Environmental and Experimental Botany 135 (2017) 1–12

Contents lists available at ScienceDirect

Environmental and Experimental Botany

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

Original research article

Unravelling the strategies used by the wild tomato species *Solanum pennellii* to confront salt stress: From leaf anatomical adaptations to molecular responses

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ARTICLE INFO

Article history:
Received 29 September 2016
Received in revised form 23 November 2016
Accepted 5 December 2016
Available online 7 December 2016

Keywords:
Solanum lycopersicum
Solanum pennellii
Salt stress
Halophytes
Stomata
Anatomical adaptations
Na^+ transporters
Aquaporins

ABSTRACT

Despite the genus *Solanum* include wild species with a high level of tolerance to salinity, the adaptation processes used by these halotolerant species are still poorly understood. Here, the differential responses to salinity of cultivated tomato *S. lycopersicum* (*Sl*) and the salt tolerant species *S. pennellii* (*Sp*) were studied in order to advance in the understanding of the strategies used by the latter one to confront salt stress, and allow identifying key tolerance traits which could be further introduced in tomato. Several physiological traits, as well as the anatomical structure of leaf and the expression of genes involved in water and Na^+ homeostasis, were analysed throughout the time period required by *Sp* to adapt to salinity (100 mM NaCl for 7 days). In this period, *Sp* was able to reduce water loss by regulating transpiration through reduced stomatal density and aperture. Other anatomical adaptations in *Sp* leaves, including larger and more turgid cells occupied by a giant vacuole, were associated to higher water and Na^+ accumulation. Interestingly, a higher basal expression of *PIP2;1* aquaporin was also found in leaf of *Sp* compared to *Sl*, which could be one of the causes of its better hydric status under salinity. With respect to the genes involved in Na^+ homeostasis, our results suggest that *SpHKT1;2* in combination with *SpSOS1* play an important role in Na^+-translocation from root to shoot, and therefore, in the determination of the includer behaviour in the wild species, which is in concordance with the higher transcript levels of Na^+ vacuolar transporters *SpNHX3* and *SpNHX4* in *Sp* leaves. Overall, these results highlight the necessity of a coordinated regulation of anatomical, physiological and molecular mechanisms in the salt tolerance of the wild species *Sp*.

1. Introduction

Development of crop plants tolerant to stress is vital to meet the growing food demand through sustainable agriculture. Salinity is responsible for important yield losses in crops, among them tomato (*Solanum lycopersicum*), a horticultural crop of the highest agro-economic importance. Although production losses are difficult to estimate, salt-affected soils constitute nearly 10% of the land surface (950 Mha) and 50% of all irrigated land (230 Mha) in the world (Ruan et al., 2010). This risk will increase as the population grows and therefore, one of the greatest challenges for the next years will be to increase crop production under salinity. To advance in this aspect it is necessary to know the main processes and the key genes controlling salt tolerance (Flowers et al., 2015). Despite the wealth of information available, these mechanisms are still poorly understood (Deinlein et al., 2014). This is likely to be a consequence of experimental limitations. First, salinity experiments frequently impose high stress levels and may lead to the identification of processes and genes involved in plant survival under extreme conditions (Shavrukov, 2013; Hancock et al., 2014).

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http://dx.doi.org/10.1016/j.envexpbot.2016.12.003
0098-8472/

Second, it is frequent to investigate individual or few traits when evaluating plant tolerance. However, since the salinity response is a complex process involving several interacting properties, its comprehension may need an integrated approach.

To understand the mechanisms responsible for salinity tolerance, it is important to bear in mind that salt stress comprises both osmotic and ionic stresses. Osmotic stress provokes the inhibition of water uptake as a consequence of increased salt content around the roots (Munns and Tester, 2008). One essential mechanism to cope with osmotic stress is osmotic adjustment, in which cells must accumulate solutes to balance extra osmotic pressure in the soil solution in order to maintain turgor. To achieve this balance, plants may use either an exclusion strategy, excluding saline ions and relying on organic solutes for osmotic adjustment, or a strategy named 'tissue tolerance', accumulating mostly saline ions and regulating their distribution within cell compartments. The latter is the most cost-efficient strategy (Munns and Gilliham, 2015), being the strategy generally used by the wild tomato species (Alarcon et al., 1993; Santa-Cruz et al., 1999). Other important mechanisms for avoiding osmotic stress are those directed to reduce transpirational water loss. Leaf transpiration seems to be regulated not only by stomatal closure but also by controlling stomatal density (Shabala et al., 2012). Thus, recent studies suggest that manipulating stomatal density reduces water loss in *Arabidopsis thaliana* (Franks et al., 2015; Hepworth et al., 2015). Besides regulation of transpiration, there are also other mechanisms aimed to regulate water movement through the cell. Aquaporins are considered the main protein channels for the transport of water and other small molecules through plant cell membranes (Maurel et al., 2008; Moshelion et al., 2015). The expression of aquaporin isoforms, especially those belonging to the plasma membrane intrinsic protein (PIP) and tonoplast intrinsic protein (TIP) subfamilies, is modulated in response to various stresses, including salinity and drought (Boursiac et al., 2005; Reuscher et al., 2013; Qian et al., 2015). Moreover, the manipulation of aquaporin expression in plants may result in enhanced tolerance to these abiotic stresses, as it has been shown for some members of the tomato *TIP2* (Sade et al., 2009) and *PIP2* (Li et al., 2016) subgroups.

