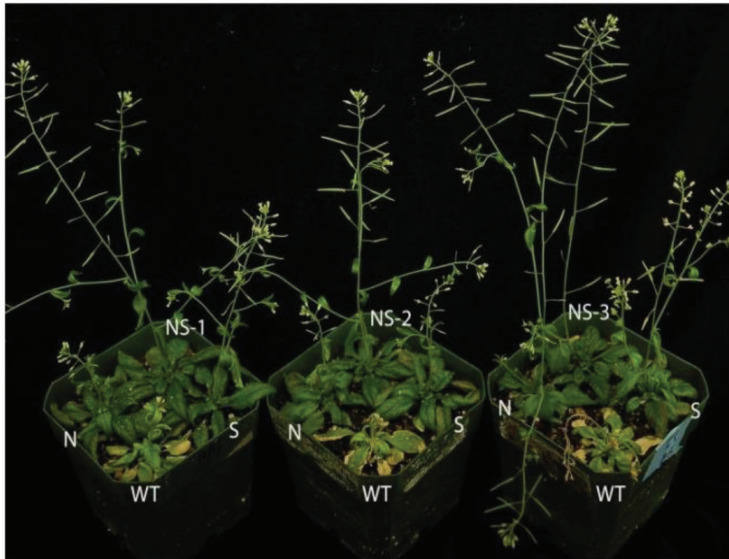
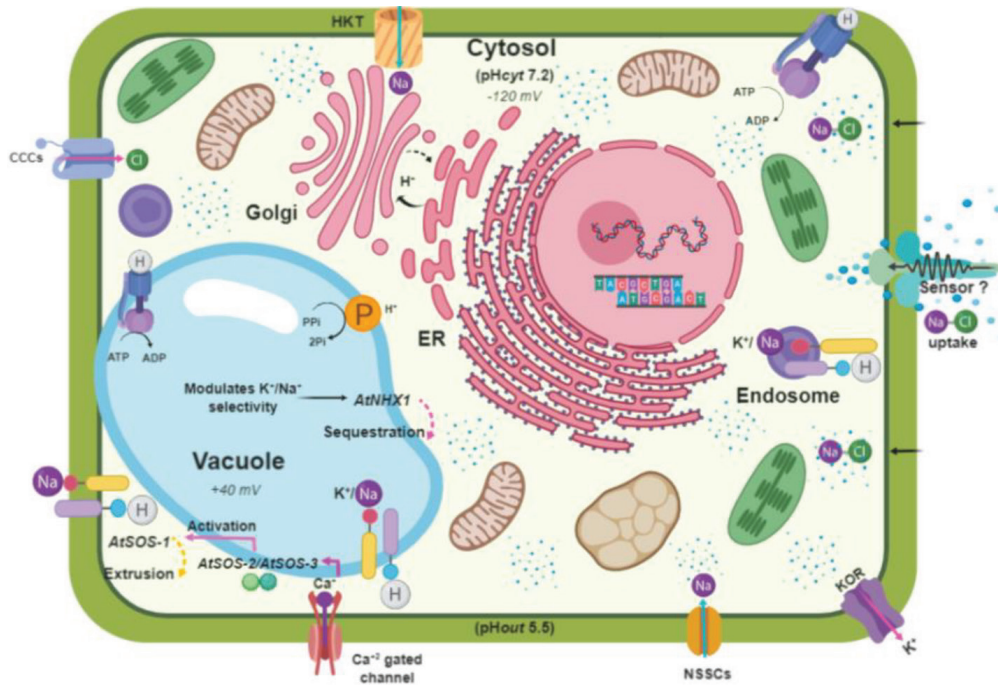


Figure 1. Upper panel, a simple cartoon illustrating Na^+ homeostasis regulated by *AtNHX1* and *SOS-1* transporters in plant cell (created with BioRender.com). Lower, phenotypes showing *AtNHX1/SOS1*-overexpression in *Arabidopsis* under 250 mM NaCl . WT, wild-type; N, *AtNHX1*-overexpressing line; S, *SOS1*-overexpressing line; NS1-2 and 3, independent *AtNHX1/SOS1*-overexpressing lines (Pehlivan et al., 2016).

capabilities of the *AtNHX1* appeared when overexpressed in *Arabidopsis* (Apse et al., 1999).

