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Overexpression of the carbohydrate binding module of strawberry expansin2 in *Arabidopsis thaliana* modifies plant growth and cell wall metabolism

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Abstract Several cell wall enzymes are carbohydrate active enzymes that contain a putative Carbohydrate Binding Module (CBM) in their structures. The main function of these non-catalitic modules is to facilitate the interaction between the enzyme and its substrate. Expansins are non-hydrolytic proteins present in the cell wall, and their structure includes a CBM in the C-terminal that bind to cell wall polymers such as cellulose, hemicelluloses and pectins. We studied the ability of the Expansin2 CBM (*CBMFaEXP2*) from strawberry (*Fragaria x ananassa*, Duch) to modify the cell wall of A*rabidopsis thaliana*. Plants overexpressing *CBMFaEXP2* were characterized phenotypically and biochemically. Transgenic plants were taller than wild type, possibly owing to a faster growth of the main stem. Cell walls of CBMFaEXP2-expressing plants were thicker and contained higher amount of pectins. Lower activity of a set of enzymes involved in cell wall degradation (PG, β-Gal, β-Xyl) was found, and the expression of the corresponding genes (*AtPG*, *Atβ-Gal*, *Atβ-Xyl5*) was reduced also. In addition, a decrease in the expression of two *A. thaliana* Expansin genes (*AtEXP5* and *AtEXP8*) was observed. Transgenic plants were more resistant to *Botrytis cinerea* infection than wild type, possibly as a consequence of higher cell wall integrity. Our results support the hypothesis that the overexpression of a putative CBM is able to modify plant cell wall structure leading to modulation of wall loosening and plant growth. These findings might offer a tool to controlling physiological processes where cell wall disassembly is relevant, such as fruit softening.

Keywords *A. thaliana* · Carbohydrate binding module · Cell wall · Expansin · Pectin · Strawberry

Introduction

The cell wall of higher plants is a complex and dynamic structure that provides mechanical support, defines cell shape, regulates growth and development and acts as a barrier against biotic and abiotic stresses (Pilling and Höfte 2003). The major polysaccharide component of primary cell wall is cellulose, which is tightly bound to hemicelluloses, and both components are imbibed in a hydrophilic pectin matrix (Darvill et al. 1980; Vincken et al. 2003).

Cell wall disassembly is the main process that leads to fruit softening during ripening (Vicente et al. 2007). An excessive firmness loss during postharvest storage of fleshy fruits such as tomato (*Solanum lycopersicum*) or strawberry (*Fragaria x ananassa*, Duch), facilitates pathogen invasion and increases postharvest decay (Amil-Ruiz et al. 2011; Cantu et al. 2008b). Several non-hydrolytic proteins

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and enzymes are actively implicated in cell wall disassembly (Cosgrove 2000a; Minic 2008), and numerous attempts to control cell wall degradation and fruit softening have involved the generation of transgenic plants with reduced expression of these enzymes. In general, the strategy of suppressing the expression of a single gene caused a limited or null effect on fruit softening (Brummell et al. 1999; Cantu et al. 2008a; Moctezuma et al. 2003). Probably, the reason was that most of the proteins involved in cell wall catabolism are encoded by multigene families and the effect resulting from the suppression of a particular gene expression is often offset by a related protein.

Several soluble cell wall proteins contain Carbohydrate Binding Modules (CBM) in their structures (Cantarel et al. 2009; Guillen et al. 2010). CBMs are auxiliary domains with autonomous folding and the capability of recognizing and binding specifically to carbohydrates (Boraston et al. 2004). Although most of CBMs bind specifically to a particular substrate, promiscuous substrate recognition has been reported in several cases (Cantarel et al. 2009; Charnock et al. 2002; Freelove et al. 2001). The overexpression of CBMs in plants has produced different phenotypes. Obembe et al. (2007a) overexpressed two bacterial CBMs in tobacco plants (*Nicotiana tabacum*), one of them considered promiscuous and the other specific according to their binding properties. The overexpression of the promiscuous CBM caused a reduction in stem elongation and delayed flowering, while the overexpression of the non-promiscuous CBM did not modify the normal plant phenotype. In *Arabidopsis thaliana*, putative CBMs have been detected in numerous enzymes involved in the catabolism of the cell wall, most of them grouped into the 22, 48 and 49 CBM families according to the classification established in CAZY data base (Cantarel et al. 2009).

Expansins (Exps) are non-hydrolytic proteins involved in relaxing the cell wall structure by disrupting non covalent bonds between cell wall polysaccharides, thus facilitating polymer slippage (Cosgrove et al. 2002; McQueen-Mason et al. 1992; McQueen-Mason and Cosgrove 1995). These proteins participate actively in processes involving cell wall remodeling, such as plant growth, organ abscission and fruit softening (Brummell et al. 1999; Cosgrove et al. 2002). Exps are one of the main families of plant cell wall proteins that contain CBMs in their structures (Choi et al. 2006; Shoseyov et al. 2006). The general Exp structure involves a signal peptide, which directs the protein to the secretory pathway, a central domain that resembles family-45 glycosyl hydrolases, and a putative CBM of approximately 80 amino acids in the extreme C-terminal. McQueen-Mason and Cosgrove (1995) isolated two cucumber Exps and proved their ability to bind different fractions of the cell wall, mainly cellulose coated with hemicelluloses. More recently it was reported that the CBM of strawberry Exp2 (*CBMFaEXP2*) binds in vitro to microcrystalline cellulose and also to oat xylan and pectins from *citrus* (Nardi et al. 2013), which suggests that *CBMFaEXP2* can be considered promiscuous as reported for other CBMs (Bolam et al. 2001; Obembe et al. 2007a).

In the present study, we have targeted the expression of *CBMFaEXP2* to *A. thaliana* cell wall to test the hypothesis that the over-expression of a promiscuous CBM would bind cell wall polysaccharides and then interfere with the binding and the activity of endogenous Exps and other cell wall degrading enzymes, thus altering cell wall metabolism. Our results indicate that *CBMFaEXP2* overexpression modifies the normal activity of several cell wall enzymes leading to modulation of cell wall loosening.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana, ecotype Columbia, as well as transformed plants obtained from this ecotype was used in this study. For root length analysis, seeds were sterilized, grown on MS medium (Murashige and Skoog 1962) and then cultivated in a growth chamber under long day regime (16/8 h light/dark cycle) at 23 °C and a photon flux density of 200 μmol m^{-2} s^{-1}. For further experiments, plants were grown on soil under similar conditions. For soil growth, seeds were sown on a mixture of sand:perlite:soil (1:1:1) and irrigated with Hoagland´s nutrient solution. Leaves and inflorescence stem tissue from 4-week-old wild type (WT) and transgenic plants were collected and frozen in liquid nitrogen and stored at −80 °C until use.

CBMFaEXP2 cloning and plasmid construction

With the aim of directing the CBM of strawberry *Exp2* (*CBMFaEXP2*, 276 bp) to *A. thaliana* cell wall, a fusion between the signal peptide of *AtEXP8* (AT2G40610; 81 bp), and *CBMFaEXP2* was constructed. First, in silico analysis was made using SignalP software (http://www.cbs.dtu.dk/services/SignalP/) to identify the signal peptide region of *AtEXP8* and PROSITE software (http://prosite.expasy.org; (Sigrist et al. 2002) to identify the CBM in *FaEXP2*. The amplification of the sequences encoding the signal peptide of *AtEXP8* and the CBM of *FaEXP2* was carried out separately by PCR. First, forward primer 5P-**GGGGACAAGTTTGTACAAAAAAGCAGGCTAA** ATGTACACTCCATCATACTTA-3P and reverse primer 5P-TGCCCGTTGATTGTGAATCTG-ATggcataggtgc ctctga- 3P were used for the signal peptide amplification. The reverse primer included 23 bp at the 5P end, which

overlapped with the beginning of CBM region (indicated in capitals). The product from the first PCR was purified and used as forward primer, and 5P-**GGGGACCACTTTGTACAAGAAAGCTGGGTA**CTAGAATTGACCGCCTGAAAACGT-3P as reverse primer for the amplification of the CBM region. *FaEXP2* full length (**Accession number AF159563**) was used as template. Recombination sites attB1 and attB2 (indicated in bold), were engineered into the upstream region of the forward primer for the signal peptide and into the downstream region of the reverse primer for the CBM, respectively. A stop codon in the reverse primer for the CBM region was included (underlined). The product was then extended by PCR to yield a single fragment that was inserted into pDONR vector (Invitrogen) using the enzyme BP Clonase (Invitrogen). The sequence obtained was verified at the Macrogen USA sequencing service. The *CBMFaEXP2* cDNA was then cloned downstream the CaMV35S promoter in the binary vector cTAPi (Rohila et al. 2004) by recombination between pDNOR aatL sites and the attR sites of the destination vector using the enzyme LR clonase (Invitrogen).

CBMFaEXP2-GFP cloning and plasmid construction

The construct *CBMFaEXP2* described above was used as template to build this second construct without the stop codon, to allow the fusion with the GFP included in the binary vector pB7FWG2 (Karimi et al. 2002). *CBMFaEXP2-GFP* construct was confirmed by sequencing.

A. thaliana transformation

The *CBMFaEXP2* and *CBMFaEXP2-GFP* constructs were introduced into *Agrobacterium tumefaciens* GV3101 strain cells by using the freeze–thaw procedure (Holsters et al. 1978). *A. thaliana* plants were grown to the flowering stage and the transformation was carried out by the floral dip method (Clough and Bent 1998). For CBMFaEXP2 plants, six independent homozygous lines were selected, which contained single insertions as estimated by their 3:1 (resistant:susceptible) segregation patterns on 0.5 % (v/v) phosphinothricin (ppt) solution (BASTA®). Lines named p1, p3 and p5 were used for phenotype characterization and further experiments. Negative segregant plants were also isolated and used as control plants (WT).

