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Chapter 6. General discussion

6.1. Development of salt-tolerant crops

One of the great challenges of the 21st century is to secure food production and fresh water availability for the increasing world population, which is forecast to reach 9.5 billion by 2050 (UN 2012). Salt-tolerant crops could contribute in this challenge by reducing the pressure on fresh water and agricultural lands (Rozema and Flowers 2008). This thesis has focussed on the development of salt-tolerant crops by exploring the mechanisms underlying salt tolerance. Salt tolerance is a trait that depends on several morphological, physiological and biochemical characteristics encoded by many different genes. The combination of these characteristics determines the degree of salt tolerance. Highly salt-tolerant plants, such as Salicornia dolichostachya, are able to grow at seawater salinity (Chapter 2), which makes them interesting to study adaptation mechanisms to salinity. Knowledge of these mechanisms will accelerate the development of salt-tolerant crops, regardless of whether the development will be based on the domestication of halophytes, breeding of conventional crops, or genetic engineering. In this final chapter, the main findings of this thesis, potential research avenues and questions based on the work presented, and the prospects for salt tolerance research are discussed.

6.1.1. Morphological and physiological traits underlying the growth response of Salicornia dolichostachya to salinity

The family of the Amaranthaceae includes a high number of halophytes such as the species of the genus Salicornia (Flowers 1985). These halophytes use Na\(^+\) for osmotic adjustment and can accumulate vast amounts of Na\(^+\) without growth reductions (Flowers and Colmer 2008). The growth optimum of S. dolichostachya was at 300 mM NaCl in the external environment, where its growth rate was 92 mg g\(^{-1}\) d\(^{-1}\) (Chapter 2). This is a much higher level of salt tolerance than that of the well-studied model halophyte Eutrema halophylum (= Thellungiella halophyla) (Inan et al. 2004; Orsini et al. 2010), which shows reduced growth rate at 100 mM NaCl compared to zero salinity (Inan et al., 2004). At its
growth optimum *S. dolichostachya* accumulated approximately 400 mM Na\(^+\) in its shoot cells, and interestingly, at 50 mM NaCl the shoot Na\(^+\) concentration was already high, around 300 mM (Chapter 2). This indicates that *S. dolichostachya* is highly efficient in the uptake and retention of Na\(^+\) even at low salinities. Probably Na\(^+\) uptake and compartmentalisation is a constitutive trait in *S. dolichostachya*, which ecologically can be explained by the fact that *S. dolichostachya* grows in the lower salt marsh (Huiskes et al. 1985; Rozema et al. 1987; Kadereit et al. 2007; Teege et al. 2011), where NaCl concentrations in the soil are always high and fluctuating around seawater salinity. Likely, this is connected with the observation in Chapter 2 that 0 and 5 mM NaCl in the external environment was insufficient to maintain growth of *S. dolichostachya*, which suggests a Na\(^+\) requirement for growth in this species. The precise nature of this apparent physiological Na\(^+\) dependency is unknown. In any case, it is likely that it reflects the constitutive nature of one or more component traits of the salt tolerance syndrome. Their operation in the absence of salt apparently causes metabolic or nutritional disturbances, eventually leading to growth arrest and mortality (Katschnig et al. 2013; Rozema and Schat 2013).

Compatible solutes increase in a variety of plant species in response to salinity (Ashraf and Foolad 2007). Especially glycophytes rely often for a large part on the accumulation of compatible solutes to adjust their water potential, which is an energetically expensive strategy (Munns and Tester 2008). Salt accumulating dicotyledonous halophytes use Na\(^+\) and Cl\(^-\) as ‘cheap’ osmolytes and compatible solutes to balance the osmotic potential within the cell (Flowers and Colmer 2008). *S. dolichostachya* probably uses mainly the compatible osmolyte glycine betaine (Weretylnik et al. 1989). The cellular glycine betaine concentrations that I measured in Chapter 2 were at all external salinities sufficient to balance the water potential within the cell, assuming that Na\(^+\) and Cl\(^-\) were compartmentalised inside the vacuole and glycine betaine was present inside the cytoplasm. However, the glycine betaine concentrations did not increase with increased salinity (Chapter 2). It is possible that glycine betaine is relocated from the vacuole to the cytoplasm in response to salinity, which would allow a faster adjustment of the cellular water potential than de novo synthesis. Concentration measurements of glycine betaine in the different cellular compartments would be interesting. As compatible solutes are often mentioned as a way to increase salt tolerance in crops (Ashraf and Foolad 2007), it would be useful to establish how they exactly function in osmotic adaptation.
6.1.2. Na⁺ compartmentalisation into the vacuole

