

# 1 **Engineering Salinity Tolerance in Plants: Progress and Prospects**

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## 26 27 28 **Abstract**

29 Soil salinity exerts significant constraints on global crop production, posing a serious challenge  
30 for plant breeders and biotechnologists. The classical transgenic approach for enhancing salinity  
31 tolerance in plants revolves by boosting endogenous defence mechanisms, often via a single gene

32 approach, and usually involves the enhanced synthesis of compatible osmolytes, antioxidants,  
33 polyamines, maintenance of hormone homeostasis, modification of transporters and/or  
34 regulatory proteins, including transcription factors (TFs) and alternative splicing events.  
35 Occasionally, genetic manipulation of regulatory proteins or phytohormone levels confers  
36 salinity-tolerance, but all these may cause undesired reduction in plant growth and/or yields. In  
37 this review, we present and evaluate novel and cutting-edge approaches for engineering salt  
38 tolerance in crop plants. First, we cover recent findings regarding the importance of regulatory  
39 proteins and transporters, and how they can be used to enhance salt tolerance in crop plants. We  
40 also evaluate the importance of halobiomes as a reservoir of genes that can be used for  
41 engineering salt-tolerance in glycophytic crops. Additionally, the role of microRNAs as critical  
42 post-transcriptional regulators in plant adaptive responses to salt stress are reviewed and their use  
43 for engineering salt-tolerant crop plants is critically assessed. The potentials of alternative  
44 splicing mechanisms and targeted gene-editing technologies in understanding plant salt-stress  
45 responses and developing salt-tolerant crop plants is also discussed.

46

47 **Keywords**

48 CRISPR/Cas9, halobiome, ion transporters, microRNAs, regulatory elements, salinity stress,  
49 transcription factors

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51

## 52 **1 Introduction**

53 Extreme soil salinity is one of the most destructive abiotic stresses for global agriculture as it can  
54 lead to the degradation of arable soils, particularly those that are heavily irrigated (Akram et al.  
55 2017; Kumar et al. 2017a). Over 800 million ha of land are estimated to be affected by salinity  
56 worldwide and 32 million ha of dryland agriculture is thought to be salt-affected (FAO 2015).  
57 Salinity stress drastically reduces agricultural productivity via adverse impacts on seed  
58 germination, plant growth and development, plant vigour and crop yields (Cheeseman 2015).  
59 The reduced plant growth caused by high soil salinity is due to salinity-induced water stress,  
60 oxidative stress, nutritional imbalances, ion toxicity, membrane disorganization, reduced cell  
61 division and expansion, disruption of key metabolic processes, and genotoxicity. The goal of  
62 most studies dealing with salinity tolerance is to boost the capacity of crop plants to maintain  
63 growth and productivity when cultivated on saline soils (Cao et al. 2018; Kumar et al. 2018). As  
64 salinization of cultivated land is an increasing global problem, understanding the biological  
65 impacts of salt exposure on plants and the development of salt-tolerant crop plants is urgently  
66 required (Munns et al. 2012; Latef et al. 2017; Nguyen et al. 2017). Of the various approaches  
67 that have been and are being utilized to enable crop production in salinity affected areas, crop  
68 improvement by breeding is perhaps one of the best strategies to accomplish this goal. The use of  
69 conventional plant breeding methods resulted in significant yield improvement in some crops  
70 when grown on saline soils and this resulted in the development of salinity-tolerant crops.  
71 However, conventional plant breeding approaches are most often lengthy, laborious and are  
72 dependent on access to germplasm with sufficient genetic variability (Wani et al. 2016). For  
73 some crops, genetic engineering strategies provide a viable alternative to conventional plant  
74 breeding and are now becoming more widely used throughout the world to produce salt-tolerant  
75 cultivars.

76 In the past few decades, plant genetic engineering approaches for the production of salt-  
77 tolerant plants revolved around manipulating single genes from a diverse range of metabolic  
78 pathways, including compatible-solute synthesis, ion-homeostasis and antioxidant  
79 synthesis/metabolism, as well as signaling/regulatory elements including transcription factors  
80 (TFs) (Cabello et al. 2014; Zhang et al. 2016). Although these single-gene manipulation  
81 strategies achieved some success, both strategies have disadvantages and limitations. Single-gene  
82 manipulation does not seem to be ideal because salinity tolerance is a complex trait probably

83 influenced by several genes and factors concomitantly. On the other hand, modifying signaling  
84 and regulatory pathways requires precise and detailed knowledge on their functioning, and often  
85 plants with improved tolerance do not perform well under unstressed conditions. In contrast to  
86 drought, soil salinity is a stressor that is constantly present in the soil, however, the intensity of  
87 the stress as well as the response of the plants at different developmental stages or organs also  
88 greatly varies. Therefore, identifying novel approaches to produce salt-tolerant plants, such as  
89 co-expression of multiple-genes, epigenetic control of gene-expression and targeting post-  
90 transcriptional modifications (small/micro-RNAs) are required (Shriram et al. 2016; Kumar et al.  
91 2017b). Although many reviews were published focusing on transgenic strategies that can  
92 potentially be used to produce salt-tolerant crop plants, in this review, we discuss and critically  
93 evaluate from a practical perspective both developments in conventional plant breeding and  
94 novel genetic engineering methods that can be used to develop salt-tolerant crops. Besides  
95 evaluating the potential genes from various metabolic pathways that confer salt-tolerance in both  
96 model and crop plants, we also discuss how genetic modification of signalling/regulatory  
97 elements (including TFs), epigenetic control of gene-expression, post-transcriptional  
98 modifications (small/micro-RNAs), and genome editing technologies help to produce plants with  
99 targeted genetic engineering (CRISPR-Cas).

100

## 101 **2 Engineering regulatory elements for increased salt tolerance**

102 As plants live in highly variable environments, they have evolved mechanisms that enable them  
103 to rapidly respond to changing environmental conditions including soil salinity. It is necessary to  
104 understand these mechanisms at the whole-plant, organ, tissue, cellular, physiological and  
105 molecular levels. When plants encounter adverse conditions such as salinity, a variety of genes  
106 with various functions can be upregulated or downregulated, and any resultant changes in  
107 developmental and/or physiological processes can contribute to stress tolerance (Tran and  
108 Mochida 2010; Mochida et al. 2011). While some of these upregulated or downregulated genes  
109 encode proteins that play critical roles in stress associated growth or metabolic changes, many  
110 other genes encode regulatory proteins such as TFs, which control salt-sensing and signal  
111 transduction pathways and the expression of a range of salinity stress-responsive genes (Gupta  
112 and Huang 2014; Li and Tran 2017; Mann et al. 2019). TFs play a vital role in connecting the  
113 salt-sensory pathways to the various genes required for plant salt-tolerance (Nishiyama et al.

114 2012). These genes play critical roles in regulating plant stress responses, to various abiotic  
115 stress factors including salinity (Joshi et al. 2016; Wang et al. 2016a). Several studies reported  
116 the development of transgenic plants with modified TF expression that improved salt-tolerance  
117 (Joshi et al. 2016). Also, the TF families ERF/AP2, bZIP, MYB, MYC, NAC, WRKY, and zinc-  
118 finger proteins were shown to have regulatory roles associated with plant stress-responses  
119 (Kazan, 2015; Sun et al. 2016).

120 As demonstrated in many studies of various plant species, stress-responsive TFs function  
121 in conjunction with the promoter regions to regulate the expression of salt-stress responsive  
122 genes involved in salt-tolerance. For example, transgenic *Oryza sativa* lines over-expressing  
123 OsDREB2A were relatively tolerant to salinity compared to their wild-type counterparts  
124 (Mallikarjuna et al. 2011). Expression of the rice TF SALT-RESPONSIVE ERF1 (*SERF1*),  
125 resulted in root-specific stimulation in response to salt and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) treatments  
126 (Schmidt et al. 2013). In the absence of SERF1, disruption of a salinity-induced MAPK cascade  
127 involved in acquisition of salt-tolerance occurs. It was shown that SERF1 binds to the promoters  
128 of *MAP3K6*, *MAPK5*, *DREB2A*, and *ZFP179* genes, and constitutive overexpression of *SERF1*  
129 enhances salinity tolerance in rice plants (Schmidt et al. 2013). In addition, transgenic  
130 *Arabidopsis* plants expressing TF gene *OsAP21* and *SbAP37* exhibited better growth than wild-  
131 type plants under salt/drought/temperature stress conditions (Jin et al. 2013; Maheshwari et al.  
132 2017). In a similar study, the *HhBREB2* gene belonging to AP2/EREBP TF family was isolated  
133 from *Halimodendron halodendron*. This gene was placed into the A-5 cluster of the DREB  
134 subfamily, on the basis of its similarity to the AP2/ERF domain. Overexpression of the  
135 *HhBREB2* gene in *Arabidopsis* led to increased salt and drought tolerance in the transgenic  
136 plants, thus providing evidence that *HhBREB2* is an important TF involved in the regulation of  
137 salinity associated signalling in plants (Ma et al. 2015b).

138 MYB-type TFs play diverse roles in plant development and plant responses to abiotic  
139 stress. Transgenic rice plants overexpressing *OsMYB2*, an R2R3-type MYB gene, were more  
140 tolerant to salt, cold, and dehydration than wild-type plants (Yang et al. 2012). *OsMYB2*-  
141 overexpressing plants accumulated greater amounts of soluble sugars and proline, and exhibited  
142 enhanced upregulation of genes encoding proline synthesis and transporter proteins than wild-  
143 type plants. Greater upregulation of stress-related genes such as *OsLEA3*, *OsRab16A* and  
144 *OsDREB2A*, were noticed in *OsMYB2*-overexpressing plants. Besides, overexpression of

145 *OsMYB48-1*, a novel MYB-related TF in rice plants, enhanced the tolerance to drought stress  
146 imposed by mannitol and PEG, and to salinity stress (Xiong et al. 2014). It was shown that the  
147 *ARS1* gene, which encodes an R1-MYB type TF, had increased expression in the leaves of salt  
148 stressed tomato plants and that the role of *ARS1* was related to reduced transpirational water loss  
149 in plants under salinity stress (Campos et al. 2016). Another family of TFs shown to be involved  
150 in salt-tolerance are the NAC proteins (*NAM*, no apical meristem; *ATAF*, Arabidopsis  
151 transcription activation factor and *CUC*, Cup-shaped cotyledon). This TF mega-family was one  
152 of the first plant-specific TF families to be identified and in addition to their involvement in the  
153 regulation of plant development, NAC proteins are believed to be involved in various abiotic  
154 stress responses, including salinity and drought (Tran et al. 2010; Rahman et al. 2016). The role  
155 of the rice *SNAC1* gene was investigated using transgenic wheat where the *SNAC1* gene was  
156 placed under the control of a maize ubiquitin promoter (Saad et al. 2013). It was concluded that  
157 drought and salt-tolerance were enhanced in these transgenic wheat plants (Saad et al. 2013). A  
158 NAC gene from wheat was also characterized for a possible role in drought, salinity and freezing  
159 tolerance, where a *TaNAC67-GFP* gene fusion was introduced into *Arabidopsis* under the  
160 control of *CaMV-35S* promoter and transgenic plants were examined at both physiological and  
161 morphological levels under various abiotic stresses (Mao et al. 2014). These transgenics showed  
162 improved tolerance to drought, salt and freezing stresses (Mao et al. 2014). In another study,  
163 *TaNAC29* isolated from wheat was introduced into *Arabidopsis* using the *Agrobacterium*  
164 *tumefaciens*-mediated floral dip method (Huang et al. 2015). The greenhouse-grown transgenics  
165 showed enhanced tolerance to salt and drought stresses (Huang et al. 2015). Likewise, *SNAC1*  
166 gene from rice was transferred using *A. tumefaciens* into *Boehmeria nivea* and the resulting  
167 transgenics displayed increased tolerance towards salt and drought stresses, both at the seedling  
168 and fiber maturation stages (An et al. 2015). A classic stress-responsive NAC TF *CarNAC4* from  
169 chickpea was characterized and its function was studied in transgenic *Arabidopsis*. Transgenics  
170 exhibited enhanced tolerance to drought and salinity, by stimulating other stress-responsive  
171 genes including *RD29A*, *ERD10*, *COR15A*, *COR47*, *KINI* and *DREB2A* (Yu et al. 2016), thus  
172 confirming the role of *CarNAC4* as a TF involved in the regulation of salt and drought stresses.  
173 Thus, the above studies confirmed the importance of *NAC* genes in salinity and drought stress  
174 tolerance. However, genetic engineering of stress related regulatory networks can result in salt  
175 and drought tolerance in crop plants, but this approach has the potential to cause pleiotropic

176 effects and reduce yields in plants grown in the absence of stress or eventually in the presence of  
177 other (e. g. biotic) stresses. Moreover, these methods have the disadvantage of not yet available  
178 for several important crops including wheat (*Triticum aestivum*). To overcome any potentially  
179 negative impacts associated with engineering, TFs alongside the use of conditional or tissue-  
180 specific promoters should be considered (Cabello et al. 2014). In addition, methodological  
181 developments have to be achieved in case of genetically and molecularly more challenging  
182 crops.

