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# Promoter activity of genes encoding the Specific Tissue protein family in the reproductive organs of *Medicago truncatula*

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## Abstract

The "Specific Tissue" (ST) are proteins of unknown function present only in some plant families, mainly *Fabaceae* and *Asteraceae*. They are included in the PF10950 protein family and characterized by the presence of at least one domain of unknown function (DUF)2775. In this work we studied the involvement of the six members of the *Medicago truncatula* ST family (ST1 to ST6) in the development of flowers, fruits, and seeds by analysing the activity of their promoters (*pST*) after the construction of *M. truncatula* transgenic plants expressing the β–glucuronidase (*GUS*) reporter gene under the control of the six *pSTs*. The GUS activity was analysed in whole flowers and fruits and also in histological sections of these organs. The *pST* expression in the reproductive organs was mainly associated with the vascular bundles, especially throughout fruit development. These results pointed to an important role of ST proteins during the reproductive development stage, related to nutrient mobilization during the fruit and seed formation, that could be facilitated by their presence in the pod vascular bundles, as well as in the connective tissue of the anthers (ST3, ST4, ST6), in the placenta, the funiculus, and the outer parts of the developing seed (ST2, ST3, ST6). The observations made in this study were in agreement with the functions previously established for the three groups of *M. truncatula* ST proteins, as in the proposed function for ST1 in the transport and assimilation of nutrients, or the involvement of ST4, ST5, and ST6 in floral defence.

*Additional key words*: barrel medic, DUF2775, flowers, fruits, nutrient mobilisation, vascular bundles.

## Introduction

The Specific Tissue proteins (STs) are proteins present in specific plant families, mainly *Fabaceae* and *Asteraceae* (Albornos *et al.* 2012a). These proteins have a signal peptide, an intermediate region with conserved characteristics and several tandems repeats of 25/26 amino acids. The main feature of STs is the presence of at least one DUF2775 domain (PF10950) (Albornos *et al.* 2012a).

The ST proteins have been related, according to their gene expression profile, to biotic interactions as symbiosis (Liu *et al.* 2007, Gaude *et al.* 2012) and to responses to abiotic stresses (Muñoz *et al.* 1997, Hernández-Nistal *et al.* 2010, Albornos *et al.* 2017), although their function remains unknown. Some STs have been associated with developmental processes such as early fruit morphogenesis (Fernández *et al.* 2007, Wechter *et al.* 2008), cell elongation (De Vries *et al.* 1985, Muñoz *et al.* 1997) or germination (Hernández-Nistal *et al.* 2006). Also, a role as storage proteins or their relationship with changes in the plant nutritional status, have been proposed (De Vries *et al.* 1985, Albornos *et al.* 2014, 2017).

The ST proteins are encoded by multigenic families, the biggest one with six members in barrel medic (*Medicago truncatula*). Thus the *M. truncatula ST* gene family has gained interest as a study model. Each member of the family might have a specific role, and hence their expression would be differentially regulated throughout development and/or under diverse stimuli, as it has been previously confirmed (Albornos *et al.* 2017, 2018).

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*Abbreviations*: CRE - *cis*-acting regulatory element; eGFP - enhanced green fluorescent protein; DUF - domain of unknown function; F1 - developing flower which appears green and closed; F2 - developing flowers with sepals and petals, yellow and closed; F3 - fully developed flower at/or after anthesis; F4 - flower after anthesis showing a curved carpel and elongated style; FB - floral bud; Fr1 - developing fruit showing one or more complete spirals, with no spines and not stacked; Fr2 - developing fruit showing barrel morphology with spines, still not hardened; Fr3 - developing fruit showing barrel morphology with spines, already hardened; GUS - β–glucuronidase; *pST*- ST promoter; ST- specific tissue; X-GlcA - 5-bromo-4-chloro-3-indolyl β–D-glucuronic acid.

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All the ST proteins enter the secretory pathway owing to the above-mentioned presence of a signal peptide, and most of them are possibly targeted to the extracellular compartment (Albornos *et al.* 2012a). Regarding the *M. truncatula* ST family, it has been established that all the six members are located in the cell wall, although ST2, ST3, and ST6 have a double location both in the cell wall and in the cytoplasm (Albornos *et al.* 2017, 2018).

In previous reports we have separated the *M. truncatula* ST proteins in three functional groups (Albornos *et al.* 2017, 2018). Thus, ST1, which is the only protein included in the first group, might participate in processes affected by nutritional status; the second group integrates ST2 and ST3, that seem to act when the availability of water is limited, not only due to environmental factors but also in physiological controlled desiccation processes such as the seed maturation (Albornos *et al.* 2017), while ST4, ST5, and ST6 (third group) might be involved in specific biotic interactions through different signalling pathways (Albornos *et al.* 2018) being their RNAs specifically and differentially up-regulated by methyl jasmonate (MeJA), ethylen (ET), and salicylic acid (SA). However, the specific functions of each ST protein are not completely established yet, and some results indicated discrepancies between the amount of ST transcripts in *M. truncatula* and the activity of the ST promoters (*pST*) when they were expressed in *Arabidopsis thaliana*, mainly during its reproductive phase (Albornos *et al.* 2017, 2018).

An involvement of the ST proteins in the development of flowers, fruits, and seeds has been reported in different species (Williams *et al.* 1990, Fernández *et al.* 2007). Also, in *M. truncatula*, it has been described that all the *pST* present some *cis*-acting regulatory elements (CREs) associated with the reproductive development and *in vivo*, the *pSTs* in *Arabidopsis* transgenic plants become transiently active at some reproductive structures (Albornos *et al.* 2017, 2018). This indicated a different function for each ST in these organs and leaded us to deepen the study.

Although the activity pattern of several plant promoters is conserved even between angiosperms and gymnosperms (Germain *et al.* 2012) indicating the universal functionality of CREs, differential performance has also been reported (Terzaghi and Cashmore 1995, Hernandez-Garcia and Finer 2014). Thus, taking into account the different types of structure and development of flowers and fruits between *A. thaliana* and *M. truncatula*, together with the above-mentioned discrepancies, we have considered the necessity to study the activity of the *pSTs* in transgenic plants of *M. truncatula*. Thus, the aim of this work was to construct *M. truncatula* transgenic plants expressing the β-glucuronidase reporter gene (*GUS*) under the control of the different *pSTs,* and to study their activity during reproductive development. In order to have a clearer picture of this activity and considering that the specificity of *pST* activation could be manifested at the tissue level, we have analysed not only the GUS activity in whole flowers and fruits but also within the tissues after making histological sections of these organs.

## Materials and methods

**Plants and growth conditions:** *Medicago truncatula* Gaernt. ecotype Jemalong line 2HA seeds were chemically scarified with 95 % (v/v) $H_2SO_4$, surface sterilized with 5 % (m/v) chlorine solution for 2 min, placed in Petri dishes with modified Fahräeus medium supplemented with $NH_4NO_3$ to a final 1 mM N concentration (Fahräeus-N) (Barker *et al.* 2006), and stratified at 4 °C in the dark for 2 d. Seeds were germinated in the dark at 25 ºC for 16 to 24 h.