Compartmentalisation of Na⁺ inside the vacuole prevents the accumulation of detrimental levels of Na⁺ inside the cytoplasm and provides adaptation to the low osmotic potentials of the outside environment. Therefore, enhancing the vacuolar compartmentalisation capacity is a well-aspired strategy to increase salt tolerance in crops (Apse et al. 1999). The Na⁺, K⁺/H⁺ antiporter NHX1 is involved in Na⁺ compartmentalisation (Gaxiola et al. 1999; Apse and Blumwald, 2007) or K⁺ compartmentalisation into the vacuole (Venema et al. 2002), depending on the prevailing concentration. The vacuolar H⁺-pumps, the V-H⁺-ATPase and the V-H⁺-PPase, provide the electrochemical gradient for the Na⁺, K⁺/H⁺ antiporter (Gaxiola et al. 2007; Apse et al. 1999) (see Fig. 1 Chapter 8 Summary). Transgenic overexpression of NHX1 has been claimed to enhance salt tolerance in different species (Apse et al. 1999; Zhang and Blumwald 2001): however, so far, the observed increases in salt tolerance are not very impressive. Because S. dolichostachya grows optimal at high internal Na⁺ concentrations (up to 400 mM), and Na⁺ concentrations above 200 mM in the cytoplasm are most probably as toxic for S. dolichostachya as for any glycophytic species (Greenway and Osmond, 1972; Flowers et al. 2014), I assume that S. dolichostachya must have highly efficient mechanisms for Na⁺ accumulation and storage. The activity of the V-H⁺-ATPase, the V-H⁺-PPase and the Na⁺, K⁺/H⁺ antiporter were at the same level in S. oleracea and S. dolichostachya at the 200 mM NaCl treatment (Chapter 3). Furthermore, when treated with 200 mM NaCl, S. dolichostachya grew better than S. oleracea. This indicates that S. dolichostachya might have a more efficient system of Na⁺-retention inside the vacuole than S. oleracea, or that the water potential equilibrium within the cell was better regulated in S. dolichostachya than in S. oleracea. A more efficient Na⁺-retention inside the vacuole might be accomplished through altered regulation of the slow- and fast-activating vacuolar channels as found for Quinoa (Chenopodium quinoa) (Bonales-Alatorre et al. 2013), and/or by the fluidity of the tonoplast membrane, dependent on the saturation of the fatty acid chains (Leach et al. 1990). For genetic engineering of salt tolerance, it might therefore be of interest not only to consider the transporters that accommodate Na⁺ compartmentalisation into the vacuole, but also the transporters or traits that prevent leakage form the vacuole.
6.1.3. SOS1 in *S. dolichostachya*

Differences between halophytes and glycophytes in gene expression levels and location might give indications for genes and traits important in salt tolerance of halophytes (Rozema and Schat 2013). In Chapter 4, I investigated potential candidate salt tolerance genes in *S. dolichostachya* in comparison with *S. oleracea*. The strongly enhanced expression of SOS1 in *S. dolichostachya* in comparison with *S. oleracea*, and the absence of detectable expression of the *AtHKT1;1* ortholog in *S. dolichostachya* were remarkable (Chapter 4). This might be a consequence of the ‘accumulation strategy’ for salt tolerance in *S. dolichostachya*, which implicates the use of Na\(^+\) as a major osmolyte in the shoot. SOS1-mediated Na\(^+\) loading into the xylem might provide sufficient Na\(^+\) ions reaching the shoot, whereas the prevention of Na\(^+\) reaching the shoot by HKT-mediated Na\(^+\) retrieval from the xylem is not necessary. However, it is possible that *S. dolichostachya* has more than one *HKT1* paralog. To answer this question, a full genome assembly of *S. dolichostachya* would be helpful. Furthermore, because the Na\(^+\) transporters in *S. dolichostachya* in theory could have a different transport capacity or transport different ions than their orthologs in *A. thaliana*, characterisation of these transporters in *S. dolichostachya* would also be necessary.

Expression of genes in different locations might lead to differences in the function of the gene (Moller et al. 2009; Plett et al. 2010). GUS expression from the partial *SdSOS1* promoter led to staining in the stele of mature roots parts in *A. thaliana* (Chapter 4), whereas GUS expression from the *AtSOS1* promoter leads to staining mainly in the root tips (Shi et al. 2002). In the xylem parenchyma cells, *SOS1* transports Na\(^+\) into the xylem, which affects Na\(^+\) accumulation in the shoot (Shi et al. 2002; Shabala et al. 2005; Jha et al. 2010); in the root tip epidermal cells, *SOS1* transports Na\(^+\) from the root into the rhizosphere (Shi et al. 2002; Shabala et al. 2005; Jha et al. 2010)(see Fig. 1 Chapter 1 General introduction), which lowers the Na\(^+\) levels inside the root tip. These differences in *SOS1* expression between *S. dolichostachya* and *A. thaliana*, and the implied differences in *SOS1* function based on the location of expression, provides evidence for the more general hypothesis that halophytes and glycophytes potentially use the same genes for different purposes by expressing these genes at a different level and in different locations.