Confocal laser scanning microscopy (CLSM)

The subcellular localization of GFP was examined under CLSM using a Leica CLSM equipped with krypton-argon laser and a HCX PL APO CS 63X/1.4 numerical aperture oil-immersion objective. Seeds from CBMFaEXP2-GFP plants were grown on soil and selected with ppt 3 d post germination. Eleven days old selected seedlings were put on slides, incubated with 7.5 $\mu g\ ml^{-1}$ propidium iodide solution (Sigma) and mounted under glass cover slips. Acquisition and image processing was carried out using LAS AF Lite 2.2.1 and Image Pro Plus version 4.5.0.29.

Phenotypic analysis

The flowering time was determined as the day on which the first flower opens. For root length analysis, seedlings were initially grown in petri dishes on MS medium with agar 0.8 % (w/v). At the 5th day, snapshots were taken with a camera with a frame grabber unit. Roots of approximately 30 seedlings per transgenic line and WT were measured. For seed size determination, seeds were taken from different batches to account for batch variation. Images of seeds were captured using a Nikon SMZ800 stereomicroscope connected to a digital camera. Root length and seeds length and perimeter were measured using Image Pro Plus software, version 4.5.0.29.

Mechanical properties of *A. thaliana* inflorescence stems and rosette leaves

The in vitro extension assay of stem and rosette leaves sections was performed using Texture Analyzer equipment in tension mode, fitted with miniature tensile grips (TA.XT2, Stable Micro Systems Texture Technologies, Scarsdale, NY). To analyze inflorescence stems, plants were grown until inflorescences were 12 cm in length. Then, the upper section of 5 cm in length (without including the bolt) was secured between the grips and extended at a constant speed of 0.05 mm s^{-1} until breakage. In the case of rosette leaves, plants were grown for 4 weeks and the 4th, 5th and 6th leaves (in order of appearance) were used. Two sections of 1 cm^2 were obtained per leaf. The section was taken parallel to the long axis of the leaf blade, excluding the central vein. The extension test was performed at a constant speed of 0.1 mm s^{-1} until breakage; the force (Kg) developed at each distance (mm) was recorded.

Quantification of cell size and cell wall thickness

Stem samples (10 mm length) were taken from the zone at 30 mm above the rosette leaves, and fixed in 70 % (v/v) FAA (formaldehyde-acetic acid–ethanol) (Johansen 1940). Transversal sections were taken by using a microtome; sections were stained with 80 % (w/v) safranin, mounted in gelatin-glycerin and examined under a bright field microscope (Leitz SM-Lux; 400×) equipped with a Moticam 2300 camera. Acquisition and image processing was carried out using Motic Imagen plus 2.0 software. For each stem segment, five images of the interfascicular tissue were

taken at approximately equidistant positions around the stem. For each image, wall thickness was measured as half of the thickness between two adjacent cell walls. A total of 300 measurements were obtained per each WT and transgenic line analyzed. The same images were used to determine the cell surface area using Image-Pro Plus software version 4.5.0.29; 300 cells per line were measured.

Isolation of cell wall polysaccharides

Cell wall polysaccharides were obtained as alcohol insoluble residues (AIR) according to d´Amour et al. (1993) with modifications. Ten grams of tissue from rosette leaves or inflorescence stems were homogenized in an Omnimixer with 40 ml absolute ethanol. In the case of the inflorescence stem tissue, a prior homogenization with $N_{2\ (l)}$ was carried out. Then, tissues were boiled with reflux for 30 min. The homogenate was filtered and the residue was washed three times with 15 ml of absolute ethanol. The residue was dried overnight at 37 °C and weighed. Total AIR content was expressed as milligrams of AIR per 100 mg of tissue.

Extraction and quantification of pectins, hemicelluloses and cellulose

First, one hundred milligrams of AIR from each tissue (rosette leaves or inflorescence stems) were extracted in 90 % (v/v) DMSO for 48 h at room temperature to dissolve starch and eliminate its interference with polysaccharide analysis (Carpita and Kanabus 1986). Thereafter, polyuronides were isolated according to Rosli et al. (2004). Uronic acid concentrations were estimated by the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen 1973) using galacturonic acid as standard. Hemicellulose and cellulose fractions were obtained according to Rosli et al. (2004) and estimated as glucose according to d´Amour et al. (1993).

Enzyme activities

Polygalacturonase activity was measured as described previously (Villarreal et al. 2010). The amount of galacturonic acid released was determined with 2-cyanoacetamide (Gross 1982). Pectin-methylesterase activity was measured according to Vicente et al. (2005). The mixture was incubated at 37 °C, monitoring the OD at 620 nm every 10 s for 10 min and using galacturonic acid as standard. Total Polygalacturonase and Pectin-methylesterase activities were expressed as µmoles of galacturonic acid released per hour and per gram of tissue. β-Xylosidase, β-galactosidase and α-L-Arabinofuranosidase activities were measured according to Villarreal et al. (2010). The amount of p-nitrophenol released was determined measuring OD at 410 nm and comparing with a calibration curve prepared from p-nitrophenol. Results were expressed as µmoles of p-nitrophenol released per hour and per gram of tissue.

RNA isolation and RT-PCR

Total RNAs were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The confirmation and semi-quantitative expression analysis of transgenic lines was done by RT-PCR, using specific primers for the fusion constructed, being the forward primer used 5′-TTCCTCCAAGGAACTCATGG-3′ and the reverse primer 5′-AGAATTGACCGCCTGAAAAC-3′. *A. thaliana* β-actin2 mRNA was also determined using the forward primer 5′-AATCTCCGGCGACTTGACAG-3′ and the reverse primer 5′-AAACCCTCGTAGATTGGCACAG-3′. The amplification conditions for β-actin were 15, 18, 21 and 24 cycles. In the case of CBMFaEXP2, the amplification was performed under the same conditions, except for the numbers of cycles that were 16, 20, 24 and 28. Products of semiquantitative RT-PCR were analyzed by electrophoresis in agarose gels 1.2 % (w/v) and the intensity of bands obtained was quantified using Gel-Pro Analyzer version 6.3.

Real-time PCR assays

First strand cDNA from *A. thaliana* rosette leaves was used for Real-time PCR assays. Primer sequences used are shown in Supplementary Table S1. The amplification reactions were performed in a Step One Plus Real-Time PCR System (Applied Biosystems). In each experiment, the relative expression level corresponds to mean of three biological replicates, normalized against the expression level of *UBQ10* (AT4G05320) gene, and using WT sample as the calibrator with a nominal value of 1. Expression levels were calculated according to the method described by Pfaffl (2001) and expressed in arbitrary units ± Standard Error (SE).

Botrytis cinerea growth conditions and infection

Conidia of *B. cinerea* isolated from strain B05.10 were plated out on potato dextrose agar supplemented with 40 mg ml^{-1} of powder obtained from dried tomato leaves. Plates were kept from 7 to 10 days in darkness at room temperature for sporulation. For susceptibility assays, conidia were harvested with sterile water containing 0.02 % (v/v) Tween-20 and then filtered and quantified with a hemacytometer. The inoculum concentration was adjusted to 5×10^4 conidia per ml with PDB medium, supplemented with 10 mM sucrose and 10 mM KH_2PO_4. Conidia were incubated for 2–3 h at room temperature without shaking

(Flors et al. 2007). For inoculation, two 5 µl droplets per leaf were placed on the adaxial surface of attached leaves of 4-week-old *A. thaliana* WT and transgenic plants, which were incubated in trays covered with PVC film to obtain high humidity conditions. After 48 or 72 h, the leaves were detached, pictured with a digital camera and the size of the necrotic area developed in response to *B. cinerea* infection was measured with the Image-Pro Plus V 4.1 software (Media Cybernetics LP, Bethesda, MD, U.S.A.). Two leaves from each of thirty-two plants corresponding to WT, p1, p3 or p5 lines (a total of 64 leaves per line) were inoculated with *B. cinerea*. After 48 or 72 h, 32 leaves from each line were detached and the necrotic area analyzed as described above.

For *B. cinerea* growth rate analysis, petri dishes containing agar 0.8 % (w/v) and the AIRs obtained from CBM-FaEXP2 and WT rosette leaves were prepared. A preliminary assay was done to optimize the amount of AIRs to be added. For this, a 9 mm^2 agar plug was transferred from the edge of a 5-day-old actively growing culture of *B. cinerea* to the solidified plates with agar 0.8 % (w/v) and 0.1, 0.2, 1 or 2 mg ml^{-1} of AIR. Plates were then cultivated at 24 °C, and the fungus growth area was measured after 48 and 72 h (Supplemental Fig. S3b). Further experiments were performed using 1 mg ml^{-1} of AIR, and the fungus growth area was measured after 72 h. Three independent AIR extractions were performed for each transgenic line and WT and four technical replicates per line were made. The *B. cinerea* growth area was measured as described above for necrotic area measurement.

Statistical analysis

Each experiment was done three times and two or three independent replications for each analysis were used. Data were analyzed by ANOVA, and the means were compared by the Dunett test, using WT plants as control, at a significance level of $P \leq 0.05$, with the exception of the appearance of the inflorescence stem. In this case, data were analyzed by a two way ANOVA test and means compared by Bonferronis test at a significance level of $P \leq 0.05$. Statistical analysis was done using the GraPh-PadPrism version 5.03 software program (GraPhPad, San Diego, CA, USA).

