CELL BIOLOGY AND MORPHOGENESIS

Inducible expression of *Pisum sativum* xyloglucan fucosyltransferase in the pea root cap meristem, and effects of antisense mRNA expression on root cap cell wall structural integrity

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Abstract Mitosis and cell wall synthesis in the legume root cap meristem can be induced and synchronized by the nondestructive removal of border cells from the cap periphery. Newly synthesized cells can be examined microscopically as they differentiate progressively during cap development, and ultimately detach as a new population of border cells. This system was used to demonstrate that *Pisum sativum* L. fucosyl transferase (*PsFut1*) mRNA expression is strongly expressed in root meristematic tissues, and is induced >2-fold during a 5-h period when mitosis in the root cap meristem is increased. Expression of *PsFut1* antisense mRNA in pea hairy roots under the control of the CaMV35S promoter, which exhibits meristem localized expression in pea root caps, resulted in a 50–60% reduction in meristem localized endogenous *PsFut1* mRNA expression measured using whole mount in situ hybridization. Changes in gross levels of cell wall fucosylated xyloglucan were not detected, but altered surface localization patterns were detected using whole mount immunolocalization with CCRC-M1, an antibody that recognizes fucosylated xyloglucan. Emerging hairy roots expressing antisense *PsFut1* mRNA appeared normal macroscopically but scanning electron microscopy of tissues with altered CCRC-M1 localization patterns revealed wrinkled, collapsed cell surfaces. As individual border cells separated from the cap periphery, cell death occurred in correlation with extrusion of cellular contents through breaks in the wall.

Keywords Fucose · Cell walls · Hairy roots · Root cap meristem

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Introduction

The purpose of this study was to characterize expression of *Pisum sativum* fucosyltransferase (*PsFut1*) mRNA during primary cell wall synthesis, and to examine the impact of gene silencing using transgenic clonal pea hairy roots. The plant cell wall consists of a primary wall, middle lamella and, in some cells, a secondary wall (Albersheim et al. 1994; Carpita et al. 2001; Lerouxel et al. 2006; McCann and Roberts 1991). These interacting parts are comprised of the complex polysaccharides cellulose, hemicellulose and pectin, and more than 100 proteins (Bauer et al. 1973; McQueen-Mason et al. 1992; Pauly et al. 1999; Somerville et al. 2004; Varner and Lin 1989; Wen et al. 2007; Zhu et al. 2006). Dissecting how the cell wall is assembled is complicated by the fact that its synthesis begins with primary wall assembly during the early moments of cell division and continues throughout the life of the cell in response to developmental and environmental stimuli (Cosgrove 2005; Carpita and Gibeaut 1993; Dhugga 2005; Freshour et al.

1996; Johansen et al. 2006; Ookawara et al. 2005; Varner and Lin 1989). Proteins within the cell wall include cell wall modifying enzymes that alter wall structure as the cell matures (Cassab and Varner 1988; Guillen et al. 1995; Maldonado-Mendoza et al. 2005; Romo et al. 2005; Van Loon et al. 2006; Ye et al. 2006). Even in a mature plant cell, a brief treatment with an elicitor triggers cross-linking that fundamentally changes the relationship of proteins to the rest of the cell wall components (Brisson et al. 1994).

Pea and other legumes have been used for decades as a model to establish fundamental principles of cell wall composition, structure, and function (Brisson et al. 1994; Callaghan and Benziman 1984; Guillen et al. 1995; Hayashi 1989, 1991; Kauss and Hassid 1967; Keller et al. 1988; Lin et al. 1991; Satiat-Jeunemaitre et al. 1992; Selvendran 1975; Takeda et al 2002; Terry et al. 1981; York et al. 1984). Within pea cell walls, xyloglucan comprises ca 20% of the weight of the cell wall (Hayashi 1989). In addition to a structural role, soluble components of xyloglucan such as fucosylated xyloglucan oligosaccharides have been shown to exhibit hormone-like signalling activities during cellular elongation in pea (Fry 1989; Fry et al. 1993a; Popper and Fry 2005; York et al. 1984). *Pisum sativum* xyloglucan fucosyltransferase (PsFUT1) is among a small number of cell wall biosynthetic enzymes whose function has been characterized (Faik et al. 1997). This microsomal enzyme was isolated from pea epicotyls and shown to synthesize an alpha-1,2 fucose:galactose linkage by in vitro fucosylation of xyloglucan from tamarind seeds, with GDP-fucose as a donor (Perrin et al. 1999). The PsFUT1 sequence was used to identify and evaluate a related gene family in *Arabidopsis* (Perrin et al. 2003; Sarria et al. 2001; Vanzin et al. 2002). Isolation of the *PsFut1* gene from pea and confirmation of its biochemical activity and substrate specificity was carried out (Faik et al. 2000). To date, *PsFut1* expression patterns and the impact of gene silencing in pea have not been evaluated.