To obtain leaf explants for genetic transformation, 4 germinated seeds were planted in Magenta boxes containing SHb10 medium (Schenk and Hildebrandt 1972) and incubated for 3 weeks in a growth chamber (*Aralab*, Lisbon, Portugal) at a temperature of 25 °C, a 16-h photoperiod, and an irradiance of 200 - 300 μmol $m^{-2}$ $s^{-1}$ (provided by a mix of cool-white and red fluorescent tubes).

For growing plants in pots, germinated seeds were transferred to a mixture of soil:*Vermiculite* (3:1), and grown in the chamber described above for 15 d and afterwards transferred to the greenhouse. Flowers and fruits at different developmental stages were collected from plants growing in the greenhouse and immediately submerged in 90 % cold acetone in order to perform GUS staining (see below). The stages of flowers and fruits analysed were: floral bud (FB); flower 1 (F1): the sepals cover the developing flower which appears green and closed; flower 2 (F2): developing flowers with sepals and petals, yellow and closed; flower 3 (F3): fully developed flower at/or after anthesis; flower 4 (F4): flower after anthesis showing a curved carpel and elongated style; fruit 1 (Fr1): developing fruit showing one or more complete spirals, with no spines and not stacked; fruit 2 (Fr2): developing fruit showing barrel morphology with spines, still not hardened and fruit 3 (Fr3): developing fruit showing barrel morphology with spines, already hardened.

***Medicago truncatula* pST::eGFP-GUS transgenic plants:** The six *pSTs* were cloned into pKGWFS7 destiny vector as indicated in Albornos *et al.* (2017, 2018), using *Gateway*™ cloning technology to construct the *pST::eGFP-GUS* transgenes. Binary vectors were introduced into *Agrobacterium tumefaciens* hypervirulent strain AGL1 by electroporation. *M. truncatula* cv. Jemalong 2HA was transformed and regenerated *via* somatic embryogenesis as described in Chabaud *et al.* (2003). Briefly, wounded leaf explants were cocultured for 3 d with the agrobacteria, decontaminated, and subsequently transferred to callus inducing medium and embryo inducing medium with kanamycin selection. Every callus developed in the selection media was isolated so the embryos arising from each one come from independent transformation events. The somatic embryos generated were committed to form stems and leaves and, later roots. Once the plantlets developed *in vitro* a good root system they were acclimated in pots and transferred to the greenhouse (generation T1) until the seeds (generation T2) could be collected.

Prior to the acclimation, regenerated plants were

checked for the presence of the transgene. To obtain gDNA, one trifolium was collected, immediately frozen in liquid N and ground using a *MM 400 Mixer* mill (*Retsch*, Haan, Germany). The powder was used for gDNA extraction by the CTAB method as described in Doyle and Doyle (1987) using 0.5 $cm^3$ of extraction buffer [2 % (m/v) CTAB, 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA]. The pellet was resuspended in 0.05 $cm^3$ of *MilliQ* sterile water and 2 $mm^3$ were used for the PCR using the primer forward 5′-CATCGGCGGGGGTCATAACG-3′ that annealed in the kanamycin gene and the primer reverse 5′-TGTGGTCGGGGTAGCGGCTG-3′ that annealed in the enhancer green fluorescent protein (*eGFP)* using the *Kappa* Taq DNA polymerase (*Kappa Biosystems*, Wilmington, MA, USA).

**Staining GUS:** *M. truncatula* transgenic plants bearing the construction *pST::eGFP-GUS* were used to study the promoter activity by histochemical localization of GUS enzymatic activity that produce blue colour when hydrolysing the 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-GlcA) (*Duchefa*, Haarlem, The Netherlands) substrate performed as described by Albornos *et al.* (2012b).

Plant material was incubated overnight at 37 °C in GUS staining solution containing 1 mM X-GlcA, 50 mM Na phosphate buffer, pH 7.0, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide and 0.2 % (v/v) *Triton X-100* and GUS-stained tissues were cleared in 70 % (v/v) ethanol. Activity of GUS was assayed in wild-type (WT) and transgenic plants in flowers and fruits along its development from adult plants growing in the greenhouse.

Images were acquired using a *Leica M205 FA* stereomicroscope equipped with a *Leica DFC495* camera (*Leica Microsystems*, Mannheim, Germany).

Experiments were performed in at least three plants of three independent lines. Only when several independent transgenic lines displayed the same pattern of expression of the reporter gene was it consider as positive. Wild type control plants showed no GUS activity (data not shown).

**Histological analysis:** After performing the GUS staining as described above, flowers and fruits were fixed in FAA solution (formalin : acetic acid : 50 % ethanol, 5:5:90) during 72 h. Samples were then dehydrated in increasing concentrations of ethanol and xylene/ethanol and embedded in paraffin (*Paraplast Plus*, *Sigma*, St. Louis, USA) embedding medium at 60 °C. The embedded tissues were sliced into 12 µm sections using a microtome (*HM310*, *Microm*, Walldorf, Germany) and fixed to slides with 0.04 % (m/v) poly-lysine. Sections were deparaffinized with xylene and mounted in *Entellan* (*Merck*, Darmstadt, Germany). Photographs were taken using a microscope *Leica DM 4000 LED* equipped with a digital camera *Leica DFC550* and using *LAS v. 4.3* software (*Leica Microsystems*).

## Results

*Medicago truncatula* transgenic plants pST::GUS carrying the *pST::eGFP-GUS* transgene (hereafter *pST::GUS*) were generated to determine the activity of these promoters. They were obtained as indicated in Materials and methods,