183

### 284 **3 Manipulating ion transport and transporters**

185 Salt stress impacts plant growth and crop productivity by causing hyperosmotic stress, resulting  
186 in decreased water potential which limits water uptake and thus cell expansion, and on a longer  
187 time-scale by hyperionic stress, i.e. toxicity due to excess  $\text{Na}^+$  and  $\text{Cl}^-$  ion concentrations  
188 (Maathuis et al. 2014). While  $\text{Cl}^-$  is an essential micronutrient, it can be toxic to plants at higher  
189 concentrations (Khare et al. 2015). Information on  $\text{Cl}^-$  transport and genetic engineering  
190 specifically for  $\text{Cl}^-$  tolerance is inadequate and so is not discussed below. Although the  
191 mechanisms involved in plant salt-tolerance are not yet fully understood, it is clear that the  
192 control of water movement through aquaporin channels (Martinez-Ballesta and Carvajal 2014),  
193 and ion transport and transporters play crucial roles in plant salt-tolerance and that the  
194 mechanisms that regulate water and ion transport may be suitable targets for the production of  
195 salt tolerant crops (Rahman et al. 2017).  $\text{Na}^+$  and  $\text{Cl}^-$  ions are first taken up by the outer root  
196 cells, then transported to the root xylem and finally from the root to the shoot, where they may be  
197 stored in vacuoles or in the apoplastic space, or possibly recirculated back to the root system or  
198 to older leaves that are less active (Figure 1). In some salt tolerant plant species,  $\text{Na}^+$  and  $\text{Cl}^-$  ions  
199 can also be excreted via special structures, which take the form of glands or bladders (Shabala et  
200 al. 2014). Salt-tolerance mechanisms also include mitigation of the effects of ion-induced stress  
201 by the production of osmoprotectants and compatible solutes (glycine betaine, mannitol,  
202 ononitol, trehalose, polyamines), proteins (stress and heat shock proteins, late embryogenesis  
203 abundant proteins, signalization and hormone modulating factors), antioxidants and antioxidant  
204 enzymes that prevent oxidative damage (Gupta and Huang 2014). However, production of the  
205 above can have high energy costs and thus reduce the crop yields. Key factors associated with  
206 the prevention of ion toxicity under salt stress are (i) restriction of  $\text{Na}^+$  and  $\text{Cl}^-$  uptake, (ii)

207 restriction of Na<sup>+</sup> and Cl<sup>-</sup> transport (iii) maintenance of beneficial ion homeostasis e.g. that of K<sup>+</sup>,  
208 which is an essential nutrient for plant growth and development. For example, a high K<sup>+</sup>/Na<sup>+</sup>  
209 ratio can be maintained by inhibition of NaCl-induced K<sup>+</sup> efflux from the cytoplasm (Kumar and  
210 Khare 2016).

211 Although the biochemical and biotechnological targeting of the Na<sup>+</sup> uptake system of plants at  
212 the root level (i.e. soil-epidermis interface) would seem very promising, there are only few  
213 attempts related to it. This is probably due to (i) the complexity of Na<sup>+</sup> uptake involving several  
214 not well characterized components like non-selective cation channels (*NSCC*) and high-affinity  
215 potassium transporters (*HKT*) and (ii) its interference with the uptake of other essential cations,  
216 especially K<sup>+</sup> (e.g. at *AKT1*, *AtHAK5* transporters). The passive influx of Na<sup>+</sup> into the cytoplasm  
217 cannot be fully prevented under salt stress; therefore, other salt tolerance mechanisms evolved in  
218 plants to decrease ion toxicity within the cells. These include its (i) active export (futile cycling)  
219 from root cells by plasma membrane (PM) Na<sup>+</sup>/H<sup>+</sup> antiporters (e.g. *SOS1*, *NHAI* or *NHXLP*), or  
220 its (ii) exclusion from the cytoplasm to other intracellular compartments (like vacuoles, plastids  
221 or endosomes) with the help of NHX-type vacuolar Na<sup>+</sup>(K<sup>+</sup>)/H<sup>+</sup> antiporters both at the root and  
222 the shoot (especially leaf) level or both mechanisms (Tables 1 and 2). The SOS system also has  
223 important roles in the complex regulatory networks activated under stress (Ji et al. 2013). PM  
224 located H<sup>+</sup>-ATPases (e.g. *AHA1/2/3* - **Figure 1**) and H<sup>+</sup>-pyrophosphatases (Wang et al. 2016b)  
225 are important to regulate ion uptake at the root level or exclusion in leaf mesophyll cells. In  
226 many species salt tolerance mechanisms also include long-distance transport processes or  
227 especially their inhibition (**Figure 1**).

228 This review focuses on recent studies that involve the genetic modification of plants with single  
229 or multiple ion transport components to improve salt tolerance (**Figure 1; Tables 1 and 2**). For  
230 Na<sup>+</sup> and Cl<sup>-</sup> transport and transporters in plants following reviews give excellent information  
231 (Hasegawa 2013; Maathuis et al. 2014; Nieves-Cordones et al. 2016; Almeida et al. 2017). Most  
232 of the transporters have tissue- and organ-dependent expression patterns in many species.  
233 Several homologs of them (e.g. *NHX* and *HKT*) are present and differently expressed in various  
234 intracellular compartments, and the expression of different transport components is often  
235 mutually regulated in a complex manner (Plett et al. 2010; Yadav et al. 2012; Gouiaa and  
236 Khoudi, 2015; Ma et al. 2017; Hamamoto et al. 2015; Almeida et al. 2017) and also depending  
237 on the developmental stage and the strength of the stress (Zhang et al. 2017). Therefore, the



238 exact impact of genetic engineering of transport components needs to be carefully assessed and  
239 analyzed for each species and in the complex context of plant nutrition.

240

### 241 **3.1 Membrane transport and salt stress tolerance**

242 PM proton (H<sup>+</sup>)-ATPases, vacuolar membrane H<sup>+</sup>-ATPases, PM and vacuolar membrane H<sup>+</sup>-  
243 pyrophosphatases (H<sup>+</sup>-PPases) constitute proton pumps in plants. Besides proton pumps, Na<sup>+</sup> and  
244 K<sup>+</sup> transporters also play a vital role during salt stress tolerance. Their functions and the  
245 transgenics developed utilizing these genes are briefly described below.

246

#### 247 **3.1.1 Proton pumps and salt stress tolerance**

248 Plant H<sup>+</sup> pumps play primary roles for the transport of ions and solutes across cell membranes. In  
249 plants, three major H<sup>+</sup> transport proteins [PM H<sup>+</sup>-ATPase, vacuolar H<sup>+</sup>-ATPase and PM and  
250 vacuolar H<sup>+</sup>-pyrophosphatases (later called VPPases)] were detected and found to be associated  
251 with salt stress tolerance. It is known that H<sup>+</sup>-pumps produce an electrochemical potential  
252 gradient which is the motive force that is essential for root nutrient uptake, stomatal aperture,  
253 phloem loading and cell growth (Blumwald et al. 2000; Gaxiola et al. 2007; Mansour 2014).

254

##### 255 **3.1.1.1 Plasma membrane H<sup>+</sup>-ATPase**

256 PM H<sup>+</sup>-ATPases or P-type ATPases (P-ATPases) are encoded by a large gene family. These  
257 transporters couple ATP hydrolysis with H<sup>+</sup> transport at the membrane level (Gaxiola et al. 2007,  
258 Fuglsang et al. 2010). Mansour (2014) noticed that high activity of P-ATPase under salt stress  
259 repolarizes the NaCl-induced depolarization of PM and is associated with salt stress tolerance.  
260 Indeed, higher P-ATPase enzyme activity and salt stress tolerance was noticed in halophytes  
261 (Mansour 2014). Sun et al. (2009) pointed out P-type ATPases reduce Na<sup>+</sup> influx and K<sup>+</sup> efflux  
262 and maintain proper K<sup>+</sup>/Na<sup>+</sup> levels and is also associated with the movement of stomatal aperture  
263 (Zhang et al. 2001; Gaxiola et al. 2007). Bose et al. (2015) found out that PM H<sup>+</sup>-ATPase  
264 activity is higher in halophytes in comparison with glycophytes under salt stress conditions  
265 indicating its role in salinity tolerance. Vitart et al. (2001) observed a reduction in growth in a  
266 PM H<sup>+</sup>-ATPase *aha4* mutant *Arabidopsis* when exposed to salt stress. Gevaudant et al. (2007)  
267 demonstrated increased salt tolerance in transgenic tobacco plants expressing a PM H<sup>+</sup>-ATPase,  
268 lacking the auto-inhibitory domain. However, the ubiquitous and not tissue specific expression of

269 the active proton pump only increased salt tolerance during germination and early growth of  
270 seedlings, and caused altered development of the adult plants (Gevaudant et al. 2007) and cell  
271 expansion in another experiment (Niczyj et al. 2016), and thus does not represent a promising  
272 tool to develop salt tolerant crops. A PM H<sup>+</sup>-ATPase4 gene (*PMA4*) isolated from *Nicotiana*  
273 *plumbaginifolia* when overexpressed in tobacco showed no difference in growth under normal  
274 conditions, but, impaired sucrose translocation, stomatal opening, plant growth and male fertility  
275 were noticed when the endogenous and the transgene PMA4 were co-suppressed (Zhao et al.  
276 2000). Zhang et al. (2014) overexpressed the PM H<sup>+</sup>-ATPase of the salt-tolerant *Chloris virgata*  
277 (*ChvPMA* and *ChvPMAΔC*) in genetically modified yeast and found enhanced resistance to salt  
278 and lower pH conditions. Moreover, the yeast overexpressing *ChvPMAΔC* displayed better  
279 growth than *ChvPMA* at an external pH 4.0 in the presence of NaCl. Transgenic *A. thaliana*  
280 containing the transgene *ChvPMAΔC* exhibited better root growth than plants containing  
281 *ChvPMA* in the presence of NaCl stress (Zhang et al. 2014). Recently, Fan et al. (2018)  
282 overexpressed a PM H<sup>+</sup>-ATPase (*SpAHA1*) isolated from the halophyte *Sesuvium portulacastrum*  
283 in *Arabidopsis thaliana* that conferred salt tolerance by improving seed germination ratio, root  
284 growth, and biomass of transgenics. In addition to improved ion homeostasis, transgenic plants  
285 displayed lower oxidative stress (Fan et al. 2018). The above studies indicate the important yet  
286 not fully understood roles of PM H<sup>+</sup>-ATPase in imparting salt stress tolerance.

