Environmental and Experimental Botany 99 (2014) 59–66

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Expression levels and promoter activities of candidate salt tolerance genes in halophytic and glycophytic Brassicaceae

Ismat Nawaz[a,*,1], Mazhar Iqbal[a,2], Henk W.J. Hakvoort[b], Mattijs Bliek[a], Bert de Boer[c], Henk Schat[a]

[a] Department of Genetics, Faculty of Earth and Life Sciences, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands
[b] Department of System Ecology, Faculty of Earth and Life Sciences, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands
[c] Department of Structural Biology, Faculty of Earth and Life Sciences, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 5 July 2013
Received in revised form
22 September 2013
Accepted 9 October 2013

Keywords:
Brassicaceae
NHX1
Promoter swapping
Salt tolerance
SOS1
VATD

ABSTRACT

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

Expression of *NHX1* genomic DNA under the *C. x hollandica NHX1* promoter in the *A.t.nhx1* mutant background yielded, irrespective of the genomic DNA source, 20-fold and 2-fold enhanced expression levels, in comparison with those in wild-type *A. thaliana* and *C. x hollandica*, respectively. This suggests that the high expression level in *C. x hollandica* is completely explained by altered *cis*-regulation of this gene. Promoter swap experiments showed that the *C. x hollandica SOS1* and *VATD* promoters were five-fold and two-fold more active than the corresponding *A. thaliana* promoters, respectively. However, particularly in the case of *VATD*, this is not sufficient to explain the difference in the wild-type expression levels between *C. x hollandica* and *A. thaliana*.

1. Introduction

On the basis of its level of salt tolerance, a species can be classified as a halophyte or a glycophyte. Halophytes are defined as plants that can complete their life cycle under continuous exposure to high concentrations of salt (Flowers and Colmer, 2008). Halophytes are widely but unevenly spread over higher plant families and orders (Flowers et al., 1977). They exhibit, in a phylogenetically biased way, a broad variety of adaptations to salt, including specific morphological structures, such as salt glands or bladder cells. The physiological determinants of the superior salt tolerance in halophytes are poorly known and, most probably, also subject to phylogenetic bias (Flowers and Colmer, 2008). There is strong evidence that salt tolerance in halophytes within the Poales order is associated with enhanced levels of selectivity for K^+ over Na^+ (Flowers and Colmer, 2008), leading to Na^+ exclusion and the maintenance of high cellular K^+ levels under salt exposure (Colmer et al., 2006). Dicotyledonous halophytes exhibit much lower degrees of K^+/Na^+ selectivity and accumulate Na^+ often to high levels in their tissues, using it as a 'cheap' osmolyte (Flowers et al., 1977; Flowers and Colmer, 2008). Since cytoplasmic Na^+ tolerance does not seem to exist in halophytes, it is therefore often believed that at least salt accumulating halophytes must have evolved enhanced capacities for Na^+ compartmentalization at the levels of organs, tissues, cells and organelles (Flowers and Colmer, 2008). All halophytes must also be capable to synthesize and accumulate 'compatible solutes', to achieve osmotic adjustment of the cytoplasm and organelles other than the vacuole. There is a huge variation, even within plant families, in the types of compatible solutes used by halophytes. It has often been suggested that halophytes should exhibit enhanced capacities for compatible solute accumulation, but there is no

---

* Corresponding author. Tel.: +92 311 6691956; +31 923 155407451.
E-mail addresses: saraneva1109@gmail.com, mazz1238@yahoo.com (I. Nawaz), mazz366@gmail.com (M. Iqbal), h.w.j.hakvoort@vu.nl (H.W.J. Hakvoort), m.bliek@vu.nl (M. Bliek), a.h.de.boer@vu.nl (B. de Boer), h.schat@vu.nl (H. Schat).

[1] Current address: Department of Biosciences, COMSATS Institute of Information Technology, Islamabad, Pakistan.

[2] Current address: Depatment of Environmental Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

0098-8472/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.envexpbot.2013.10.006

hard evidence either in favor or against this hypothesis. In general, glycophytes tend to accumulate various compatible solutes under exposure to a broad variety of stresses, including salt, drought, frost, or even heavy metal toxicity (Munns and Tester, 2008).

Except for the recently proposed halophyte model species, *Thellungiella halophila* (Inan et al., 2004; Gong et al., 2005), the molecular mechanisms of salt tolerance have been investigated almost exclusively in glycophytes thus far, in particular the plant genetics model, *Arabidopsis thaliana*. These studies have revealed a number of genes that appeared to be essential for wild-type-level salt tolerance in *A. thaliana*, including those encoding the Na^+ transporter SOS1, which is a Na^+ effluxing plasma membrane-located Na^+/H^+ antiporter (Shi et al., 2000), NHX1, a tonoplast Na^+/H^+ antiporter (Pardo et al., 2006), and HKT1, a plasma-membrane-located Na^+ influxer, supposed to be a $Na^+(K^+)/H^+$ antiporter (Rubio et al., 1995), or a $Na^+(K^+)$ channel (Horie et al., 2009; Kronzucker and Britto, 2011), and suggested to resorb Na^+ from the xylem. HKT1 is also supposed to promote Na^+ retranslocation to the root via the phloem (Berthomieu et al., 2003). Other genes supposed to be essential for normal salt tolerance in glycophytes are those encoding the plasma-membrane H^+-ATPase, the tonoplast H^+-ATPase, *VAT*, and the tonoplast H^+-pyrophosphatase (*V-PPA*), because their gene products are responsible for the maintenance of the electric potential or pH gradient required for passive or secondary active Na^+ transport by HKT1, or SOS1 and NHX1, respectively (Vera-Estrella et al., 2005; Martinez-Atienza et al., 2007; Silva and Geros, 2009).