The root cap in pea and other legumes provides a convenient model to examine the role of altered gene expression in plants because normal development can be induced and synchronized nondestructively (Feldman 1984; Hawes et al. 2003). Root cap development is controlled by an extracellular signal secreted from border cells (Hawes and Lin 1990). Within 5 min after removing border cells from the cap periphery by gentle agitation of the root tip in water, increased mitosis can be detected within the root cap meristem concomitant with a global switch in gene expression throughout the cap (Brigham et al. 1998). Mitosis in the meristem remains at an elevated level for 5 h as a new set of 3,500 ± 500 cells is made to replace the harvested border cells, and then returns to baseline values (Brigham et al. 1998; Hawes and Lin 1990). Newly synthesized cells differentiate progressively through specialized cell layers dedicated to starch synthesis, gravity sensing, mucilage production and cell separation, and these developmental stages can be readily distinguished morphologically (Feldman 1984). A new set of border cells is present on the cap periphery after 24 h, at which time cap turnover ceases and mitosis remains blocked at the G2M phase of the cell cycle indefinitely, until cap turnover is again induced. Stage-specific localized expression of specific genes associated with processes including cell division, cell wall synthesis, starch synthesis, and cell wall separation has been profiled using this system (Brigham et al. 1998; Wen et al. 1999, 2004, 2005, 2007; Woo and Hawes 1997; Woo et al. 1999, 2003, 2007), The use of transgenic hairy roots allows efficient use of gene silencing in clonal tissue that can be amplified rapidly for detailed cellular, molecular and chemical analysis (Hu and Du 2006).

In this study, we measured the expression of *PsFut1* mRNA in pea root caps during the induction of mitosis and cap turnover, and evaluated the impact of *PsFut1* antisense mRNA expression in transgenic clonal hairy roots.

Materials and methods

Plant material

Pea (*Pisum sativum* cv Little Marvel, Royal Seed Company) seeds were surface sterilized with 95% ethanol for 10 min followed by immersion in 5.25% sodium hypochlorite for 30 min, as described (Hawes and Lin 1990). Seeds were rinsed 5× with sterilized water and seeds that floated to the surface, were discoloured, or obviously damaged were culled during a 6-h period of imbibition in sterile distilled water (Hawes and Lin 1990). Imbibed seeds were placed onto 1% water agar (Bacto™ Agar, Becton Dickinson and Company) in sterilized magenta jars, and then were incubated at 24°C for 2–3 days or until the stem was 1–2 cm high. Germinated seedlings were transferred to 24°C in a 16 h light intensity growth chamber for two more days.

Construction of sense and antisense *pBIPsFut1* transgenes constructs

The 1,698 bp *PsFut1* gene fragment in pGEMT-easy vector was amplified using polymerase chain reaction (PCR), with primers containing created restriction sites for cloning. The PCR-amplified fragment was digested by corresponding restriction enzymes and then inserted in both sense and antisense orientations under the control of the cauliflower mosaic virus (CaMV) promoter in vector pBI121 whose *uid*A gene was removed. The resulting constructs *pBIPsFut1S* and *pBIPsFut1A* were mobilized into *Agrobacterium*

rhizogenes R1000nal through triparental mating using p*RK2013* as helper strain and kanamycin as selectable marker (Wen et al. 1999; Woo et al. 1999).

Southern and Northern blot analysis

Southern blot analysis of pea genomic DNA was carried out according to standard procedures (Sambrook et al. 1989). *PsFut1* mRNA levels in root tips at different time points after removing border cells were detected by RNA blot analysis using ^{32}P-labelled single strand *PsFut1* transcript as probe. A single strand RNA probe (riboprobe) was synthesized according to Maxiscript in vitro transcription kits (Ambion, Austin, TX). Northern blot analysis was also carried out to compare *PsFut1* mRNA levels in wild type hairy roots with that in hairy roots expressing *PsFut1* antisense mRNA. A single strand RNA probe (riboprobe) was synthesized according to Maxiscript in vitro transcription kits (Ambion, Austin, TX). Samples were processed by standard procedures, as described (e.g. Wen et al. 1999; Woo et al. 1999).

Transgenic clonal root culture development and maintenance

Hairy root clones were transformed using full-length sense and antisense mRNA, as described (Wen et al. 1999). Individual hairy roots induced by *A. rhizogenes* with the pBI*PsFut1* antisense plasmid, and hairy roots induced by the *A. rhizogenes* R1000nal strain (wild type) were grown on Gamborg's B-5 (B5) basal medium, supplemented with carbenicillin (50 μg/ml) to eliminate *A. rhizogenes*. Transformed roots were selected by plating individual, excised roots (ca 1 cm in length) onto medium containing kanamycin; cultures then were amplified by growth on B5 medium for 2 weeks.

Transformation of individual hairy root lines was confirmed by PCR amplification of the target sequence. Total chromosomal DNA was isolated from the hairy root clones using the DNeasy plant mini kit (Qiagen). Kanamycin primers (left and right) were used and PCR conditions of 1×: 95°C 5 min, 35×: 95°C 30 sec, 50°C 30 sec, 72°C 30 sec, 1×: 72°C 5 min for the pBI*PsFut1* antisense samples. PCR products were analyzed by 1% agarose gel electrophoresis. Hairy root lines were maintained on B5 medium at 24°C in the dark and subcultured by transferring individual root tips onto fresh medium at 2 to 4-week intervals.

Cell wall analysis

Oligosaccharide mass profiling (OLIMP) (Lerouxel et al. 2002) was used to compare wild type hairy roots from clonal lines transformed with R1000nal only, or from two independent lines expressing *PsFut1* antisense mRNA (*PsFut1*AS1 or *PsFut1*AS2). Excised roots, root tips (1–2 mm from the apex), or border cells were stored and transported in reaction tubes with ethanol. Root material was transferred to a screw capped Eppendorf tube together with a metal ball and transferred into liquid nitrogen. Tissue was ground in a Retsch MM200 grinder (Retsch GmbH & Co., KG, Haan, Germany) for 1 min at 25 Hz. The ground cell wall material was resuspended in 70% ethanol and centrifuged. The pellet was washed once in chloroform: methanol (1:1 v/v) and dried. The pellet was incubated with xyloglucanase to release xyloglucan fragments for 17 h at 37°C. The enzyme digest was centrifuged and the supernatant was analyzed by MALDI-TOF MS to characterize the formed xyloglucan oligosaccharides (Lerouxel et al. 2002; Pauly et al. 2001a, b). The spectra were statistically analyzed for differences in peak areas.