287

### 288 **3.1.1.2 Vacuolar membrane H<sup>+</sup>-ATPase (V-ATPase)**

289 It is a multisubunit enzyme complex (encoded by many genes), composed of two subcomplexes  
290 (V1 and V0) that help in H<sup>+</sup> translocation (Gaxiola et al. 2007). Jiang et al. (2010) and Bassil and  
291 Blumwald (2014) noted that V-ATPase provides the driving force for the vacuolar  
292 compartmentalization of Na<sup>+</sup>. V-ATPase is highly abundant in the tonoplast and its activity is  
293 modulated in order to cope with environmental changes (Ratajczak 2000). Enhanced V-ATPase  
294 activity as well as upregulation of transcript levels of some subunits was reported under salt  
295 stress by Kirsch et al. (1996) and Silva and Geros (2009). Klychnikov et al. (2007) found out that  
296 14-3-3 proteins (activators of PM ATPases) interact with some of the subunits (VHA-A) in a  
297 phosphorylation dependent way in *Hordeum vulgare* for the regulation of V-ATPase activity.  
298 Dietz et al. (2001) showed the functional significance of V-ATPase in the ability of plants to  
299 show resistance to abiotic stress. Golldack and Dietz (2000), Baisakh et al. (2008) demonstrated

300 upregulation of V-ATPase during early stages of salt stress indicating its involvement in the  
301 process. In response to salt stress, *VHA-A* transcript was upregulated in tobacco (Narasimhan et  
302 al. 1991), sugarbeet (Lehr et al. 1999) and wheat (Golldack et al. 2001). Higher transcript levels  
303 of *VHA-B* were noticed under salt stress in *Mesembryanthemum crystallinum* (Golldack and  
304 Dietz 2000), and wheat (Wang et al. 2011). Likewise, *VHA-C* gene was found triggered by salt  
305 stress in *M. crystallinum* (Kluge et al. 2003), and *Pennisetum glaucum* (Tyagi et al. 2005).  
306 However, studies on the role of orthologous expression of genes encoding V-ATPase subunits on  
307 the ability of plants to withstand salt stress are scarce though RNAi mutants were studied by  
308 Padmanaban et al. (2004). Gaxiola et al. (2001) pointed out that overexpression of *VHA* would  
309 not be easy since it consists of multisubunit complex that needs to be expressed at the correct  
310 level. Baisakh et al. (2012) showed enhanced salt stress tolerance of rice transgenics expressing a  
311 vacuolar H<sup>+</sup>-ATPase subunit c1 (*SaVHAc1*) gene from the halophyte grass *Spartina alternifolia*.  
312 They noticed high accumulation of Na<sup>+</sup> levels in roots and leaves, and yet the plants were not  
313 affected by the toxic Na<sup>+</sup> ions. This could be due to the sequestration of Na<sup>+</sup> ions at the tonoplast  
314 by Na<sup>+</sup>-H<sup>+</sup>-antiporter that was energized by a proton motive force created by the overexpression  
315 of the above gene as pointed out by Apse et al. (1999). Schumacher et al. (1999) demonstrated  
316 that V-ATPase is involved in plant development and signaling as evident from the V-ATPase  
317 mutant, *det3*, which reduced subunit C transcript. Xu et al. (2011), Wang et al. (2011) and Zhang  
318 et al. (2014b) overexpressed subunit *CI* of VHA from *Limonium bicolor*, subunits *B* and *E* of  
319 VHA from *Triticum aestivum*, respectively, and found improved salt tolerance in tobacco (Xu et  
320 al. 2011) and *Arabidopsis* (Wang et al. 2011; Zhang et al. 2014b). It is clear from the studies of  
321 Baisakh et al. (2012) that *SaVHAc1* (isolated from *Spartina alterniflora*) is involved in cell  
322 expansion and maintenance of net photosynthesis (i.e. higher chlorophyll content in transgenics  
323 in comparison with wild-type plants) with higher root and leaf growths and yields under salt  
324 stress. Zhang et al. (2013) showed that RNAi-directed downregulation of vacuolar H<sup>+</sup>-ATPase  
325 subunit- $\alpha$  results in enhanced stomatal aperture and density in rice. He et al. (2014) reported high  
326 expression levels of *A*, *C*, *D*, *F* and  $\alpha$ -subunits under salt stress conditions. They also  
327 overexpressed wheat V-H<sup>+</sup>-ATPase subunit genes that imparted salt tolerance significantly to the  
328 transgenic *Arabidopsis thaliana* plants. Further, Dong et al. (2015) found that ectopic expression  
329 of subunit *A* of *MdVHA-A* (vacuolar H<sup>+</sup>-ATPase subunit A) isolated from *Malus domestica*  
330 enhanced the salt tolerance in tobacco. Wang et al. (2016a) showed that overexpression of a

331 vacuolar-type HC-ATPase C subunit gene from *Iris lactea* (*IrlVHA-c*), enhanced the salt  
332 tolerance in tobacco. Liu et al. (2018) demonstrated that the gene encoding subunit A of the  
333 vacuolar H<sup>+</sup>-ATPase from cotton (*GhVHA-A*) plays an important role in conferring tolerance to  
334 water deficit. The above studies also infer that the genes isolated from halophytes could be  
335 effectively utilized for alleviating salt stress in the crop plants.

### 336 **3.1.1.3 PM and vacuolar membrane bound H<sup>+</sup>PPases**

337 In plants, pyrophosphate (PPi) is produced as a by-product during the activation or  
338 polymerization steps of many biosynthetic pathways. It is hydrolyzed by soluble  
339 pyrophosphatase (H<sup>+</sup>-PPase) enzyme in the plastids (Weiner et al. 1987). On the other hand,  
340 cytosol of higher plants contains very little PPi. Zhen et al. (1997) found out that cytosolic PPi  
341 acts as an energy source for energizing the vacuolar membrane via PPi-dependent proton pump.  
342 H<sup>+</sup>-PPases are hydrophobic single subunit proteins unlike that of PM and V-ATPases. They  
343 generate H<sup>+</sup> gradient across the vacuole, Golgi and PM using the energy generated during  
344 hydrolysis of PPi (Gaxiola et al. 2007). While Silva et al. (2009) showed that V-H<sup>+</sup>-PPase  
345 activity decreases when plants are exposed to salt stress, enhanced activity was also recorded in  
346 several taxa (Parks et al. 2002; Queirós et al. 2009). H<sup>+</sup>-PPases are of two types (type I and type  
347 II). For their activity, type I H<sup>+</sup>-PPases require cytosolic K<sup>+</sup> and are sensitive to inhibition by  
348 Ca<sup>2+</sup>. On the other hand, type II H<sup>+</sup>-PPases are insensitive to K<sup>+</sup> ions but Ca<sup>2+</sup> sensitive. Type I  
349 H<sup>+</sup>-PPases acidify the vacuole (Gaxiola et al. 2007) and are also noticed in the PM  
350 (Alexandersson et al. 2004). Maeshima (2000) showed that vacuolar H<sup>+</sup>-PPase activity is  
351 upregulated under salt stress conditions. Gene that encodes vacuolar H<sup>+</sup>-pyrophosphatase  
352 (*VPPase*) was cloned both from dicots (*Arabidopsis*) as well as monocots (*Sorghum bicolor*) and  
353 overexpressed in diverse plants like *Arabidopsis*, finger millet etc. (Guo et al. 2006; Anjaneyulu  
354 et al. 2014; Pizzio et al. 2015). Such transgenics displayed both salt and drought stress tolerance  
355 (Sarafian et al. 1992, Gaxiola et al. 2001, Guo et al. 2006, Pizzio et al. 2015). The tolerance to  
356 salt and water stresses in these transgenic plants is due to increased solute uptake into vacuoles  
357 as evident from the measurements of Na<sup>+</sup> and K<sup>+</sup> ions (Anjaneyulu et al. 2014). The above  
358 studies infer that VPPase is an important gene involved in H<sup>+</sup> translocation and abiotic stress  
359 tolerance. The regulation of V-H<sup>+</sup>-ATPase as well as V-H<sup>+</sup>-PPase activities by salt stress is  
360 reported, but correlative pattern is missing for activation or deactivation of these proton pumps in  
361 response to salt stress and water deficit. It appears that P-ATPases, V-ATPases and H<sup>+</sup>-PPases

362 are regulated by a common mechanism and 14-3-3 proteins are known to regulate many  
363 membranes localized proteins such as ion pumps (Bunney et al. 2002). Taken together, all the  
364 three ion pumps seem to play a pivotal role during salt stress in higher plants, but their  
365 engineering is challenging (especially in case of the multisubunit V-ATPase). Also, their use to  
366 improve crop yields under glasshouse or field conditions remains questionable.

367

### 368 **3.2 Na<sup>+</sup>-H<sup>+</sup>-antiporters (NHX), sodium-proton exchangers (NHE), and salt stress**

369 Transport of Na<sup>+</sup> into the plant cells is mediated by non-selective cation channels (*NSCCs*) that  
370 do not have selectivity for cations. Demidchik and Maathuis (2007) pointed out that these are  
371 permeable to many monovalent cations at the PM tonoplast level. *NSCCs* are of many types such  
372 as depolarization-activated (voltage-dependent), hyper-polarization-activated (voltage-  
373 independent), reactive oxygen species (ROS)-activated *NSCCs*, amino acid-activated *NSCCs*,  
374 cyclic nucleotide-gated (CNG) *NSCCs*, etc. Apse and Blumwald (2007) and Zhang et al. (2010)  
375 suggest that Na<sup>+</sup> influx across the PM occurs via *NSCC/VIC* in root cortical cells.

376 *NHX* transporters are modulated by and hence associated with salt stress, long-distance transport  
377 of Na<sup>+</sup> from root to shoot, protein targeting and trafficking and even functioning of stomata as  
378 pointed out by Bassil et al. (2011a, 2011b), Barragan et al. (2012), and Wu et al. (2016). *AtNHX1*  
379 is the first reported plant transporter that mediates Na<sup>+</sup> transport into vacuoles (Apse et al.,  
380 1999). Further, *AtNHX1* or salt overly sensitive pathway (SOS) gene *AtSOS1* overexpression in  
381 tomato resulted in better salt tolerance (Apse et al. 1999, Shi et al., 2003). At the same, it was  
382 shown that salt or Na<sup>+</sup> does not accumulate in the fruit, but accumulates only in the foliage of  
383 tomato (Zhang and Blumwald, 2001; Shi et al., 2003). Liu and Zhu (1998) noticed that a calcium  
384 sensor homolog is required for salt tolerance. Later, Liu et al. (2000) demonstrated that in  
385 *Arabidopsis thaliana*, *SOS2* gene encodes a protein kinase required for salt stress tolerance.  
386 Subsequent studies revealed that the regulation of Na<sup>+</sup>/H<sup>+</sup> exchange in *A. thaliana* is carried out  
387 by the SOS pathway (Qiu et al. 2004). Besides *NHX* family members, *NHE*-type protein family  
388 members (both PM and organellar membrane-bound) were identified in plants that may perform  
389 regulation of intracellular pH and cell volume (Counillon and Pouyssegur 2000). The roles of all  
390 *NHX* members (8 in most eukaryotes) during salt stress were amply demonstrated in many  
391 transgenics (Apse et al. 1999; Apse and Blumwald 2007; Shi et al. 2008; Kronzucker and Britto  
392 2011; Bassil et al. 2011a; Yadav et al. 2012). But the numbers of *NHE* members that exist in

393 higher plants except in *S. bicolor* (9), the functions of *NHE* family members (during salt stress),  
394 and their tissue specific expressions are not completely known though redundant functions are  
395 suggested. Recently, Kumari et al. (2018) showed that their tissue specific expressions under salt  
396 and drought stress conditions in *Sorghum bicolor*, suggesting their tissue specific role during  
397 abiotic stress. Further, *NHX*-like proteins were detected earlier, and one such protein bound to  
398 the PM was isolated from *S. bicolor* and overexpressed in tomato (Kumari et al. 2017).  
399 Transgenic tomato plants displayed better salt stress tolerance in comparison with wild-type  
400 plants. But it is obscure how many such *NHX*-like proteins exist in higher plants, where they are  
401 localized intracellularly and whether they have any role in development and abiotic stress  
402 tolerance.

403

### 404 **3.1.3 Transport of Na<sup>+</sup> in chloroplasts and salt stress tolerance**

405 In C<sub>4</sub> plants, Na<sup>+</sup> is transported into the chloroplasts for the synthesis of phosphoenolpyruvate  
406 (PEP) for photosynthetic CO<sub>2</sub> fixation. In C<sub>4</sub> plants, a Na<sup>+</sup>/pyruvate symporter BASS2 (bile  
407 acid/sodium symporter family protein 2), helps in co-transport of Na<sup>+</sup> and pyruvate (Figure 1).  
408 Na<sup>+</sup> is exported out by a sodium hydrogen antiporter *NHDI* in such plants (Furumoto et al.  
409 2011). On the other hand, in C<sub>3</sub> plants, Na<sup>+</sup> accumulation in chloroplasts inhibits photosynthesis  
410 and thus may be detrimental to their survival. Müller et al. (2014) used knock out mutants  
411 (*NHDI*) of *A. thaliana* and demonstrated that such plants are not able to export Na<sup>+</sup>,  
412 photosynthesis is impaired in them and they are unable to tolerate NaCl stress. This indicates that  
413 Na<sup>+</sup> homeostasis in chloroplasts may be important for plant survival as well as to their  
414 photosynthetic activity and thus productivity and yield.

415 It appears that halophytes overcome stomatal limitation by switching to CO<sub>2</sub> concentrating  
416 mechanism under saline conditions. Number of chloroplasts also increases per cell in halophytes  
417 and salt entry into chloroplast stroma appears necessary for the formation of grana and PSII  
418 (Bose et al. 2017). Such a phenomenon has not yet been reported in glycophytes so far.  
419 Halophytes accumulate more Cl<sup>-</sup> ions and use it in functional roles but not glycophytes. Several  
420 studies characterized a total of 53 salt-responsive genes encoding chloroplast localized proteins.  
421 This implies that multiple pathways like thylakoid membrane organization, activity of PS II,  
422 assimilation of CO<sub>2</sub>, photorespiration, ROS scavenging, osmotic and ion homeostasis and ABA

423 biosynthesis and signaling are triggered in chloroplasts in response to salt stress (Suo et al.  
424 2017).