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

There is no common opinion on the type of molecular changes, which underly high-level salt tolerance in halophytes to date. In attempts to genetically engineer improved salt tolerance in glycophyte crops, many investigators have used cDNAs of halophytic origin (Ashraf and Akram, 2009), which apparently reflects the belief that structural changes at the protein level could be responsible, at least in part, for the superior salt tolerance in halophytes. The effects of transgenes from halophytic and glycophytic sources have only seldomly been compared in a single experiment, but the few studies available to date unequivocally suggest that the transgene source is irrelevant for its effect in the host (Chang-Qing et al., 2008; Li et al., 2008). This suggests that non-synonymous mutations in the coding regions of particular genes may not be primarily responsible for the superior salt tolerance in halophytes. Indeed, it is more likely that halophytes and glycophytes basically use the same set of genes to cope with salt, but express them in a different way, most likely through altered *cis*-regulation (Wittkopp et al., 2004) or, such as established for heavy metal tolerance in metallophytes, a combination of altered *cis*-regulation and gene copy number expansion (Hanikenne et al., 2008).

Reports on direct comparisons of gene expression patterns between halophytes and glycophytes are remarkably scarce to date, which hampers a deeper understanding of the salt tolerance mechanisms in halophytes. Extensive transcriptome comparisons are only available for *T. halophila* and *A. thaliana*, which share sufficient DNA identity to allow the use of *A. thaliana*-based cDNA microarrays (Taji et al., 2004; Gong et al., 2005). However, there are reasons to believe that *T. halophila* might not be the ultimate halophyte model. First, it has the slow maximum growth rate typical of a “stress tolerator” (Grime, 1979), which is not apparent in coastal halophytes (Flowers and Colmer, 2008; Rozema and Schat, 2013). Second, although it seems to survive at sea water salinity level for a fairly long period, its growth rate is already severely inhibited at relatively low salinity (Inan et al., 2004), which is also different from coastal halophytes (Flowers and Colmer, 2008). Moreover, *T. halophila* is also tolerant for several other stresses, like temperature extremes, or drought (Bressan et al., 2001; Inan et al., 2004), which is, again, often not the case in coastal halophytes, and may lead to difficulties in distinguishing specific ‘salt tolerance genes’ from (other) ‘stress tolerance genes’.

In view of the above, it would be interesting to gain much more information on the expression patterns of candidate salt tolerance genes in halophytes other than *T. halophila*, and in glycophytes other than *A. thaliana*. In this study we address the question whether enhanced expression of *SOS1*, *NHX1*, or the genes encoding the D subunit of tonoplast H^+-ATPase (*VATD*), could contribute to the superior salt tolerance in halophytes in comparison with various glycophytes. A second aim was to compare the concentrations and root-to-shoot allocation patterns of Na^+ and K^+ among and within the halophytes and glycophytes and, in so far possible, to infer possible correlations with salt tolerance and gene expression patterns. To facilitate gene identification we confined our selection of halophytes and glycophytes to the Brassicaceae family. We selected a coastal halophyte, *Cochlearia x hollandica* (Pegtel, 1999), which is the allohexaploid hybrid of *C. anglica* and *C. officinalis* (Koch et al., 1998), a continental inland halophyte, *Thellungiella botschantzevii* (German, 2008), the glycophytes *Thlaspi arvense* and *A. thaliana*, as well as *Cochlearia danica* and *Brassica oleracea*, which could be expected to be relatively salt-tolerant glycophytes, in view of their more or less coastal distribution patterns. We checked the supposed halophyte/glycophyte status of these species by growing them with and without NaCl in the nutrient solution. Finally, to assess the potential role of *cis*-regulatory alterations in the evolution of differential candidate salt tolerance gene expression between halophytes and glycophytes, we isolated and cloned the upstream (partial) promoter sequences of *SOS1*, *NHX1*, and *VATD* from the most salt tolerant species, *C. x hollandica*. We expressed the gDNA coding regions or cDNA from *C. x hollandica* and *A. thaliana* in the corresponding *A. thaliana* mutant background or wild-type, both under the *A. thaliana* and the *C. x hollandica* supposed promoter sequences and compared the expression levels of the transgenes.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of *C. x hollandica* were collected from a ‘green beach’ at the island of Voorne, The Netherlands (51.91N, 4.05E). Seeds of *C. danica* were collected from a foredune at the island of Texel, The