AtNHX1 protein has a shorter hydrophilic C-terminal tail (in contrast to *SOS1*), and many other different homologous proteins associated with it in the *Arabidopsis* genome. This has caused difficulty in finding homologue of Na^+/H^+ carrier in *Arabidopsis AtNHX1* (Zhang and

Shi, 2013). In addition to that, a phosphorylated form of *AtNHX1* could not be detected in the cell (Feki et al., 2011; Jia et al., 2018).

All this research was determined for *AtNHX1* after its discovery in 1999 and it has been adapted to targets for agricultural production by overexpressing this gene into yeast, *Arabidopsis* and *Festuca* (Qiao et al., 2007; Hamaji

et al., 2009; Xiaowei et al., 2018), cotton (He et al., 2005), switchgrass (Huang et al., 2017) wheat (Xue et al., 2004), *Fagopyrum esculentum* (Chen et al., 2008), soybean (Li et al., 2010), tomato (Zhang and Blumwald, 2001; Leidi et al., 2010), brassica (Zhang et al., 2001), peanut (Asif et al., 2011), jatropha (Jha et al., 2013), tobacco (Zhang et al., 2008; Zhou et al., 2011), petunia (Xu et al., 2009), rice, maize (Ohta et al., 2003; Chen et al., 2007; Amin et al., 2016) cowpea (Mishra et al., 2014; Mishra et al., 2015), mustard (Rajagopal et al., 2007), cumin (Pandey et al., 2016), castor (Patel et al., 2015), mungbean (Sahoo et al., 2016; Kumar et al., 2017), potato (Charfeddine et al., 2019). When it is overexpressed in *Brassica napus*, *Fagopyrum esculentum* and *Lycopersicon esculentum* the growing capacity, flowering, and seed production rate increased even under 200 mM NaCl. The toxic effects of the salt were eased by proline accumulation and the content of essential nutrients was not affected by the high salt concentration (Zhang and Blumwald, 2001; Zhang et al., 2001; Chen et al., 2008). In *Gossypium hirsutum*, overexpression of the gene provided more biomass, better quality, and more cotton fibre than wild-type cotton plants, with increased nitrogen assimilation and photosynthetic performance (He et al., 2005). The damage ratio was found to be less in transgenic plants when *OsNHX1* was overexpressed in *Oryza sativa* (Chen et al., 2007). In 2004, a similar result was reported with the *BnNHX1* gene of canola and tobacco. While wild-type plants were not robust, transgenic plants overall grew better under 200 mM salt stress, and no physiological damage was detected in flowers and seeds (Wang et al., 2004). The *AeNHX1* (*Agropyron elongatum NHX1*) gene in another work was also transferred to Arabidopsis and a grass species, *Festuca arundinacea*, that showed improvements in osmotic balance, photosynthesis, and salt tolerance (Qiao et al., 2007).

Same family member wheat *TaNHX2* was overexpressed in alfalfa (*Medicago sativa*). The activity of the tonoplast H^+ -ATPase, H^+ -phosphophosphatase (PPase) along with ATP-dependent proton pumps in the generated transgenics has been found to be higher than that of the wild plants, repressed the salt stress induction of this gene (Zhang et al., 2014). Although most of these studies confirmed that *AtNHX1* can improve salt tolerance, one report claimed otherwise by showing counterevidence (Yang et al., 2009). They created *AtNHX1* + *SOS3*, *SOS2* + *SOS3*, and *SOS1* + *SOS2* + *SOS3* multi gene expression cassettes along with single *NHX1* expressing ones. Yet, they claimed no significant increase in salt tolerance of more than 100 independent Arabidopsis transgenics overexpressing only *NHX1* in their assay conditions compared to WTs contrary to vast majority of all studies using *SOS1* and/or *NHX1* show positive results. The argument in their work was basically different lab testing conditions could

cause different tolerance levels or *NHX1* as antiporter has a modest effect in tolerance.

3.2. Milestone studies of *SOS1* in plants

There are 3 mechanisms that cooperate with each other to inhibit the Na^+ accumulation in the cytoplasm: inhibition of Na^+ uptake in the cell, activation of Na^+ efflux from the cell, and Na^+ accumulation in the vacuoles (Niu et al., 1995). The *SOS1* that serves as a Na^+/H^+ exchanger on the plasma membrane, which sends Na^+ directly out of cell via the second mechanism (Shi et al., 2000; Qiu et al., 2002). It is involved in sodium loading and unloading during long distance transport in xylem and phloem (Zhu, 2003) and provides salt tolerance by limiting Na^+ toxicity in the xylem and stem. Plants are not able to simply discard and get rid of Na^+ from each cell, but need to systematically relocate sodium from the leaves to the roots.