Accession numbers

The accession numbers of genes analyzed here are the following: AT3G10740 (*αAra1*), AT3G19620 (*AtβXil5*), AT3G14310 (*AtPME3*), AT2G40610 (*AtEXP8*), AT3G29030 (*AtEXP5*), AT2G04378 (*AtβGal*), AT1G2346 (*At*PG), AT4G05320 (*AtUbq10*). Sequence data can be found in The *A. thaliana* Information Resource (TAIR).

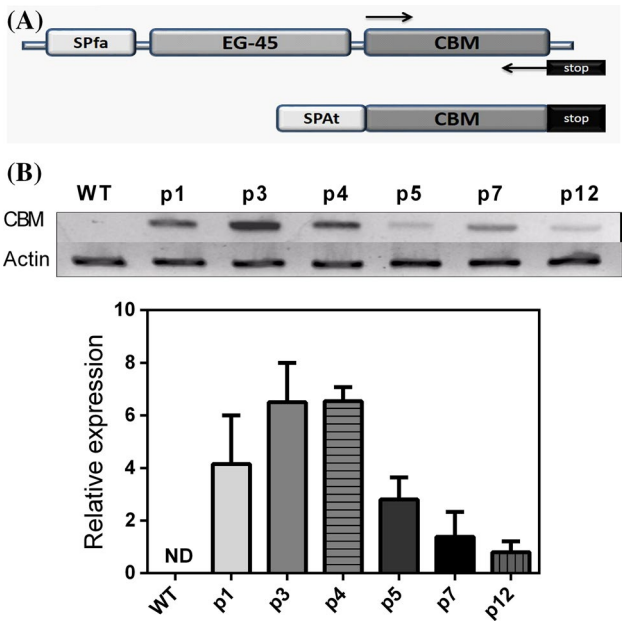

Fig. 1 **a** Modular organization of *FaEXP2* and the fusion made for *A. thaliana* transformation (*CBMFaEXP2*). *Light gray boxes* represent the signal peptides of strawberry (SPfa) and *A. thaliana* (SPAt). EG-45 corresponds to the family–45 endoglucanase-like domain found in *FaEXP2* and *dark gray boxes* represent the carbohydrate binding module (CBM). *Arrows* indicate the location of the primers used for amplification of the CBM region. **b** Amplification of *CBM-FaEXP2* by RT-PCR assay in selected transgenic lines. The primers used flanked the zone of the fusion between the signal peptide and CBMFaEXP2. Twenty-four cycles were used for amplification of CBMFaEXP2 while 18 cycles were used for β-actin amplification. The RT-PCR assay was performed three times and bands intensities were quantified using Gel-Pro Analyzer software. *Bars* at the bottom panel indicate the relative intensity of the corresponding bands (CBM/Actin); ND: No detected

Results

Construction of the *CBMFaEXP2* fusion gene and transformation of *A. thaliana*

In order to direct the protein CBMFaEXP2 from strawberry to the secretory pathway of *A. thaliana*, we engineered a signal peptide of *A. thaliana Exp8* at the N-terminal of *CBMFaEXP2* (Fig. 1a). Following *A. thaliana* transformation and selection with phosphinothricin (ppt), a total of 12 independent lines were obtained. The presence of the transgene was confirmed by PCR amplification of genomic DNA with specific primers (Supplemental Fig. S1). The T_1 transgenic plants were screened for homozygous lines with a single insertion as estimated by their 3:1 (resistant:susceptible) plant proportion, using ppt as selection marker. Six lines were obtained and the transgene expression level was evaluated by RT-PCR performed

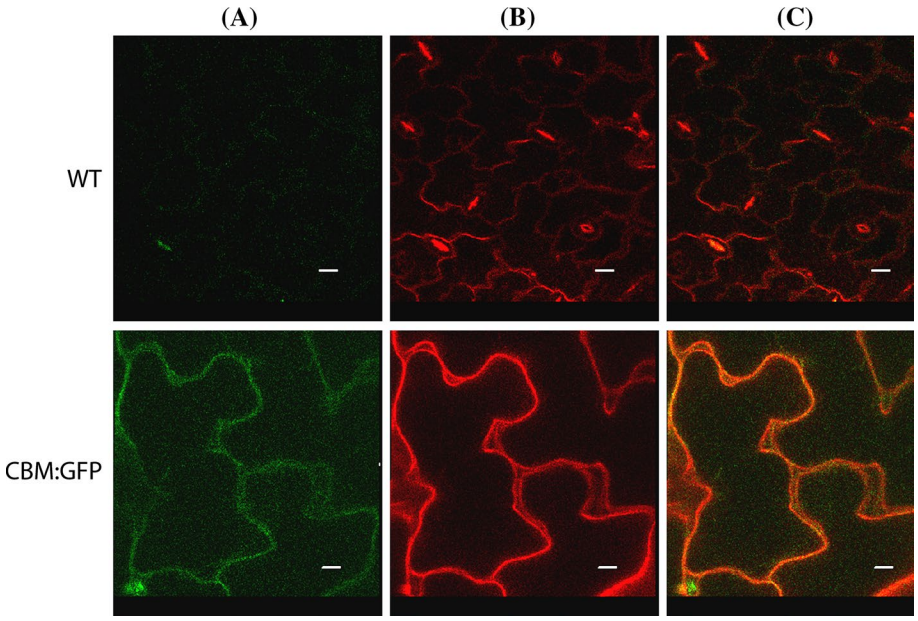

Fig. 2 Subcellular localization of CBMFaEXP2-GFP fusion protein in leaf epidermal cells. **a** Green fluorescence (GFP), **b** red fluorescence (propidium iodide) **c** merged image. *Bar* = 10 microns

with RNA extracted from rosette leaves (Fig. 1b). For further experiments we chose a high-expression line (p3), a medium-expression line (p1) and a low-expression line (p5).

Subcellular localization of *CBMFaEXP2-GFP*

To investigate if the signal peptide used was functional directing the construct to the secretory pathway, *A. thaliana* plants transformed with the construction *CBMFaEXP2-GFP* were generated and subcellular localization of CBMFaEXP2 was tested by monitoring the GFP fluorescence. Eleven days-old seedlings selected with ppt were examined by CLSM. Five different plants showed identical localization patterns, characterized by GFP fluorescence at the cell wall of mesophyll cells. GFP fluorescence in CBMFaEXP2-GFP plants was clearly distinguishable from auto-fluorescence in controls (WT) (Supplemental Fig. S2). To confirm that GFP signal observed in mesophyll cells was specifically located in the cell wall, seedlings were incubated with propidium iodide (PI) before microscopic analysis. In this case, because of the dye was not able to reach the mesophyll cells, epidermal cells were analyzed instead. The fluorescence of GFP was clearly higher as compared with the auto-fluorescence of the cell wall of WT plants (Fig. 2a). Figure 2b shows the PI fluorescence, and in the merged image (Fig. 2c) it can be noticed the co-localization of GFP and PI signals in epidermal cells of transgenic plants. These results demonstrate that the signal peptide used was able to direct CBMFaEXP2 from strawberry to the secretory pathway of *A. thaliana*.

Phenotypic analysis of *CBMFaEXP2* plants

In order to determine if the expression of *CBMFaEXP2* was able to modify the plant phenotype, a detailed analysis was carried out using the growth stages defined by Boyes et al. (2001). Morphological characteristics of principal growth stage 0–3.9 of transgenic lines (comprising from seed germination until complete growth of rosette leaves) did not differ from those of WT. Plant cultivation in soil did not reveal differences during the rosette development, including number of leaves at bolting and rosette diameter (data not shown). Instead, we observed differences in plant height, being plants overexpressing *CBMFaEXP2* taller than the WT (Fig. 3a). Therefore, we analyzed in more detail the principal growth stage 5 (inflorescence emergence stage). The stage 5.1 was altered in transgenic plants, since the main bolt was visible significantly earlier in comparison with the WT (Fig. 3b). All the transgenic plants showed the first flower bud between 27 and 28 d post germination (DPG), depending on the line analyzed, whereas it took two additional days (30 DPG) to WT plants (Fig. 3b). Also, the mean stem elongation rate in the three transgenic lines was around 22.5 ± 4.2 to 25.8 ± 5.6 mm d^{-1}, compared to a rate of 13.5 ± 3.2 mm d^{-1} for the WT. (Figure 3c). As a result, the three transgenic lines showed earlier flowering, measured as the first flower opened, by about 2–3 days when compared to the WT (Fig. 3d).