Whole mount in situ hybridization and immunolocalization

Whole mount in-situ hybridization (WISH) analysis of pea hairy roots was carried out as described (Brigham et al. 1998; Woo and Hawes 1997; Woo et al. 1999). pGEMT-*PsFut1* plasmids were extracted using standard procedures (Sambrook et al. 1989) from *E. coli* grown overnight in Luria broth at 37°C.

Sense and antisense mRNA probes were prepared using the Digoxigenin (DIG)-UTP RNA labelling kit, according to the manufacturer's instructions (SP6/T7, Cat. #1 175 025). Root tips of wild type hairy roots transformed with R1000 *Agrobacterium rhizogenes* and hairy roots induced by *A. rhizogenes* containing the pBI*PsFut1* antisense plasmid were excised 1.5 cm from the apex, then fixed and processed as described (Brigham et al. 1998). Both sense and antisense DIG-labelled probes (100–500 ng probe/mL) were used for gene expression analysis. Colorimetric detection was carried out with the DIG nucleic acid detection kit (Roche Cat. # 1 175 041).

Whole mount immunolocalization with the CCRC-M1 antibody was carried out as described in Freshour et al. (2003). The secondary antibody used is goat anti-mouse IgG–fluorescein isothiocyanate conjugate (Sigma, catalog No. F-0257). All the procedures of immunofluorescent labelling were done on unfixed roots cut (1.5-cm long with the root tip) in microfuge tubes with the change of corresponding buffers or reaction solutions. Unlabelled roots were included as negative controls to illustrate background yellow autofluorescence and its distinction from the bright green fluorescence seen in response to fluorescein labelling.

Results

PsFut1 genes in Pisum sativum

PsFut1 encodes a 1,2-fucosyltransferase, which shares 56–67% amino acid identity with its orthologue AtFut1 in Arabidopsis (Faik et al. 2000). The Arabidopsis FUT gene family includes nine additional closely related sequences (AtFut2–10) (Sarria et al. 2001). Southern blot analysis of pea genomic DNA at high stringency hybridization conditions revealed the existence of a small family of 2–3 sequences closely related to PsFut1 (Fig. 1).
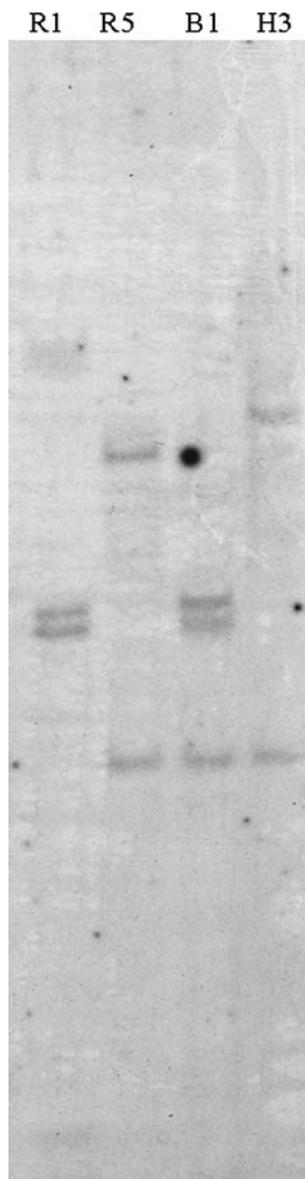

Fig. 1 Genomic Southern analysis of pea sequences related to Psfut1. Pea genomic DNA were digested with EcoRI (R1), EcoRV (R5), BamHI (B1), or HindIII (H3) and probed with ³²P-labelled Psfut at 55°C
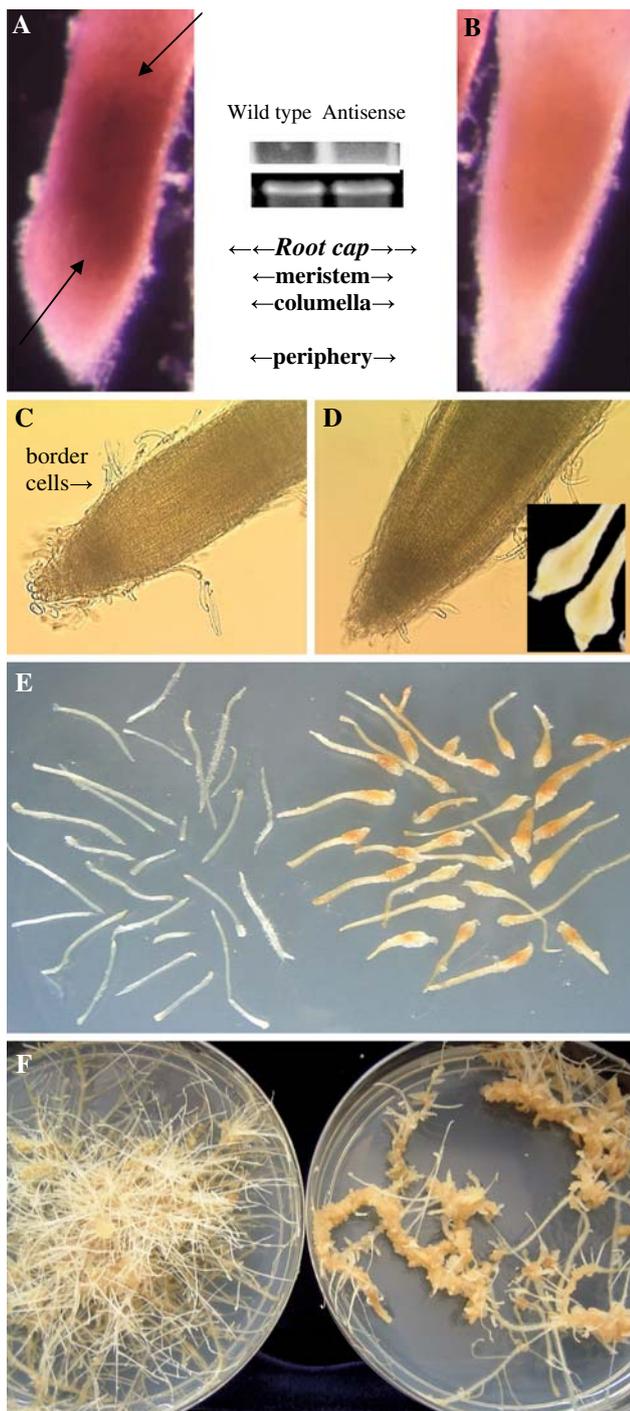
Increased meristem-localized PsFut1 expression in correlation with induction of mitosis