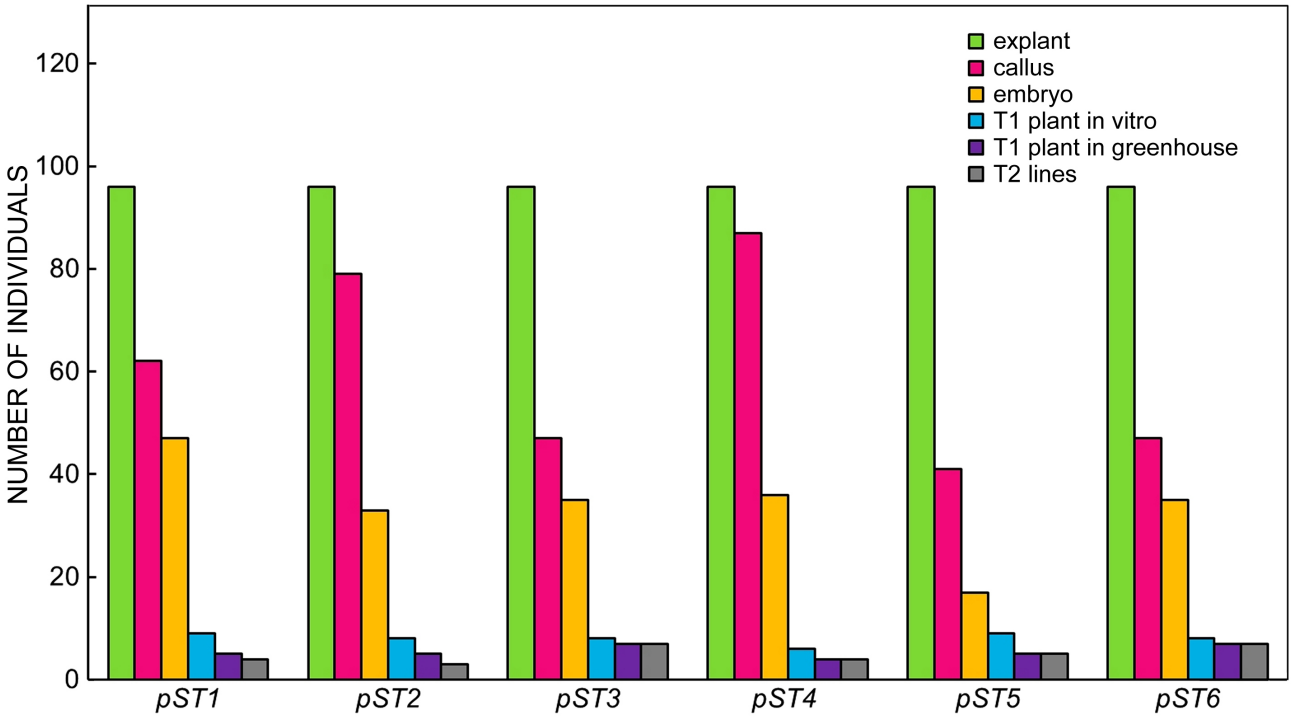


Fig. 1. The percentage of individuals that survived in each phase of the regeneration of the transgenic plants.

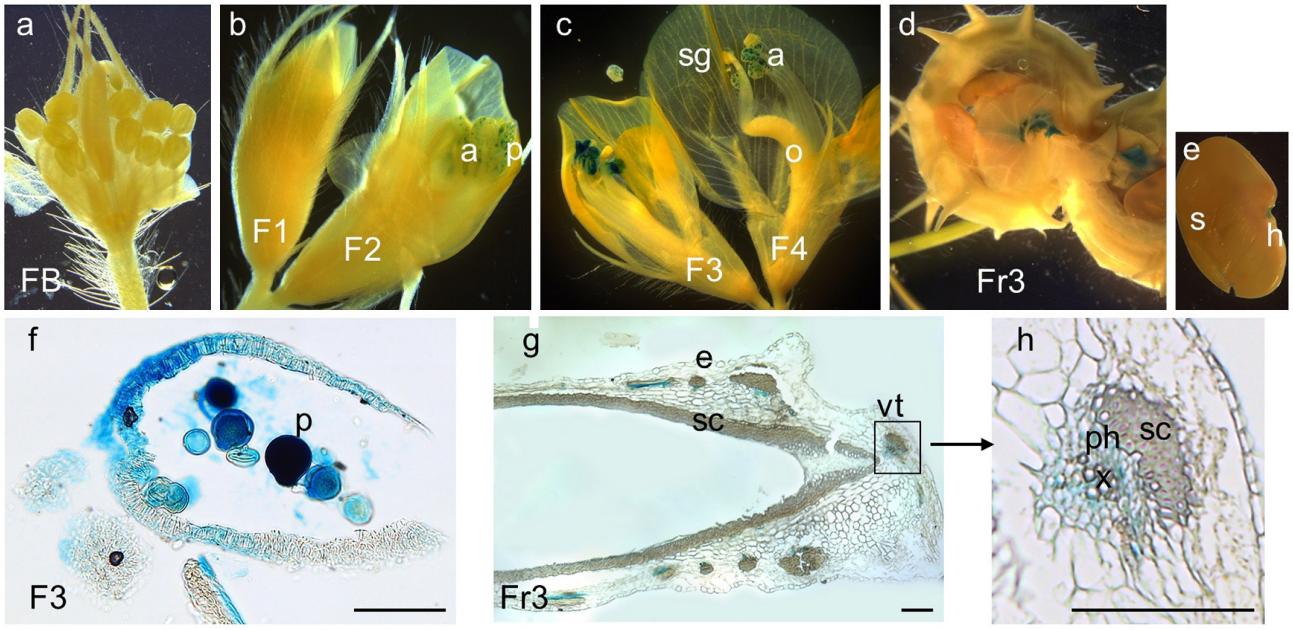


Fig. 2. Activity of *Medicago truncatula pST1* in reproductive organs. Histochemical β–glucuronidase (GUS) staining during reproductive development of *M. truncatula* pST1::GUS plants, sorted according to developmental stage (*A-E*) and histological sections after GUS staining (*F-H*). *A* - floral bud, FB, *B* - developing flowers F1 and F2, *C* - fully developed flower F3 and flower after anthesis F4, *D* - green pod Fr3, *E* - developing seed in Fr3, *F* - F3 anther section, *G* - Fr3 pod section, *H* - Fr3 pod section detail. The *blue* colour indicates the zones with GUS activity driven by *pST1*; a - anther, h - hilum, o - ovary, p - pollen, ph - phloem, s - seed, sc - sclerenchyma, sg - stigma, v - vascular tissue, x - xylem; *scale bars* = 100 µm.

using the AGL1 strain of *A. tumefaciens*. The main difficulty was their regeneration from the cells transformed after the co-cultivation with the agrobacteria (Fig. 1). The limiting step in the regeneration was to obtain plants from somatic embryos. Only between 14 and 19 % of the embryos were able to develop plants, of which 55 - 87 % survived. At the end of the process, the regeneration rates achieved ranged between 3.1 % (*pST2*) and 7.3 % (*pST3* and *pST6*), considering only those plants that produced seeds once acclimatized (called T2 lines).

Before the acclimation of the plants, it was verified by PCR that they had incorporated the transgene, acclimatizing only those plants. Non-transformed regenerated plantlets were rarely found (data not shown). The transgenic plants that survived the acclimatization (generation T1) were grown in the greenhouse until they produced seeds (generation T2).

The *M. truncatula* pST::GUS transgenic plants were used to study the activity of the promoters in different tissues and stages of flowers and fruits during the reproductive development by locating the GUS activity with the substrate X-GlcA that produces blue stain. To facilitate the visualization of the activity profiles of each *pST*, the photographs of the transgenic lines with more intense colour were selected, being shown, in most cases, only those organs in which GUS activity was observed. Since the specificity of activation of *pST* could occur at the tissue level, histological sections from flowers and fruits were also made once the enzymatic reaction of GUS was carried out.

The activity of *pST1* was specifically observed in pollen. While the staining could not be detected in floral buds (FB) with the stamen shorter than the carpel (Fig. 2*a*), it was observed in the pollen grains from closed flowers (F2) with well-developed petals (Fig. 2*b*); the pollen labelling remained after fertilization when the fruit began to develop (F4) (Fig. 2*c*). Once the pod reached the characteristic barrel morphology (Fr3), the labelling could be detected in the funiculus and in the seed hilum (Fig. 2*d* - *e*). Histological sections of anthers of F3 clearly showed GUS staining not only in pollen grains but also in the epidermis of the anthers (Fig. 2*f*). Pod sections revealed *pST1* activity in the vascular bundles of Fr3 (Fig. 2*g*,*h*), which could not be noted when the whole fruits were observed.

