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Expression profiles and hormonal regulation of tobacco expansin genes and their involvement in abiotic stress response

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ABSTRACT

Changes in the expression levels of tobacco expansin genes *NtEXPA1*, *NtEXPA4*, *NtEXPA5*, and *NtEXPA6* were studied in different organs of tobacco (*Nicotiana tabacum* L.) as well as in response to phytohormone and stress treatments. It was shown that *NtEXPA1*, *NtEXPA4* and *NtEXPA5* transcripts were predominantly expressed in the shoot apices and young leaves, but almost absent in mature leaves and roots. The *NtEXPA6* mRNA was found at high levels in calluses containing a large number of undifferentiated cells, but hardly detectable in the leaves of different ages and roots. In young leaves, expression levels of *NtEXPA1*, *NtEXPA4* and *NtEXPA5* genes were induced by cytokinins, auxins and gibberellins. Cytokinins and auxins were also found to increase *NtEXPA6* transcripts in young leaves but to the much lower levels than the other expansin mRNAs. Expression analysis demonstrated that brassinosteroid phytohormones were able either to up-regulate or to down-regulate expression of different expansins in leaves of different ages. Furthermore, transcript levels of *NtEXPA1*, *NtEXPA4*, and *NtEXPA5* genes were increased in response to NaCl, drought, cold, heat, and 10 μM abscisic acid (ABA) treatments but reduced in response to more severe stresses, i.e. cadmium, freezing, and 100 μM ABA. In contrast, no substantial changes were found in *NtEXPA6* transcript level after all stress treatments. In addition, we examined the involvement of tobacco expansins in the regulation of abiotic stress tolerance by transgenic approaches. Transgenic tobacco plants with constitutive expression of *NtEXPA1* and *NtEXPA5* exhibited improved tolerance to salt stress: these plants showed higher growth indices after NaCl treatment and minimized water loss by reducing stomatal density. In contrast, *NtEXPA4*-silenced plants were characterized by a considerable growth reduction under salinity and enhanced water loss. Our findings indicate that expression levels of all studied tobacco expansins genes are modulated by plant hormones whereas NtEXPA1, NtEXPA4, and NtEXPA5 expansins may be involved in the regulation of stress tolerance in tobacco plants.

1. Introduction

Expansins are a group of proteins that are capable to modify the mechanical properties of plant cell walls by a nonenzymatic mechanism. There are four classes of plant expansins: α-expansins, β-expansins, expansin-like proteins A and expansin-like proteins B (Kende et al., 2004; Sampedro and Cosgrove, 2005). These proteins are encoded by large multigene families and are widely distributed in plant species. For example, 36, 38, and 56 expansin genes were identified in the genomes of *Arabidopsis thaliana*, tomato *Solanum lycopersicum*, and rice *Oryza sativa*, respectively (Cosgrove, 2015; Lu et al., 2016). Significance of such a wide variety of expansin genes in plant genome is currently unclear. One can assume that each of them may fulfill a specific function (Cosgrove, 2015; Marowa et al., 2016). There may be a difference between the spatial and temporal expression patterns of different expansins, and some of these proteins appear to be tissue- or organ-specific (Lu et al., 2016). Numerous studies have reported the involvement of expansins in the growth promotion of different plant organs, in particular, roots. Thus, studies of loss-of-mutations of expansin gene *AtEXPA5* from *A. thaliana* revealed that *expA5-1* plants (*AtEXPA5* gene null mutants) had shorter roots and hypocotyls, and reduced number of rosette leaves (Park et al., 2010). RNA silencing technology was used to study the function of *AtEXPA7*, which is expressed specifically in

Abbreviations: NtEXPA, expansin genes of *Nicotiana tabacum*; ABA, Abscisic acid; BAP, 6-benzylaminopurine; EBL, 24-epibrassinolide; IAA, Indoleacetic acid; GA_3, Gibberellic acid; MeJA, Methyl jasmonate; NAA, 1-naphthaleneacetic acid.

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the root hairs of *A. thaliana* (Lin et al., 2011). In *AtEXPA7-RNAi* transformants, a reduction in the *AtEXPA7* transcript level resulted in the suppression of root hair elongation which indicates a positive role for expansins in root hair growth (Lin et al., 2011). Expansins are also involved in the regulation of growth and development of stems and leaves. For example, they are capable not only to induce the development of new leaves but also to determine their position by affecting meristematic cells (Fleming et al., 1997). Furthermore, expansins may have an important role for development of other organs besides roots and leaves. Expression studies of nine expansin genes of potato (*Solanum tuberosum* L.) revealed high expression of *EXPA1* gene in young growing potato tubers (Jung et al., 2010). In gladiolus (*Gladiolus grandiflorus* Andrews), GgEXPA1 expansin was expressed in stamen filaments, gynoecium styles and expanding leaves, but not in tissues where expansion had ceased (Azeez et al., 2010). In general, a lot of studies have shown that the activity of expansins correlates with the cell expansion, and these proteins are expressed predominantly in the intensively growing organs (Marowa et al., 2016).

Expression of expansin genes is subjected to fine-tune regulation by phytohormones and environmental conditions (Marowa et al., 2016). Thus, expansin gene expression is regulated by auxins (Jung et al., 2010; Lu et al., 2016), which stimulate cell wall acidification, thus increasing the activity of α-expansins (McQueen-Mason et al., 1992). However, several studies have questioned that auxin-induced cell wall acidification contributes to growth elongation, and demonstrated that fungal phytotoxin fusicoccin, but not IAA, causes cell extension through acidification of the cell walls. This fusicoccin-induced acid growth is presumed to be mediated by expansin-induced cell wall loosening process (Kutschera and Wang, 2016). Cytokinins are plant hormones that have a broad spectrum of regulatory activity, including the regulation of expansin expression (Lee et al., 2008; Li et al., 2014; Lu et al., 2016). Furthermore, expression of expansins can be induced not only by auxins and cytokinins, but also by brassinosteroids (Park et al., 2010), gibberellins (Azeez et al., 2010; Lu et al., 2016), ethylene (Trivedi and Nath, 2004), methyl jasmonate (MeJA) (Han et al., 2012), and ABA (Zhang et al., 2014).