425 Tonoplast localized  $K^+$  channel (*TPK1*) was found crucial for maintaining intracellular  $K^+/Na^+$   
426 ratio in *Arabidopsis*. Latz et al. (2013) showed that salt stress triggers phosphorylation of the  
427 *Arabidopsis* vacuolar  $K^+$  channel *TPK1* by calcium-dependent protein kinase (*CDPK*) and  
428 modulates cytosolic  $K^+$  influx under NaCl stress conditions. Carraretto et al. (2016)  
429 demonstrated that *TPK3* is localized to the chloroplast membranes and mediates  $K^+$  homeostasis,  
430 an important event for plant fitness. It is essential for us now to understand the intricate  
431 mechanisms how these transporters move both cations and anions such as  $Na^+$ ,  $K^+$  and  $Cl^-$  across  
432 the chloroplast envelope membrane and thylakoids (reviewed in Szabó and Spetea 2017) and  
433 their precise regulation of PSI and PSII activities under salt stress conditions.

434

#### 435 **3.1.4 Potassium transporters and salt stress**

436  $K^+$  is an essential nutrient for plant metabolism, therefore, the detrimental effects of salt stress  
437 are often associated with disturbances in the  $K^+$  uptake and thus intracellular  $K^+$  homeostasis or  
438 the  $K^+/Na^+$  ratio (Amtmann and Beilby 2010, Srivastava et al. 2020). PM located  $H^+$  pumps are  
439 involved in creating a pH gradient and thus contribute to the maintenance of membrane potential  
440 providing the driving force for essential ion ( $K^+$ ) uptake through voltage-gated channels and also  
441 for the exclusion of  $Na^+$  in exchange for  $H^+$  on plasma membrane located  $Na^+/H^+$  exchangers  
442 such as *SOS1* (Bose et al. 2015). On the other hand, voltage-independent channels with increased  
443  $K^+/Na^+$  selectivity and thus reduced  $Na^+$  uptake may contribute to improved salt tolerance as  
444 shown in the case of the ‘salt excluder’ *Thellungiella salsuginea* (previously *T. halophila*) when  
445 compared with *Arabidopsis*, its glycophyte relative (Volkov et al. 2004; Volkov and Amtmann  
446 2007, Amtmann and Beilby 2010). Biochemical and biotechnological targeting of these channels  
447 to restrict  $Na^+$  uptake is also beneficial for the  $K^+/Na^+$  homeostasis as it does not impact the  
448 negative membrane potential and thus the selective uptake of  $K^+$  via inward rectifying  $K^+$   
449 channels (Amtmann and Beilby 2010). Influx of  $Na^+$  (or  $K^+$ ) into the root cells may also occur on  
450 some members of the high-affinity potassium ( $K^+$ ) transporter (*HKT*) family (Kronzucker and  
451 Britto 2011). Data on cultivars with different salinity tolerance have shown that genetic  
452 modification (i.e. partial deletion or point mutations, etc.) of *HKT1* (now termed *HKT2;1*) may

453 contribute to improved  $K^+/Na^+$  selectivity, altered activity and thus increased salt tolerance  
454 (Cotsaftis et al. 2012).

455 Transgenic expression of a selective,  $K^+$ -inward-rectifying channel (KIRC) of the halophyte  
456 *Puccinellia tenuis* (*PutAKT1*) in *Arabidopsis* resulted in decreased shoot and root  $Na^+$  content  
457 and thus enhanced salt tolerance (Ardie et al. 2010), while under salt stress the level of *AKT1*  
458 expression was in general downregulated especially in salt excluder cultivars (Golldack et al.  
459 2003, Fuchs et al. 2005). These and other data (Wang et al. 2007) indicate that some halophytes  
460 can tolerate salt by being able to maintain high  $K^+$  uptake and simultaneously restrict  $Na^+$  influx  
461 to the root cells (Ardie et al. 2010).

462 Taken together, a high number of ion transport components (alone or simultaneously with other  
463 components) have been genetically modified to improve salt tolerance (**Tables 1, 2**). However,  
464 no GM crop has been commercialized on the basis of these data (Fita et al. 2015). This is due to  
465 the fact, that complex interactions, mutual and tissue- or cell-specific regulations and  
466 competitions of the uptake systems of essential ions need to be considered and may all need to be  
467 targeted individually or simultaneously but specifically for enhanced salt tolerance. Therefore,  
468 due to the complex nature of plant ion homeostasis and regulatory networks that operate it, the  
469 development of transgenics or crop plants with better tolerance is quite challenging and not very  
470 promising at the moment in this field.

471

#### 472 **4 RNA interference (RNAi) technologies for the production of salt-tolerant plants**

473 RNAi is a biological process by which small RNA (sRNA) molecules stop the translation of  
474 targeted mRNAs and thereby inhibit or alter gene expression. RNAi is regarded as an important  
475 mechanism involved in the post-transcriptional regulation of gene expression in plants (Shriram  
476 et al. 2016; Kumar et al. 2017b). Plants have evolved a complex set of interfering non-coding  
477 RNA (ncRNA) species, including the microRNAs (miRNAs) and small interfering RNAs  
478 (siRNAs), to silence targeted genes very precisely and without affecting the expression of other  
479 genes. In this review, only miRNAs are covered. These small ncRNAs are the products of  
480 dsRNAs cleaved by DICER or DCL enzyme-mediated systems. ncRNAs when coupled with  
481 RISC and argonaute proteins lead to RNAi (Saurabh et al. 2014). RNA mediated gene silencing  
482 plays pivotal roles in orchestrating the expression, stability and inheritance of plant genomes, as  
483 well as in defenses against biotic and abiotic stressors. Numerous investigations demonstrated



484 that RNAi can function as a master regulator of the regulatory networks associated with the  
485 expression of stress related genes (Sunkar et al. 2012; Lotfi et al. 2017). Selected salt responsive  
486 plant miRNAs, their targets and the traits regulated by the respective miRNAs are presented in  
487 **Figure 2**.

488 RNAi is emerging as a powerful tool for targeted gene silencing or as a means to alter gene  
489 expression for the improvement of crop plants. Owing to the development of other genomic tools  
490 such as deep sequencing and degradome analysis, coupled with computational tools, an  
491 increasing number miRNAs involved in growth/developmental processes, and stress-responses in  
492 plants are being identified and analysed for their functionality. Major databases and  
493 computational tools to identify miRNAs and predict their target genes are presented in **Table 3**.  
494 Approaches to regulate the expression of genes by the overexpression or knock-down of specific  
495 miRNAs offer a novel and potentially effective approach to engineer salinity tolerance into crop  
496 plants are gaining momentum (Zhang 2015; Khare et al. 2018). Recent studies have confirmed  
497 that manipulating miRNA gene(s) significantly improves crop plant tolerance to environmental  
498 stresses (Leng et al. 2017; Yang et al. 2017). Over-expressing or repressing stress responsive-  
499 miRNAs or their targets, miRNA-resistant target genes and miRNAs all have potential for  
500 increasing the stress tolerance of crop plants. Overexpression of the *gma-miR172* transcript  
501 improved salt tolerance of *Arabidopsis* plants, with higher germination and cotyledon greening  
502 rates, and longer roots (Li et al. 2015). Transgenic plants showed 73-78% germination rate, with  
503 72-77% seedlings turning green, at 150 mM NaCl. In contrast, wild-type plants showed only  
504 63% germination and 57.6% greening rates, at the same NaCl level (Li et al. 2015) and it has  
505 been demonstrated that salt stress inhibits chlorophyll biosynthesis (Abdelkader et al. 2007). Pan  
506 et al. (2016) reported roles for *miR172a* in enhanced salinity tolerance and long-distance stress  
507 signalling. They investigated the growth of *miR172a* hairy root lines and *miR172a* deficient lines  
508 under 200 mM NaCl salt stress. Overexpression of *osa-miR528* in transgenic *Agrostis stolonifera*  
509 resulted in shortened internodes, a higher number of tillers, better water retention, membrane  
510 integrity, and K<sup>+</sup> homeostasis, improved salt tolerance and higher antioxidant enzyme activities  
511 compared to wild-type plants (Yuan et al. 2015). Furthermore, ectopic expression of *miR398*  
512 genes was associated with oxidative stress tolerance in grapevine (Leng et al. 2017). In contrast,  
513 Yang et al. (2017) showed that the overexpression of *osa-miR171c* reduced the salt tolerance of  
514 transgenic rice. These authors concluded that *osa-miR171c* was involved in the regulation of

515 stomatal development, several physiological changes, the expression of stress-related genes and  
516 ABA-dependent signalling pathways, and that it was by regulating these processes that *osa-*  
517 *miR171c* influenced the salt tolerance of rice (Yang et al. 2017). The above studies demonstrate  
518 the potential of miRNA-based manipulations to enhance plant salt-tolerance and the fact that  
519 genes encoding miRNAs represent potential targets for engineering salt tolerance in glycophytic  
520 crop plants. However, further research is required to field evaluate these transgenics for their  
521 yield potential and also to better understand species-specific differences in miRNA-transformed  
522 transgenic plants.

523

## 524 **5. QTLomics of salt tolerance**

525 Identification and analysis of quantitative trait loci (QTL) has progressed phenomenally towards  
526 marker development and crop breeding applications (Ismail and Horie 2017; Cobb et al. 2019). It  
527 has become feasible to characterize at the molecular level the genes involved in novel traits  
528 associated with abiotic stress tolerance, especially salt tolerance. This has become possible  
529 owing to the extensive physiological and molecular studies on tolerance to various abiotic (ionic  
530 and/or osmotic stresses) and information gateway on stress specific as well as shared stress  
531 adaptation mechanisms. Successful efforts have been made for the identification of important,  
532 novel QTLs in rice, cotton, soybean and other crops. A significant finding has been the  
533 identification of salt-tolerance associated QTLs, especially seedling stage Saltol QTL which is  
534 associated with  $\text{Na}^+/\text{K}^+$  homeostasis under salt stress (Ren et al. 2005; Thompson et al. 2010).  
535 Another QTL which is contained within Saltol is the SKC1 associated with shoot  $\text{K}^+$   
536 concentration (Ren et al. 2005). In rice, a QTL, Saltol, was introgressed into an elite salinity  
537 sensitive rice cv Pusa Basmati 1121 for achieving seedling-stage salt stress tolerance. The newly  
538 bred line showed superior agronomic performance and better seedling stage tolerance to salinity  
539 (Babu et al. 2017a). Several rice breeding programs across the globe have successfully adopted  
540 this QTL for marker assisted breeding programs aimed at developing tolerant lines.

541 QTLs other than Saltol from Hasawi rice genotype have also been used for adoption into  
542 breeding programs to derive rice inbred lines which led to the construction of a genetic map and  
543 the identification of twenty new QTLs for salt tolerance (Bizimana et al. 2017). In addition to  
544 rice, QTLs have also been analyzed for breeding salt tolerance in other crops. SSR markers have  
545 been used to scan crop genomes to identify markers associated with salt tolerance in cotton

546 (Zhao et al. 2016) and cucumber (Kere et al. 2017). In maize, Luo et al. (2017) mapped the  
547 QTLs for salt tolerance using a doubled haploid population and high-density SNP markers. The  
548 authors isolated major QTLs for salt tolerance and identified two candidate genes involved in ion  
549 homeostasis. In soybean, Guan et al. (2014) used fine-mapping method in land races and wild  
550 germplasm to isolate *GmSALT3*, a salt tolerance-associated gene. This gene was shown to be  
551 localized to the endoplasmic reticulum and encoded a cation/H<sup>+</sup> exchanger family transporter.  
552 There has been steady progress in the information on crop genome sequence and accessibility to  
553 genome datasets and this is expected to lead the molecular breeding of salt tolerance (Morton et  
554 al. 2019). Crop breeding for salt tolerance can be primarily achieved through combining  
555 mapping based genomic and phenotypic data, meta analysis of QTLs and detection of QTLs for  
556 different tissue and stage specific tolerance traits. Extensive genome-wide diversity  
557 investigations need be conducted to explore allelic diversity at Saltol and other major traits of  
558 interest to guide marker assisted salt tolerance breeding (Ismail and Horie 2017).

559

## 560 **6 Alternative splicing and salt stress tolerance**

561 Multiple mature mRNAs may arise from one gene because of alternate splice site options. This  
562 has been documented since long, but their role during stress tolerance was not known until  
563 recently. A wide array of mature RNAs may arise due to intron retention, exon skipping, and  
564 also alternative 5' or 3' splicing sites (Syed et al. 2012; Reddy et al. 2013). As pointed out by  
565 Gracheva et al. (2011), alternatively spliced transcripts can yield proteins with different  
566 structures or metabolic functions. Further, their cellular locations may also alter as demonstrated  
567 by Kriechbaumer et al. (2012). Surprisingly, such alternatively spliced variants compete with  
568 normal variants and therefore protein function interference has been noticed in a dominant  
569 negative manner (Seo et al. 2011, Pose et al. 2013). Thus, alternative splicing can increase both  
570 transcriptome and proteome diversity.

