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Short communication

Medicago truncatula stress associated protein 1 gene (*MtSAP1*) overexpression confers tolerance to abiotic stress and impacts proline accumulation in transgenic tobacco

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ABSTRACT

Stress associated proteins (SAP) have been already reported to play a role in tolerance acquisition of some abiotic stresses. In the present study, the role of MtSAP1 (*Medicago truncatula)* in tolerance to temperature, osmotic and salt stresses has been studied in tobacco transgenic seedlings. Compared to wild type, *MtSAP1* overexpressors were less affected in their growth and development under all tested stress conditions. These results confirm that MtSAP1 is involved in the response processes to various abiotic constraints. In parallel, we have performed studies on an eventual link between *MtSAP1* overexpression and proline, a major player in stress response. In an interesting way, the results for the transgenic lines did not show any increase of proline content under osmotic and salt stress, contrary to the WT which usually accumulated proline in response to stress. These data strongly suggest that MtSAP1 is not involved in signaling pathway responsible for the proline accumulation in stress conditions. This could be due to the fact that the overexpression of *MtSAP1* provides sufficient tolerance to seedlings to cope with stress without requiring the free proline action. Beyond that, the processes by which the *MtSAP1* overexpression lead to the suppression of proline accumulation will be discussed in relation with data from our previous study involving nitric oxide.

Introduction

Plants are frequently subjected to environmental changes which lead to important consequences for the development and the survival. To cope with these constraints, plants have developed some adaptive responses and complex cellular mechanisms involving many actors, some of which are still unknown (Zhu, 2002; Vinocur and Altman, 2005; Huang et al., 2012).

In a recent past, a new stress associated protein (SAP) family has been described to be involved in the stress response in plants (Mukhopadhyay et al., 2004; Kanneganti and Gupta, 2008; Ben Saad et al., 2010; Dixit and Dhankher, 2011). SAP are characterized by the presence of A20/AN1 zinc finger domains. The A20 zinc-finger (ZnF) domain is characterized by multiple Cys_2/Cys_2 finger motifs whereas the AN1-type ZnF domain contains six conserved cysteines and two histidines which potentially coordinate two zinc atoms (Linnen et al., 1993). In animals, the role of A20/AN1 proteins in the regulation of the immune response has been well established (Huang et al., 2004; Diatchenko et al., 2005). However, in plants, although several studies have showed their involvement in the abiotic stress tolerance acquisition, their precise function remains unknown.

Recently, we have identified the first SAP (MtSAP1) in the plant model legume *Medicago truncatula* (Gimeno-Gilles et al., 2011). The impact of *MtSAP1* overexpression in transgenic tobacco seedlings has been shown to allow a better growth development in normal culture conditions, but also to induce salt and osmotic stress tolerance (Charrier et al., 2012). It was also noted in this previous study that tolerance could be linked to high levels of nitric oxide (NO) observed in the transgenic lines under normal and stress conditions. It has been suggested that *MtSAP1* overexpression could be associated with this higher NO production that would enable seedlings to reach a high protection level to prepare them to cope with abiotic stresses.

Abbreviations: MS, Murashige and Skoog; NO, nitric oxide; ROS, reactive oxygen species; SAP, stress associated protein; WT, wild type.

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One of the common responses of many plant species exposed to different abiotic stresses is the accumulation of compatible organic solutes such as proline (Verbruggen and Hermans, 2008; Szabados and Savoure, 2010). Free proline accumulation has been shown to be involved in various protection processes such as osmotic adjustment, protection against reactive oxygen species (ROS) and stabilization of protein and membrane structures (Handa et al., 1986; Kishor et al., 2005; Ashraf and Foolad, 2007). The protecting roles of proline have been notably described in higher plants subjected to salt and osmotic stresses (Yoshiba et al., 1995; Khedr et al., 2003).

In order to demonstrate that *MtSAP1* overexpression conferred tolerance to a large variety of environmental stresses, extreme temperatures (cold and heat) but also salt and osmotic stresses at very drastic concentrations have been tested. Following this, a possible link between *MtSAP1* overexpression and free proline contents has been investigated with the intention to address whether *MtSAP1* could be involved in signaling pathway inducing the proline accumulation.

Materials and methods

Plant material and growth conditions

Experimentations were carried out on *Nicotiana tabacum* cv. Xanthi (obtained from UMR PMS, University of Angers, France). Transgenic tobacco plants overexpressing *MtSAP1* were obtained as described in Charrier et al. (2012). Following sterilization, wild type (WT) and transgenic tobacco seeds were sown on solid half-strength Murashige and Skoog medium (MS 0.5×) without sucrose and at pH 5.7 (Murashige and Skoog, 1962). After stratification on MS 0.5× medium during two days at 4 °C, seeds were placed under controlled and optimal growth conditions during 21 days (Charrier et al., 2012). Physiological growth parameters such as biomass, primary root length and leaf number were measured (i) under drastic osmotic (D-mannitol; 400 mM) and salt stress (NaCl; 250 mM) during 21 days in a solid MS 0.5× medium and (ii) under heat (42 °C) and cold (4 °C) treatments for 7 days on tobacco seedlings grown previously on MS 0.5× medium during 21 days.