Using the PsFut1 sequence as a probe in Northern blot analysis, a single band was detected in root tips throughout the ~24-h period of root cap development from the onset of mitosis through maturation and separation of a complete set of new border cells (Fig. 2). There was a >2-fold increase in mRNA levels for several hours after removal of border cells (Fig. 2). This pattern of temporal expression is correlated with the period when new cells are being synthesized in the root cap (Brigham et al. 1998).

To evaluate spatial patterns of PsFut1 mRNA expression in hairy roots, WISH was conducted (as in Woo and Hawes 1997; Brigham et al. 1998; Woo et al. 1999). The most intense labelling with PsFut1 antisense mRNA in root tips of wild type hairy roots was localized within the apical and root cap meristematic regions (Fig. 3a, arrows). This pattern of localized expression is similar to that of reporter genes expressed in pea hairy roots under the control of the CaMV35S promoter, with moderate localized expression in the apical and root cap meristems but little or no expression in the root cap columella, periphery or border cells (Brigham et al. 1998; Nicoll et al. 1995). This promoter therefore was used to control expression of PsFut1 antisense mRNA in transgenic hairy roots to examine the
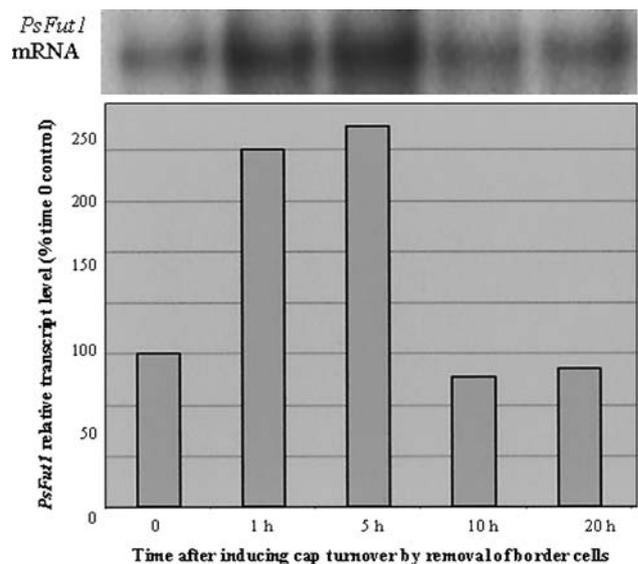
Fig. 2 Correlation between increased PsFut1 expression and induction of mitosis, which is optimal for 4–5 h after cap turnover is induced (Brigham et al. 1998). Density quantification of the signals obtained from the Northern blot analysis (top panel) was done using the NIH image software program (bottom panel). Relative level was determined based on the ratio of the density values obtained for the signal of interest compared with that of an actin control signal for the same evaluated RNA, and was normalized based on setting Time 0 as 100%

and vector only control lines. When pea stems are inoculated with *A. rhizogenes* expressing pBI121, 100% of roots that develop are transformed with the Ri plasmid, and 40–50% also carry the transgene of interest and can be clonally propagated (Nicoll et al. 1995). In the current study, positive control hairy roots expressing CaMV35S::*uidA* were obtained at a frequency of ∼40% (45/105; 52/115; 77/160) in three independent experiments. In contrast, frequency of transformation with *PsFut1* antisense mRNA was only 4% (3/94; 8/190; 2/55).

Three transformed lines (*PsFut1*AS1, *PsFut1*AS2, and *PsFut1*AS3) were successfully amplified in culture. Density quantification of mRNA levels in PsFut1AS1 (Fig. 3b) and the other two lines (not shown) revealed that expression was reduced by 60–70% in individual hairy roots expressing *PsFut1* antisense mRNA, when compared with expression in wild type hairy roots (Fig. 3a). Northern blot analysis revealed similar reductions in expression (Fig. 3a, b, inset).