In addition, CBM-expressing plants weighed more than the WT on both fresh and dry basis (data not shown). When analyzed separately, it was found that the weight increase was due to a heavier aerial tissue, while no differences were observed in rosette leaves either as fresh or dry

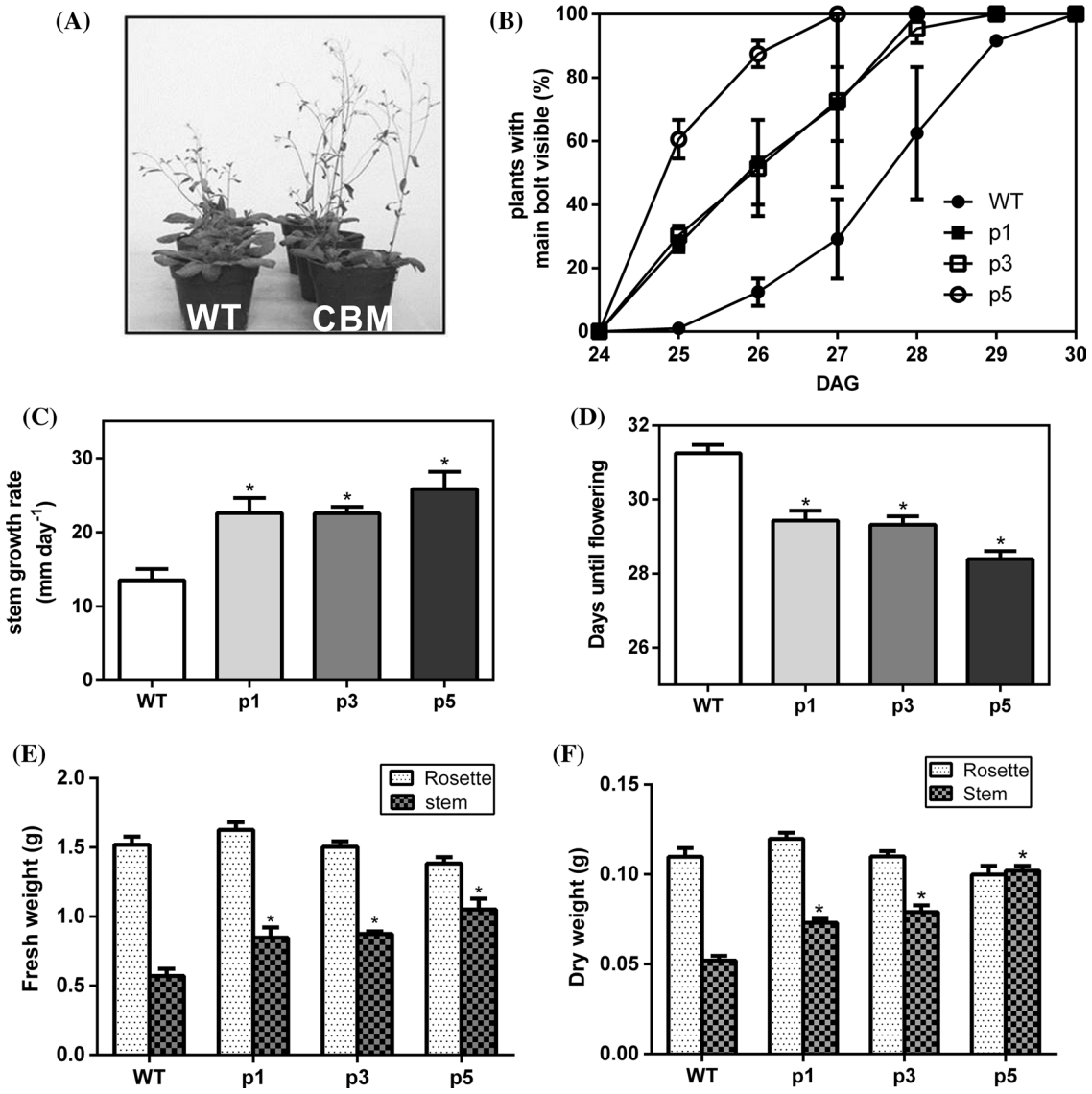

Fig. 3 Phenotypic analysis of CBMFaEXP2 transgenic plants. **a** Phenotype of 5 weeks old CBMFaEXP2 and WT plants. **b** Percentage of plants with the main bolt visible in transgenic and WT plants. Plants were grown in soil and the day of the appearance of inflorescence stem was recorded. DAG: days after germination. **c** Inflorescence stem growth rate in transgenic and WT plants. **d** Time until flowering in CBMFaEXP2 plants compared with WT. **e** Fresh weight (grams) of rosette leaves and stem of transgenic and WT plants. **f** Dry weight (grams) of rosette leaves and stem of transgenic and WT plants. Results are mean ± SD from three independent replicates. p1; p3 and p5 represent the three independent transgenic lines selected. *Asterisks* indicate significantly different from WT (One way ANOVA; post test: Dunnet $P \leq 0.05$)

weight basis (Fig. 3e, f). Inflorescence stem thickness was also measured but no differences were observed (data not shown).

We also observed a differential root growth at the cotyledon fully opened stage (growth stage 1.0), being roots significantly shorter in the transgenic plants (Fig. 4). When the seed phenotype was analyzed, a significant increase of length and perimeter was found in transgenic seeds (Fig. 5). There was no significant difference at the seed germination rate between transgenic and WT plants (data not shown).

With regard to growth stages beyond 8.0 (from silique ripening to complete senescence), the transgenic plants phenotype resembled that of the WT (data no shown).

Extension assay with *CBMFaEXP2* inflorescence stems and rosette leaves

In addition to the altered growth of the inflorescence stem, the transgenic lines appeared to have weaker stems. Therefore, their mechanical resistance was tested in

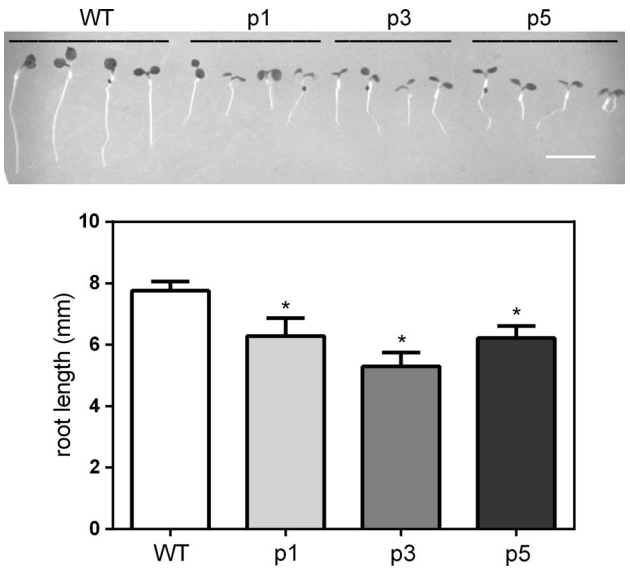

Fig. 4 Root phenotype of CBMFaEXP2 transgenic plants. Photograph was taken 5 days after germination on MS medium plates. Results are mean ± SD from three independent replicates (n = 15). p1; p3 and p5 represent the three independent transgenic lines selected. *Asterisks* indicate significantly different from WT (One way ANOVA; post test: Dunnett $P \leq 0.05$). *Bar* = 5 mm

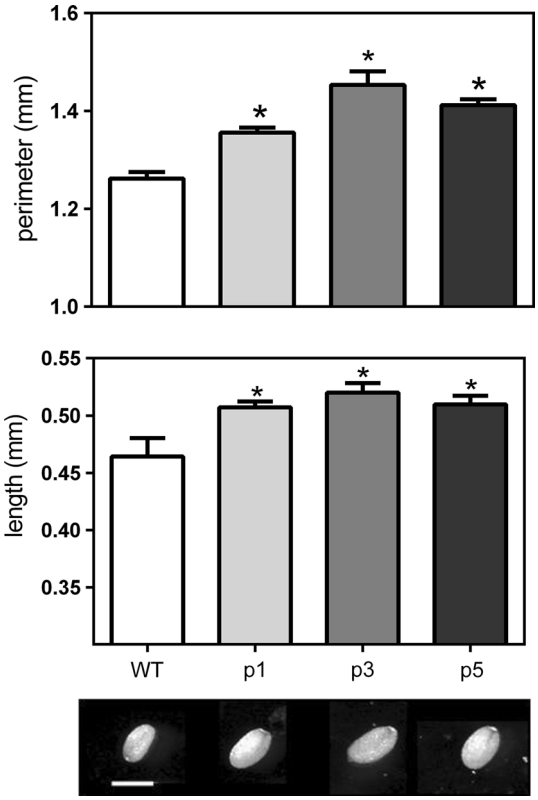

Fig. 5 Seed phenotype of CBMFaEXP2 transgenic plants. Results are mean ± SD from three independent batches. Approximately 50 seeds per batch of transgenic and WT lines were measured. p1; p3 and p5 represent the three independent transgenic lines selected. *Asterisks* indicate significantly different from WT (One way ANOVA; postest: Dunnett; $P \leq 0.05$). *Bar* = 0.5 mm

CBMFaEXP2 and WT plants. Extension assays were performed with a Texture meter to measure two parameters: the force necessary to break the tissue, and the displacement until breakage. A typical strain profile obtained for the inflorescence stem is shown in Fig. 6a. The force required to break the stem of transgenic plants was approximately half of the force needed for the WT (Fig. 6b). In addition, tissue displacement until breakage was significantly higher for the inflorescence stem of the transgenic lines in comparison with the WT (Fig. 6c). The extension assay was performed with rosette leaves sections as well (Fig. 6 d–f). We chose to work with the 4th, 5th and 6th rosette leaves (in order of appearance). Figure 6d shows an example of the strain profiles obtained for this tissue. Even though no differences were detected in the force needed to cause breakage for CBM-expressing plants and WT (Fig. 6e), the tissue displacement until breakage was significantly higher for two of the three transgenic lines (p3 and p5) (Fig. 6f).

Effect of *CBMFaEXP2* on cell size and cell wall structure and composition

The microscopic analysis of the inflorescence stem revealed that cell walls in the interfascicular region from the three lines overexpressing *CBMFaEXP2* were thicker than those from the WT (Fig. 7a, b). The cell size was estimated by measuring the surface cell area of interfascicular region, and data obtained were grouped into three categories: small (10–60 μm^2), intermediate (60–110 μm^2), and large (110–160 μm^2) (Fig. 7c). Almost 80 % of the cells of WT plants were grouped into the small cell size category, while only 40–60 % (depending on the transgenic line analyzed) of cells from CBM expressing plants were included in this category. The opposite trend was observed in the intermediate and large groups: less than 20 and 5 % of stem interfascicular cells from WT ranked, respectively, in these categories while in p1 line the corresponding percentages were 40 and 25 %. The two other lines showed similar trend to p1, though differences were not statistically significant regarding the WT (Fig. 7c). It is worth to mention that a similar effect on cell wall thickness and cell size was observed in epidermal cells from rosette leaves (data not shown).