During the development of the flowers, the *pST2* was only activated in the stamens (Fig. 3*a*) being the blue colour specifically located in the apical zone of the filaments and especially in the place where they join the anther but not in the pollen grains (Fig. 3*b*). This activity remains in the anthers after the fertilization, when the carpel has given rise to the pod (Fig. 3*c*). During the early stages of fruit formation (Fr1), *pST2* activity was also found in the peduncle (Fig. 3*c*) as well as in the developing seeds (Fig. 3*d*). In more advanced stages of the fruit development (Fr3), when the pod presents thorns and begins to harden, blue staining was maintained in the join between the fruit and the peduncle (Fig. 3*e*) and was also found in the external vascular bundle, the median carpellary bundle (Fig. 3*f*), but not in the inner ones (Fig. 3*g*). Histological sections of anthers show that blue staining is limited to the internal layers of the connective tissue (Fig. 3*h*). Regarding

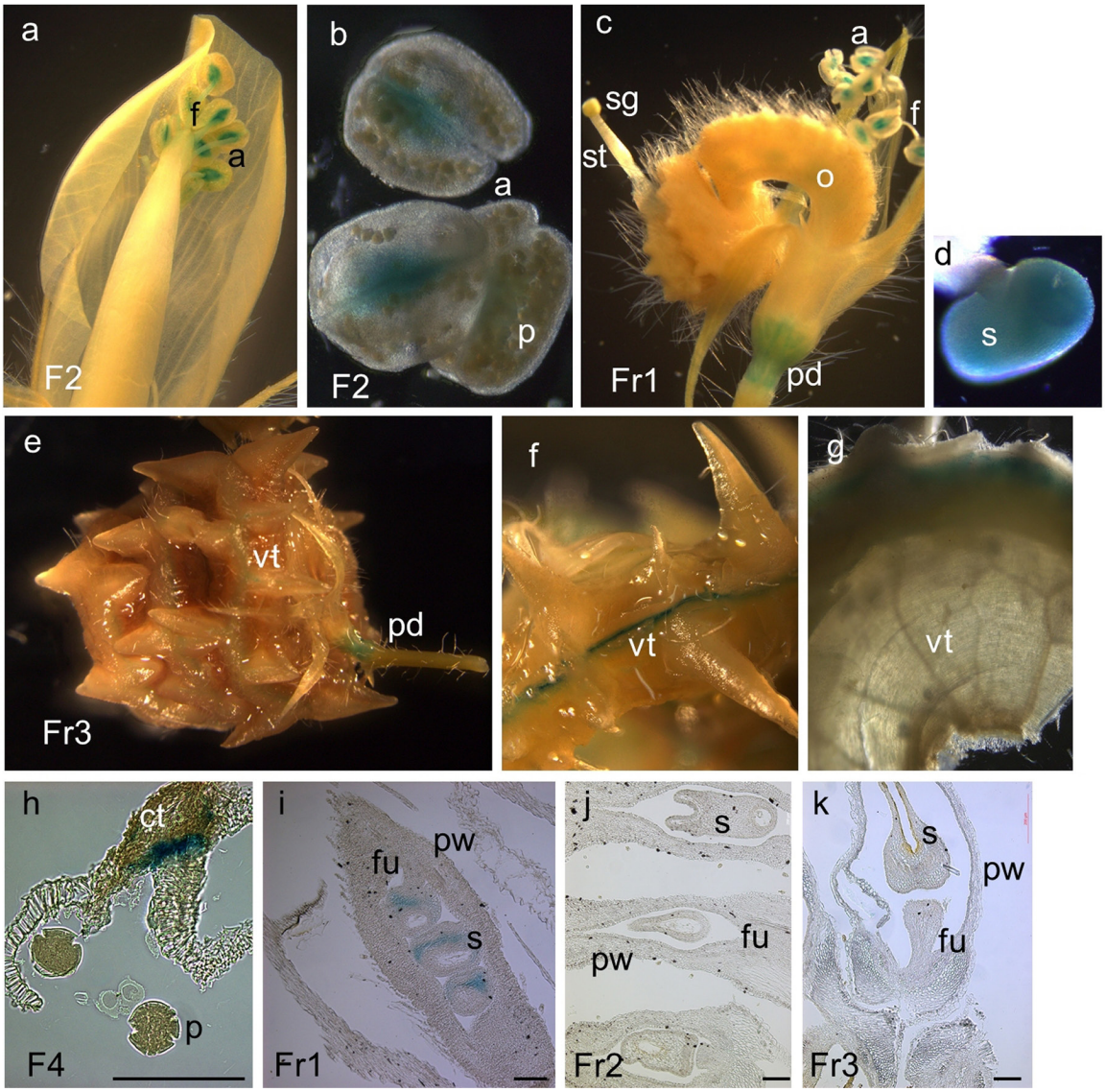


Fig. 3. Activity of *Medicago truncatula pST2* in reproductive organs. Histochemical β–glucuronidase (GUS) staining during reproductive development of *M. truncatula* pST2::GUS plants, sorted according to developmental stage (*A-G*) and histological sections after GUS staining (*H-K*). *A* - Developing flower F2, *B* - F2 anthers *C* - developing fruit Fr1, *D* - developing seed in Fr1, *E* - green pod Fr3, *F,G* - Fr3 pod detail, *I* - developing fruit Fr1 and seeds section, *J* - Fr2 pod and seeds section, *K* - Fr2 pod and seeds section. The *blue* colour indicates the zones with GUS activity driven by *pST*; a - anther, ct - connective tissue, f - filament, fu - funiculus, o - ovary, p - pollen, p - peduncle, pw - pod wall, s - seed, sg - stigma, st - style,; vt - vascular tissue; *scale bars* = 100 µm.

the developing fruit and seed sections, the blue labelling was exclusively found in the seeds at the beginning of fruit development, Fr1 (Fig. 3i); this staining could not be appreciated in histological sections from more developed pods, Fr2 and Fr3 (Fig. 3j and *k*, respectively). *pST2* was neither active in the tissues of the pod wall nor in the funiculus throughout fruit development (Fig. 3*i*,*j*,*k*).

During flower formation, the *pST3* was activated in pollen and anthers. A clear staining was observed in the anthers of F2 and F3 when the petals and the carpel were fully formed and the style was not folded (Fig. 4*a*). The staining began to disappear shortly after fertilization (F4) when the carpel begins to elongate to form the fruit (Fig. 4*b*,*c*). The *pST3* activity could be appreciated in the seeds from the beginning of their development (F4) until the fruit reached its definitive morphology (Fr2) (Fig. 4*c-e*) and showed no staining at the final stage of their formation (Fr3) (Fig. 4*f*,*g*). Pods appeared stained throughout its development, from Fr1 to Fr3 (Fig. 4*d-f*), with the GUS activity located on the placenta (box in the Fig. 4*d*).