Retrieving or loading Na^+ into or from the xylem depends and also differs under severe or mild salt conditions (Shi et al., 2002; Shi et al., 2003). Except for Na^+ tolerance, the mechanism balances pH, membrane vesicle traffic, vacuole functions, and interaction with ABA (Ohta et al., 2003). Salt Overly Sensitive 1 (*SOS1*) in this context is reported to be important for Na^+ and K^+ balance of the cell (Wu et al., 1996). Forward genetics was first used to identify the gene in Arabidopsis salt-hypersensitive mutants (Zhu, 2003). Expression analysis showed that it is stimulated by salt stress yet not by ABA in vegetative tissues, root tips, and parenchyma cells around the vascular tissue of stems in Arabidopsis (Shi et al., 2000; Shi et al., 2002; Guo et al., 2009; Hamam et al., 2016). Following identification of the gene in Arabidopsis (*SOS1* overexpression alone led to salt tolerance in Arabidopsis) its homologs from rice (Martinez et al., 2007), wheat (Xu et al., 2008), tomato (*SISOS1*) (Olias et al., 2009) *Thellugiella halophila ThSOS1* (Oh et al., 2009), and soybean (Chen et al., 2017) were characterized. Furthermore, *sos1* mutation has been shown to be resistant to an herbicide (paraquat) that causes the formation of ROS, which has a role in oxidative stress tolerance (Zhang and Shi, 2013).

Key regulation pathway is Ca^{+2} dependent, however, regulation is also possible via ethylene (Quan et al., 2017), transcription factors *WRKY*, *NAC*, and *bZIP* (Ji et al., 2013) as well as ROS (Feki et al., 2017). Yue et al., (2012) reported that *SOS1* overexpression plants could tolerate salt stress by maintaining high K^+/Na^+ balance in transgenic tobacco plants. The *Oryza sativa OsSOS1* was reported to be the functional equivalent of *SOS1* which can suppress *atsos1*'s salt sensitivity. The *SOS1* plants showed inhibited development under the influence of high Na^+ and low K^+ . The *SOS1* homologue of Arabidopsis's close relative *Thellungiella halophila ThSOS1* was on the other hand found to be more tolerant to high salt concentrations (Kant et al., 2006). An inactivating mutation in *ThSOS1* gene

resulted in excessive sensitivity to salt in this halophyte (Oh et al., 2009). Sequence analysis showed that the SOS1 consisted of a fragment of approximately 700 amino acids encoding 12 transmembrane domains in the N-terminal portion, whereas the C-terminal part of the gene encodes a 127-kDa protein with a long hydrophilic cytoplasmic tail (Shi et al., 2000). The transmembrane portion of the protein exhibited significant sequence similarities with the animal, bacterial, and fungal plasma membrane Na⁺/H⁺ antiporters (Zhu et al., 1998). Double mutant analysis indicated that SOS1 is activated by SOS3 and SOS2 association followed by Ca⁺² signal for NaCl response (Qiu et al., 2002) or ethylene pathway (Quan et al., 2017), which suggested that they were in the same signalling pathway for salt tolerance. The SOS1 is the only plasma membrane protein that has been identified in plants that extrudes Na⁺ ions to the apoplast from the cytoplasm and is one of the best candidates for Na⁺ sensing due to its long tail residing in the cytoplasm. It has been first studied by Shi et al. (2000). Subsequently, it was characterized in wheat (Xu et al., 2008), tomato (Olias et al., 2009), the moss *Physcomitrella patens* (Fraile-Escanciano et al., 2010), rice (Martinez-Atienza et al., 2007), and *Festuca arundinacea* (Ma et al., 2013). Overexpression of *GmsSOS1* also showed increased salt tolerance in *Atsos1-1* mutant Arabidopsis plants (Nie et al., 2015). Rather than full length, truncated SOS1 (without C-terminal tail) also was shown to confer NaCl tolerance in *Triticum durum* (Feki et al., 2014).