571 A large body of emerging evidences suggest that there is a dynamic regulation of pre-  
572 mRNA splicing which has been correlated with stress tolerance. A plethora of splicing factors  
573 have been noticed in higher plants. Notably, in *A. thaliana*, Sm-like conserved protein 5 (LSm5)  
574 has been recognized, which promotes inaccurate selection of splice sites in the genome of plants  
575 (Cui et al. 2014). Another splicing factor *PRP31* has been found later which is involved in  
576 transcriptional gene silencing and stress response in *A. thaliana* (Du et al. 2015). They reported

577 that under cold stress, *PRP31* is critical for pre-mRNA splicing. This splicing factor ensures  
578 modulation of cold-responsive gene expression in *A. thaliana*. The experiments conducted by Du  
579 et al. (2015) infer that splicing machinery has a wide array of functions to perform such as pre-  
580 mRNA splicing, gene regulation, transcriptional gene silencing and also abiotic stress response  
581 in higher plants. Thus, alternative splicing has been playing crucial roles at post-transcriptional  
582 level during abiotic stress tolerance. Another component of spliceosome called Ski-interacting  
583 protein (SKIP/SNW1) functions not only as a splicing factor but also as a transcriptional co-  
584 activator for induced genes (Chen et al. 2011; Wang et al. 2012; Feng et al. 2015). Interestingly,  
585 a DEAD box helicase has been found essential for pre-mRNA splicing, cold-responsive gene  
586 regulation, and cold tolerance in *A. thaliana* (Guan et al. 2013). Feng et al. (2015) found that  
587 SKIP controls alternative splicing under salt-stress environment by interfering with the cleavage  
588 of 5' and 3' splice donor and acceptor sites during stress conditions. How the mRNA splicing  
589 machinery modulates salt stress responses in plants has been answered only partially.

590 Not only salt stress, but also other stresses like temperature stress can produce splice  
591 variants. Alternative splicing has been found to incorporate premature termination codons  
592 (PTCs) into transcripts. Such transcripts are usually degraded, else produce truncated proteins in  
593 plants (Kalyna et al. 2012; Ottens and Gehring 2016). Dehydration responsive element-binding  
594 (DREB) protein 2B was found regulated under drought stress conditions by alternative splicing  
595 (Matsukura et al. 2010). One of the isoforms, *OsDREB2B1*, which retains an exon functions  
596 under normal conditions. The second isoform *OsDREB2B2* produces a functional protein. This  
597 functional protein is triggered under both drought and high temperature stresses and also  
598 promotes the expression of several genes involved in stress tolerance (Matsukura et al. 2010).  
599 Further, Sugio et al. (2009), Staiger and Brown (2013), and Cheng et al. (2015), demonstrated  
600 that in *Arabidopsis* and rice the heat shock transcription factor A2 (*HSFA2*) helps the  
601 introduction of a PTC into the splicing isoform *HSFA2-II* and creates truncated protein under  
602 normal conditions. Contrarily, the alternative splice isoform *HSFA2-I*, instead encoded a protein  
603 with transcription activation activity and was also triggered by heat stress. In *A. thaliana*, and  
604 rice this splice isoform activated heat stress-responsive genes under high temperature conditions  
605 (Cheng et al. 2015, Sugio et al. 2009). High temperature stress imposed in grapevines by Jiang et  
606 al. (2017) resulted in the alternative splicing of 70% of the genes. Among the various splice  
607 events, intron retention was frequent. Not surprisingly, intron retention events were high at

608 higher temperatures (40 °C and 45 °C) than at lower (35 °C), indicating that intron retention is a  
609 key posttranscriptional regulation event. Simultaneously, the levels of RNA-binding proteins  
610 were also increased with an increase in temperature. Their investigations revealed that a  
611 multiprotein bridging factor1c and *HSFA2* were associated with heat tolerance by modulating  
612 alternative splicing and translational levels. The findings of Gu et al. (2018) highlighted the  
613 critical role being played by another spliceosomal protein U1A which is involved in alternative  
614 splicing as well as a regulatory role in hostile environments. Thus, alternative splicing, a  
615 universal phenomenon appears to play many critical roles during abiotic stress conditions.  
616 However, it is vital to find out the components that transduce the signal to splice the pre-mRNA  
617 molecules and we need to improve our understanding on how these are involved in salt tolerance  
618 or how they could be used to produce plants with improved tolerance and acceptable yields  
619 under field conditions.

620

## 621 **7 Exploring halobiomes as a pool of genes for the production of salt-tolerant plants**

622 The halobiome can be referred to as a group of halophilic and/or halotolerant bacteria, algae,  
623 fungi and plants (halophytes) that are able to withstand hypersaline environments. Hence, the  
624 halobiome can be regarded as a potential reservoir of genes associated with salt-tolerance, which  
625 could be used to engineer salt-tolerant crop plants. Salt-tolerance in halophytes involves  
626 structural and developmental adaptations that include succulence, leaf shedding and the presence  
627 of salt secreting assemblies such as salt glands or salt hairs (Shabala et al. 2014). Many  
628 halophytes achieve tolerance using cellular mechanisms that include reduced Na<sup>+</sup> influx,  
629 vacuolar compartmentalization of it or elimination of Na<sup>+</sup> ions through PM anti-porters (Flowers  
630 and Colmer 2015). While all plants have cellular mechanisms that can potentially confer a  
631 degree of salt-tolerance, in halophytes these mechanisms are expressed at higher levels than in  
632 glycophytes.

633 Hence, quantitative not necessarily qualitative difference in the expression of key genes  
634 or intrinsically more active proteins involved in salt tolerance is often the basis for better survival  
635 in halophytes (Himabindu et al. 2016). Overexpression of salt tolerance genes such as *SOS*,  
636 *NHX*, *HKT* etc., isolated from glycophytes or their halophytic gene homologs, showed that the  
637 genes from halophytes generally provide a better salinity tolerance than those isolated from  
638 glycophytes (Volkov 2015). Selection of effective promoters and regulatory sequences are the

639 key to achieve good levels of transgene expression and recent studies show that the promoter  
640 regions and the *cis*-regulatory elements of various stress-inducible genes found in halophytes are  
641 strongly expressed under saline conditions. For example, promoter regions of the *SIBADH* and  
642 *SIPLEAMT* genes of *Suaeda liaotungensis* (Zhang et al. 2008; Li et al. 2016), the *TsVPI* from  
643 *Thellungiella halophila* (Sun et al. 2010), and *SbGSTU* gene from *Salicornia brachiata* (Tiwari  
644 et al. 2016) showed the ability to cause a multi-fold upregulation of their associated genes in  
645 plants under salt stress. In addition, genes from halophytes including TFs (*MYB*, *NAC*, *DREB*)  
646 associated with enzymatic or non-enzymatic antioxidants (BADH, APX, SOD etc.) and  
647 antiporter genes (*NHX*, *HKT*, *SOS*) were successfully used to enhance salt-tolerance in  
648 glycophytic plants. **Table 4** provides a summary of halophyte genes used in transgenesis-  
649 experiments.

650 In general, halophytes are plants used for isolating genes implicated in salt tolerance,  
651 however, some other salt tolerant non-plant organisms are also explored for this purpose. Genes  
652 encoding enzymes involved in the production of some osmoprotectants, as well as antioxidative  
653 enzymes and some ribosomal proteins from non-plant halobionts were successfully transferred  
654 into plants, resulting in salt-tolerance (Table 5). Three genes, *ectA* (2,4-diaminobutyric acid  
655 acetyltransferase), *ectB* (2,4-diaminobutyric acid aminotransferase) and *ectC* (L-ectoine  
656 synthase), isolated from the halophilic bacterium *Halomonas elongata*, were expressed in  
657 transgenic plants (Moghaieb et al. 2011). The resultant transgenic lines exhibited improved  
658 ectoine synthesis and accumulation, and the plants grew better under saline conditions (100-300  
659 mM NaCl) with improved biomass production and photosynthetic rates, and reduced lipid  
660 peroxidation. Also, the genes encoding glycine sarcosine methyltransferase (*GSMT*) and  
661 sarcosine dimethylglycine methyltransferase (*SDMT*), involved in the synthesis of glycine  
662 betaine (GB), were isolated from the halotolerant cyanobacterium *Aphanothece halophytica* and  
663 expressed in rice (*Oryza sativa*) plants (Niu et al. 2014). These rice transgenics accumulated high  
664 levels of GB, and performed better under saline conditions in comparison with wild-type plants.  
665 Ribosomal proteins from the extremely halophilic fungus *Aspergillus glaucus* were also shown  
666 to alleviate salt stress when expressed in *Arabidopsis thaliana* and *Nicotiana tabacum* (Liu et al.  
667 2014; Liang et al. 2015).

668 In addition to the successful application of transgenic technologies, the information  
669 generated by whole genomes and transcriptome analysis of organisms from the halobiome could

670 be of great value for crop improvement. The genomes of several halotolerant organisms were  
671 sequenced including the halophytes like *Thellungiella salsuginea* (Wu et al. 2012), *T. parvula*  
672 (Dassanayake et al. 2011), the halophilic methanogenic archaea *Methanohalophilus mahii* SLP<sup>T</sup>  
673 (Spring et al. 2010), the halophilic archaeon *Halococcus hamelinensis* (Burns et al. 2012) and the  
674 halophilic bacterium *Halomonas elongate* (Lafi et al. 2016), and these genomes could provide  
675 candidate genes for future transformation studies in the future. However, it is important to note  
676 that the transformation of crop plants using a single gene approach might not provide total  
677 tolerance with high yield potential, as abiotic stress tolerance is often controlled by multiple  
678 genes (Abe et al. 2012). While omics and transgenic approaches were demonstrated to mitigate  
679 the negative effects of salinity, introducing halotolerant bacterium into salt contaminated fields  
680 was proved to be beneficial for the growth of *Pisum sativum* (Ali et al. 2015), *Lycopersicon*  
681 *esculentum* (Fan et al. 2016), *Arachis hypogaea* (Sharma et al. 2016), *Chenopodium quinoa*  
682 (Yang et al. 2016) and *Triticum aestivum* (Raheem and Ali 2015). Therefore, exploring  
683 halobiomes to identify and isolate genes that confer salt-tolerance could be a promising approach  
684 to enable crop plants to be grown in saline soils, but also simpler (but maybe more expensive and  
685 laborious) agricultural methods like introduction of special soil bacteria and fungi may be also  
686 interesting (Aroca and Ruiz-Lozano 2012; Shrivastava and Kumar 2015).

687

## 688 **8 Plant genome editing for producing salt-tolerant plants**

689 Genome editing (GE) methods have enabled specific and predictable modifications to be tailored  
690 into crop genomes and these techniques are now revolutionizing plant breeding and crop  
691 improvement efforts (Puchta 2017; van der Wiel et al. 2017). GE methods utilize customized  
692 nucleases to introduce mutations (insertions or deletions) or precisely change gene sequences  
693 (Jain, 2015). Several GE methods, e.g. zinc finger nucleases (*ZFNs*), transcriptional activator-  
694 like effector nucleases (*TALENs*) and clustered regularly interspaced short palindromic repeat  
695 (CRISPR)-Cas9 (CRISPR-associated nuclease 9) are now readily available (Voytas 2013; Kumar  
696 and Jain 2015). These sequence-specific nucleases induce double-strand breaks (DSBs) in DNA  
697 at specific sites in the genome which are subsequently followed by DNA repair processes (non-  
698 homologous end joining or homology-directed repair) and result in genome modifications such  
699 as targeted mutagenesis, gene insertion, or gene replacement. While the non-homologous end

700 joining mode creates insertions or deletions, the homology-directed mode can achieve precise  
701 modifications such as gene replacement or gene insertion.

702 The GE technology was successfully used on a variety of important crop plants (Puchta, 2017).  
703 However, there have been few studies using GE to develop salt-tolerant plants. Due to the  
704 complexity of the salt tolerance trait and the need to manipulate multiple genes, often in multiple  
705 pathways, it is essential to introduce site-directed changes with no apparent off-target effects in  
706 order to minimize unintended yield penalties (Osakabe et al. 2016; Shi et al. 2017). The *ARGOS8*  
707 genomic sequence was edited using the CRISPR-Cas method and resultant maize lines exhibited  
708 ubiquitous and higher levels of expression in most tissues and growth phases. The authors  
709 suggested that this precise means of modifying specific genes and altering their expression could  
710 be used to develop tolerant plants with high grain yields (Shi et al. 2017). Lou et al. (2017)  
711 reported the generation of mutations in the gene for ABA signalling ‘osmotic stress/ABA-  
712 activated protein kinase 2’ (*SAPK2*) to study its functional role, and observed that *sapk2* mutants  
713 exhibited an ABA-insensitive phenotype. Their study suggests that this gene could be useful to  
714 improve salt and associated osmotic stress tolerance. GE can also be used to study the effects of  
715 gene modifications on plant functionality and aid in designing plants with specific modifications  
716 tailored to the trait of interest.

