Environmental and Experimental Botany 132 (2016) 140–153

Contents lists available at ScienceDirect

Environmental and Experimental Botany

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

Survive or die? A molecular insight into salt-dependant signaling network

Alice Trivellini[a,*], Mariella Lucchesini[b], Antonio Ferrante[c], Giulia Carmassi[b], Guido Scatena[a], Paolo Vernieri[b], Anna Mensuali-Sodi[a]

[a] Institute of Life Sciences, Scuola Superiore Sant'Anna, Pz. Martiri della Libertà 33, Pisa 56127, Italy
[b] Department of Agriculture, Food and Environment, Via del Borghetto 80, 56124 Pisa, Italy
[c] Department of Agricultural and Environmental Sciences, Università degli Studi di Milano, I-20133, Italy

ARTICLE INFO

Article history:
Received 13 June 2016
Received in revised form 15 July 2016
Accepted 15 July 2016
Available online 20 July 2016

Keywords:
Antirrhinum majus L.
NaCl stress
RNAseq
Transcriptome
ABA
Ethylene
Snapdragon
Ca2+ signaling
Cell cycle progression

ABSTRACT

The response of plants to salt stress involves dynamic changes in growth and signaling leading to successful adaptation or death. To elucidate how these opposed events are coordinated we identified a salt-tolerant (*obesifruticosa*) and a salt-sensitive (*aestiva) Antirrhinum majus* mutants using shoots as sensitive indicator of stress magnitude. A series of physiological tests were performed that compared the response after 6 h and 3 days of these contrasting mutants grown in agar under a single (200 mM) NaCl concentration, including shoot area, root length, relative water content, plant height, and overall biomass accumulation. Additional measurements of ABA content, chlorophyll degradation, ethylene production, net photosynthesis rates and Na^+, K^+, Ca^{2+}, and Mg^{2+} content were also reported. RNA-seq analysis was performed on the two mutants after 6 h and 3 days under 200 mM NaCl. A total of 9199 transcripts were found to be differentially expressed in response to NaCl treatment in the two mutants. A large collection of known genes, including MAPKs, CDKs, CDPKs, CIPKs, various transcription factors, various ion transport proteins, and various genes involved in ABA and ethylene signaling pathways were described in detail that displayed differential expression profiles. Overall these data provided evidences of a putative osmotic tolerance sensing and signaling mechanism through a better integration and transduction of environmental cues into growth programs. The reprogramming of calcium-signaling components, generates specific stress signatures affecting differentially the salinity tolerance traits, such as tissue tolerance and anion exclusion. Interestingly, the hormones ABA and ethylene may action as a positive regulators of salt acclimation by the modulation of their signal transduction pathway.

1. Introduction

High salinity is considered to be the major environmental factor limiting plant growth and productivity (Munns and Tester, 2008).

High NaCl levels expose the plants to two distinct stress components: an osmotic and an ionic (Munns and Tester, 2008). As a result of osmotic stress, water potential is reduced and a complex response involved in limiting cellular damages and reaching a new homeostasis, is triggered in plants, through the coordination of several physiological changes such as stomata closure, alterations of cell growth and photosynthesis inhibition (Zhu, 2002). The ionic component of salt-stress is attributed to the toxic effects of Na^+ and Cl^-, increasing the levels of Na^+ and Cl^- in the cytosol which imbalances the intracellular K^+/Na^+ ratio and the homeostasis of other ions like Ca^{2+} (Blumwald et al., 2000). The mechanisms involved in sensing and transmitting both osmotic and Na^+ are extremely important to cope with salinity stress and those sensory modalities are crucial for adaptation (Deinlein et al., 2014; Roy et al., 2014). Three main mechanisms of salinity tolerance exist in plants: osmotic tolerance involved in limiting shoot growth with a not well understood sensing and signaling mechanisms; then ion exclusion by reducing the accumulation of toxic ions in the cytosol, using translocation and remobilization systems; and tissue tolerance which involves the sequestration of toxic ions into the vacuoles (Roy et al., 2014; Julkowska et al., 2016). Plant hormones are known to play key roles in regulating ionic homeostasis and plant salt tolerance (Wu et al., 2008; Ferrante et al., 2011). For example, salt-induced abscisic acid (ABA) levels activates ABA-dependent signaling pathways (Zhu, 2002), which in turn controls the salt-stress responses at transcriptome level, leading to

* Corresponding author.
E-mail address: alice.trivellini@gmail.com (A. Trivellini).

http://dx.doi.org/10.1016/j.envexpbot.2016.07.007
0098-8472/

adaptation. Also ethylene and its signaling pathways play crucial role in plant salinity stress adaptation, as shown by the increased salt tolerance of transgenic plants overexpressing ethylene response factors (ERFs) and others mutants deficient for ethylene sensitivity having, on the contrary, higher salt-sensitivity (Zhang et al., 2012; Achard et al., 2006).

Here, we used forward genetics with modern genomics to discover molecular and physiological traits that contribute significantly to salinity acclimation in *Antirrhinum majus* L. *A. majus* is a glycophyte perennial native to the Mediterranean region with a large range of mutants available at IPK gatersleben germplasm bank (https://gbis.ipk-gatersleben.de/). This species was used as a model system to study the morphology and the symmetry of flowers (Schwarz-Sommer et al., 2003) and in our work it was studied to identify novel acclimations to salinity stress.

