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Hormonal responses of nodulated *Medicago ciliaris* lines differing in salt tolerance

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ABSTRACT

Hormonal changes in two *Medicago ciliaris* lines differing in salt tolerance (TNC 1.8 being more tolerant than TNC 11.9) were studied as possible regulators of growth and symbiotic nitrogen fixation (SNF). After 21 days of saline treatment (100 mM NaCl), four major phytohormones (abscisic acid, ABA; the cytokinin *trans*-zeatin, *t*-Z; the auxin indole-3-acetic acid, IAA; and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid, ACC) were analysed. Salinity differently affected hormone concentrations in vegetative tissues and nodules in both lines. Principal component analysis (PCA) revealed that growth and SNF parameters under salinity were inversely correlated to the amount of reduced nitrogen (Nred) allocated to the roots and nodules, to the sucrolytic activity (TSA) in the roots, and to the *t*-Z and ABA concentration in the nodules. However, those parameters were positively associated along PC1 to the Nred and TSA in the leaves and all the hormones analysed in the roots. Interestingly, the ACC concentration of all organs was positively associated with vegetative growth and with SNF under salinity, as a putative regulator of the symbiotic-parasitic relation with the bacteria. The influence of hormonal changes in relation to plant growth, SNF and symbiotic relation is discussed.

1. Introduction

Excessive soil salinity is a great concern since it causes both water stress and ion toxicity, which disrupts the integrity of cellular membranes, the activities of the various enzymes, the function of photosynthetic apparatus and many other processes namely symbiotic nitrogen fixation (SNF) in legume species (Munns and Tester, 2008; Manchanda and Garg, 2008). These species form a mutually beneficial relationship with nitrogen-fixing soil bacteria referred to as ‘rhizobia’. The physiological processes responsible for inhibition of SNF by salinity are not well known, although many studies analysed SNF under drought conditions (González et al., 2001; Vadez and Sinclair, 2001; Gálvez et al., 2005; Marino et al., 2007). It has been suggested that at least two major mechanisms are involved in the drought-induced inhibition of SNF: (i) an impairment of long-distance transport of nitrogen compounds which is likely to provoke a nitrogen feedback regulation of SNF; and (ii) an impairment of metabolic carbon flux in nodules, resulting in a shortage of carbon substrate for SNF. More recently, the use of a partially droughted split-root system provided evidence that SNF activity under drought is mainly controlled at the local level rather than by a systemic nitrogen signal (Marino et al., 2007). However, since the shoot responds homogenously to the partial root drying (Sobeih et al., 2004; Marino et al., 2007), it cannot be ruled out that other kind of systemic signal coming from the shoot could influence the local SNF activity, as for example hormonal balances.

Successful bacterial infection is established between host plant and rhizobia through a complex set of signals and reciprocal communication between both partners as well as environmental factors regulate the formation and functioning of such organs. In this sense, plant regulatory processes, such as those facilitated by phytohormones, play important roles. However, hormones have been mostly studied in relation to nodule formation and development rather than in relation to the regulation of SNF activity, especially under abiotic stress conditions.

Ethylene and its precursor ACC (1-aminocyclopropane-1-carboxylic acid) are negative regulators of nodulation influencing the earliest stages (Ma et al., 2003). The application of exogenous ethylene or ACC suppresses nodulation, and conversely, application of chemical inhibitors of ethylene perception (i.e. Ag^+) or biosynthesis (i.e. amino ethoxyvinyl glycine) can increase nodule numbers (reviewed in Ma et al., 2002). Consistent with a negative role for endogenous ethylene, mutants of rhizobia defective in rhizobitoxine, a metabolite that inhibits the activity of the key ethylene

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biosynthetic enzymes ACC synthase and ACC deaminase, display decreased nodulation on specific legume hosts (Duodu et al., 1999; Yuhashi et al., 2000).

Abscisic acid (ABA) is another negative regulator of nodulation (Bano and Harper, 2002; Suzuki et al., 2004; Liang et al., 2007). Thus, *Medicago truncatula* seedlings overexpressing *ABA-insensitive1-1* gene from *Arabidopsis* that suppresses the ABA-signalling pathway, exhibited ABA insensitivity as well as a hypernodulating phenotype (Ding et al., 2008). Besides, it has been more recently shown that the mutant *enhanced nitrogen fixation1* (*enf1*), which shows lower endogenous ABA concentration by comparison with the wild-type *Lotus japonicus* seedlings, not only showed increased root nodule number but also enhanced nitrogen fixation (Tominaga et al., 2009). The depressive effect of ABA on nodulation was linked to the abolition of both Nod factor-induced calcium spiking and gene expression to coordinate Nod factor and cytokinins (CKs) signalling during the regulation of nodulation (Ding et al., 2008).

It has been suggested that cortical responses leading to nodule primordia formation are mostly the function of CKs and auxin signalling (Oldroyd and Downie, 2008). It has long been observed that nodule initiation is associated with suppression of lateral root formation (Nutman, 1948) probably following changes in ABA/CKs ratio and their opposite effects on the inception of cell division associated with nodules and lateral roots formation. In contrast to ABA, CKs inhibit cell division in the pericycle, the predominant cell type involved in lateral root initiation, and promote it in the cortex, the predominant cell type involved in the formation of the nodule (Ding et al., 2008).