The possible effect of *CBMFaEXP2* overexpression on cell wall composition was analyzed. The total cell wall (as alcohol insoluble residue, AIR) and its main components were quantified from rosette leaves and inflorescence stems (Table 1). In the case of rosette leaves, transgenic lines had approximately 20 % more AIR content than WT, while no difference was found in inflorescence stems (Table 1). The

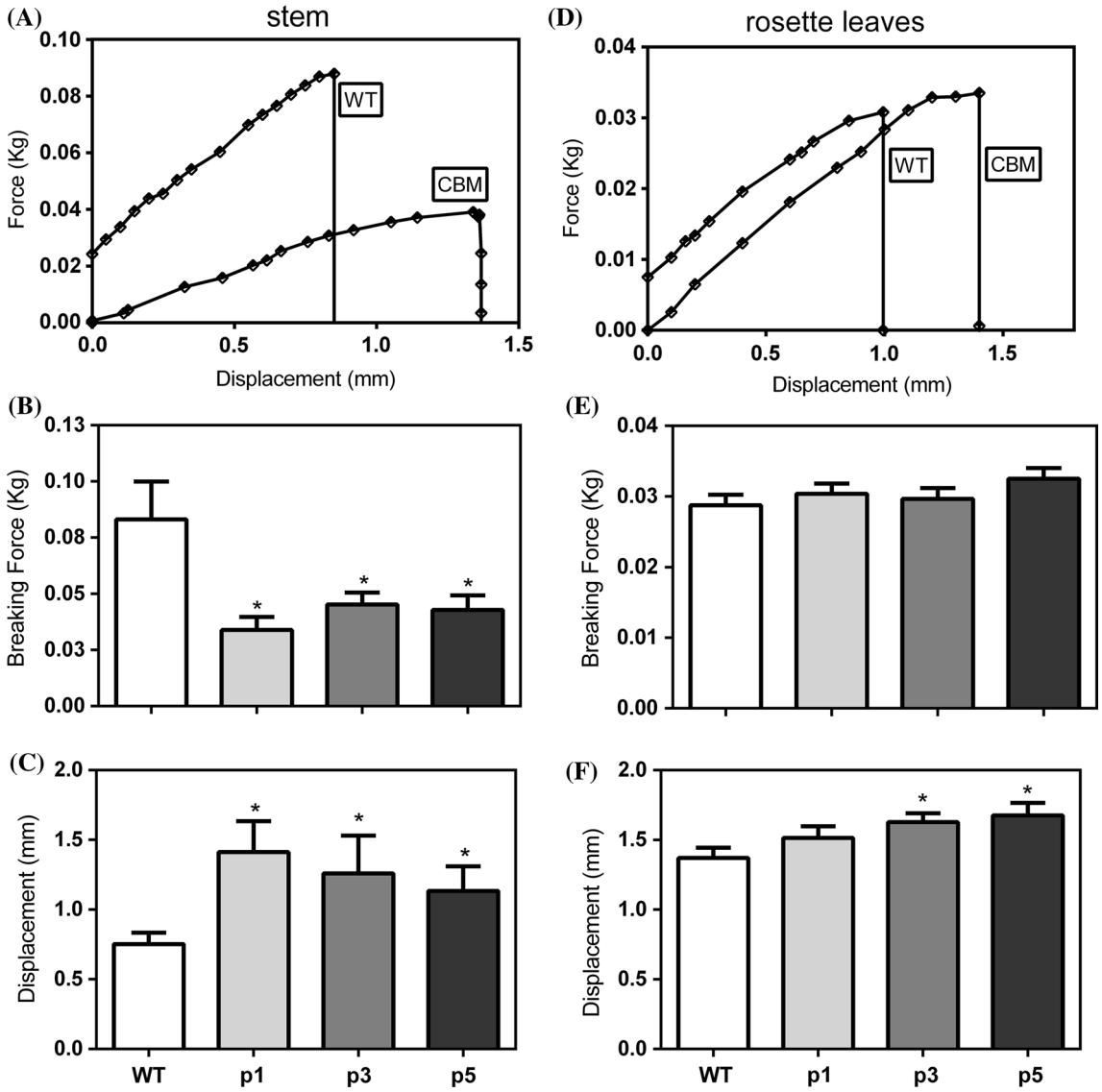

Fig. 6 Mechanical properties of CBMFaEXP2 plants. **a** Example of the curves of force versus displacement obtained for CBMFaEXP2 and WT inflorescence stem. **b** Mean values for maximum force before breakage for the CBMFaEXP2 and WT inflorescence stem. **c** Mean values for displacement until breakage for the transgenic lines and WT inflorescence stem. **d** Example of the curves of force versus displacement obtained for CBMFaEXP2 and WT rosette leaves. **e** Mean values for maximum force before breakage for the CBMFaEXP2 and WT rosette leaves. **f** Mean values for displacement until breakage for the transgenic lines and WT rosette leaves. Results are mean ± SD of three replicates. p1; p3 and p5 represent the three independent transgenic lines selected. *Asterisks* indicate significantly different from WT (One way ANOVA; post test: Dunnett; $P \leq 0.05$)

AIR from both tissues was extracted sequentially with different solutions to quantify cell wall fractions. The HCl extraction allowed obtaining pectin enriched fractions (Nara et al. 2001). After quantification, a significant increase in pectin content was detected in both tissues, being the effect higher for rosette leaves (Table 1). Further treatment with NaOH allowed the hemicelluloses extraction, while the remaining solid was considered as cellulose. It was observed a reduction in both hemicellulose and cellulose fractions in the case of rosette leaves. In the case of stems, the hemicellulose content was slightly higher in p1 and p3 lines, whereas there was a minor decrease in the cellulose content in one of the transgenic lines analyzed (p1) (Table 1).

Effect of *CBMFaEXP2* on cell wall enzymatic activities

To further characterize cell wall changes in plants expressing *CBMFaEXP2*, the activity of enzymes acting on different cell wall components was measured in rosette leaves extracts.

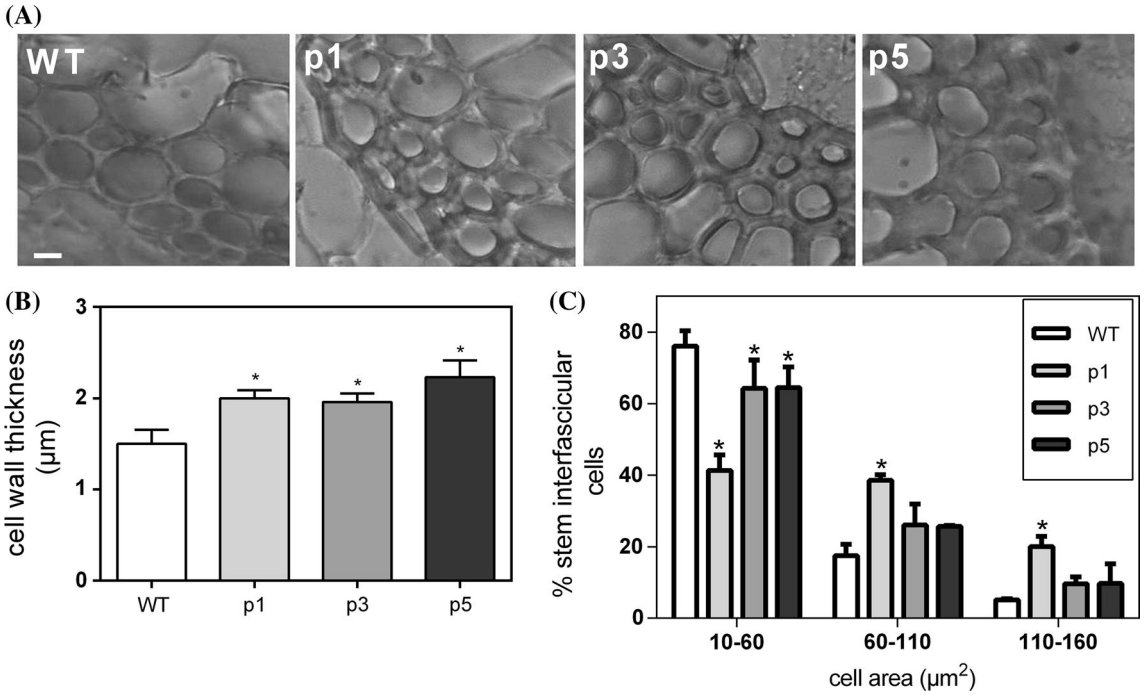

Fig. 7 Effect of *CBMFaEXP2* on cell wall thickness and cell size. **a** Micrographs of stems interfascicular tissue showing a thicker cell wall in transgenic lines. **b** Cell wall thickness quantification as half of the thickness between two adjacent cells. **c** Cell size distribution of interfascicular tissue in transgenic and WT plants. Results are mean ± SD. 320 measurements of wall thickness were obtained per each WT and transgenic line analyzed. Surface areas of 300 cells per line were measured. p1; p3 and p5 represent the three independent transgenic lines selected. *Asterisks* indicate significantly different from WT (One way ANOVA; post test: Dunnett; $P \leq 0.05$). *Bar* = 10 µm