The F3 anther sections confirmed *pST3* activity in pollen and especially in the connective tissue (Fig. 4*h*). The histological analysis of the pods at different stages of development (Fig. 4*i-k*) showed the GUS activity associated to seeds from early development phases (Fr1) in funiculus, raphe, and chalaza. In Fr1 blue staining was strong in the placenta (Fig. 4*j*) and extended from the funiculus through the integuments region, and in Fr3

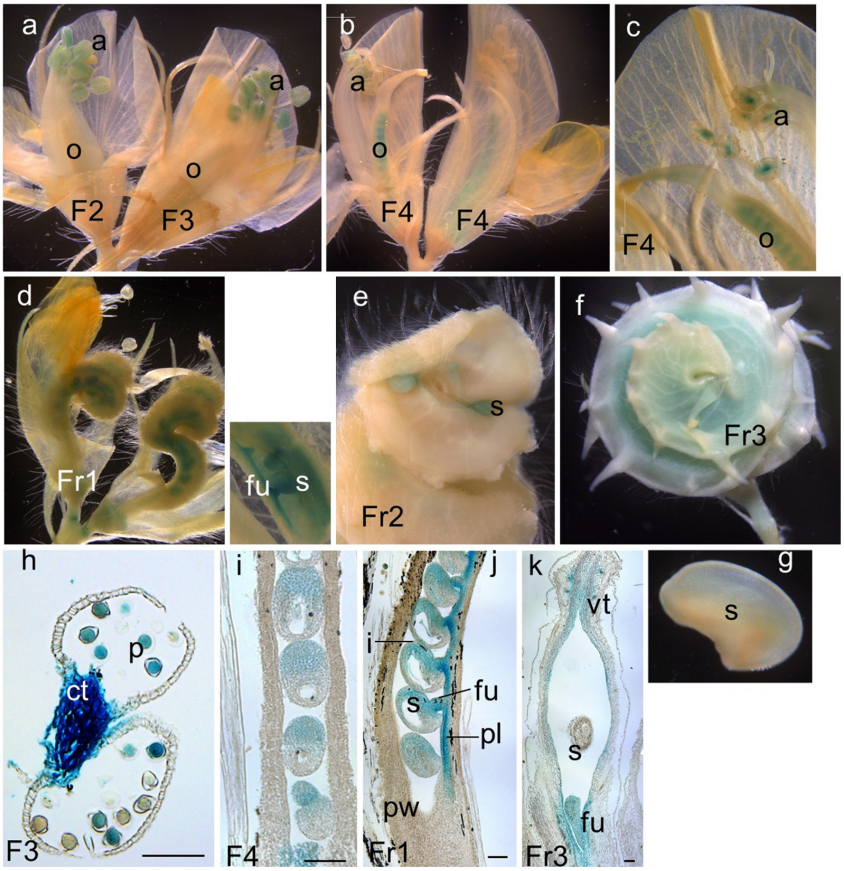


Fig. 4. Activity of *Medicago truncatula pST3* in reproductive organs. Histochemical β– glucuronidase (GUS) staining during reproductive development of *M. truncatula* pST3::GUS plants, sorted according to developmental stage (*A-G*) and histological sections after GUS staining (*H-K*). *A* - Developing flower F2 and fully developed flower F3, *B,C* - mature flower F4 after anthesis, *D* - developing fruit Fr1, *E* - developing seeds inside the fruit Fr2, *F* - green pod Fr3, *G* - developing seed in Fr3, *H* - anther section in F3, *I* - early developing fruit section in F4, *J* - developing fruit Fr1 section, *K* - green pod Fr3 section. The *blue* colour indicates the zones with GUS activity driven by *pST3*; a - anther, ct - connective tissue, fu - funiculus, i - integuments, o - ovary, p - pollen, pl - placenta, pw - pod wall, s - seed, vt - vascular tissue; *scale bars* = 100 µm.

the activity was shown in vascular bundles, endocarp, and also in funiculus (Fig. 4*k*). It should be noted that funiculus activity was maintained in Fr3 but seed activity disappeared (Fig. 4*k*).

The *pST4* was the only one showing activity in FB (Fig. 5*a*) and this activity decreased rapidly as the development progressed. Besides, in F2 the promoter was activated in anthers and filaments (Fig. 5*b*) and, once fertilization occurred, fruit showed GUS staining, first in the apical zone and later all over the organ (Fig. 5*c-f*). *pST4* was also active in the funiculus, but not in the developing seeds of Fr1 (Fig. 5*d*,*e*). During fruit development, the blue coloration was maintained from Fr1 until hardening (Fr3), but no staining was observed in the developing seeds (Fig. 5*e*,*f*). Histological sections in F3 anther revealed that promoter activity was localized only in the connective tissue (Fig. 5*g*). Sectioned Fr1 showed GUS staining in the endocarp, especially in the area where the funiculus, which was also strongly coloured, is attached to the pod wall (Fig. 5*h*). In more developed pods (Fr3), sections revealed high activity of *pST4* in the endocarp and also in the vascular bundles (Fig. 5*i*,*j*).

The *pST5* did not show any activity through the flower development (Fig. 6*a*) nor in the early developing fruits (Fr1) (Fig. 6*b*). In more advanced stages of fruit development, Fr3, when the green pod presents thorns and begins to harden staining could be seen only in the vascular bundles (Fig. 6*c*,*d*,*e*) and this specific activity was clearly confirmed in the histological sections (Fig. 6*f*,*g*).

Developing flowers F1 showed *pST6* directed GUS staining at the base of the anthers and in the ovules when the petals had not yet fully developed and the style appeared folded (Fig. 7*a*) and remained active in F3 (Fig. 7*b*) and during the formation of the fruit and early stages of seed formation (Fig. 7*c-g*). Transiently, during fertilization and in the early stages of fruit formation, the style showed blue coloration (Fig. 7*c-f*). The staining in the ovules was later observed in the seeds (Fig. 7*d-f*) until the fruit began to harden, once the spiral was completely developed. In Fr3, the blue colour was located in the outer vasculature (Fig. 7*g*) but no staining was detected in the seeds (Fig. 7*h*).

The cross section of the anthers from F3 allowed to determine that *pST6* was lightly activated in the

