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BRIEF COMMUNICATION

Expression of a yeast polygalacturonase gene in *Arabidopsis thaliana*

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Abstract

Polygalacturonases are enzymes involved in plant cell wall growth and reorganization. Transgenic *Arabidopsis thaliana* plants with a *Saccharomyces cerevisiae* endopolygalacturonase gene (*PGU1*) were obtained. The yeast gene was properly expressed in the plants as it has been shown by RT-PCR as well as by the increase in the endopolygalacturonase activity. The transgenic plants showed conspicuous malformations in early stages of development probably due to a weak cell adhesion. On the other hand, adult plants exhibited almost no phenotypic differences as compared to the wild type plants, this suggesting the appearance of some mechanisms on the plant side to counteract the effect of the overexpressed polygalacturonase.

Additional key words: cell wall, RT-PCR, *Saccharomyces cerevisiae,* transgenic plants.

The plant cell wall is a highly dynamic structure that undergoes changes in composition and configuration in response to functional requirements (Somerville *et al.* 2004). Cell walls are composed by cellulose microfibrils embedded in a matrix of polysaccharides and glycoproteins (Carpita and Gibeaut 1993, O'Neill and York 2003). Pectins are defined by the presence of uronic acids although they are composed of homogalacturonans and rhamnogalactouronans (Ridley *et al.* 2001). Pectins are involved in the control of cell wall porosity, cell adhesion, plant defence and so on (Solecka *et al.* 2008).

A great number of enzymes with different functions are present in the cell walls The best characterized being exo- and endo-polygalacturonases, pectate lyase, pectin methylesterase, and β-galactosidase (Fry 2004, Veronesi *et al.* 2007). Polygalacturonases were first identified over 35 years ago and have been suggested to be involved in the disassembly of pectin that accompanies many stages of plant development, particularly those that require cell separation (Bonghi *et al.* 1992). The large number of polygalacturonase genes in plants complicates the study of the expression pattern of these enzymes. For instance, in *Arabidopsis thaliana* there are at least 50 putative polygalacturonase-coding genes.

Few studies involving the constitutive expression in plants of polygalacturonases from different origins have been done (Osteryoung *et al.* 1990, Atkinson *et al.* 2002, Capodicasa *et al.* 2004) and some of them have shown the diverse consequences that these transformations may have on the plant. So, the over-expression of a tomato ripening-involved polygalacturonase in tobacco plants did not have a significant effect on phenotype as far as the growth of transgenic plants (Osteryoung *et al.* 1990). However, the over-expression of an endopolygalacturonase from *Aspergillus niger* in tobacco caused different and conspicuous effects, including a dwarf phenotype (Capodicasa *et al.* 2004). Furthermore, the expression of a fruit-specific apple endopolygalacturonase gene under a constitutive promoter led to a range of novel phenotypes. These phenotypes included silver-coloured leaves and premature leaf shedding due to reduced cell adhesion in leaf abscission zones. Mature leaves had malformed and malfunctioning stomata that perturbed water relations and contributed to a brittle leaf phenotype (Atkinson *et al.* 2002).

In order to obtain a better knowledge of polygalacturonases as well as their role in the cell wall structure and metabolism, we have over-expressed the *Saccharomyces cerevisiae PGU1* gene in *A. thaliana*.

PGU1 is an endopolygalacturonase-encoding gene

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Abbreviations: RT-PCR - reverse transcriptase- polymerase chain reaction;
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first cloned by Blanco *et al.* (1998). We over-expressed the gene in *A. thaliana* under the control of 35S CaMV promoter and its effect on phenotype was studied. The selection of *A. thaliana* was made because it is a model plant (Huang *et al.* 2009) and its genome is fully characterized.

Arabidopsis thaliana L. ecotype Columbia seeds were surface sterilized with 1 % sodium hypochlorite solution for 10 min at room temperature and thoroughly washed with sterile water. Seeds were soaked at 4 ºC for 2 - 3 d before germination. Following this, seeds were sown in Petri dishes and grown under a 16-h photo-period (irradiance of 65 μmol m^{-2} s^{-1}) and temperature of 25 ºC.

The gene *PGU1* (GenBank Acc. No. Z49653) was ligated to pG3.3 at the EcoRI site under the control of the constitutive promotor CaMV 35S. pG3.3 is a vector derived from pBin19 (Bevan 1984). The construction pG3.3 *35S::PGU1* was introduced into *Agrobacterium tumefaciens* strain EHA105 (Hood *et al.* 1993) by electroporation (200 Ω, 1.44 kV, 5 ms) and then into *A. thaliana* by the floral dipping method (Clough and Bent 1998). Transgenic plants were selected in Murashige and Skoog (MS) medium with kanamycin (50 μg cm^{-3}).