The expression level of genes can be enhanced either by copy number expansion (Hanikenne et al. 2008), or *cis*-regulatory or trans-regulatory alterations (Yamaguchi-Shinozaki and Shinozaki 2005). The differences in *SOS1* expression between *S. dolichostachya* and *S. oleracea* could not be explained by *SOS1* gene copy number variation;
however, some putative cis-regulatory elements containing binding sites for transcription factors that are induced by osmotic stress were exclusively present or present in higher numbers in the AtSOS1 promoter compared with the ShSOS1 promoter (Chapter 5). This would indicate that binding of stress inducible transcription factors to the AtSOS1 promoter, maybe in combination with the SOS2-SOS3 complex (Qiu et al. 2002), might be responsible for AtSOS1 up-regulation in response to salt treatment. However, the constitutive SOS1 expression of Salicornia is not likely to originate from constitutive binding of normally inducible transcription factors to cis-regulatory DNA elements. Constitutive SdSOS1 expression might be caused by the absence of a binding site for a negative regulator, or, conversely, enhanced AtSOS1 transcript concentrations in response to salt treatment might as well result from a higher stability of the transcript in the presence of salt (Shi et al. 2003; Chung et al. 2008).

In the context of SOS1 regulation, it would be of interest to study differences in SOS2 and SOS3 expression between S. dolichostachya and S. oleracea in response to salinity. This would give additional information about the regulation of SdSOS1 in Salicornia. Another interesting aspect regarding SOS1 regulation is its unusually long cytoplasmatic tail. Within this tail A. thaliana has a 500 bp sequence that causes instability in the absence of Na⁺. It would be interesting to establish if Salicornia also possesses this sequence, and if this sequence confers the same instability to Salicornia SOS1 mRNA as shown for AtSOS1 mRNA (Shi et al. 2003; Chung et al. 2008). The cytoplasmic tail of SOS1 might also be interesting regarding the sensing of Na⁺. How Na⁺ is sensed by plants is not known yet. It could be sensed intercellularly or intracellularly. In theory, the cytoplasmic tail of SOS1 could be involved in sensing (Shi et al. 2000), as shown for glucose sensing in two glucose transporters with comparably long cytoplasmic tails (Özcan et al. 1998). In any case, either as a transporter or a sensor, or both, SOS1 is extremely important in salt tolerance, as demonstrated by the pronounced salt-hypersensitivity of sos1 mutants (Wu et al. 1996).
6.1.4. The Salicornia brachystachya genome

The big advantages of de novo draft genome assembly based on short reads are the limited cost and the relative short time span in which the de novo draft assembly can be generated. Several short read assemblers are publicly available (Kajitani et al. 2014; Simpson et al. 2009; Zerbino and Birney 2008; Luo et al. 2012). Our de novo genome draft assembly of S. brachystachya covered approximately 35% of the genome (Chapter 5). However, this 35% is expected to contain many coding regions, as coding regions tend to contain less repeated stretches than non-coding regions, which makes them easier to assemble. As cloning of orthologous genes and their adjacent promoter regions can be highly time consuming, I advocate that deep sequencing technologies would be a faster strategy. Moreover, much more information besides sequences of orthologous genes can be gained from de novo draft genome assemblies. Salt tolerance research could greatly benefit from the disclosure of the genome sequences of halophytes of different phylogenetic origins. Besides genome assemblies, the development of transformation protocols for halophytes would also be rewarding.

6.1.5. Conclusions and prospects

To develop plants with a higher salt tolerance, it would be helpful to unravel the genetic determinants of salt tolerance (Rozema and Schat 2013). This will enable the use of modern marker systems for breeding as well as the development of salt-tolerant crops through genetic engineering. Moreover, the domestication of halophytes could profit from the knowledge of the genetic determinants, so that during domestication the salt tolerance traits will be retained. As salt tolerance is a multigenic trait, probably with epistatic intergenic interaction effects, the development of salt-tolerant crops has shown to be a difficult task in the past (Flowers and Flowers 2005, Ashraf and Akram 2009). Minor increases in salt tolerance have been claimed by the overexpression of single genes (Apse et al. 1999; Shi et al. 2003), which indicates that different salt tolerance genes can have at least partially additive individual effects, which might in turn imply that pyramiding of such ‘salt tolerance’ genes might be a fruitful strategy. Only one major breakthrough for increased salt tolerance, by introgression of a locus originating from ancestral bread wheat into durum wheat, has been reported in the literature (Munns et al. 2012). As research techniques advance and
become more accessible, the discovery of mechanisms and genes contributing to salt tolerance might accelerate, which will speed up the development of salt-tolerant crops. Halophyte research can greatly contribute to further progress in salt tolerance research, because they offer possibilities to discover mechanisms and alleles for high salt tolerance enabling crops to be cultivated with seawater irrigation. Comprehensive knowledge of the molecular mechanisms of salt tolerance in a selection of taxonomically divergent halophytes will open new prospects that can be utilised in the development of salt-tolerant crops.