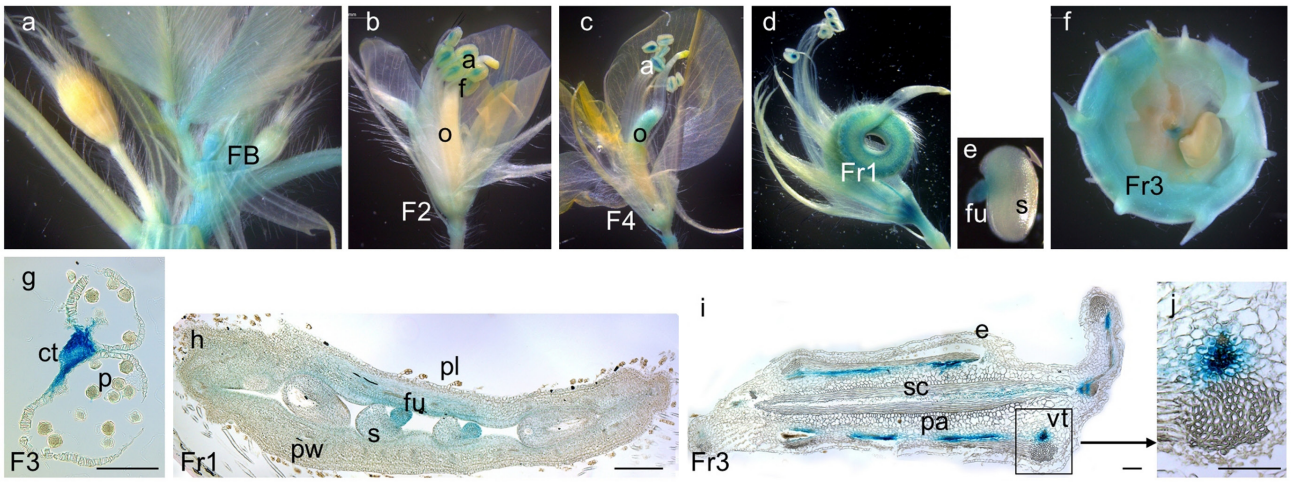


Fig. 5. Activity of *Medicago truncatula pST4* in reproductive organs. Histochemical β–glucuronidase (GUS) staining during reproductive development of *M. truncatula* pST4::GUS plants, sorted according to developmental stage (*A-F*) and histological sections after GUS staining (*G-J*). *A* - Floral buds (FB), *B* - developing flower F2, *C* - mature flower after anthesis F4, *D* - early developing fruit Fr1, *E* - detail of developing seed in Fr1, *F* - green pod Fr3 with seeds, *G* - F3 anther section, *H* - early developing fruit Fr1 section, *I* - green pod Fr3 section, *J* - Fr3 vascular tissue detail. The *blue* colour indicates the zones with GUS activity driven by *pST4*; a - anther, ct - connective tissue, e - epidermis, f - filament, fu - funiculus, o - ovary, p - pollen, pa - parenchyma, pl - placenta, pw - pod wall, s - seed, sc - sclerenchyma, vt - vascular tissue; *scale bars* = 100 µm.

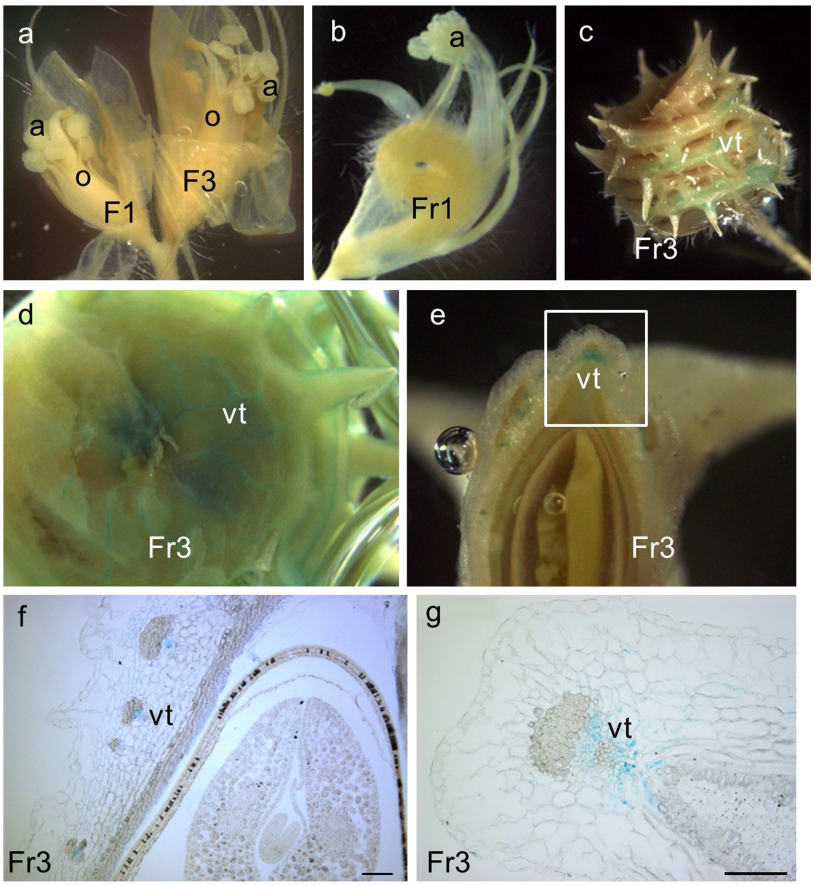


Fig. 6. Activity of *Medicago truncatula pST5* in reproductive organs. Histochemical β–glucuronidase (GUS) staining during reproductive development of *M. truncatula* pST5::GUS plants, sorted according to developmental stage (*A-E*) and histological sections after GUS staining (*F-G*). *A* - Developing F1 and mature F3 flowers, *B* - early developing fruit Fr1, *C,D* - green pod Fr3, *e* - Fr3 transversal detail, *F* - Fr3 section with developing seed, *G* - Fr3 section vascular tissue detail. The *blue* colour indicates the zones with GUS activity driven by *pST5*; a - anther, o - ovary, vt - vascular tissue; *scale bars* = 100 µm.