The presence of the transgene *PGU1* in the transformed plants was confirmed by polymerase chain reaction (PCR). DNA was extracted from young leaves from *35S::PGU1* or wild-type plants. The tissue was homogenized at room temperature. Then, 0.7 cm^3 of extraction buffer (200 mM Tris-HCl pH 8.0, 250 mM NaCl, 25 mM EDTA, and 0.5 % sodiumdodecylsulphate, SDS) was added and vortexed for 5 s. The extracts were centrifuged at 18 000 *g* for 1 min and the supernatant was transferred to a new microcentrifuge tube. Isopropanol (0.6 cm^3) was added, vortexed briefly and immediately centrifuged at 18 000 *g* for 5 min. The supernatant was removed and the pellet dried and re-suspended in 0.02 cm^3 of deionized water. The forward and reverse primers used for *PGU1* were GATTTCTGCTAATTC ATTACTTAT and AACAGCTTGCACCAGATC, respectively.

Total RNA from leaves was extracted with *RNeasy® Plant Mini Kit* (*Qiagen*, Hilden, Germany) according to the manufacturer recommendations. Total RNA was reverse transcribed using the *First Strand cDNA Synthesis Kit* for reverse transcriptase (RT)-PCR (*Roche Farma*, Basel, Switzerland). The cDNA obtained was used as template in semi-quantitative RT-PCR. The PCR amplification was performed with gene-specific primers (Table 1). *At5g23860* coding for β-tubulin was used as control for all the studied genes. The cDNA was amplified using *SYBR-Green® PCR Master* kit (*Applied Biosystems*, San Francisco, USA) containing an *AmpliTaq Gold* polymerase on a *iCycler* (*BioRad*, Berkeley, USA). Real-time quantification was based on the ratio of Ct value for the control gene per the Ct for the studied gene.

Leaves from 7- and 21-d-old plants grown on agar and on soil, respectively, were homogenised (0.45 g cm^{-3}) in 40 mM sodium acetate buffer pH 5.0 containing 13 mM EDTA, 1 M NaCl, 10 g dm^{-3} PVP, 10 mM β-mercaptoethanol and 5 U protease inhibitor cocktail (*Sigma-Aldrich*, St. Louis, USA) and incubated in agitation for 10 h at 4 ºC. The extracts were centrifuged 10 min at 15 000 *g* at 4 ºC, the supernatants were filtrated and precipitated with saturated ammonium sulphate. Following centrifugation 15 min at 15 000 *g* at 4 ºC the pellet was dissolved in 40 mM sodium acetate buffer pH 5.0, and dialyzed for 6 h against the same buffer. Polygalacturonase activity was measured by reducing end-group production as described by Lever (1972).

RT-PCR showed presence of PGU1 gene in transgenic plants (Fig. 1). Transgenic plants (7-d-old) grown in Petri dishes in the presence of kanamycin showed a rather abnormal phenotype; the leaf borders were quite deteriorated with signs of necrosis (Fig. 2*B*). Necrosis was not observed neither in plants transformed with just the plasmid pG3.3 and grown in the same conditions nor in the wild type plants (Fig. 2*A*). These plants were transplanted to soil, where the effect was still apparent for the second and the third pair of leaves with evident signs of necrosis in the leaf borders and some alterations in the growth pattern (Fig. 2*D*). The leaves of transgenic plants were longer and narrower as compared to wild-type plants (Fig. 2*C*). An effect of either the transformation method or the kanamycin gene expression on this phenotype was ruled out since transgenic plants transformed with plasmid pG3.3 alone showed the wild-type phenotype in selective medium with kanamycin. Furthermore, an effect based on the site of the insertion should be ruled out since all the transformed plants resistant to kanamycin showed a similar phenotype. In later stages (4^{th} and 5^{th} leaf pair) the phenotype of *PGU1* transgenic plants became progressively similar to the wild-type. In fact, in adult plants there were not phenotypic differences in terms of flowers, leaves and siliques as well as growth rate between the transgenic and wild type plants.

The expression level of *PGU1* gene was studied in

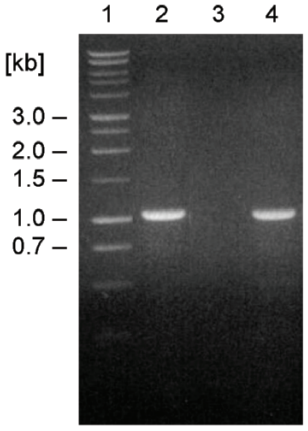

Fig. 1. PCR to detect the presence of the *PGU1* transgene in the transformed plants. *Lane 1* - markers, *lane 2* - plasmid pG3.3 containing *35S::PGU1*, *lane 3* - DNA extracted from wild type plants, *lane 4* - DNA from plants transformed with *35S::PGU1*.

Table 1. Primers used for semi-quantitative real time PCR.