Table 1 Effect of *CBMFaEXP2* on cell wall composition

	AIR[a]	Pectin[b]	Hemicellulose[c]	Cellulose[d]
WT rosette	4.26 ± 0.05	11.88 ± 3.56	11.66 ± 2.74	25.25 ± 2.27
p1 rosette	5.2 ± 0.37*	20.12 ± 2.92*	8.75 ± 2.01	19.17 ± 3.61*
p3 rosette	4.98 ± 0.11*	17.01 ± 2.82*	7.87 ± 1.47*	20.01 ± 2.97*
p5 rosette	5.52 ± 0.23*	17.43 ± 1.97*	7.01 ± 3.24*	21.68 ± 0.92*
WT stem	9.33 ± 2.25	13.11 ± 1.39	3.94 ± 0.31	18.70 ± 2.15
p1stem	9.92 ± 2.30	15.6 ± 0.87*	5.18 ± 0.76*	15.62 ± 1.55*
p3 stem	10.35 ± 1.33	16.04 ± 1.70*	4.81 ± 1.27*	18.92 ± 1.53
p5 stem	10.26 ± 1.23	14.52 ± 0.93	4.33 ± 0.69	16.48 ± 1.67

[a] Mean ± SD of alcohol insoluble residue (AIR) expressed in terms of mg per 100 mg Fresh Weigth^{-1}. Three independent replicates were done (n = 15 plants per replicate)

[b] Mean ± SD of pectin content estimated as uronic acid expressed in mg per 100 mg AIR^{-1}

[c,d] Mean ± SD of hemicellulose and cellulose content respectively estimated as glucose expressed in terms of mg per 100 mg AIR^{-1}

[b,c,d] Three independent replicates were done and each individual measurement was done twice. *Asterisks* indicate significantly different from WT at $P \leq 0.05$ (One way ANOVA; post test: Dunnett). p1; p3 and p5 represent the three independent transgenic lines selected

Polygalacturonases (PGs; EC 3.2.1.15) catalyze the hydrolysis of glycosidic bonds between galacturonic acid residues in pectins. Total PG activity was lower in the transgenic lines when compared to WT (Fig. 8a). Pectin-methylesterases (PME; EC 3.1.1.11) act immediately before PG, catalyzing the pectin de-esterification. In this case, similar activity was found in transgenic and WT plants (Fig. 8b). β-Galactosidases (β-Gal; EC 3.2.1.23) and α-arabinofuranosidases (α-Ara; EC 3.2.1.55) remove residues from the side chains of cell wall components. β-Gal

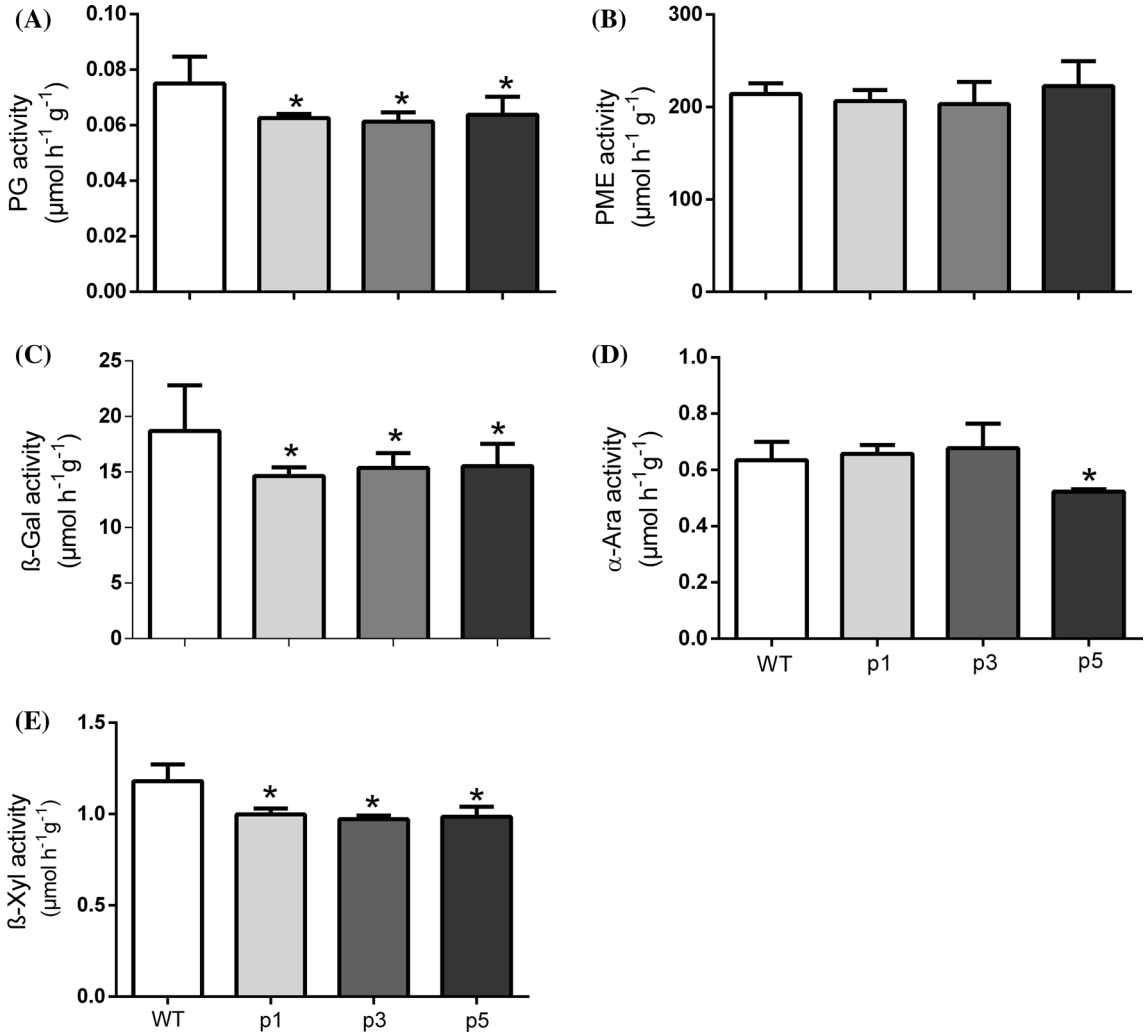

Fig. 8 Effect of *CBMFaEXP2* on **a** PG, **b** PME, **c** ß-Gal, **d** α-Ara and **e** ß-Xyl activities. Results are mean ± SD of three replicates from pools of 10 g of tissue. p1; p3 and p5 represent the three independent transgenic lines selected. *Asterisks* indicate significantly different from WT (One way ANOVA; post test: Dunnet; $P \leq 0.05$)

removes non-reducing terminal galactosyl residues from pectins and α-Ara catalyzes the hydrolysis of terminal α-L-arabinofuranosyl residues from pectic and hemicellulosic polysaccharides. Total β-Gal activity was lower in the three transgenic lines analyzed in comparison with WT, whereas the α-Ara activity decreased only in one transgenic line (p5) (Fig. 8c, d). ß-xylosidases (ß-Xyl; EC 3.2.1.37) are involved in xylans degradation. A significant decrease in ß-Xyl activity was found in the three transgenic lines analyzed (Fig. 8e).

Expression of genes involved in cell wall catabolism

Plants overexpressing *CBMFaEXP2* showed substantial alterations in phenotype, cell wall composition, cell wall structure and enzymes activities. In order to explore the possible causes of these alterations, we measured the expression levels of a set of genes involved in cell wall catabolism.

Regarding pectin catabolism, we found a two-fold decrease in the expression levels of *AtPG* (AT1G2346) and a significant reduction in *Atβ-Gal* (AT2G04378) gene expression. In agreement with total PME enzyme activity results, the expression levels of *AtPME3* (AT3G14310) were similar in CBM-expressing and WT plants (Fig. 9). A significant reduction in *Atβ-Xyl5* (AT3G19620) expression was observed in transgenic plants. Similar results were obtained for *Atα-Ara1* (AT3G10740), being its expression lower in two of the three transgenic lines (p3 and p5) (Fig. 9).