Another important role in the control of nodulation has been demonstrated for auxins since nodule formation requires high auxin levels for initiation of cell division and establishment of nodule primordium (Mathesius et al., 1998; van Noorden et al., 2006). Experimental evidence comes from work with inhibitors of polar auxin transport, in which nodule-like structures (pseudonodules) expressing early nodulin genes were induced (Scheres et al., 1992; Wu et al., 1996). Conversely, exogenous auxins injected into the shoot inhibited nodulation in soybean Bragg and its supernodulating mutant *nts382* (Olsson, 1988) indicating that the auxin/CKs balance in the root cortex is a key control factor for the induction of cortical cell division and nodule organogenesis (Mathesius et al., 1998).

Long distance signals in the form of hormones or their precursors are also believed to be involved in the control of shoot growth under salt stress (Munns and Tester, 2008; Albacete et al., 2008; Ghanem et al., 2008; Pérez-Alfocea et al., 2010). Changes in phytohormone concentration triggered by salt stress likely regulate assimilate partitioning between source and different sink tissues (Hartig and Beck, 2006; Pérez-Alfocea et al., 2010). Although some studies suggest that ABA accumulation inhibits growth (Albacete et al., 2008; Ghanem et al., 2008), others report a positive role for ABA since ABA-deficient mutants are more sensitive to salt stress than wild genotype, and the expression level of responsive and biosynthetic ABA genes is higher in the salt-tolerant *Thellungiella halophylla* than in its sensitive relative *Arabidopsis thaliana* (reviewed in Xiong, 2007). Ethylene and its precursor ACC have been suggested to play a role in the onset of salt-induced senescence in tomato (Ghanem et al., 2008). By contrast, increasing shoot CKs levels via transgenic IPT overexpression or grafting onto salt tolerant rootstocks has been positively correlated with shoot growth and delay of salt-induced leaf senescence in tobacco (Rivero et al., 2007) and tomato (Albacete et al., 2009; Ghanem et al., 2011). In legumes, the mechanisms of phytohormone regulation under abiotic stress remain relatively obscure. It is uncertain whether salinity-induced changes in nodule phytohomone concentrations can directly suppress SNF or whether alteration of whole plant phytohormone status affects plant growth and C status which in turn affects SNF process and the plant–bacteria interaction. The role of phytohormones in salt tolerance may be illuminated by comparing hormonal responses of salt tolerance between plant species or genotypes differing in salt tolerance. Accordingly, this study evaluated whether hormonal changes can help to explain differential growth and SNF responses under salt stress in two nodulated lines of *Medicago ciliaris* differing in salt tolerance (Ben Salah et al., 2009, 2011).

2. Materials and methods

2.1. Biological materials and growth conditions

The culture of pasture legumes is of special interest, since they undergo symbioses with dinitrogen-fixing bacteria, which improve soil fertility and quality. These plants are good candidates for the improvement of marginal or degraded lands with low fertility and/or high salinity like Sabkha edges. In such areas in Tunisia, these species live in association with halophytes and can produce, in rainy years, up to 40% of the vegetative cover. These plants promote halophyte growth through enriching the soil with nitrogenous compounds (Abdelly et al., 1995). *M. ciliaris* is the most salt tolerant of the annual medics (Medicago spp.) and can tolerate up to 100 mM of NaCl in presence of NO_3^- (Abdelly et al., 1995).

Seeds of two lines of *M. ciliaris* were kindly provided by the Laboratory of Legumes (LL) in the Center of Biotechnology at the technopole of Borj Cedria in Tunis (CBBC). These lines originated from local populations from the edge of saline depressions of Enfidha (TNC 1.8) and non-saline habitats in Mateur (TNC 11.9), and they have been catalogued as tolerant and sensitive to salinity (100 mM NaCl), respectively, according to biomass and SNF related parameters (Ben Salah et al., 2009, 2011). Seeds were scarified with concentrated H_2SO_4 for 40 min, were then washed 10 times with sterile distilled water and placed on sterile agar medium at 25 °C in the dark. Three-day-seedlings were transferred into pots filled with sterile vermiculite and were inoculated with 1 mL (about 10^8/mL) of *Sinorhizobium medicae* CI 1.12/E22 strain suspension, which was kindly provided by the same laboratory. Experiments were performed in the greenhouse during May to June under controlled conditions: 27/19 °C temperature and at relative humidity of 40–80% day/night, day length ranged from 13 to 14 h. Plants were regularly irrigated with N-free Hewitt nutrient solution: KH_2PO_4 (1.6 mM), $MgSO_4$ (1.5 mM), K_2SO_4 (1.5 mM), $CaSO_4$ (3.5 mM), H_3BO_3 (4 μM), $MnSO_4$ (4 μM), $ZnSO_4$ (1 μM), $CuSO_4$ (1 μM), $CoCl_2$ (0.12 μM), $(Na)_6(Mo)_7O_{24}$ (0.12 μM). Urea (2 mM N) was added to the nutrient solution only during the first week of irrigation. Salt treatment (100 mM) was applied a month later. The plants were harvested 3 weeks after the start of salt treatment (at late vegetative stage, 54 DAS). Plants were uprooted carefully and washed with distilled water. Plants were separated into shoots, roots and nodules and all plant parts were oven dried at 60 °C for 7 days and dry weight were determined. Nitrogen content was determined as described in Ben Salah et al. (2009).

2.2. Hormone analysis

Hormones were mostly extracted and analysed as previously described (Albacete et al., 2008; Ghanem et al., 2008). Cytokinins (zeatin, Z), abscisic acid, indole-3-acetic acid (IAA) and ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) were determined using a HPLC/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a micro-well plate autosampler and a capillary pump, and connected to an Agilent Ion Trap XCT Plus mass