In each hairy root line expressing *PsFut1* antisense mRNA, similar morphological changes were observed. Emerging root tips of wild type hairy roots (Fig. 3c) appeared to be morphologically similar to root tips of antisense lines (Fig. 3d). However, as the root elongated, a bulge developed within the region of elongation in the antisense roots (Fig. 3d, inset). Whereas slight swelling developed at a frequency of <1% (4/484; 1/199; 1/260; 0/128) on any given plate with wild type hairy roots of varying developmental stage, gross swelling was present on nearly 50% (46/99; 122/250; 97/199; 184/310) of antisense hairy roots under similar conditions and at the same developmental stages (Fig. 3e).

Cultured wild type pea hairy roots exhibit normal growth, development, and border cell production indefinitely (Fig. 3f, left). In contrast, hairy roots expressing *PsFut1* antisense mRNA converted to undifferentiated callus after 2–4 weeks (Fig. 3f, right).

The *PsFut1AS1* antisense clonal line has continued to express the same phenotype for >3 years: new root tips

impact of reduced meristem localized expression of *PsFut1* on root cell wall structure and function.

Deleterious effect of *PsFut1* antisense mRNA on recovery of transgenic hairy roots

Hairy roots expressing full length *PsFut1* antisense mRNA were induced by inoculation with *A. rhizogenes*, and the frequency of transformation was compared with wild type

continue to emerge and to appear normal initially, but elongating roots develop bulges within the region of elongation and convert to undifferentiated callus.

The *PsFut1*AS2 and *PsFut1*AS3 lines ceased to grow after 6 and 8 months in culture, respectively, at which time all roots developed into callus which turned brown, and ceased to develop further.

Altered patterns of fucosylated xyloglucan surface labelling in hairy roots expressing *PsFut1* antisense mRNA

To determine if these phenotypic changes were correlated with gross changes in fucosylated xyloglucan, oligosaccharide mass profiling (OLIMP) was used to compare products of cell wall digests from wild type and antisense hairy root tissues (Fig. 4). Levels of fucose containing xyloglucan oligosaccharides released by enzymatic digestion of excised root tips, region of elongation, or detached border cells of wild type or antisense hairy roots were not statistically distinct (Fig. 4). Because the test lines exhibited only partial inhibition of expression within the meristem, this result was not unexpected.

Changes in surface labelling patterns were detected using CCRC-M1, a monoclonal antibody that specifically recognizes α-L-fucosylated xyloglucan (Freshour et al. 1996, 2003; Puhlmann et al. 1994). A yellow autofluorescence that occurs in pea roots under some conditions (Fig. 5a) is readily distinguishable from the bright green fluorescence seen using fluorescein (Fig. 5b) (Hawes 1983). The results revealed strong positive responses on all
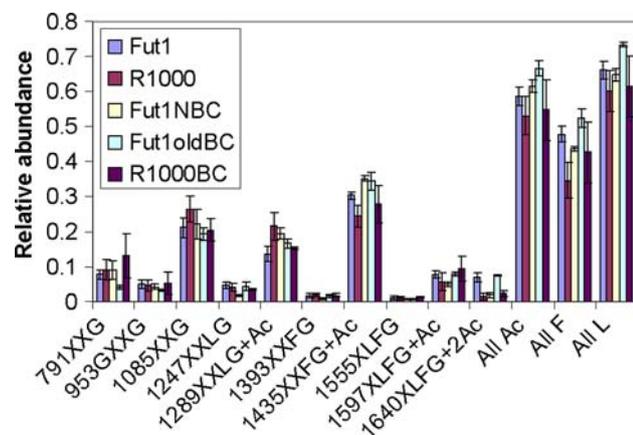
Fig. 4 Xyloglucan composition (relative amounts) of wild type hairy root tips (*R1000*), antisense root tips (*FUT1*), wild type border cells (*R1000BC*), border cells from a new (<1 week) culture of antisense hairy roots (*Fut1NBC*), and border cells from an older (>3 weeks) culture of antisense hairy roots (*Fut1OldBC*) as analyzed by OLIMP (*n* = 3). The various xyloglucan oligosaccharides (XXG, XXXG, etc.) are presented in their one-letter code (Fry et al. 1993b) including mono- or di-*O*-acetylated species (+OAc, +2OAc)

wild type pea hairy roots (Fig. 5b). These results were similar to patterns seen on *Arabidopsis* roots (Freshour et al. 1996, 2003). Surface labelling of pea hairy root tips expressing *PsFut1* antisense mRNA, by contrast, was variable among individual roots, with patterns distinct from that of wild type. There was an overall reduction in fluorescence in emerging root tips (Fig. 5c), with localized areas of very intense labelling at the periphery alternating with dark, non-reactive regions (arrow).

These data suggest that a change in distribution of fucosylated xyloglucan epitope occurred on the root surface in the antisense lines. The results obtained from pooled samples of complex tissues might obscure transient or localized changes in wall structure leading to phenotypic effects (Freshour et al. 2003). Individual hairy roots with altered CCRC-M1 labelling were subjected to a comparative microscopic analysis to assess possible phenotypes that were not obvious macroscopically.