Gene	Acc. number	Forward (5'-3')	Reverse (5'-3')
PGU1	Z49653	TCCACTTTGTGCGCTTTTGCG	TCCATTCCTTGTACTGAAATGTGGTT
PGIP1	NM120769	ACCCTCCTCAAGATCAAGAAATCTCTAAACAAC	GGTTCTTGAGTTTGGCGATGGTGG
β-tubulin	NM122291	TCGACGGGAAGATACCAAGGAGAGAA	TCCTTACGAACAACATCGAGAACGGAATCG

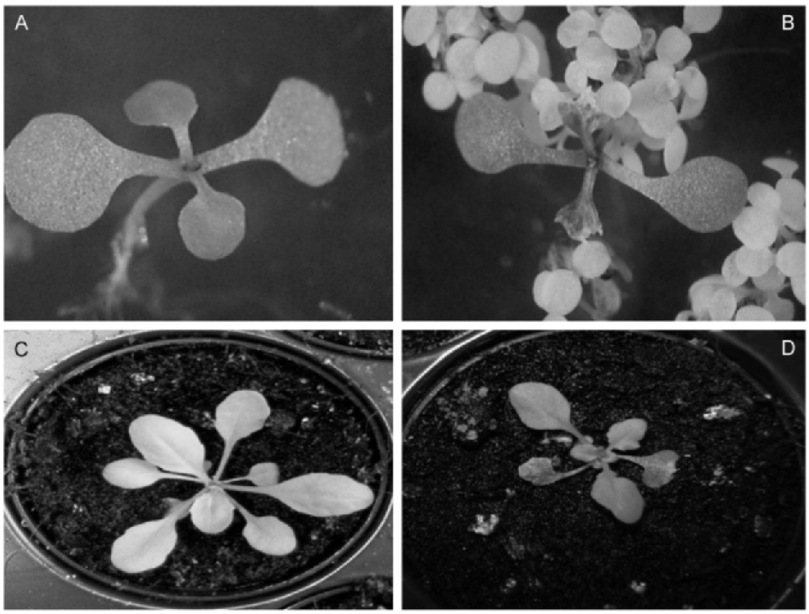

Fig. 2. Morphology of 7-d-old *Arabidopsis* plants growing in Petri dishes with MS medium supplemented with kanamycin. *A* - Wild type plants growing in MS medium without kanamycin. *B* - Plants transformed with *35S::PGU1*. Morphology of 15-d-old transgenic plants after transplantation to soil. *C* - Wild-type *A. thaliana* plant transferred into soil. *D* - Plants transformed with *35S::PGU1* transferred into soil.

35S::PGU1 transgenic 21-d-old plants, using semi-quantitative real time RT-PCR. The ratio of Ct value for the tubulin (*At5g23860.1*) per the Ct for *PGU1* was 1.24 ± 0.05. Thus, the *PGU1* gene in transgenic plants expressed and its transcript level was higher than that of the tubulin, the constitutive gene used as reference.

The polygalacturonase activity was determined in leaf crude extracts from 7- and 21-d-old wild-type plants and *35S::PGU1* transgenic plants. The polygalacturonase activity was higher in the transgenic plants as compared with the wild type, the ratio being *ca*. 1.5 in the young plants (7-d-old) and slightly decreasing in 21-d-old plants. Thus, the *PGU1* gene was properly expressed in the transgenic plants and transgenic plants always showed the higher polygalacturonase activity than the wild-type.

To check if the absence of phenotype in adult plant might be caused by the induction of polygalacturonase inhibitor (PGIP), as a consequence of the introduction of a foreign polygalacturonase (D´Ovidio *et al.* 2004), its expression in transgenic plants was also studied by quantitative real-time PCR. The gene expression was normalized to that of tubulin, a constitutive *Arabidopsis* gene. The expression of *PGIP1* in *35S::PGU1* transgenic plants was not significantly different from wild-type plants.

The *35S::PGU1* transgenic plants showed damaged leaves with evident signs of necrosis and conspicuous malformations in early stages of development that has also been reported for other polygalacturonase transgenic plants (Atkinson *et al.* 2002, Capodicasa *et al.* 2004). This effect was not caused by an unspecific effect of T-DNA insertion since no phenotype appeared when plants were transformed with the void plasmid. Moreover, the phenotype of the different transgenic lines obtained was similar, this being in clear agreement with the idea that the effect was indeed caused by the polygalacturonase expression.

The transgenic plants showed an important increase in the polygalacturonase activity, especially in the younger plants when the leaf malformations were more apparent. However, the more mature stage of development was, the less phenotypic differences were visible, suggesting the triggering of some mechanisms on the plant, thus counteracting the stressing effect of the over-expressed polygalacturonase. A similar lack of effect of hetero-

logous polygalacturonase over-expression has been found in tobacco plants (Osteryoung *et al.* 1990). The lack of effect of the yeast polygalacturonase in the mature plants might be explained in terms of different susceptibility of cell walls to its action, probably based on structural differences of pectins. However, other unknown factors cannot be ruled out.

The expression of *PGIP* genes has been described as a general mechanism of plants to prevent the action of microbial polygalacturonases (Di Mateo *et al.* 2006), but the *PGIP* trancripts levels did not show significant differences between the *35S::PGU1* transgenic plants and the wild type. Thus, the absence of a clear effect on the phenotype of the mature plants cannot be explained only in terms of PGIP action and different structural features between young and mature leaves as far as pectins, can be recalled to explain the behaviour shown here.

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