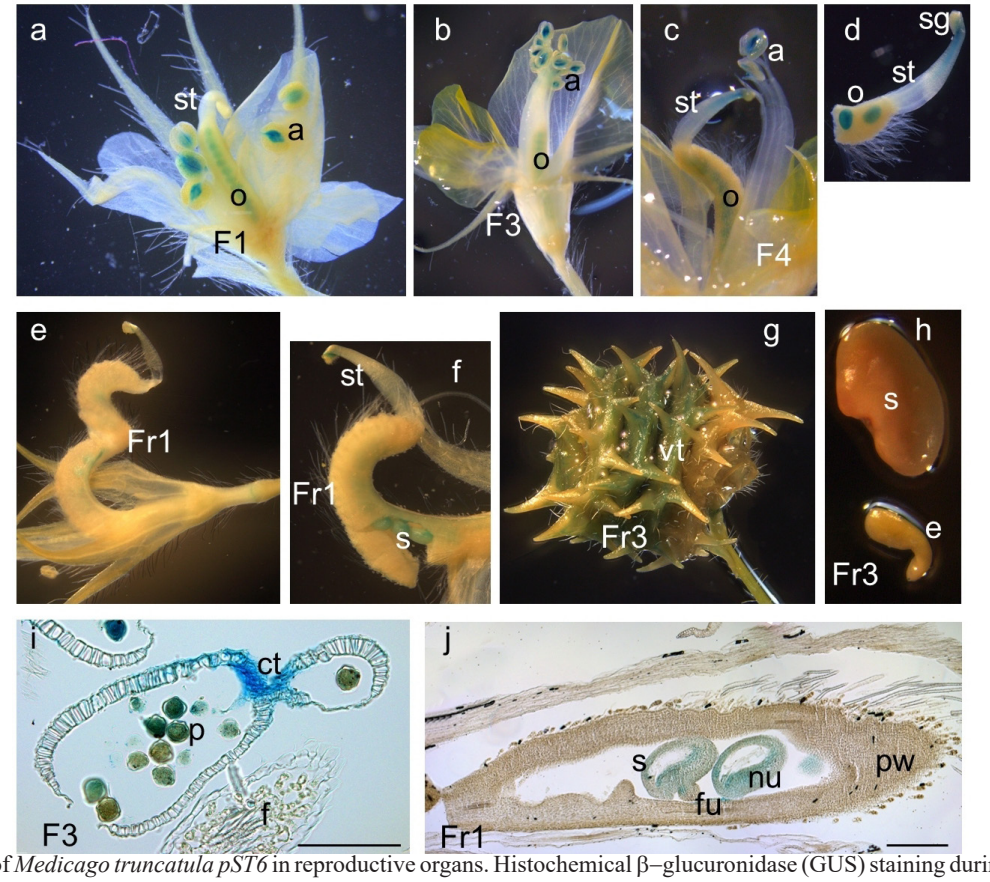


Fig. 7. Activity of *Medicago truncatula pST6* in reproductive organs. Histochemical β–glucuronidase (GUS) staining during reproductive development of *M. truncatula* pST6::GUS plants, sorted according to developmental stage (*A-H*) and histological sections after GUS staining (*I*,-*J*). *A* - Developing flower F1, *B* - mature flower F3, *C* - flower after anthesis F4, *D* - detail of early developing seed in F4, *E,F* - developing fruit Fr1, *G* - green pod Fr3, *H* - developing seed and embryo inside Fr3, *i* - F3 anther section, *J* - developing fruit Fr1 section. The *blue* colour indicates the zones with GUS activity driven by *pST6*; a - anther, ct - connective tissue, e - embryo, f - filament, fu - funiculus, nu - nucellus, o - ovary, p - pollen, pw - pod wall, s - seed, sg - stigma, st - style, vt - vascular tissue; *scale bars* = 100 μm).

parenchyma, in certain pollen grains and in some regions of the epidermis (Fig. 7*i*). When analysing the GUS activity in the histological sections of Fr1, it was observed that, although the pod and the funiculus did not show blue coloration, it was found in the seeds, mainly in the region of the nucellus (Fig. 7*j*).

## Discussion

Although different ST proteins have been related to developmental processes such as early fruit morphogenesis (Fernández *et al.* 2007, Wechter *et al.* 2008), the putative function of STs during flower, fruit, and seed development remains controversial depending on the species (Williams *et al.* 1990, Muñoz *et al.* 1997, Fernández *et al.* 2007, Albornos *et al.* 2014). The fact that the ST sequences found in cDNA libraries made from mRNA isolated from seeds represent the second highest percentage after roots, and that the ST mRNAs have also been found in other reproductive organs such as flowers and fruits (Albornos *et al.* 2012a), point to a function of these proteins during the reproductive period of the plants.

The activity of the promoters of the six members of the *M. truncatula* ST family (*pST*) during the reproductive development was previously studied in *Arabidopsis thaliana* transgenic plants carrying the *pST::GUS* transgene and some discrepancies were found between the detected *pST* activity and the corresponding transcription (Albornos *et al.* 2017, 2018). Inevitably, any promoter outside its native context may give rise to a different activity, either due to the loss of the influence of distal elements acting on the promoter in its original location or due to the effect of foreign elements in the new region in which the transgene is inserted (Hernandez-Garcia and Finer 2014). In the aforementioned study, the different models of flower and fruit development in *A. thaliana* and *M. truncatula* could also account for the imperfect correlation between promoter activity and transcript accumulation.

To overcome the problems derived from using a heterologous plant species and to deepen in the study of the ST family during reproductive development, we have carried out the study of the activity of the *ST* promoters in *M. truncatula* transgenic plants expressing the *GUS* gene driven by the six *pSTs*. In spite of the difficulty in developing these plants, which is reflected in the regeneration rates indicated in Fig. 1, it has been possible to obtain several lines of the transgenic plants containing

any of the 6 *pST::GUS* transgenes. Having generated several transgenic lines allows us to compare and assess the results obtained and provides an interesting tool in the study of the ST family.

Despite the *pST* activity being well conserved in flowers and seeds of *Arabidopsis* with respect to the pattern observed in *M. truncatula*, the activity in the pod differs from that observed in the siliques (Albornos *et al.* 2017, 2018). Mainly, in the homologous system we could find the activation of every *pST* in the pod vasculature and sometimes in other fruit tissues where it was silent in *Arabidopsis*.

As a common feature to all the *pSTs,* the activity in the reproductive organs is associated with the vascular bundles, especially over the fruit formation, where there was activation in the pod vasculature at different developmental stages (*pST1* to *pST6*) (Figs. 2 to 7) and sometimes the activity was found in the funiculus (*pST1*, *pST3*, and *pST4*) (Figs. 2, 3, 4). The low activity of *pST1* specifically located in the vascular tissue (Fig. 2*g*,*h*) could only be detected after sectioning the tissue, while it was not evident in the whole fruit.

This association of *pST* with vascular cylinder relates the ST proteins with cells close to the vascular bundles, such as the transfer cells (Gunning and Pate 1969) that facilitate the arrival of nutrients. Transfer cells, among other sites, are characteristic of maternal-filial exchange zones during seed formation (Talbot *et al.* 2001, Royo *et al.* 2007). In fact, the differentiation of transfer cells has been well described in *M. truncatula* pod vascular bundles (Wang and Grusak 2005). Also in the pods, the *pST3* and *pST4* promoters were activated in the inner epidermis of the endocarp at the final stages of their development (Figs. 4*i*, 5*h*), where Wang and Grusak (2005) have described the formation of invaginations of the cell wall towards the cytoplasm, characteristic of the transfer cells. In legumes, these transfer cells facilitate the exchange of solutes between the seed covers and the embryo (Offler and Patrick 1993) and, in addition, the pods can function as a temporary storage of nutrients that are later transferred to the developing seed (Setia *et al.* 1987). In short, the activation of the *pST* in the proximity of the vascular bundles, especially in the pod (Figs. 2 to 7) indicates their relationship with transfer cells that enable the arrival of nutrients to the new organs, which would support a function of STs in the storage and/or mobilization of reserves as has previously been indicated (Albornos *et al.* 2014).

In *M. truncatula,* it has been established that *ST* transcription and *pST* activity are usually much higher in roots than in shoots (Albornos *et al.* 2017, 2018). It is known that developing roots are a sink for P and photoassimilated carbon which are transported from the shoot to the root (Marschner *et al.* 1996, Wissuwa *et al.* 2005). Later in plant development, in the reproductive phase, flowers and fruits become the major sink organs and therefore there is a shift in the nutrient allocations (Peng and Li 2005). Thus, the presence of ST transcript and proteins in flowers and fruits in different species would support a role in nutrient transport and exchange during fruit development. In pea, *PsaST3* was described as a pod specific mRNA (Williams *et al.* 1990) and in chickpea, the protein CarST1 was immunolocalized to green pod endocarp (Albornos *et al.* 2014). Also, research on the development of fleshy fruits, such as grapes (Waters *et al.* 2005, Fernández *et al.* 2007) and watermelon (Levi *et al.* 2006, Wechter *et al.* 2008) showed that ST proteins could be involved in early fruit morphogenesis, as transcripts accumulate before maturation (Fernández *et al.* 2007) and decline in the mature fruit (Wechter *et al.* 2008).

In addition to these common characteristics found for the *pST* activities during the reproductive development, our present results reinforce and sometimes supplement the functions previously established for the three functional group of ST proteins described in *M. truncatula*.