Altered surface shape in root caps of hairy roots expressing PsFut1 antisense mRNA, revealed by SEM

Emerging root tips which appeared to be normal macroscopically or when examined at low magnification (Fig. 3c, d) but whose CCRC-M1 surface labelling patterns were altered compared with wild type (Fig. 5), were examined using SEM (Fig. 6). Root tips of wild type hairy roots retained size, shape, and contour after fixation and processing for SEM (Fig. 6a). In contrast, root tips from the *PsFut1*AS1 antisense hairy root line revealed obvious distortions in structure and mechanical integrity (Fig. 6b), with some localized areas collapsed (Fig. 6b, arrow). Similar results were reported previously in trichomes of *Arabidopsis mur2* mutant plants lacking fucosylated xyloglucan due to a mutation in *AtFUT1* (Vanzin et al 2002). Whereas the trichomes appeared normal macroscopically, SEM analysis revealed wrinkled, collapsed surfaces. Similar results were seen with border cells (Fig. 6c, d). Whereas individual border cells from wild type hairy roots retained their shape after processing and fixation for SEM (Fig. 6c), border cells from antisense lines appeared collapsed and wrinkled (Fig. 6d).

Failure of cell wall structural integrity in individual border cells from hairy roots expressing *PsFut1* antisense mRNA

As border cells undergo cell separation at the cap periphery, they can be collected and examined as populations of single cells. Border cell viability in wild type pea hairy roots is $90 \pm 8\%$, and border cell walls remain intact even after cells die (Hawes 1983). In contrast, no viable border cells were observed on any of the roots
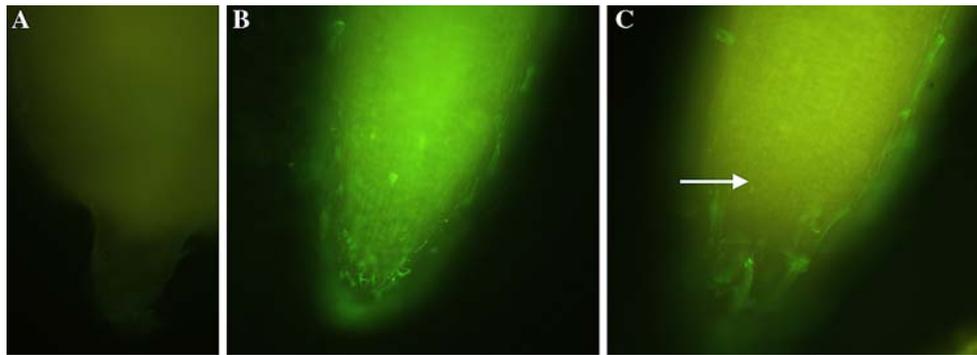
Fig. 5 Aberrant localization of surface fucosylated xyloglucan using CCRC-M1 in root tips of hairy roots expressing *PsFut1* antisense mRNA. **a** Control hairy root expressing *PsFut1* antisense mRNA, without exposure to CCRC-M1; a yellow autofluorescence is evident. **b** Root tip from wild type pea hairy root; strong labelling with CCRC-M1 occurred uniformly over the entire root cap. The *bright green* fluorescence represents fluorescein-based immunolabelling. **c** Root tip from individual hairy roots expressing *PsFut1* antisense mRNA; *bright green* fluorescence indicating a positive reaction was evident but varied in intensity among and within individual roots, with some regions exhibiting only yellow autofluorescence (*arrow*)

Fig. 6 Scanning electron microscopic comparison of root tips (**a**, **b**) versus border cells (**c**, **d**) from wild type hairy roots (**a**, **c**) versus PsFut1 antisense mRNA lines (**b**, **d**)
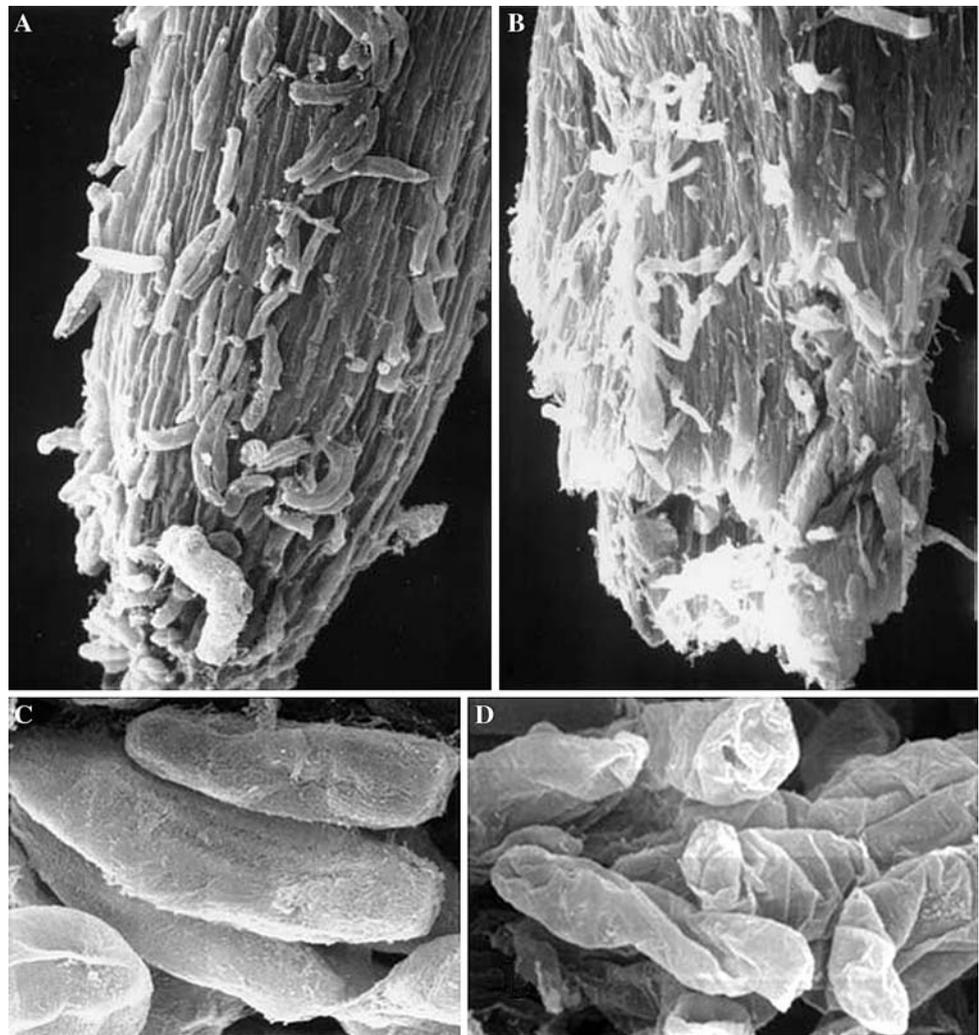
from antisense lines at any stage of development; viability among border cell samples from 45 roots examined was zero (0/1,937 cells).