717 Genes associated with salt-tolerance could be specifically targeted using the CRISPR-Cas9  
718 system as this approach was used to confer tolerance to other abiotic stresses as detailed below.  
719 Osakabe et al. (2016) used truncated gRNAs (*tru-gRNAs*) and the CRISPR-Cas9 system for  
720 accurate site-modification, while Merlot et al. (2007) induced mutation in an abiotic stress  
721 tolerance gene encoding *OST2 (AHA1)* in *Arabidopsis*. Two dominant mutations in the *ost2*  
722 locus resulted in the constitutive activity of guard cell proton pumps and inhibited stomatal  
723 responses to ABA (Merlot et al. 2007). In addition, it was found that the use of *tru-gRNA* guided  
724 Cas9, driven by a germline specific promoter, increased heritability in successive generations,  
725 and the mutant plants exhibited altered stomatal response and hence this approach could be used  
726 as a means to produce crops that can tolerate drought and/or salinity.

727 Salt-tolerance involves the controlled expression of multiple genes and the coordination of  
728 regulatory, signalling and metabolic pathways. Moreover, functional redundancy among the  
729 genetic pathways controlling salinity and other stress response pathways makes it difficult to  
730 achieve total knockout of functionality. In this regard, it was advocated that multiplex genome



731 editing (Zhou et al. 2014; Ma et al. 2015) can be employed to deduce the functions of multiple  
732 functionally redundant genes involved in the same biological process, such as abiotic stress  
733 responses (Jain 2015). To this end, multiple gRNAs could be used to accomplish the targeted  
734 mutagenesis for traits like salt tolerance, homology-directed repair (HDR) may be an efficient  
735 method to induce specific change(s) in salt responsive pathways. HDR-mediated gene targeting  
736 can also be used for pyramiding of several genes involved in a stress response pathway or  
737 regulatory network (Jain 2015). For multiplex gene editing in rice, Wang et al. (2018) developed  
738 a simplified single transcriptional unit (*SSTU*) CRISPR system using FnCpf1, LbCpf1 or Cas9 to  
739 target eight genes in the Late Embryogenesis Abundant (LEA) family, and generated a higher  
740 proportion of heterozygous and chimeric mutants induced by Cpf1 albeit with lower editing  
741 efficiency. Though phenotypic nature of mutations has not been shown in this work, this study  
742 can be helpful for multiplex genome editing in plants for stress tolerance.

743         It is now becoming evident through several reports of genome-wide association studies  
744 that single base-pair changes constitute much of the variation in elite traits (Zhang et al. 2018).  
745 This has formed a basis for base editing as a novel strategy for inducing point mutations in crops.  
746 The recent advancement in the CRISPR/Cas9-mediated genome-editing is the base-editing  
747 technology obviating the need for a DNA repair template (Komor et al. 2016). The methods use  
748 different enzyme resource such as Cas9 nickase or dead Cas9 which can be conjoined with base-  
749 converting enzymes (Hess et al. 2017). The cytosine (C) to uracil (U) conversion is mediated by  
750 cytidine aminases, and this could result in C•G which could ultimately become T•A substitution  
751 (Gaudelli et al. 2017). The G•C substitutions are created from the conversion of adenine (A) to  
752 inosine (I) by adenine deaminases which is treated by polymerases as G, and hence the A•T to  
753 G•C substitution. Presently the base editors for adenine and cytosine are reported to bring about  
754 transition mutations of all the four bases in plants (Kang et al. 2018, Li et al. 2018a, Hua et al.  
755 2019). The cytidine deaminase-based gene editing also has shown additional advantages of  
756 inducing nonsense mutations to disorder and knockout gene functions (Billon et al. 2017).  
757 Towards this, CRISPR/Cpf1 system is a promising tool for gene editing especially AT-rich  
758 regions (Begemann et al., 2017; Tang et al., 2017; Wang et al., 2017; Li et al., 2018b). The Cpf1  
759 nucleases from *Francisella novicida* (FnCpf1) and *Lachnospiraceae* bacterium ND2006  
760 (LbCpf1) have shown good potential for introducing accurate gene insertions and indel  
761 mutations at the target site in the rice genome (Begemann et al. 2017). Both Cpf1 produced up to

762 8% high frequency of targeted insertions as compared to most often *Cas8* mediated genome  
763 editing methods. With such an unprecedented arsenal of gene and base editing tools and  
764 precision, plant gene engineering could undoubtedly lead to modifying genes involved in the  
765 responses of plants to different stresses including production of salt-tolerant crop plants with  
766 better yields.

767

## 768 **9 Conclusion and future outlook**

769 It is important to emphasize that studies investigating the TFs or other factors associated with  
770 salinity stress should be carefully designed and evaluated in order not to describe and target less  
771 relevant factors associated with the salt shock response (i.e. osmotic shock caused by exposure to  
772 a single, high concentration of salt, which only occurs in nature due to rare climatic events such  
773 as after a tsunami). Of greater importance are the salt-tolerance related processes that occur in  
774 plants that are grown in fields where salt concentrations are gradually increasing over time  
775 (Shavrukov 2013). The genes and TFs affected by salt shock versus gradual increases in field salt  
776 levels may greatly vary and the latter seems to be agronomically relevant with respect to the  
777 breeding of salt-tolerant crops. Similarly, breeding practices using selection for salt stress  
778 tolerance at seedling stage (and under laboratory conditions) may be less relevant from the  
779 agricultural point of view. Therefore, field trials using proper controls are needed to reliably  
780 evaluate yields of different cultivars and thus to successfully improve crop productivity (Roy et  
781 al. 2014).

782 Land plants have evolved many different salt-tolerance mechanisms and thus different plant  
783 species have different transporters, gene expression patterns and mechanisms to regulate ion  
784 fluxes. Unfortunately, our understanding of the ion transporters that are involved in the uptake,  
785 exclusion, sequestration and transport of salt and thus salt-tolerance is still limited for most of the  
786 crops. There are also many uncertainties, nomenclatural problems, contradictions based on  
787 hastily made generalized statements, and yet unanswered questions with respect to salt-tolerance.  
788 It appears that there are no general rules in salt tolerance mechanisms, or at least that salt-  
789 tolerance mechanisms are likely to vary greatly from plant species to species (Nieves-Cordones  
790 et al. 2016), cultivar to cultivar, population to population, and individual to individual, and  
791 between organs, tissues and cells and also depending upon alternative splicing events. The  
792 experimental and/or field treatments used and plant growth conditions can also have greater

793 impact how plants respond to salt. Furthermore, it has to be considered that the same ion  
794 transporter can have different effects on, for instance, shoot  $\text{Na}^+$  accumulation when modified  
795 (down- or upregulated) in different cell types (e.g. root epidermal cells vs. xylem cells) and  
796 therefore the expression of ion transporter/transport components may need to be regulated at the  
797 cell specific level (e.g. Møller et al. 2009). Also, minor differences in transcription or  
798 protein/gene structure of a transporter can have great impact on a plant's ability to control ion  
799 fluxes and thus tolerate salt stress (e.g. Cotsaftis et al. 2012). This outlines the need for further  
800 functional analyses and characterization of various ion transporters from various organisms, an  
801 improved understanding of their regulation and cell- or tissue-specific expression patterns, as  
802 well as the need to characterize halobionts as potential sources of genes or TFs providing  
803 improved salt tolerance in glycophytes (Volkov 2015; Assaha et al. 2017; Mishra and Tanna  
804 2017). The real impact of such approach is demonstrated by the fact that alfalfa plants  
805 overexpressing *Salsola soda* *NHX* gene could grow in up to 400 mM NaCl over 50 days (Li et al.  
806 2011). Improved and enhanced ion sequestration in the vacuole under salt stress decreases the  
807 solute potential of the cells and thus alleviates the osmotic component of salt stress by  
808 facilitating the water uptake (Lv et al. 2008). Therefore, in spite of excellent promising studies  
809 and the accumulation of an avalanche of information, engineering ion transport components for  
810 improved salt tolerance is a complex and demanding task, especially if the aim is to develop crop  
811 plants that are both salt-tolerant and possess agronomically important traits (i.e. yield) when  
812 grown under commercially relevant field conditions. This is not an easy task even with newest  
813 genetic engineering tools or using transcription factors, because alterations in ion homeostasis  
814 often result in yield burden. Not surprisingly, to the best of our knowledge, no GM crops with  
815 increased salinity tolerance have been commercialized (Fita et al. 2015). Our recent searches in  
816 major relevant public databases (USDA APHIS - [https:// www. aphis. usda.gov / aphis / ourfocus](https://www.aphis.usda.gov/aphis/ourfocus)  
817 [/biotechnology/ permits- notifications- petitions](https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/permits-notifications-petitions); ISAAA - [http:// www.isaaa.org /gm approval](http://www.isaaa.org/gm-approval-database/)  
818 [database/](http://www.isaaa.org/gm-approval-database/)) resulted in no records for 'salt stress' in ISAAA and a few records in APHIS which  
819 requested permission for field trials of some potentially salt tolerant GM crops (e.g. soybean,  
820 Chinese silvergrass), but no data on potentially commercialized and cultivated salt tolerant GM  
821 crops have been found. However, certain field-based studies where overexpression of gene(s) has  
822 improved the salinity tolerance and growth performance of transgenic plants in saline fields  
823 (Paspula et al. 2011; Schilling et al. 2014) should be looked upon as lead for developing salt-

824 tolerant crops at commercial levels. In addition to a combination of genetic engineering and  
825 conventional breeding tools, other practices like for instance the domestication or increased use  
826 of halophyte plants (e.g. quinoa) as crops on saline soils (Fita et al. 2015) look promising.

827

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838

## 839 **Conflict of Interest**

840 Authors declare that there is no conflict of interest

841

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**Table 1.** Single ion transporter genes (from glycophyte sources) recently genetically engineered to improve salt tolerance.

Source organism	Gene	Transgenic host (additional information)	Reference
<b>Plasma membrane bound high-affinity potassium (K<sup>+</sup>) transporter (HKT)</b>			
<i>Arabidopsis thaliana</i>	<i>AtHKT1;1</i>	<i>Arabidopsis thaliana</i> (specific expression in root cortex and epidermis cells)	Plett et al. (2010)
		<i>Oryza sativa</i> (specific expression in root cortex and epidermis cells)	Plett et al. (2010)
<i>Hordeum vulgare</i>	<i>HvHKT2;1</i>	<i>Hordeum vulgare</i> (overexpression)	Mian et al. (2011)
<i>Triticum turgidum</i> ssp. <i>Durum</i> (original source: <i>T. monococcum</i> )	<i>TmHKT7</i> a.k.a. <i>TmHKT1;4-A2</i> <i>TmHKT8</i> a.k.a. <i>TmHKT1;5-A</i>	<i>Triticum aestivum</i>	James et al. (2011)
<i>Oryza sativa</i> cv. <i>Indica</i>	<i>OsHKT1;1</i> and its promoter	<i>Oryza sativa</i> cv. <i>japonica</i>	Campbell et al. (2017)
<i>Glycine max</i>	<i>GmHKT1;4</i>	<i>Nicotiana tabacum</i>	Chen et al. (2014)
<i>Triticum monococcum</i>	<i>TmHKT1;5-A</i>	<i>Triticum turgidum</i> ssp. <i>durum</i>	Munns et al. (2012)
<b>Plasma membrane bound Na<sup>+</sup> insensitive K<sup>+</sup> transporter</b>			
<i>Oryza sativa</i> cv. <i>Nipponbare</i>	<i>OsHAK5</i>	<i>Nicotiana tabacum</i> cv. <i>BY2</i> cells	Horie et al. (2011)
<b>Plasma membrane Na<sup>+</sup>/H<sup>+</sup> antiporter system</b>			
<i>Arabidopsis thaliana</i>	<i>AtSOS1</i>	<i>Arabidopsis thaliana</i> (overexpression) <i>Nicotiana tabacum</i> cv. <i>Xanthi-nc</i>	Yang et al. (2009) Yue et al. (2012)
<i>Artemisia japonica</i>	<i>AjSOS1</i>	<i>Chrysanthemum morifolium</i> <i>Arabidopsis thaliana</i>	Gao et al. (2016) Gao et al. (2016)
<i>Chrysanthemum crissum</i>	<i>CcSOS1</i>	<i>Chrysanthemum morifolium</i>	An et al. (2014a); Gao et al. (2016)
<i>Chrysanthemum morifolium</i>	<i>CmSOS1</i>	<i>Arabidopsis thaliana</i> <i>Chrysanthemum morifolium</i> (overexpression)	Gao et al. (2016) Gao et al. (2016)
<i>Crossostephium chinense</i>	<i>CrcSOS1</i>	<i>Arabidopsis thaliana</i> <i>Chrysanthemum morifolium</i>	Gao et al. (2016) Gao et al. (2016)
<i>Gossypium hirsutum</i>	<i>GhSOS1</i>	<i>Arabidopsis thaliana</i>	Gao et al. (2016)
<i>Sorghum bicolor</i>	<i>SbNHXLP</i>	<i>Solanum lycopersicum</i>	Chen et al. (2017) Kumari et al. (2017)
<b>Plasma membrane Na<sup>+</sup> pumping ATPase</b>			
<i>Physcomitrella patens</i>	<i>PpENAI</i>	<i>Oryza sativa</i>	Jacobs et al. (2011)
<b>NHX-type vacuolar Na<sup>+</sup>(K<sup>+</sup>)/H<sup>+</sup> antiporter</b>			
<i>Arachis hypogaea</i>	<i>AsNHX1</i>	<i>Nicotiana tabacum</i>	Zhang et al. (2017)
<i>Arabidopsis thaliana</i>	<i>AtNHX1</i>	<i>Actinidia deliciosa</i>	Tian et al. (2011)

