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Research article

Three members of *Medicago truncatula* ST family (MtST4, MtST5 and MtST6) are specifically induced by hormones involved in biotic interactions

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ABSTRACT

In this work, we study the function of the *Medicago truncatula* ST4, ST5 and ST6 proteins that belong to a protein family of unknown function characterized by the DUF2775 domain. Thus, we analyse their promoter sequence and activity, their transcript accumulation, and their subcellular location. The analysis of the three promoters showed different combination of *cis*-acting regulatory elements and they presented different activity pattern. Throughout development only *ST6* mRNAs have been detected in most of the stages analysed, while *ST4* was faintly detected in the roots and in the flowers and *ST5* was always absent. The addition of MeJA, ET and SA revealed specific responses of the STs, the *ST4* transcript accumulation increased by MeJA; the *ST5* by MeJA and ET when applied together; and the *ST6* by ET and by SA. Finally, the ST4 and ST5 proteins were in the cell wall whereas the ST6 had a dual location. From these results, we can conclude that the *ST4*, *ST5* and *ST6* RNAs are specifically and differentially up-regulated by MeJA, ET and SA, plant regulators also involved in the plant defence, pointing that ST4, ST5 and ST6 proteins might be involved in specific biotic interactions through different signalling pathways.

1. Introduction

The family of the ST proteins (from ShooT specific (Vries et al., 1983), and usually named Specific Tissue (Albornos et al., 2012)) is only present in specific taxonomic groups of the plant kingdom, like Fabaceae and Asteraceae, but absent in others such as Brassicaceae (Albornos et al., 2012). The first members of the ST family were reported in *Pisum sativum* and *Cicer arietinum* (Muñoz et al., 1997; Vries et al., 1985, 1983; Williams et al., 1990). The ST family is associated with the DUF2775 domain that is the core sequence of these proteins. All the ST proteins have a signal peptide, an N-terminal region displaying conserved features, and several units of a consensus sequence of 25/26 amino acids arranged in tandem. The consensus sequence consists of a well-conserved hexapeptide (positions 1 to 6), four partially conserved amino acids (from 7 to 10) and a totally conserved tyrosine at position 11; the rest of the sequence (from position 12 and on) is more variable. The differences in the putative glycosylation pattern associated to the four amino acids in positions 7 to 10 were used to establish a classification of the ST proteins in three groups (see Albornos et al. (2012) for a deeper description).

A holistic in silico study was carried out to characterize the ST family (Albornos et al., 2012) and a broad expression profile of the *ST* genes was outlined analysing expressed sequence tags (EST) deposited in the databases (Albornos et al., 2012). It stands out the high abundance of *ST* mRNAs in the roots and/or radicles, being also notably the accumulation of the *ST* transcripts in seeds, leaves and epicotyls/hypocotyls or stems.

The function of the ST proteins remains unknown, although in accordance with their gene expression profile, they have been related to biotic interactions as symbiosis (Gaude et al., 2012; Liu et al., 2007) and to abiotic stress (Albornos et al., 2017; Hernández-Nistal et al., 2010; Muñoz et al., 1997). Also, different STs have been related to developmental processes such as early fruit morphogenesis (Fernandez et al., 2007; Wechter et al., 2008), cell elongation (Muñoz et al., 1997; Vries et al., 1985) or germination (Hernández-Nistal et al., 2006). Finally, a putative role as vegetative N storage and/or with changes in the plant nutritional status has been proposed (Albornos et al., 2014, 2017; Vries et al., 1985).

M. truncatula is a well-established model legume, highly suitable to investigate the function of STs as it has the biggest *ST* gene family. The six members that belong to the *M. truncatula* ST multigenic family (*MtST1* [Medtr4g069810], *MtST2* [Medtr3g116440], *MtST3*

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Abbreviations

AM	arbuscular mycorrhiza
Arabidopsis	*Arabidopsis thaliana*
CRE	*cis*-acting regulatory element
DUF	domain of unknown function
EST	expressed sequence tag
gDNA	genomic DNA
GFP	green fluorescent protein
GUS	β-glucuronidase
Hpi	hours post imbibition
LRE	light regulated elements
MS	Murashige and Skoog media
nIA	normalized and integrated absorbance
ORF	open reading frame
PI	propidium iodide
p35S::ST-GFP	transgenic arabidopsis plants carrying *p35S::ST-GFP* transgene
pST	promoter of *Medicago truncatula ST* gene
pST::GUS	transgenic arabidopsis plants carrying *pST::GUS* transgene
sqRT-PCR	semi quantitative reverse-transcription polymerase chain reaction
ST	ShooT specific/Specific Tissue
TF	transcription factor
TSS	transcription start site

[Medtr3g116430], *MtST4* [Medtr3g034640], *MtST5* [Medtr3g034610] and *MtST6* [Medtr3g107810]) might have different or complementary roles in the plant physiology as indicated by the results so far (Albornos et al., 2012; Benedito et al., 2008).