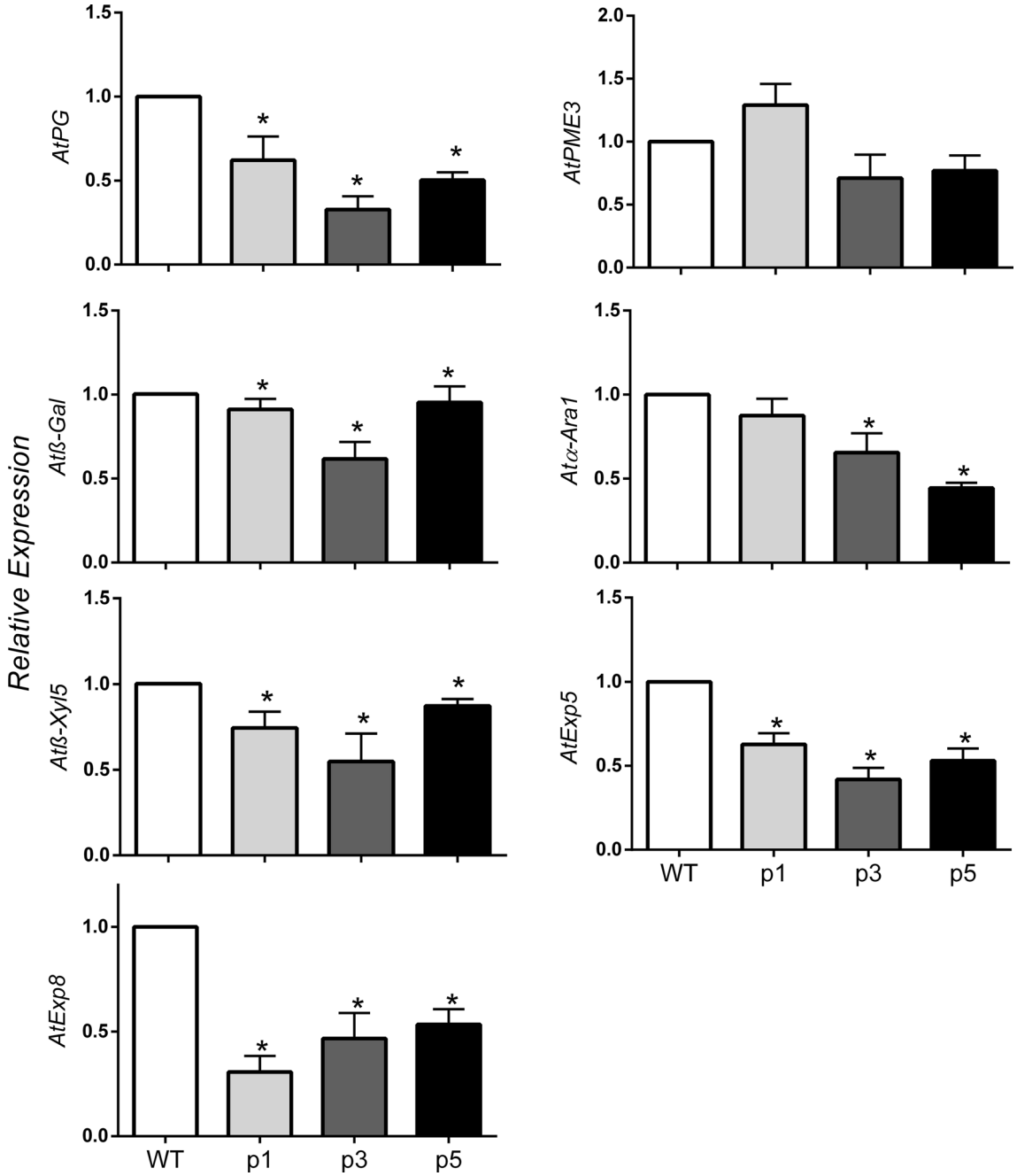

Fig. 9 Relative expression of genes involved in cell wall catabolism in transgenic CBMFaEXP2 plants. Real Time assay was used to analyze the mRNAs abundance of *AtPG*, *PME3*, *Atß-Gal*, *Atα-Ara*, *Atß-Xyl*, *AtExp5* and *AtExp8*. Results are mean ± SEM of three replicates. p1; p3 and p5 represent the three independent transgenic lines selected. *Asterisks* indicate statistically significant differences in the expression of each gene between transgenic lines and WT (One Way ANOVA, postest: Dunnet; $P \leq 0.05$)

In order to find out if the mRNA accumulation of Exps was affected in the transgenic plants, we analyzed the expression of two *A. thaliana* Exp genes: *AtExp5* (AT3G29030) and *AtExp8* (AT2G40610). The amount of both transcripts was significantly reduced (between 50 and 75 %, depending on the line analyzed) in the transgenic lines in comparison with the WT (Fig. 9).

Susceptibility to *Botrytis cinerea* infection

Since overexpression of *CBMFaEXP2* in *A. thaliana* modified cell wall metabolism, we decided to examine whether transgenic plants showed altered susceptibility to infection with the necrotrophic fungus *B. cinerea*. To this aim, leaves of CBM-expressing plants and WT were inoculated

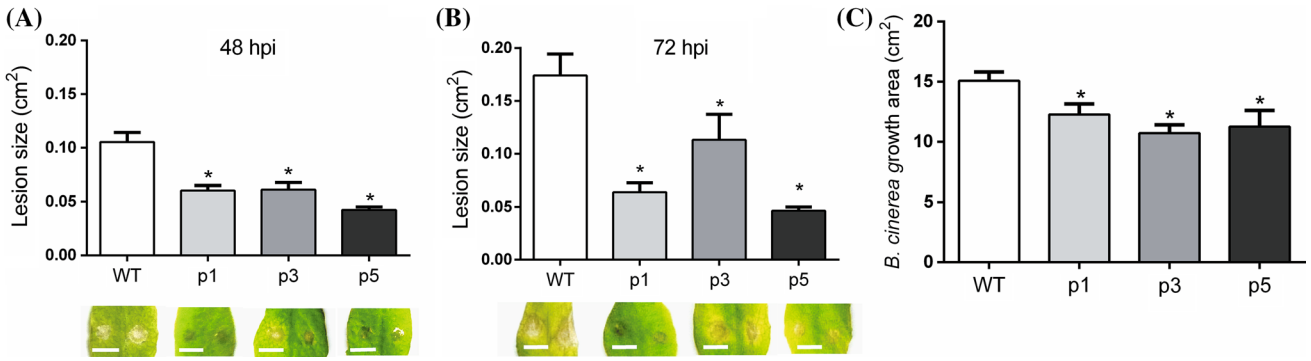

Fig. 10 *Botrytis cinerea* infection of *A. thaliana* plants overexpressing *CBMFaEXP2*. Leaves from WT and CBMFaEXP2 plants were inoculated with *B. cinerea* conidia and the lesion size was determined 48 **a** and 72 h post-inoculation (hpi). **b**. Results are mean ± SEM of four replicates. **c** Growth of *B. cinerea* (72 h) in petri dishes containing agar 0.8 % (w/v) and 1 mg ml^{-1} of alcohol insoluble residues (AIRs) obtained from rosette leaves of transgenic and WT plants. p1; p3 and p5 represent the three independent transgenic lines selected. *Asterisks* indicate statistically significant differences between each transgenic line and WT (One way ANOVA, post test: Dunnet; $P \leq 0.05$). *Bar* = 0.2 cm

with fungal conidia. Lesions were visible 48 h post inoculation (hpi); then, leaves were photographed and the lesion size was measured. A significant reduction of the infection area in the three lines analyzed compared to WT plants was found (Fig. 10a). The analysis of lesion size was repeated 72 hpi and the same tendency was observed, being the lesion size smaller in the leaves from transgenic lines (Fig. 10b). Supplemental Fig. S3a shows the general status of transgenic and WT leaves at 48 and 72 hpi. In addition, the capacity of *B. cinerea* to grow on agar plates containing cell wall material (AIRs) from rosette leaves of WT and transgenic lines as the only carbon source, was assayed (Fig. 10c). The radial growth of *B. cinerea* was lower on plates containing AIRs from p1, p3 and p5 transgenic lines regarding WT (Fig. 10c).

Discussion

It has been proposed that Exps induce cell wall extension by disrupting non-covalent bonds at the interface between cellulose microfibrils and hemicelluloses (McQueen-Mason and Cosgrove 1995). Although Exps lack hydrolytic activity (McQueen-Mason and Cosgrove 1995), they would play a crucial role in the hydrolysis of cell wall polysaccharides by detaching glucans from the cellulose surface, making it more accessible to the action of other enzymes, such as endo-glucanases (Cosgrove 2000c; Levy et al. 2002) and transglycosylases (Redgwell and Fry 1993; Schröder et al. 2004). The mode of action proposed for Exps includes first, a binding step to the cellulose surface through their CBM and then the interaction between the central domain and hemicelluloses at the microfibril surface or in the matrix between microfibrils (Cosgrove 2000b). In the present work, we have overexpressed the CBM from the strawberry Exp2 (*CBMFaEXP2*) in *A. thaliana* and analyzed the effects on plant phenotype and cell wall metabolism.

Overexpression of *CBMFaEXP2* alters *A. thaliana* growth as well as cell wall structure and composition.

The overexpression of CBMs could affect plant morphology differently depending on the species and, perhaps, on the binding specificity of the CBM. In this sense, it has been reported that the overexpression of a promiscuous bacterial CBM tandem in tobacco plants reduced stem elongation, prolonged juvenility, and delayed flower development (Obembe et al. 2007a). However, no visible altered phenotype was observed in plants overexpressing a less promiscuous bacterial CBM tandem in tobacco. The authors proposed that the promiscuous CBM would bind to different polysaccharide types, then affecting more extensively the cell wall metabolism (Obembe et al. 2007a). Safra-Dassa et al. (2006) overexpressed a bacterial CBM from *Clostridium cellulovorans* in potato and reported that transgenic plants had increased stem growth rate, which correlated with an increase of cell size.

Regarding CBMs from plant resources, there are few reports in the literature. In that sense, Obembe et al. (2007b) targeted a putative potato expansin CBM to the cell wall of tobacco plants, and reported an abnormal enlargement (radial expansion) of cells from xylem and phloem fibres. In addition, the cell walls of the vascular tissue were observed to be thinner in the transgenic plants as compared to the control; although no differences in cellulose content or marked changes in plant morphology was observed in transgenic plants. On the other hand, (Boron et al. 2015) overexpressed the expansin AtEXLA2, member of the Expansin-like A family in *A. thaliana*, and observed a growth-promoting effect in roots and etiolated

hypocotyls and thicker cell walls in transgenic lines regarding WT. It is worth to mention that CBMFaEXP2 shares only 69 % of sequence identity with the putative expansin CBM from potato used by (Obembe et al. 2007b) and 27 % with the putative CBM from AtEXLA2 (Boron et al. 2015). Moreover, in a previous work, we demonstrated that CBMFaEXP2 is able to bind not only cellulose but xylan and pectin in vitro (Nardi et al. 2013). Taken together, we proposed that differences in CBM sequences identities and different binding specificity of each particular CBMs could explain the different results found.