		<i>Arabidopsis thaliana</i> (overexpression)	Liu et al. (2010)
		<i>Arachis hypogaea</i>	Asif et al. (2011)
		<i>Beta vulgaris</i>	Liu et al. (2008)
<i>Hordeum vulgare</i>	<i>AtNHX3</i>	<i>Solanum tuberosum</i>	Bayat et al. (2010)
<i>Malus domestica</i>	<i>HvNHX2</i>	<i>Malus domestica</i> (overexpression in rootstocks of grafted plants or in calli, respectively)	Li et al. (2010), (2013); Sun et al. (2017)
	<i>MdNHX1</i>	<i>Arabidopsis thaliana</i>	Sun et al. (2017)
<i>Solanum torvum</i>	<i>StNHX1</i>	<i>Glycine max</i>	Chen et al. (2014)
<i>Triticum aestivum</i>	<i>TaNHX3</i>	<i>Nicotiana tabacum</i>	Lu et al. (2014)
<i>Vigna radiata</i>	<i>VrNHX1</i>	<i>Arabidopsis thaliana</i>	Mishra et al. (2014)
<b>NHX-type endosomal K<sup>+</sup>/H<sup>+</sup> antiporter</b>			
<i>Solanum lycopersicum</i>	<i>LeNHX2</i>	<i>Arabidopsis thaliana</i>	Rodriguez-Rosales et al. (2008)
		<i>Solanum lycopersicum</i> (overexpression)	Huertás et al. (2013)
<i>Triticum aestivum</i>	<i>TaNHX2</i>	<i>Glycine max</i>	Cao et al. (2011)
		<i>Solanum lycopersicum</i>	Yarra et al. (2012)
		<i>Medicago sativa</i>	Zhang et al. (2012)
<b>Vacuolar H<sup>+</sup>-ATPase subunits</b>			
<i>Iris lactea</i>	<i>IrlVHA-c</i> (C subunit)	<i>Nicotiana tabacum</i>	Wang et al. (2016)
<i>Limonium bicolor</i>	<i>LbVHA-c1</i> (C1 subunit)	<i>Nicotiana tabacum</i>	Xu et al. (2011)
<i>Malus domestica</i>	<i>MdVHA-A</i>	<i>Nicotiana tabacum</i>	Dong et al. (2015)
<i>Triticum aestivum</i>	<i>TaVB</i> (B subunit)	<i>Arabidopsis thaliana</i>	Wang et al. (2011)
	<i>W36</i> (putative E subunit)	<i>Arabidopsis thaliana</i>	Zhang et al. (2014c)
	Several subunit genes	<i>Arabidopsis thaliana</i>	He et al. (2014)
<b>Vacuolar H<sup>+</sup>-pyrophosphatase</b>			
<i>Arabidopsis thaliana</i>	<i>AVP1</i>	<i>Agrostis stolonifera</i> L.	Li et al. (2010)
		<i>Arabidopsis thaliana</i> (overexpression)	Undurraga et al. (2012)
		<i>Arachis hypogaea</i>	Qin et al. (2013)
		<i>Gossypium hirsutum</i>	Pasapula et al. (2011)
		<i>Hordeum vulgare</i>	Schilling et al. (2014)
		<i>Saccharum officinarum</i>	Kumar et al. (2014)
<i>Populus trichocarpa</i>	<i>PtVPI.1</i>	<i>Populus davidiana</i> × <i>P. bolleana</i>	Yang et al. (2015)
<i>Sorghum bicolor</i>	<i>SbVPPase</i>	<i>Eleusine coracana</i>	Anjaneyulu et al. (2014)

**Table 2.** Two or more ion transporter genes simultaneously genetically engineered to improve salt tolerance.

Targets/Genes	Source organism(s) (genes)	Transgenic host	Reference
Plasma membrane Na <sup>+</sup> /H <sup>+</sup> antiporter and NHX-type vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Arabidopsis thaliana</i> (co-overexpression of <i>AtSOS1</i> and <i>AtNHX1</i> )	<i>Arabidopsis thaliana</i>	Pehlivan et al. (2016)
Plasma membrane Na <sup>+</sup> /H <sup>+</sup> antiporter 'system'	<i>Arabidopsis thaliana</i> ( <i>AtSOS1</i> + <i>AtSOS2</i> + <i>AtSOS3</i> )	<i>Festuca arundinacea</i>	Ma et al. (2014)
Plasma membrane Na <sup>+</sup> /H <sup>+</sup> antiporter and a MYC-like bHLH transcriptional activator	- <i>Chrysanthemum crassum</i> ( <i>CcSOS1</i> ) - and <i>Chrysanthemum dichrum</i> ( <i>CdICE1</i> )	<i>Chrysanthemum morifolium</i> 'Jinba'	Song et al. (2014)
NHX-type vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter and vacuolar H <sup>+</sup> -pyrophosphatase	<i>Arabidopsis thaliana</i> ( <i>AtNHX1</i> and <i>AVP1</i> )	<i>Gossypium hirsutum</i>	Shen et al. (2015)
	<i>Pennisetum glaucum</i> ( <i>PgNHX1</i> ) and <i>Arabidopsis thaliana</i> ( <i>AVP1</i> )	<i>Solanum lycopersicum</i>	Bhaskaran and Savithramma (2011)
	<i>Oryza sativa</i> ( <i>OsNHX1</i> and <i>OsVPI</i> )	<i>Oryza sativa</i> (overexpression)	Liu et al. (2010)
	<i>Suaeda salsa</i> ( <i>SsNHX1</i> ) and <i>Arabidopsis</i> ( <i>AVP1</i> )	<i>Oryza sativa</i>	Zhao et al. (2006)
	<i>Suaeda corniculata</i> ( <i>ScNHX1</i> and <i>ScVP</i> )	<i>Medicago sativa</i>	Liu et al. (2013)
	<i>Triticum aestivum</i> ( <i>TNHX1</i> and <i>TVPI</i> )	<i>Arabidopsis thaliana</i>	Brini et al. (2007)
	<i>Triticum aestivum</i> ( <i>TNHXS1</i> and <i>TVPI</i> )	<i>Solanum lycopersicum</i> <i>Nicotiana tabacum</i>	Khoudi et al. (2009) Gouiaa et al. (2012)
	<i>Zygophyllum xanthoxylum</i> ( <i>ZxNHX</i> and <i>ZxVPI-1</i> )	<i>Solanum lycopersicum</i>	Gouiaa and Khoudi (2015)
		<i>Beta vulgaris</i>	Wu et al. (2015a)
		<i>Lotus corniculatus</i>	Bao et al. (2014)
		<i>Medicago sativa</i>	Bao et al. (2016)

**Table 3.** List of major databases/repositories and tools developed in recent years and available for prediction or identification of non-coding RNAs (including miRNAs) and their targets

Database/ Tool	Description	Web link	Reference
miRBase	A biological database which acts as an archive of miRNA sequences and annotations from more than 220 organisms including plants	<a href="http://www.mirbase.org/">http://www.mirbase.org/</a>	Kozomara and Griffiths-Jones (2014)
RNAcentral	A ncRNA sequence database coordinated by European Bioinformatics Institute and associated with 25 different databases	<a href="http://rnacentral.org/">http://rnacentral.org/</a>	RNAcentral Consortium (2017)
PMTED	A target expression prediction database exclusively for plant miRNAs	<a href="http://pmted.agrinome.org/">http:// pmted.agrinome.org/</a>	Sun et al. (2013)
miRPlant	A program with tools to predict novel plant miRNAs	<a href="https://sourceforge.net/projects/mirplant/">https://sourceforge.net/projects/mirplant/</a>	An et al. (2014)
miRDeep-P	A computational tool for analyzing the miRNA transcriptome in plants.	<a href="https://sourceforge.net/projects/mirdp/">https://sourceforge.net/projects/mirdp/</a>	Yang and Li (2011)
P-SAMS	A plant sRNA maker site especially for amiRNAs and syn-tasiRNA	<a href="http://p-sams.carringtonlab.org">http://p-sams.carringtonlab.org</a>	Fahlgren et al. (2016)
PmiRKB	Plant miRNA knowledge base	<a href="http://bis.zju.edu.cn/pmirkb/">http://bis.zju.edu.cn/pmirkb/</a>	Meng et al. (2010)
NONCODE	An integrated knowledge database dedicated to ncRNAs from various organisms including <i>Arabidopsis</i>	<a href="http://www.noncode.org/">http://www.noncode.org/</a>	Zhao et al. (2016)
PNRD	A plant ncRNA database with 25739 entries of 11 ncRNA-types from 150 plant species	<a href="http://structuralbiology.cau.edu.cn/PNRD/index.php">http://structuralbiology.cau.edu.cn/PNRD/index.php</a>	Yi et al. (2015)
plantDARIO	Web-based tools and platform for quantitative and qualitative analyses of RNA-seq data in plants	<a href="http://snostrip.bioinf.uni-leipzig.de/index.py">http://snostrip.bioinf.uni-leipzig.de/index.py</a>	Patra et al. (2014)
miRge	Tool for processing sRNA-seq data to determine miRNA entropy	<a href="http://atlas.pathology.jhu.edu/baras/miRge.html">http://atlas.pathology.jhu.edu/baras/miRge.html</a>	Baras et al. (2015)
PmiRExAt	A database resource that provides plant miRNA expression profile and query tool for 1859 wheat, 2330 rice and 283 maize miRNAs	<a href="http://pmirexat.nabi.res.in">http://pmirexat.nabi.res.in</a>	Gurjar et al. (2016)
miRPursuit	A pipeline to provide running end-to-end analyses of high-throughput sRNA-seq data in non-/model plants from known and novel sequences	<a href="https://readthedocs.org/projects/mirpursuit/">https://readthedocs.org/projects/mirpursuit/</a>	Chaves et al. (2017)
mirEX 2.0	A platform to explore plant miRNA expression data based on qRT-PCR and NGS	<a href="http://www.combio.pl/mirex">http://www.combio.pl/mirex</a>	Zielezinski et al. (2015)
miRA	A tool useful for identifying miRNA precursors in plants	<a href="https://github.com/mhuttner/miRA">https://github.com/mhuttner/miRA</a>	Evers et al. (2015)
miRandb	miRNA algorithmic network database, a meta-database offering online services for miRNA research	<a href="http://mirandd.ir">http://mirandd.ir</a>	Aghaee-Bakhtiari et al. (2017)