4. Pyramiding approach: Why it is a trend?

The sustainability of agricultural production cannot be achieved by changing only a single feature pertaining to salt tolerance. It requires the multiple changes to the plant genome to most efficiently manipulate biological

traits (Cuartero et al., 2009). Single gene manipulation does not have much potential to combat stress (Capell and Christou, 2004). Tolerance in phenotype involves cumulative (may be synergistic) roles of several genes due to the fact that genetic resistance to high salinity contains several quantitative traits. Researchers have successfully used single genes to improve salt tolerance, but not at a high level of tolerance. Genomic integration of multiple genes (gene pyramiding) on the other hand offers stronger opportunities for improving salt tolerance compared to single gene transfer. There are, however, difficulties in getting stable lines to express every individual gene simultaneously at the desired level because of limited vector capacity, multiple promotor/terminator requirements, etc. This strategy had been trending until the creation of “golden rice”, which expressed stacked carotenoid biosynthesis genes simultaneously to produce pro-vitamin A in its endosperm (Ye et al., 2000).

4.1. Methods and outcomes of multiple gene expression

Since 1996, almost one-third of multi-gene-transferred plant biotech studies have been carried out using a single vector with different genes (Figure 2). Even though manipulating some pathways (e.g., lignin content engineering) or coordinating multiple enzymes are challenging due to the polygenic nature of many agronomical traits, multiple trans gene assembly has additional advantage and not many drawbacks except vectoral limitations and/or T-DNA delivery. Golden rice in this context is one of the oldest examples of gene pyramiding approach which carries 2 genes *psy* (phytoene synthase) from a perennial wild daffodil and *crtI* (phytoene desaturase) from a soil phytopathogenic bacterium to modulate beta-carotene biosynthesis (Ye et al., 2000). Several reports have further shown successful expression of multiple genes in tomato,

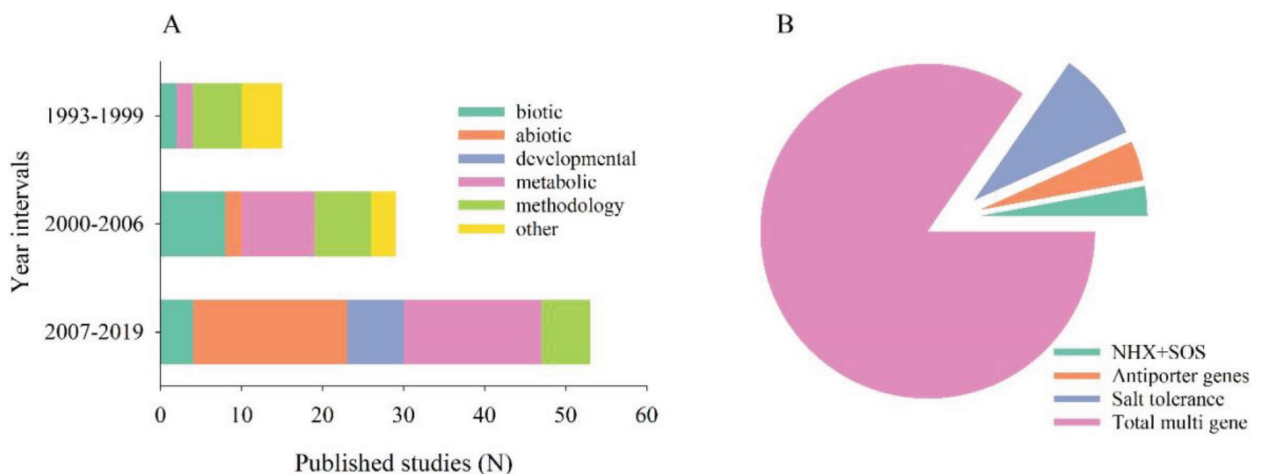


Figure 2. Published studies regarding multi gene co/overexpression in plants. A. Published studies with different goals (1993 to 2019). B. Proportion of multi gene co/overexpression studies using different genes for salt tolerance.

tobacco, Arabidopsis and Brassica in the last two decades (Wang et al., 2018).

Despite limitations in the gene pool that can be used in salinity tolerance, the design of vectors carrying several genes together has become routine in cloning (Schmidt et al., 2008). All of these methods have been successful in the past to express two or more genes simultaneously in different plant species (Naqvi et al., 2010).