It has been reported that the suppression of an Exp gene (*OsEXP4*) in rice caused shorter plants and earlier flowering than controls (Choi et al. 2003). The authors proposed that the floral structure development occurs at a low expansins level. In agreement, plants overexpressing *CBMFaEXP2* had reduced expression of two endogenous Exps genes (*AtEXP5* and *AtEXP8*; Fig. 9) and also showed early flowering pattern (Fig. 3d). Regarding the effect of CBMs on root length, a dose-dependent effect was reported in root development of *A. thaliana* plants treated exogenously with recombinant CBM from *Clostridium cellulovorans*. Root elongation was enhanced by treatment with the recombinant protein at low concentrations, whereas it was inhibited at higher CBM concentrations in a dose-dependent manner (Shpigel et al. 1998). In the case of the plants overexpressing *CBMFaEXP2*, an inhibition of root elongation was found in the three transgenic lines independently of their different expression levels (Fig. 4).

Data obtained in the present work suggest that the overexpression of an Exp CBM is able to modulate plant growth, probably due to its capacity to bind different cell wall polysaccharides and then to interfere with the normal activity of cell wall enzymes and other proteins as Exps.

We also found that the overexpression of *CBMFaEXP2* increased the total amount of cell wall (Table 1) and increased the cell wall thickness and cell size in inflorescence stems (Fig. 7a–c). Consequently, we propose that this could be due to the increase found on the pectin fraction (Table 1). In a previous work, we showed that the recombinant protein CBMFaEXP2 could bind citrus pectin in vitro (Nardi et al. 2013). It is possible to hypothesize that CBMFaEXP2 could bind other cell wall components than cellulose also in vivo and then block binding sites for cell wall hydrolytic enzymes. However, it should not be discarded that the actual effect of this CBM were on cellulose fraction (where a decrease was observed in both tissues analyzed, Table 1), which could lead to an increase in pectin fraction. Plants can sense and act in response to the functional properties of the cell wall. It has been suggested that expansins may play a role in the assembly of the cell wall by affecting cellulose synthesis or deposition (Boron et al. 2015; Zenoni et al. 2004). CBMs with high affinity for cellulose, such as a bacterial CBM from *Clostridium cellulovorans*, affect cellulose synthesis by interacting with glucans at an early stage of crystallization. By interfering with this process the CBM enables cellulose polymerization to proceed freely, and it removes feedback inhibition caused by the cellulose crystallization (Levy et al. 2002; Safra-Dassa et al. 2006; Shpigel et al. 1998).

The effects of increasing or suppressing expansins expression on the cell wall composition are variable (Brummell et al. 1999; Wang et al. 2011; Zenoni et al. 2004, 2011). Nevertheless, it is worth to mention that pectin fraction was affected most of the times, suggesting that could exist a relationship between expansin activity and pectin metabolism (Cantu et al. 2008b; Nardi et al. 2013). Furthermore, results reported here indicate substantial changes in pectin metabolism due to the overexpression of *CBMFaEXP2*.

Mechanical properties of plants overexpressing *CBMFaEXP2*

The extensibility of a tissue may be defined and evaluated by different methods (Cosgrove 1993). In our case, we have used the extensibility definition given by Wainwright et al. (1976), as the amount of strain (measured as the change in length) that a material can withstand before breaking.

The displacement until breakage for both tissues analyzed (inflorescence stem and rosette leaves sections) was increased in transgenic plants (Fig. 6c, f), indicating that tissues of *CBMFaEXP2* overexpressing plants, particularly the inflorescence stem, are more extensible than those from the WT (Fig. 6c). The higher tissue extensibility seems to be contradictory with the fact that the transgenic plants contained higher amount of total cell wall (Table 1). However, this increase was mainly due to an increase of pectin content both in rosette and stem, while the cellulose content was, in general, diminished. Several studies have identified a correlation between the cellulose content and the physical properties of tissues (Tanaka et al. 2003; Taylor et al. 2003; Turner and Somerville 1997). In this sense, Ching et al. (2006) detected a reduction in mechanical strength in corn stalks with a spontaneous mutation called “brittle stalk -2” and probed that this was strongly correlated with a reduction in the amount of cellulose. In addition, it has been reported that pectins have a major role on the mechanical properties of the primary cell wall (Abasolo et al. 2009; Ryden et al. 2003), although little is known about their contribution to tissue extensibility.

Otherwise, a direct effect caused by the accumulation of CBMFaEXP2 on the mechanical properties of the cell wall, independently of metabolic pathways, should not be discarded. The treatment with CBMs from bacterial enzymes, other than expansins, have been used to modify

the properties of cellulosic fibers, and some CBMs are able to disrupt the structure of cellulose fibers, resulting in the release of small particles without any detectable hydrolytic activity (Din et al. 1991; Shoseyov et al. 2006). The ability of full length expansins or the expansin- CBM to bind to cellulose, hemicelluloses and pectins in vitro has been reported (Cosgrove et al. 1997; Nardi et al. 2013). The presence of CBMFaEXP2 in the cell wall of transgenic lines could interfere with the normal interactions among these components, and then modify the mechanical properties of the cell wall.

CBMFaEXP2 modifies expression and enzymatic activity of cell wall enzymes

An analysis of the possible effect of CBM-FaEXP2 overexpression on transcription levels and activity of cell wall enzymes was done. Most of the enzymatic activities measured in vitro were decreased in transgenic lines (Fig. 8).

The degradation and remodeling of cell wall involves several enzymes and non-hydrolytic proteins and a common feature of their structure is its modular organization, which typically includes a catalytic domain and one or more CBMs (Boraston et al. 2004). It is possible to hypothesize that the constitutive expression of a promiscuous CBM in the cell wall would bind the substrates of enzymes acting on different cell wall carbohydrates. The ability of CBMFaEXP2 to bind different substrates in vitro (cellulose, pectins and xylan) has been reported (Nardi et al. 2013). We propose that under these conditions, the cell would sense a higher concentration of bound enzymes and proteins in the cell wall, thus decreasing the transcription levels of the corresponding gene, which would reduce the amount of the encoded proteins. The mechanism by which the cell would perform this control is unknown and need further studies.

It has been proposed an in vivo interaction between expansins and polygalacturonases (PGs) (Brummell and Harpster 2001; Brummell et al. 1999). The suppression of an expansin gene in tomato (*LeEXP1*), inhibited polyuronide depolymerization late in ripening (Brummell et al. 1999). Authors suggested that the action of Exps may be necessary to allow some component of polyuronide disassembly to occur, perhaps by controlling access of PG to its substrate. In addition, Brummell and Harpster (2001) observed that mRNA accumulation of PG was reduced throughout ripening in these antisense *LeEXP1* plants and proposed that there would be a limited access of PGs to their substrates due to the absence of Exp activity. The possible relationship between pectin metabolism and expansins is supported by data obtained in the present work, since overexpression of *CBMFaEXP2* reduced the transcript levels of two Exps and one PG gene (Fig. 9), reduced PG activity (Fig. 8a), and caused an increase of pectins in the cell wall.

According to the results obtained here, we propose that the overexpression of a promiscuous CBM in the cell wall would be a promising strategy to decrease, simultaneously, the expression and activity of several enzymes involved in cell wall catabolism.

CBMFaEXP2 overexpression reduces *A. thaliana* susceptibility to *B. cinerea*

Fungal growth could be reduced as a result of decreased penetration efficiency, possibly due to higher cell wall integrity. A correlation between cell wall composition and susceptibility to pathogen infection has been reported (Vorwerk et al. 2004). Besides, it is known that endoPGs are the major cell wall degrading enzymes secreted by *B. cinerea* to perform the infection, suggesting that pectin catabolism is critical for the fungal invasion (van Kan 2006). An increased resistance to *B. cinerea* infection in tomato plants with suppressed expression of PG has been reported (Kramer and Redenbaugh 1994). A similar effect was found in transgenic tomato fruit with suppressed polygalacturonase (*LePG*) and Exp (*LeExp1*) (Cantu et al. 2008b). Suppression of both genes dramatically reduced the susceptibility of ripe fruit to *B. cinerea*. Authors also reported a variation in the composition of cell wall of transgenic fruit and proposed that the reduced susceptibility to necrotrophic fungus was due to reduced access of fungus enzymes to the host plant cell wall substrates.

On the other hand, several degradation enzymes from *B. cinerea* include CBMs in their structures, most of them classified as CBM1 according to www.cazy.org (Cantarel et al. 2009). It is possible that the overexpression of a promiscuous CBM on *A. thaliana* cell wall interferes with fungal enzymes activities as well, avoiding efficient penetration.

Conclusions

In this study, we present new findings regarding the effect of overexpressing an Exp CBM into a plant cell wall. The overexpression of the promiscuous CBM from FaEXP2 modifies the normal growth of *A. thaliana* plants. Furthermore, CBMFaEXP2 was able to alter cell size and the structure and composition of the cell wall. A higher content of pectin in both, transgenic rosette leaves and inflorescence stems, was observed regarding WT. In agreement, the transgenic plants had a lower expression level of genes and enzyme activities (PGs and β-Gal) involved in pectin catabolism. The combination of higher amount of pectins and lower amount of cellulose found in both tissues from

transgenic plants could cause the cell wall to be more deformable and thus more extensible. Finally, changes in the cell wall metabolism due to overexpression of *CBM-FaEXP2* caused an increased resistance to *B. cinerea* infection. Taken together, our findings confirmed the hypothesis that the overexpression of a promiscuous CBM, as the one that is present in FaEXP2, can alter plant cell wall metabolism, affecting simultaneously several genes and enzymes involved in cell wall degradation. This strategy could be used to modulate the cell wall metabolism in transgenic plants for different purposes. Particularly, this approach could be useful to delay cell wall degradation during fruit ripening, and then to reduce fruit softening and susceptibility to fungal attack.

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