**Table 4.** Halophytes as sources of genes for enhanced salt-tolerance in glycophytes.

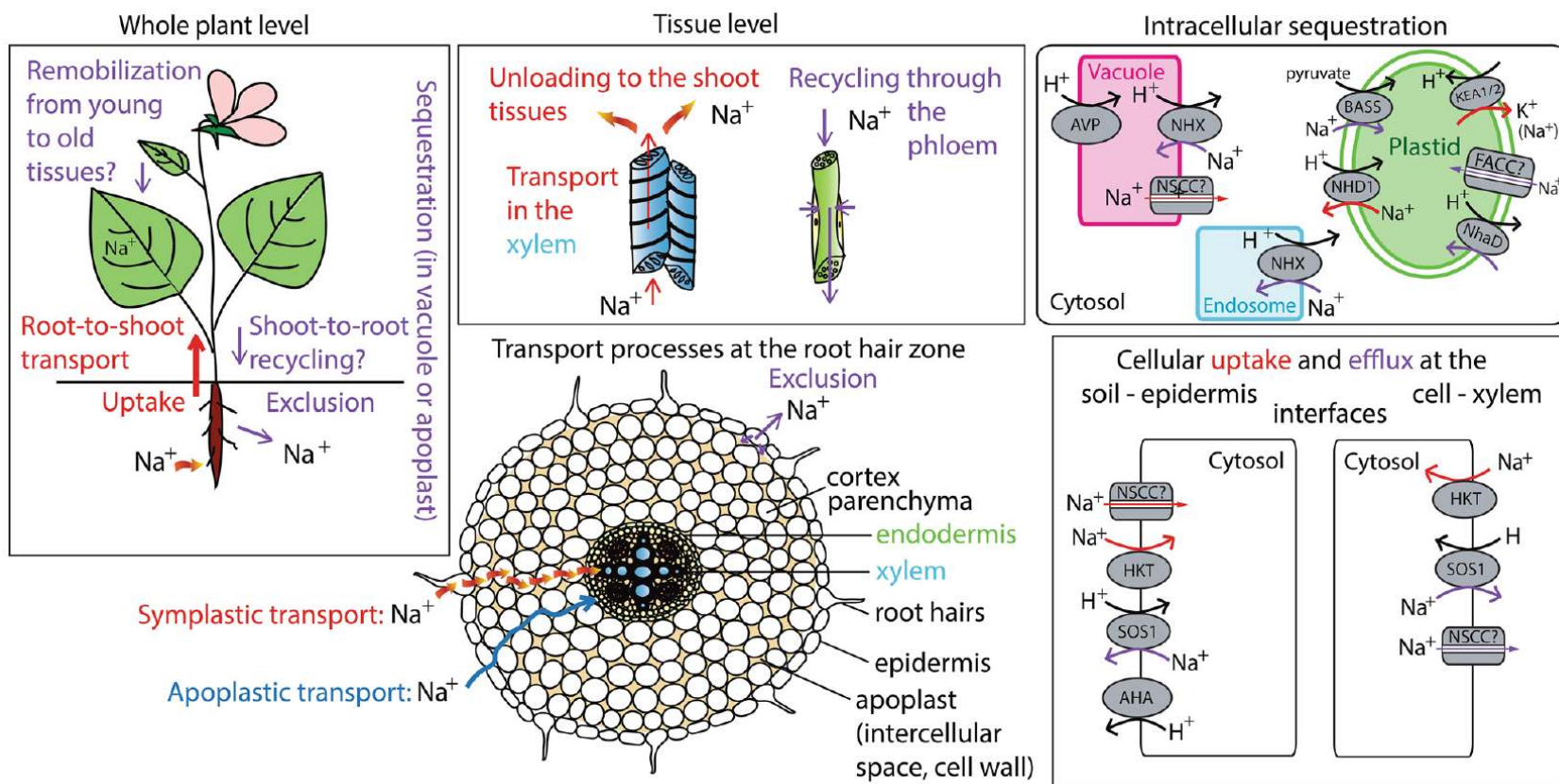
Donor halophyte	Gene	Description	Transformed glycophyte	Reference
<i>Aeluropus littoralis</i>	AINHX1	Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Nicotiana tabacum</i>	Zhang et al. (2008)
<i>Ammopiptanthus mongolicus</i>	AmVP1	Vacuolar H <sup>+</sup> -pump	<i>Arabidopsis thaliana</i>	Wei et al. (2012)
<i>Atriplex dimorphostegia</i>	AdNHX1	Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Oryza sativa</i>	Li et al. (2008)
<i>Atriplex gmelini</i>	AgNHX1	Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Oryza sativa</i>	Ohta et al. (2002)
<i>Atriplex hortensis</i>	AhBADH	Synthesis of glycine betaine	<i>Nicotiana tabacum</i>	Jia et al. (2002)
<i>Chenopodium glaucum</i>	CgNHX1	Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Oryza sativa</i>	Li et al. (2008)
<i>Chloris virgata</i>	CvPMAAC	Plasma membrane H <sup>+</sup> -ATPase	<i>Arabidopsis thaliana</i>	Zhang et al. (2014a)
	HcNHX1	Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Arabidopsis thaliana</i>	Guan et al. (2011)
<i>Halostachys caspica</i>	HcVHA-B	Vacuolar H <sup>+</sup> -ATPase, subunit B	<i>Arabidopsis thaliana</i>	Hu et al. (2012)
	HcVP1	Vacuolar H <sup>+</sup> -pump	<i>Arabidopsis thaliana</i>	Hu et al. (2012)
<i>Kalidium foliatum</i>	KfVP1	Vacuolar H <sup>+</sup> -pump	<i>Arabidopsis thaliana</i>	Yao et al. (2012)
<i>Pennisetum glaucum</i>	PgNHX1	Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Brassica juncea</i> <i>Oryza sativa</i>	Rajagopal et al. (2007) Verma et al. (2007)
	PutAKT1	Plasma membrane located K <sup>+</sup> inward rectifying channel (KIRC)	<i>Arabidopsis thaliana</i>	Ardic et al. (2010)
<i>Puccinellia tenuifolia</i>	PtNHA1	Plasma membrane Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Arabidopsis thaliana</i>	Wang et al. (2011)
	PutNHX	Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Oryza sativa</i>	Kobayashi et al. (2012)
	SbMYB15	R2R3-type transcription factor (TF)	<i>Nicotiana tabacum</i>	Shukla et al. (2015)
	SbASR1	Abscisic acid stress ripening-1	<i>Arachis hypogea</i> <i>Jatropha curcas</i>	Tiwari et al. (2015) Jha et al. (2013)
	SbNHX1	Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Ricinus communis</i> <i>Cuminum cyminum</i>	Patel et al. (2015) Pandey et al. (2016)
<i>Salicornia brachiata</i>	SbpAPX	Peroxisomal ascorbate peroxidase	<i>Nicotiana tabacum</i>	Singh et al. (2014)
	SbpAPX	Peroxisomal ascorbate peroxidase	<i>Arachis hypogea</i>	Singh et al. (2014)
	SbSDR1	Salt and drought responsive gene	<i>Nicotiana tabacum</i>	Singh et al. (2016)
	SbSOS1	Plasma membrane Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Nicotiana tabacum</i>	Yadav et al. (2012)



<i>Salicornia europaea</i>	SeNHX1	Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Medicago sativa</i>	Zhang et al. (2014c)
<i>Salix matsudana</i>	SmQR	Quinone reductase	<i>Arabidopsis thaliana</i>	Song et al. (2016)
<i>Salsola soda</i>	SsNHX1	Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Medicago sativa</i>	Li et al. (2011)
<i>Sesuvium portulacastrum</i>	SpAQP1	Aquaporin gene	<i>Nicotiana tabacum</i>	Chang et al. (2016)
	<i>SpAHA1</i>	Plasma membrane H <sup>+</sup> -ATPase	<i>Arabidopsis thaliana</i>	Fan et al. (2018)
<i>Spartina alterniflora</i>	SaVHAc1	Vacuolar H <sup>+</sup> -ATPase subunit c1	<i>Oryza sativa</i>	Baisakh et al. (2012)
<i>Suaeda liaotungensis</i>	SIBADH	Betaine aldehyde dehydrogenase	<i>Zea mays</i>	Wu et al. (2008)
	SINAC	NAC transcription factor	<i>Arabidopsis thaliana</i>	Li et al. (2014)
<i>Suaeda corniculata</i>	ScBADH	Betaine aldehyde dehydrogenase	<i>Arabidopsis thaliana</i>	Wang et al. (2016)
	ScVP	Vacuolar H <sup>+</sup> -pump	<i>Arabidopsis thaliana</i>	Liu et al. (2011)
	SsCAX1	Vacuolar H <sup>+</sup> /Ca <sup>2+</sup> transporter	<i>Arabidopsis thaliana</i>	Han et al. (2012)
<i>Suaeda salsa</i>	SsDREB	Dehydration-responsive element-binding (DREB) transcription factor	<i>Nicotiana tabacum</i>	Zhang et al. (2015)
	Ss.sAPX	Stroma ascorbate peroxidase	<i>Arabidopsis thaliana</i>	Li et al. (2012)
<i>Tamarix androssowii</i>	TaMnSOD	Manganese superoxide dismutase	<i>Populus davidiana</i> x <i>P. bolleana</i>	Wang et al. (2010)
	TsNHX1	Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Arabidopsis thaliana</i>	Wu et al. (2009)
<i>Thellungiella salsuginea</i>	TsLEA1	Late embryogenesis abundant (LEA)	<i>Arabidopsis thaliana</i>	Zhang et al. (2012)
	TsTIP1	Tonoplast aquaporin gene	<i>Arabidopsis thaliana</i>	Wang et al. (2014)
	ThSOS1	Plasma membrane Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>Arabidopsis thaliana</i>	Oh et al. (2009)
<i>Zoysia matrella</i>	ZmVP1	Vacuolar H <sup>+</sup> -pyrophosphatase (VP)	<i>Arabidopsis thaliana</i>	Chen et al. (2015)

**Table 5.** Additional sources of genes (from halobionts) for enhanced salt-tolerance in glycophytes.

Donor type	Organism	Gene	Transformed glycophyte	Improved character/s	References
Halotolerant cyanobacterium	<i>Aphanothece halophytica</i>	ApGSMT and ApDMT	<i>Oryza sativa</i> L., cv. Nipponbare	Enhancement in glycine betaine synthesis, improved growth and survival rate during stress as well as recovery stage	Niu et al. (2014)
Halophilic bacterium	<i>Halomonas elongata</i>	HeectA, HeectB and HeectC	<i>Lycopersicon esculentum</i> cv. 'Momotaro' and cv. 'Saturn'	Increased accumulation of ectoine, improved dry weight values and photosynthetic rate, decreased accumulation of MDA in leaves	Moghaieb et al. (2011)
Halophilic archaeon	<i>Natrinema altunense</i>	NaMnSOD	<i>Oryza sativa</i> L., cv. Nipponbare	Increased SOD and CAT activities, reduced superoxide radical and hydrogen peroxide levels, reduced ion leakage and MDA content	Chen et al. (2013)
Halophilic fungi	<i>Aspergillus glaucus</i>	AgRPS3aE	<i>Arabidopsis thaliana</i> (Columbia-0) <i>Nicotiana tabacum</i> cv. SR-1	Increased leaf area and root length	Liang et al. (2015)
	<i>Aspergillus glaucus</i>	AgRPL44	<i>Nicotiana tabacum</i>	Increased root length, fresh weight	Liu et al. (2014)
Halotolerant algae	<i>Dunaliella salina</i> strain Y6	Ds-A3-3 and Ds-26-16	<i>Nicotiana tabacum</i>	Increased leaf area, root length, enhanced level of SOS gene expression	Gong et al. (2014)
	<i>Chlamydomonas</i> strain W80	GPX-like protein cDNAs (GPX and GPX)	<i>Nicotiana tabacum</i> cv. Xanthi	Increased tolerance against oxidative and salt stress with reduced lipid peroxidation and increased photosynthetic and antioxidative system	Yoshimura et al. (2003)



**Figure 1. Summary of Na<sup>+</sup> fluxes and transport in plants.** Represented processes include those at the whole plant level, in various tissues and tissues interfaces, as well as within cells. Ions, e.g. Na<sup>+</sup>, from the soil can enter the root cortex via apoplastic transport through cell wall spaces and intercellular cavities. Except for the branching zone (sites of lateral root formation) and the meristematic and elongation zones, the endodermis forms a barrier that stops the apoplastic flow of Na<sup>+</sup> and forces all ions to move through the symplast into the xylem. With respect to root hairs, ions can enter the cytoplasm through specific channels and transporters, and are then transported via the symplast to the central vascular cylinder. Once loaded into the xylem, Na<sup>+</sup> is transported to the shoot, where it is unloaded from the xylem into the shoot tissues and apoplast. It is still a matter of debated whether Na<sup>+</sup> recycling can occur through the phloem back down to the root. Specific transport mechanisms are probably involved in excluding Na<sup>+</sup> flow from the xylem towards back into cortex and from epidermis cells back into the soil. Redistribution of Na<sup>+</sup> from young tissues and organs towards older ‘sinks’, organs that may be sacrificed, is another possibility that has been suggested as a salt tolerance mechanism. Processes or components are unproven at present are indicated by question marks.

**Figure 2. Selected salt responsive plant miRNAs, their targets and the traits regulated by these miRNAs** (summary diagram showing information obtained from important crops including *Oryza sativa*, *Zea mays*, *Triticum aestivum*, *Hordeum vulgare*, *Glycine max*, *Solanum tuberosum*, and *Solanum lycopersicum*).