Expansins are implicated in the growth responses of plants to adverse environments (Gao et al., 2010; Han et al., 2012; Lu et al., 2013). Thus, drought stress was shown to up-regulate the expression and activity of expansins in *Craterostigma plantagineum* plants thereby contributing to an increase in the cell wall flexibility as an adaptation to dehydration (Jones and McQueen-Mason, 2004). It has been shown that salt and osmotic stresses increased the transcript levels of expansin genes in bryophyte *Physcomitrella patens*, suggesting that these proteins were responsible for specific changes in cell morphology exhibited by *P. patens* in response to these stresses (Schipper et al., 2002). Moreover, numerous studies have reported that overexpression of expansins improved the tolerance of transgenic plants to various stresses (Cosgrove, 2015; Marowa et al., 2016).

Recent advances in genetic engineering and plant biotechnology will help to further elucidate the functions of different expansins and molecular mechanisms underlying the regulation of their expression. Tobacco (*Nicotiana tabacum* L.) is one of the model objects in functional genomics and genetic engineering of plants. Tobacco genome is not completely sequenced, however, six expansin genes *NtEXPA1–NtEXPA6* were identified (Link and Cosgrove, 1998), but their functions still remain unclear. In our previous studies, we have shown that tobacco expansins NtEXPA1, NtEXPA4, and NtEXPA5 were involved in the regulation of tobacco organ growth by affecting cell expansion (Kuluev et al., 2013, 2014a,b). The goal of the present research was to study the expression pattern of tobacco expansin genes in response to phytohormone and stress treatments in different organs of tobacco plants. In addition, we have proposed a role for expansins in the regulation of tobacco stress tolerance by performing experiments with previously generated transgenic tobacco plants with up-regulated (*35S:NtEXPA5* and *DMV:NtEXPA1*) and down-regulated (*NtEXPA4i*) expansin expression (Kuluev et al., 2013, 2014a,b). This study may put us one step closer to understanding the certain functions of expansins in the regulation of tobacco organ growth under changing environmental conditions.

2. Materials and methods

2.1. Growth conditions, treatments of wild type plants and callus production

Plants of *N. tabacum* cv. Petit Havana SR1 were grown in 450 ml pots filled with universal soil substrate "Terra vita" in a greenhouse at +27 °C under 140 mmol m^{-2} sec^{-1} photon flux density and photoperiod of 16/8 h (day/night) within 40 days (twelve leaves stage) and then subjected to phytohormone and stress treatments.

Tobacco plants were sprayed with phytohormone solutions containing Tween-20 (0.1%). The plant hormone solutions were used at following concentrations: 6-benzylaminopurine (BAP) at 50 μM; indoleacetic acid (IAA) at 5 μM, 1-naphthaleneacetic acid (NAA) at 10 μM, 24-epibrassinolide (EBL) at 1 μM; gibberellic acid (GA_3) at 5 μM; abscisic acid (ABA) at 10 μM and 100 μM; methyl jasmonate (MeJA) at 1 μM. Control plants were sprayed with 0.1% solution of Tween-20 without phytohormones. Leaves were numbered starting from the top of the plant, and the youngest, visible to the naked eye, leaf was defined as leaf no. 1. In this study, we selected shoot apices, leaves numbered 1, 2, 3, 5, 6, and 12 (cotyledon) of hormone-untreated plants which were frozen in liquid nitrogen and used to isolate total RNA. Two hours after the treatment with BAP, IAA, EBL and GA_3 shoot apices, leaves nos. 1, 2, 5, 8 were frozen in liquid nitrogen for RNA extraction. In experiments with MeJA and ABA treatments, leaves no. 3 were frozen in liquid nitrogen 4 h after the spray with corresponding compounds, and then were used to isolate total RNA.

In other experiments, tobacco plants were subjected to various kinds of stress treatment to observe the expression of tobacco expansin genes. Cadmium stress was induced by exposing plants to 100 μM cadmium supplied as $Cd(CH_3COO)_2$ for 16 h. For freezing and cold treatments, plants were exposed to a temperature of 0 or 10 °C for 6 h and 8 h, respectively. Heat stress treatment was performed by incubating tobacco plants at 42 °C for 6 h. Drought stress was induced by withholding water supply for 2 days. For salt stress treatment, tobacco plants were germinated on universal soil for 40 days, then roots were carefully washed and plants were transferred for 5 days on hydroponics solution (10% Hoagland-Arnon solution). After that, plants were incubated for 2 h on different concentrations of NaCl (20 mM, 90 mM, 170 mM, 250 mM, 350 mM, 850 mM). After stress treatments, leaves no. 3 were frozen in liquid nitrogen and used to isolate total RNA. Untreated plants served as controls.

Callus was produced from the leaves no. 5 of 40-days-old wild type (WT) tobacco plants. Leaves were cut into individual explants and placed on MS medium with addition of NAA (2.0 mg/L) and kinetin (0.2 mg/L). On the 11th day of incubation on MS medium, the calluses were fixed with liquid nitrogen and used to isolate total RNA.

2.2. RNA extraction and analysis of expansin mRNA abundance

Total RNA was isolated with Trizol reagent and the first strand of cDNA was synthesized using oligo(dT) primer and M-MuLV-reverse transcriptase (NEB, USA). Real time RT-PCR was