Proline content

Total proline content was measured (with slight modifications) from the method of Bates et al. (1973). To perform experiments on seedlings enough developed, mannitol and NaCl concentrations were lowered in order to obtain a more moderate stress (D-mannitol, 300 mM; NaCl, 200 mM). Seedlings were homogenized with 2.5 mL of sulfosalycilic acid (5%) in a mortar with a pestle. After continuous grinding, the suspension was centrifuged (10,000 × g, 12 min, 4 °C). After this centrifugation, one mL of the extract was added to 1 mL of acid ninhydrin and 2 mL of glacial acetic acid in a test tube for one hour at 100 °C. The reaction was stopped by placing the tube on ice for 5 min. Following centrifugation (10,000 × g, 5 min, 4 °C), proline content from the supernatant was determined colorimetrically by measuring the absorbance at 515 nm.

Statistical analysis of data

All data are presented as mean ± SE of values from at least three independent experiments. A one-way ANOVA (analysis of variance) test was also performed. Different letters are used to indicate means that differ significantly ($P < 0.05$).

Results and discussion

Abiotic stress tolerance acquisition by overexpression of MtSAP1

In order to observe whether *MtSAP1* overexpression confers tolerance to various abiotic stresses, transgenic seedlings were subjected to cold (4 °C), heat (42 °C) and very drastic osmotic (D-mannitol; 400 mM) and salt (NaCl; 250 mM) stress. In a general way, WT lines showed a strong reduction in their development under all the treatments mentioned above whereas transgenic seedlings seemed to be less affected by these stresses (Fig. 1). Indeed, while leaves from transgenic line seedlings were already well developed after 21 days under drastic osmotic and salt stress, WT seedlings did not grow further than the cotyledon stage (Fig. 1D). In concordance with these observations, phenotypic parameters such as the biomass, the primary root length and the leaf number decreased more strongly in WT seedlings under these treatments compared to *MtSAP1* overexpressing lines (Fig. 1A–C). With regard to the temperature stresses (cold and heat), the biomass of *MtSAP1* overexpressing lines were not reduced by low and high temperatures compared to normal growth conditions, whereas in WT seedlings, the biomass was strongly reduced under these stresses (Fig. 1A). In addition, primary root length and leaf number were more developed in transgenic lines compared to WT seedlings under cold and heat stress (Fig. 1B and C). More precisely under high temperature, a strong wilting of leaves was observed in WT seedlings whereas *MtSAP1* overexpressors appeared to be less affected by this stress. Our results are in agreement with previous studies on SAP in other species (Kanneganti and Gupta, 2008; Ströher et al., 2009; Dixit and Dhankher, 2011; Hozain et al., 2012). In particular, the overexpression of *OsiSAP8* (*Oriza sativa*) in *Nicotiana tabacum* and rice conferred tolerance to cold but also to salt and drought stress (Kanneganti and Gupta, 2008). Moreover, Dixit and Dhankher (2011) have demonstrated that *AtSAP10* (*Arabidopsis thaliana*) was involved in heavy metal and heat stress responses. Therefore, SAP, whatever the plant species, seem to be involved in the adaptive responses to a majority of abiotic stresses. Recently, it has been demonstrated that plant tolerance of adverse environmental conditions was mediated by protein ubiquitination (Lyzenga and Stone, 2012). This is strongly supported by the fact that *AtSAP5*, which encodes a protein with both A20/AN1 zinc finger domains, acts through its E3 ubiquitin ligase activity as a positive regulator of stress responses in *Arabidopsis* (Kang et al., 2011).

Impact of MtSAP1 overexpression on proline accumulation

In order to know whether *MtSAP1* induces changes in proline metabolism to prepare seedlings to cope with environmental constraints, we have measured the final product (proline content) in transgenic lines overexpressing *MtSAP1* under optimal and stress conditions. Indeed, it has been demonstrated that proline is an osmoprotectant recognized to be accumulated in response to various stresses (Ashraf and Foolad, 2007; Szabados and Savoure, 2010). The first observations under normal conditions have provided a slight increase of proline content in transgenic lines (2.13 mg g^{-1} FW) compared to WT (1.39 mg g^{-1} FW) (Fig. 2). Interestingly, this higher proline content could be correlated to a stronger production of NO observed in our previous study in *MtSAP1* overexpressors under non-stressfull conditions (Charrier et al., 2012). However, other hypotheses could be also considered, including the fact that the proline increase in transgenic lines may be due to a change in total amino acid content. Indeed, *MtSAP1* overexpression seems to have multiple effects, especially the actions of SAP in general are not fully known to date.

Afterwards, the impact of the overexpression of *MtSAP1* on the proline content has been investigated under D-mannitol and NaCl