Microscopic analysis revealed that, as border cells detached, cell walls failed. Wild type border cells have uniformly thick (~ 1 μm) cell walls whose contours can be

Fig. 7 Altered structure and function in cell walls of border cells from three independent lines (PsFut1AS1, PsFut1AS2, PsFut1AS3) of hairy roots expressing *PsFut1* antisense mRNA. **a** Border cells of pea hairy roots, like those of normal roots, have rigid cell walls up to 1 μm in diameter, the nucleus is prominent, and viability is >90%; **b** Border cells of antisense line PsFut1AS2 were extremely thin and fragile; cells from PsFut1AS3 (*inset*) were twisted and misshapen, with some wall regions having near-normal width (*arrow*), adjacent to regions with visible holes (*triangle*); **c** Border cells from PsFut1AS1 exhibited regions of structural derangement, appearing to swell (*arrows*); **d** Cytoplasmic contents were extruded through holes in the walls of PsFut1AS1 border cells
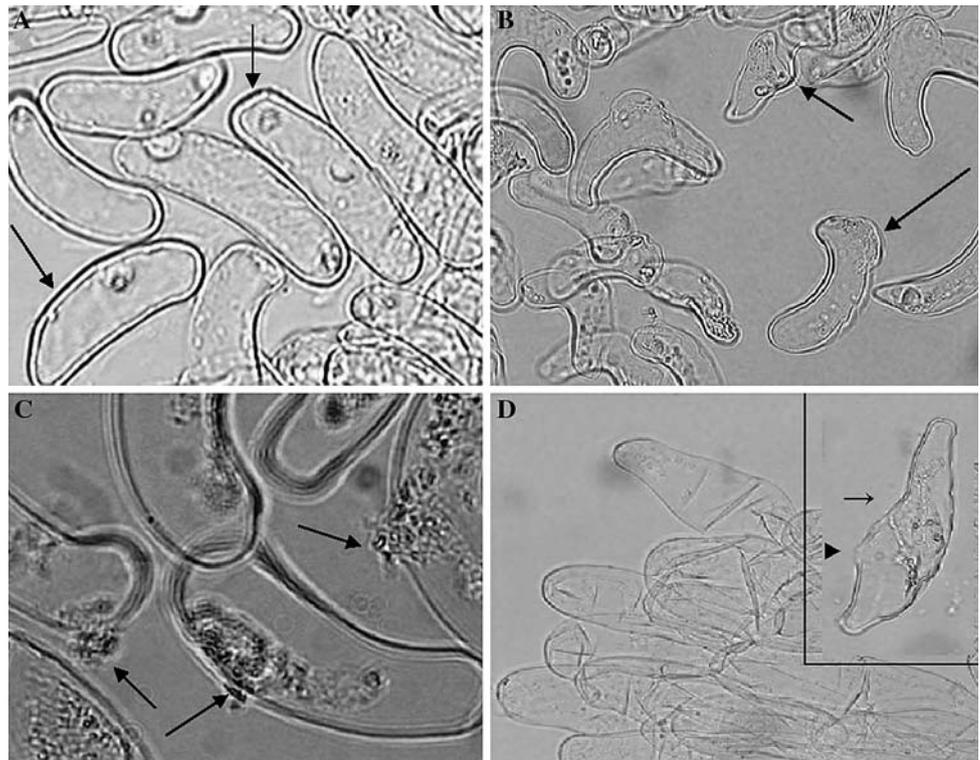
visualized (Fig. 7a, arrows). Nuclei and other cellular contents are intact and cytoplasmic streaming is readily apparent. Cell walls in all border cells (100/100) of all roots (100/100) examined from each of three independent antisense lines were morphologically distinct from those of wild type border cells (Fig. 7b–d).

*PsFut1*AS1 border cell walls exhibited irregular contours and sudden conspicuous bulges (Fig. 7b, arrows). Cytoplasm was retained in many *PsFut1*AS1 cells, but in others contents could be seen to be extruded through defects in the wall (Fig. 7C, arrows).

The lines with more extreme developmental derangement—*PsFut1*AS2 and *PsFut1*AS3—also had more severe changes in border cell wall phenotypes (Fig. 7d). *PsFut1*AS2 border cell walls were thin, the nucleus was only observed in a few cells, and most cells were devoid of cellular contents (Fig. 7d). *PsFut1*AS3 border cells were devoid of cytoplasmic bodies and were almost unrecognizable as plant cells (Fig. 7d, inset). Cell walls had some regions with normal thickness (Fig. 7d, inset, arrow) adjacent to visible breaks with cytoplasm extruded outward (Fig. 7d, inset, triangle).