Ionic stress is the second important issue plants need to face under salinity. In this regard, tolerance is determined in part by the ability of the plant to regulate Na^+ transport from root to the shoot over time, as well as the compartmentalization in cellular organelles in order to avoid toxic Na^+ concentrations in the cytoplasm (Maathuis, 2014; Shabala, 2013). Na^+ efflux is mediated by the plasma membrane Na^+/H^+ antiporter SOS1 (Hasegawa, 2013), whereas HKT transporters, particularly those belonging to class I (Platten et al., 2006), are critical determinants of Na^+ unloading from xylem vessels to other cells in the stele (Hasegawa, 2013). In tomato, two *HKT1*-like isoforms have been identified, named *SlHKT1;1* and *SlHKT1;2* (Asins et al., 2013). Compartmentalization in the vacuole of Na^+ ions is an effective mechanism to avoid the toxic effects of Na^+ in the cytosol (Maathuis, 2014). The transport of Na^+ from the cytoplasm into the vacuole occurs via tonoplast Na^+/H^+ −antiporters NHX. Four *NHX* isoforms have been identified in tomato, among them *SlNHX3* and *SlNHX4* show the strongest induction upon salinity (Galvez et al., 2012). In addition, *SlNHX3* has been associated with a QTL for Na^+ concentration in leaves (Villalta et al., 2008). However, *SlNHX2* was shown to be involved in K^+ but not Na^+ homeostasis (Huertas et al., 2013) and *SlNHX1* was associated with a QTL for Cl^- concentration in young leaves (Villalta et al., 2008).

In recent years, the use of halophytes for saline agriculture has been the subject of numerous reviews (Ruan et al., 2010; Shabala, 2013; Panta et al., 2014; Cheeseman 2015; Flowers and Colmer, 2015). Given that the diversity for stress tolerance within traditional crops is likely to be too narrow for improving plant productivity under stress conditions, it may be very interesting to advance in the knowledge of the salt tolerance mechanisms in halotolerant accessions of wild species related to tomato (Bergougnoux, 2014; Muir et al., 2014). The genus *Solanum* includes accessions of wild species like *S. pennellii*, which is endemic to Andean regions in South America and presents a high level of tolerance to salinity (Bolarin et al., 1991; Santa-Cruz et al., 1999). There are pronounced phenotypic differences between *S. lycopersicum* and *S. pennellii*, including fruits and vegetative organs. Thus, leaves exhibit marked differences in size, complexity and morphology. It has been shown that this phenotypic divergence is an important factor determining the ability of the wild species to thrive in extreme environments (Chitwood et al., 2013; Muir et al., 2014). However, there are still few studies regarding leaf morphology in tomato species and its relation to salt tolerance. Moreover, the genomic sequence of *S. pennellii* has been recently published (Bolger et al., 2014), making this species an ideal candidate for identifying key mechanisms and genes determining salinity tolerance in tomato. In this work, we dissected the anatomical, physiological and molecular responses to salinity of a tomato cultivar (cv. Moneymaker) and an halotolerant accession of the wild species *S. pennellii* (acc. PE47) with the objective to identify key traits and genes determining salinity adaptation in the wild species, which could be further introduced in domesticated tomato. Here we show the importance of the anatomical adaptations in the salt tolerance of *S. pennellii* and provide novel insights into the orchestrated regulation of water and ionic homeostasis in tomato.

2. Materials and methods

2.1. Plant material, growth conditions and salt treatment

Solanum lycopersicum cv. Moneymaker (*Sl*), a cultivar showing the typical excluder mechanism of tomato in saline medium (Martinez-Rodriguez et al., 2008), and one accession of the wild species *Solanum pennellii* (acc. PE47, *Sp*), which presents a high degree of salt tolerance and a Na^+ includer mechanism (Bolarin et al., 1991) were selected.

Two salinity experiments in the same experimental conditions were carried out, and the experimental design was selected on the basis of our previous results obtained with cv. Moneymaker (Campos et al., 2016). The plants were grown in hydroponic culture, since the uptake rates and transport of saline ions from the root to the shoot are higher and faster than in soil growth conditions and, consequently, the symptoms caused by salinity in leaves are shown much earlier in this growth condition. The salt treatment was applied for 14 days, time period sufficient for both the osmotic and toxic effects induced by salinity are clearly shown but senescence symptoms are not very evident yet (Campos et al., 2016).

The hydroponic system consisted in tanks of 50 L capacity ($219 \times 20 \times 17$ cm) filled with half-strength ($^1/_2$) Hoagland nutritive solution (Hoagland and Arnon, 1950), which was continuously aerated by means of a compressor (Puska N-150-150, with a 115 L min^{-1} flow, 10 kg cm^{-2} maximum pressure and 50 L capacity). Hydroponic solution was controlled by monitoring pH and electrical conductivity (EC), renewing the solution at least once per week. A number of 30 plants of each species were randomly distributed in three hydroponic tanks (each one containing ten plants per species) filled with control solution ($^1/_2$ Hoagland). When plants reached a stage of three-four fully-developed leaves, the salt treatment was applied. Six plants of each species (two plants per species and tank) were harvested in control (day 0) and after 1, 2, 7 and 14 days of salt treatment (DST).