Multiple gene transfer can be conducted in many ways: manual cross-pollination, sequential transformation, simultaneous transformation with different vectors carrying different genes (co-transformation), or transformation cassettes with several genes organized on a single binary vector (Rukavtsova et al., 2013). These genetic engineering studies aimed at growing tolerant plants are known as the most effective and economical approaches in improving salinity tolerance. This approach offers control of specific traits, without affecting the remaining characteristics of an elite genotype by transferring the relevant genes to the plant (Ashraf, 2010). Concurrent expression of *AtNHX1* and *SOS1* can be given as a successful example of multiple gene transfer to tolerate high NaCl (Pehlivan et al., 2016). Other examples of multiple gene transfer aimed salt tolerance are: cooverexpression of *AVP1* and *PP2A-C5* in Arabidopsis (Li et al., 2018), *sGS1:1*, and *OsGS2* cooverexpression in rice (James et al., 2018), cooverexpression of *PeDREB2a* and *KcERF* in transgenic lotus (Wang et al., 2018), cooverexpressing *ZxNHX* and *ZxVP1-1* in transgenic alfalfa (Kang et al., 2016), cooverexpression of *AVP1* and *AtNHX1* in cotton (Shen et al., 2015), cooverexpression of *HVA1* and *mtlD* gene in maize (Nguyen et al., 2013) and cooverexpression of salt responsive Protein 3-1 (*SaSRP3-1*) and *SaVHAc1* from *Spartina alterniflora L.* in rice (Biradar et al., 2018).

So far, gene transfer has frequently been used over the course of genetic manipulations. Engineered genes were mostly the genes that regulated important biochemical webs or functions in the final step of stress signalling pathways (i.e. Na^+/H^+ antiporters).

4.2. Genetic engineering of plants co-overexpressing *SOS1* and *NHX1* genes

The *NHX* pathway and *SOS* pathway might crosstalk (Zhu, 2003). However, this crosstalk might not be occurring at transcriptional level (Zhu, 2003). The H^+ -adenosine triphosphatase and the vacuolar membrane-bound proton pumps generate the proton electrochemical gradient needed for the crosstalk between *SOS1* and *AtNHX1* activities (Gaxiola et al., 2007). Vacuoles were determinants of this crosstalk as they are storage reservoirs for ion balance. The mechanism of *AtNHX1* must work in conjunction with other genes that limit the Na^+ entry or increase the excretion of Na^+ in order to reduce Na^+ concentration in the cell cytoplasm under extreme concentrations of Na^+ .

Zhu's group demonstrated that the Na^+ transporting capability of *AtNHX1* (tested from separated vesicles) is greatly dependent on *SOS2* protein by studying *sos2* mutant, which indicated potential regulation of *AtNHX1* by *SOS2* (Qiu et al., 2004). Given the active roles of these two proteins in salt tolerance, overexpression of gene combinations has been tried by researchers to obtain an inter-dependent effect for Na^+ removal in plants. In one of these examples, *AtSOS1+AtSOS2+AtSOS3* combination improved salt tolerance in Arabidopsis at 100–200 mM NaCl (Yang et al., 2009). On the other hand, Bhaskaran and Savithramma, 2011; Kumar et al., 2017; Zhao et al., 2006; Shen et al., 2015, and Pehlivan et al., 2016 showed *SOS1* and *AtNHX1* crosstalk with possible synergistic effects of pyramided gene (co)overexpression rather than single gene overexpression.

5. Debates and perspectives

The need for research and knowledge about the key genes in salt stress are possible with new discoveries, which may alleviate the issues to develop plants that are capable of surviving at sea salt level concentrations (Panta et al., 2014) or curbing global climate change. Despite advances in technology, stress tolerance development through co/over expression of gene(s) in the tolerance networks showed that quality and yield increase in field testing were extremely rare. The current peer reviewed literature suggests that trans gene-based studies were usually carried out in controlled conditions of plant growth rooms where only a few lines are tested under field conditions. As salt stress occurs in combination with more than one stress factor in real environments, tolerance cannot stand-alone by working on one gene or one condition. Nature should be mimicked by studying simultaneous stresses on major crops with the greatest acreage rather than single stress types. Even though we recognize limitations including public acceptance of the technology, we suggest that these technologies need to be rolled out much faster at larger scale for better sustainability.

The *AtNHX1* and *SOS1* pathways are potential candidates for engineering salinity tolerance to a higher extent yet it should be tried with tissue specific promoters that limit undesirable transgene expression as well as being active only in certain cell types. Except for NCL (which was homologous to animal's $\text{NCX Na}^+/\text{Ca}^{2+}$ channel) *moca1* and *SOS1* new candidates for Na^+ sensing should be added to the mix by comparing Na^+ binding motifs in mammalian ion channels and plants.

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