Discussion

The capacity to synchronously induce root cap development facilitates identification of genes associated with specific processes that occur in morphologically distinct stages (Barlow 1975). For example, subtractive hybridization of pea root caps during the 5-min window when mitosis is induced was used to identify several meristem localized genes whose expression changes in correlation with the cell cycle (Woo and Hawes 1997). *Psugt1*, a meristem localized glycosyltransferase (GT) encoding gene whose expression is induced just prior to increased mitosis, was initially suspected to be involved in cell wall synthesis but its expression pattern was not consistent with such a function (Woo et al. 1999). The encoded enzyme ultimately proved instead to be a Family 1 GT controlling cell cycle regulation (Kamra et al. 2005; Woo et al. 2003, 2007). The inducible pea root cap system also was used in conjunction with transgenic hairy roots to identify a cap periphery localized pectin methylesterase (RcPME1) and to document for the first time that PME activity is necessary for cell separation (Wen et al. 1999; Zhu et al. 2004). Starch synthase expression was found to be localized only within cells of the central columella where amyloplasts are assembled (Brigham et al. 1998).

In the current study, pea hairy roots were used to characterize *PsFut1* expression during root cap development. The results revealed, for the first time, that *PsFut1* mRNA levels increase by >2-fold in correlation with increased mitosis and remain at an increased level throughout the 5-h period while new cells are synthesized. Expression occurred at a constitutive background level throughout the maturation process as cells differentiate in the root cap. WISH analysis revealed that *PsFut1*

expression was predominantly localized within the root and root cap meristems but expression also occurred, at a lower level, within the body of the cap. These patterns are consistent with those predicted for an enzyme with roles in primary wall assembly as well as cell wall maturation.

Transgenic hairy roots expressing full length *PsFut1* antisense mRNA under the control of the CaMV35S promoter, whose expression in pea hairy roots is localized to the root cap meristem, were developed. Macroscopic appearance of root caps appeared to be normal, but when processed for SEM and examined, the tissue was found to exhibit a wrinkled, collapsed appearance. This phenotype was strikingly similar to that previously found in trichomes of *mur2 Arabidopsis* mutant plants (Vanzin et al. 2002). These surface tissues also appeared macroscopically normal, but SEM revealed a wrinkled, collapsed appearance. The *mur2* mutant was selected based on altered levels of fucose in lines successfully recovered after T-DNA insertion (Reiter et al. 1993, 1997). The plants have a T-DNA insertion in the *AtFUT1* locus and chemical analysis failed to identify measurable fucosylated xyloglucan from bulk root, shoot, stem, and flower tissue (Vanzin et al. 2002). The appearance of these tissues is macroscopically similar to that of wild type plants, and tensile strength and growth is normal (Reiter et al. 1997; Vanzin et al. 2002). It is possible that subtle defects might have been overlooked, or that deleterious effects of altered gene expression in whole plants may have masked by unknown compensatory mechanisms (Albersheim et al. 1994; Sarria et al 2001; Zablackis et al. 1996). On the other hand, different plant species or tissues have divergent cell wall structures and fucosylated xyloglucan undoubtedly plays a more critical role in some cells than in others; indeed, this epitope is naturally lacking in some plant tissues (Perrin et al. 1999).

Our data suggest that in pea, even down-regulation of *PsFut1* mRNA and/or the closely related species detected in the pea genome, is deleterious to root development. Recovery of transformed hairy root lines expressing *PsFut1* antisense mRNA was reduced dramatically compared with vector-only controls, and development in those, which were recovered, was altered. Within individual clonal pea hairy roots, the observed phenotypic changes increased in severity and ultimately proved lethal, and two of three independent lines expressing *PsFut1* antisense mRNA ceased to be culturable within 6–8 months. The mechanism for the observed changes is unclear but is not correlated with the wholesale loss of fucosylated xyloglucan reported in bulk stem, root, and floral tissues of the *mur2* mutants (Vanzin et al. 2002). Antisense mRNA under the control of the CaMV35S promoter does not completely inhibit endogenous *PsFut1* expression in the root cap meristem, and expression ceases in cells beyond the root cap meristem (Nicoll et al. 1995; Wen et al. 1999; Woo

et al. 1999). Because *PsFut1* expression occurs at a low level throughout the cap, a complete absence or even major reduction in amount of fucosylated xyloglucan would not necessarily be expected, and absolute amounts may be less important in structural integrity than accurate timing and localization of its deposition (van Hengel and Roberts 2002).

Closely related gene family members identified in Southern blot analysis could not be distinguished in Northern blot analysis of root caps but the possibility that *PsFut1* antisense mRNA expression altered the expression of other genes which contributed to the observed effects cannot be ruled out. The full range of natural substrates of most modifying enzymes, including *PsFut1*, are not known with certainty. Putative *PsFut1*-related genes in pea, even if they do prove to encode fucosyltransferases, could contribute to the observed changes by altering fucose-containing molecules that are not a component of cell wall xyloglucan (Freshour et al. 2003). Fucose is a component of pea root cap mucilage and soluble root exudates, for example (Knee et al 2001). Soluble fucosylated oligosaccharides are potent signalling molecules in mammals as well as in plants, and changes in such metabolites could account for the pleiotropic effects observed in *PsFut1* antisense hairy roots (McDougall and Fry 1989, 1990; Mejias-Luque et al. 2007; York et al. 1984).

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