A genetic screen *in-vitro* using shoots as sensitive indicator of stress tolerance for salt-stress was carried out (Claeys et al., 2014; Dinneny 2015), to selected two mutants by comparing their behaviour (sensitive *versus* tolerant). Then we investigated the physiological alterations as well transcriptional regulation by next-generation-RNA-sequencing technologies (RNA-seq) evaluating temporal dynamic changes (six h versus three days). Our observations provide understanding of how the salt stress promotes the survival or the death by investigating molecular activities underlying these outcomes.

2. Materials and methods

2.1. Plant material

Seeds of *Antirrhinum majus* (L.) were obtained from GBIS/I (http://www.ipk-gatersleben.de/en/genebank/, Genebank Information System of the IPK Gatersleben, Germany). The mutant's details are reported in Supplementary Table S1 and S2 and further information can be obtained from the above website as well as from snapdragon database (http://www.antirrhinum.net/).

2.2. Screening of A. majus mutants

In the first step experiment (Fig. S1), 62 mutants were screened for NaCl-sensitivity using a root bending assay previously described for Arabidopsis by Wu et al. (1996). The seedlings were grown under an 8:16 h, dark:light (100 μmol-1 m^{-2} s^{-1}) at 22 °C. Five day after germination, the seedlings with 1- to 4 cm-long roots were transferred in squared plates onto MS/2 half-strengh supplemented with increasing concentration of NaCl: 0, 50, 100, 200, 400 mM for the preliminary test with wild type, and 0, 100, 300 mM for the mutant screening. The plates, with seedling arranged in row, were oriented vertically with the roots pointing upward. Roots that did not show curving and apparent growth were noted, as well as the seedling color (bleaching of cotyledons or not; Table S1). Then, the mutant seedlings from control plates were picked up and the shoots micro-propagated and used for the second step experiment (Fig. S1).

2.3. In vitro growth conditions

Germinated plantlets without roots were sub-cultured on MS medium containing 0.25 mg/L BA and the developed shoots were used for salt-stress experiments. For NaCl treatments, similar size apical shoots of the selected mutants were transferred in vented Magenta® vessels (nine explants/mutant) with MS medium without both PGRs and sucrose, supplemented with NaCl at the following concentrations: 0, 100 and 200 mM. The apical shoots (explants) were sampled and collected for downstream morpho-physiological and molecular analysis, respectively after 21 d (Fig. S1, second step experiment) and 6h-3d (Fig. S1, third step experiment). At least four replicate vessels were used for each treatment.

2.4. Growth and water content

The height and water content parameters were determined after 21 days. Water content was calculated as the difference between fresh weight and dry weight of each sample. Dry weight was determined after drying the samples in ventilated oven at 72 °C for 4 days.

Height reduction and water loss were calculated using the following equation 1:

% = 100 (1–S/C) where S and C are the values of each parameter, respectively, in the salt-stressed shoot and in the controls.

2.5. Mineral content and seedling pigments

Dried samples were mineralized (60 min at 220 °C) using nitric and perchloric acids. Sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) were determined using an atomic absorption spectrometer (Varian AA 24FS, Australia): three samples, each consisting of 10 individual shoots, were analyzed for each treatment.

Total chlorophyll and anthocyanins were determined spectrophotometrically following Lichtenthaler (1987) and Kho et al. (1977) methods, respectively.

Pigment degradation percentage was calculated using equation 1 based on the measurements on the salt-stressed (S) and the control (C) plants.

2.6. Free ABA and measurements of ethylene and CO_2

Explant samples were collected, weighed, frozen in liquid nitrogen and then stored at −80 °C until analysis. ABA was determined by an indirect ELISA based on the use of DBPA1 monoclonal antibody, raised against S(+)-ABA (Vernieri et al., 1989). The ELISA was performed following Trivellini et al. (2011).

Ethylene and CO_2 concentrations were measured using an HP 6890 gas-chromatograph (Hewlett Packard, Milano, Italy) as reported in Kiferle et al. (2014). The instantaneous rate of net photosynthesis (PN; μM s^{-1} g^{-1} DW) and the ethylene release (pM s^{-1} g^{-1} FW) was calculated as reported by Kozai et al. (1986) and Kiferle et al. (2014). Air samples (2 cm^3) were taken from the headspace of culture vessels (at least five replicates, each consisting of an individual vessel).

2.7. RNA-Seq analysis and functional annotation

Tissue sample and RNA isolation – To reduce plant to plant variability, each sample was created by pooled together 12 different shoots from six different magenta growing-box, deriving from at least three independent experiments. To avoid the effects of circadian rhythm on gene expression patterns, the harvesting shoots occurred at the same time of day (after 6 h in the photoperiod). The samples from control 6 h and control 3d of each mutant were pooled together. Samples were immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted with Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Italy) according to the manufacturer's instructions. The extracted RNA was treated with RNasefree DNase I (Takara) following the manufacture protocol.

RNA purity and integrity were assessed by Agilent 2100 bioanalyzer-RNA 6000 NanoChip (Agilent Technologies) and