The *pST1* activity in *M. truncatula*, which was specifically located in the pollen of flowers in stages F2 to F4 (Fig. 2*b*,*c*,*f*) as well as in the funiculus and in the seed hilum of Fr3 fruit (Fig. 2*d*-*e*), coincides with the previously reported activity in *Arabidopsis* (Albornos *et al.* 2017) and supports the proposed function for ST1 in the transport and assimilation of nutrients (Albornos *et al.* 2017), as the funiculus provides a way for nutrients to move from the mother plant (Schulz and Jensen 1971, Meinke 1994). The fact that the *ST1* gene is differentially regulated upon changes in the nutritional status of the plant is supported by several data. Firstly, the transcript accumulation in nitrogen and phosphate starvation (Albornos *et al.* 2017); secondly the *pST1* showing CREs related to nitrogen metabolism and phosphate transporters (Albornos *et al.* 2017); and finally, the publicly available gene expression experiments using microarrays that show an increase in *ST1* transcription under limited nitrogen availability (*MtGEA*, Beneditto *et al.* 2008).

This ST1 function was reinforced by the detection of *pST1* activity in the vascular bundles after the histological sectioning of Fr3 fruits (Fig. 2*g*,*h*), and relates it to the reserve storage and mobilization in the reproductive phase, consistent with the reported accumulation of *ST1* transcripts in developing fruits and seeds (Albornos *et al.* 2017). In this sense, it is worth mentioning that the chickpea protein CarST1, belonging to the same structural group than ST1, accumulates in cotyledons of dry seeds and decreases during germination until finally disappearing in the exhausted cotyledons (Albornos *et al.* 2014) resembling the behaviour of seed storage protein.

The *pST2* and *pST3* are only activated in early stages of seed development in Fr1 and Fr2 (Fig. 3*c*, Fig. 4*d*,*e*), but no activity is found in later stage of seed formation (Fr3; Fig. 3*e*, Fig. 4*g*). However, ST2 and ST3 have been related to water stress and to physiologically controlled desiccation processes such as the seed maturation (Albornos *et al*. 2017), based on the high amount of transcripts found at the final stage of seed formation and the high amount of CREs related to the gene expression in seeds found in their promoter sequences (Albornos *et al.* 2017). The inconsistency between the strong transcript accumulation in the green seeds (Albornos *et al.* 2017) and the disappearance of the *pST2* and *pST3* activity in seeds at the final stages of their formation (Figs. 3 and 4) had already been observed when the pST activity was studied

in Arabidopsis (Albornos *et al.* 2017), which validates our previous results, and indicates that this discrepancy is not due to the different type of fruit and seed development in both species. However, our present results in *M. truncatula* allow us to have a clearer picture of the *pST* activity pattern and to explain these differences considering the specificity of the *pST* activity in the different parts of the ovules and pods during the seed formation. The high *pST3* activity that can be appreciated in the placenta, funiculus, and the external part of the developing seed points to a high transcription rate of *ST3* gene at these early stages (Fig. 4j). These high amount of transcripts could accumulate untranslated in the seeds while their development is progressing and would remain in the integuments of the seeds when they become green and mature, which can explain why mRNA can be detected (Albornos *et al.* 2017) even though at this final stage there is not *pST3* activity in green seeds, except for some staining in the funiculus (Fig. 4*k*). This can also be observed for pST2 (Fig. 3*f*,g). A high representation of *ST3* transcript and an increase of these transcripts in seed at 36 d after pollination was also reported in expression profiles from platform *Genevestigator* (Hruz *et al*. 2008) and *MtGEA* (Beneditto *et al*. 2008). A similar behaviour has been observed for *ST2* gene regarding promoter activity (Fig. 3*f*,*g*) and transcript accumulation (Albornos *et al.* 2017) indicating that it could be regulated in the same way.

Regarding to the third functional group of ST proteins (ST4, ST5, and ST6), the promoter activity of each one was different along reproductive development, except for its above-mentioned presence in vascular bundles. It is worth noting the high activity of *pST4* in floral buds (Fig. 5*a*), the only one which shows high activity at this stage of the floral development, matching the accumulation of transcripts found at this stage in the flowers (Albornos *et al.* 2018). The *pST4* activity in floral buds was also detected in *Arabidopsis* transgenic plants (Albornos *et al.* 2018), although in these species no other activity was found during the reproductive stage. However, in *M. truncatula* transgenic plants the activity of this promoter is also detected during flower and fruit development (Fig. 5) despite the absence of mRNA accumulation in these stages (Albornos *et al.* 2018) which points to the existence of extra control of the transcription beyond the promoter sequence.

No *pST5* activity was detected throughout the reproductive phase (Fig. 6), with the exception of the already mentioned faint activity in the fruit vascular bundles, which coincides with the absence of transcripts in the reproductive period and resolves the discrepancy found in *Arabidopsis* transgenic plants, where *pST5* activity was detected in developing anthers, at the base of the youngest siliques and in the seed chalazal tissue (Albornos *et al.* 2018), which could indicate a mis-regulation of this promoter in different plant species.

The *pST6* was active along flower development (Fig. 7), as it happened in *Arabidopsis* transgenic plants and coinciding with the pattern of transcripts accumulation (Albornos *et al.* 2018). The detection of GUS activity in the seeds of young fruits, mainly in the nucellar region, presents a similar pattern than the described for *pST2* and *pST3*, and again can explain the discrepancy between the low activity of its promoter and the high levels of transcripts found in the green seeds (Albornos *et al.* 2018). It is remarkable that these three proteins (ST2, ST3 and ST6) are the only ones that present a double subcellular location, both in the cell wall and in the cytoplasm (Albornos *et al.* 2017, 2018).

This third group of *M. truncatula* STs (ST4, ST5, and ST6) has been linked to the interactions with other organisms, and their transcripts are specifically and differentially up-regulated by MeJA, ET, and SA, plant regulators involved in the plant defence. Also, in publicly available gene expression experiments using microarrays we have found that *ST6* is upregulated in roots infected with *Ralstonia solanacearum* in two different genotypes (Beneditto *et al*. 2008). Although flowers are attacked by herbivores and pathogens, floral defence as compared with leaf defence, is rarely studied. Some literature describes the accumulation of defensive proteins in flowers but the signalling that regulate these defences are unknown. Thus, the pathogenesis related protein PR-1 has been reported to be accumulated constitutively during the normal development of the tobacco flower, even in the absence of microbial attack or other stresses (Lotan *et al.* 1989), although the specific function of this protein in flowers is unknown. Damle *et al.* (2005) indicated a higher accumulation of proteinase inhibitors in tomato flowers than in leaves as a possible way to counteract the feeding preference of *Helicoverpa armigera*, and more recently, Li *et al.* (2017) reported that in *Nicotiana attenuata*, early flower stages constitutively accumulate large amounts of tripsin proteinase inhibitors, and that a flower-specific sector of JA signalling regulates these constitutively expressed floral defences. In our study, the activation of *pST4*, *pST5* and *pST6* in flowers (Fig. 5, 6, 7), considering the strong induction of the *ST4*, *ST5* and *ST6* transcripts caused by the hormones involved in plant defence (Albornos *et al.* 2018), could mean that these ST proteins also participate in the defence of the reproductive organs, according to the proposed function for this ST group, although more work is necessary to establish this point.

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