The putative functions of the ST protein family of *M. truncatula* are being investigated by our group by searching the promoter sequences of *ST* genes for regulatory motifs in silico; by analysing their activation pattern in *Arabidopsis thaliana* transgenic plants; by tracking their transcript accumulation during plant development and in different growth conditions, and by determining the location of these ST proteins within the cell. Gathering all this information we could infer the putative function performed by a given *M. truncatula* ST protein. In a previous work, we reported that ST1, ST2 and ST3 are ubiquitous proteins that are separated into two functional groups (Albornos et al., 2017). Thus, ST1 might participate in processes affected by nutritional status, while ST2 and ST3 seem to act when the availability of water is limited, not only because of environmental constraints but also in physiologically controlled processes (Albornos et al., 2017).

This work intends to elucidate the possible roles of ST4, ST5 and ST6, the 3 members of the *M. truncatula* ST protein family not studied so far.

2. Materials and methods

2.1. Plant material and growth conditions

The ecotype Columbia-0 (Col-0) of *Arabidopsis thaliana* was used to generate transgenic plants, as described in Albornos et al. (2017). The analyses of the promoter activity of *ST* genes were performed in 3- and 10-d-old seedlings growing either in darkness at 25 °C (dark-grown) or with a 16 h/8 h light/dark photoperiod at 22 °C (light-grown). Different organs were collected throughout plant development to perform GUS staining (Albornos et al., 2017).

Medicago truncatula ecotype Jemalong line A17 seeds were scarified, surface sterilized, placed in Petri dishes with Fahräeus-N, stratified and grown in a chamber (Aralab, Portugal) at 25 °C with a 16 h/8 h light/dark photoperiod, as described in Albornos et al. (2017).

To perform sqRT-PCR experiments, different organs in different developmental stages were collected (Albornos et al., 2017). In summary, we analysed seedlings of 24 h post imbibition (hpi); seedlings at 3-, 6- and 10-d-old growing in the darkness and in light, in which we separated roots and aerial parts; roots from 30-d-old plants; 3 development stages of leaves, flowers and fruits as well as green seeds. The stages analysed are represented in Fig. S1 and the stages of flowers and fruits are based on Kurdyukov et al. (2014). Green seeds were collected at 24–26 d after pollination (the end of the filling phase according to Verdier et al. (2013)).

The chemical treatments were applied for 24 h to 6-d-old seedlings as indicated in Albornos et al. (2017). These chemicals were 100 µM abscisic acid (ABA), 10 µM benzylaminopurine (CK), 10 µM epibrassinolide (BL), 1 mM ethephon (ET), 100 µM giberellic acid (GA), 10 µM indolacetic acid (IAA), 250 mM mannitol; 100 µM methyl jasmonate (MeJA), 150 mM NaCl, 1 mM salicylic acid (SA), 10 µM strigolactone GR24 (SL), and MeJA combined either with ET, GA or SA. The seedlings were kept for 12 h in the darkness at 4, 25 and 37 °C, with 25 °C as the control for the temperature treatments. The starvation for N (-N) and Pi (-Pi), were performed in seedlings maintained for 7 days in Fahräeus without N supplement or Fahräeus-N media without phosphate, respectively. A transversal cut on one foliole was performed for wounding experiments (Albornos et al., 2017).

2.2. Analysis of cis-regulatory element

The promoters of *M. truncatula ST4, ST5 and ST6* genes (*pST*) were analysed, using 1000 bp upstream of the ATG start codon as described in Albornos et al. (2017) using PLACE (Higo et al., 1998) and PlantCARE (Lescot, 2002).

2.3. Cloning of promoters pST4, pST5 and pST6 and ST4, ST5 and ST6 ORFs

The promoter region sequences were obtained from phytozome v9.1 database (http://phytozome.jgi.doe.gov/pz/portal.html) and a sequence of ca. 2000 bp upstream ATG start codon was PCR-amplified using gDNA from leaves as indicated in Albornos et al. (2017), except for *pST5* clone that was obtained using gDNA from 6-d-old seedlings treated for 24 h with 100 µM MeJA and 1 mM ET.

After several unsuccessful attempts using RNA from plants growing in standard conditions, *ST4* [EMBL:LN827610], *ST5* [EMBL:LN827611] and *ST6* [EMBL:LN827612] ORFs were cloned using RNA extracted from seedlings treated with MeJA (*ST4* and *ST5*) or from roots inoculated with mycorrhiza (*ST6*) as described in Albornos et al. (2017).

Several primer pairs were designed for each cloning experiment using the online tool Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and those that actually were used for the promoters (primers 1 to 7) and the ORFs (primers 8 to 15) are listed in Table S1. The amplified products were gel-purified (NucleoSpin® Gel and PCR Clean-up by Macherey-Nagel, Germany). Different attempts were made to clone *ST4* and especially *ST5*, because the expected size of the PCR fragment differed from the obtained. In fact, additional primers from 3′ UTR were used (number 10 and 13 in Table S1 for ST4 and ST5, respectively) to obtain the ORF and to assure that the right ORFs had been cloned.

2.4. Transformation of Arabidopsis thaliana

Two reporter gene cassettes (*pST::GUS* and *p35S::ST-GFP*) were prepared using Gateway™ cloning technology (Invitrogen, USA) as reported by Albornos et al. (2017).

pENTR201.*pST* and pENTR201.*ST* vectors were selected by PCR