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Short title: Stomatal cuticular ledge formation requires FOCL1

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Formation of the Stomatal Outer Cuticular Ledge Requires a Guard Cell Wall Proline-Rich Protein

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Summary:

Plants lacking the guard cell expressed, proline-rich secreted protein FOCL1, are drought tolerant, because they fail to form a stomatal cuticular ledge and produce stomatal pores that are covered by a continuous cuticle.

Author Contributions:

J.E.G, A.J.F and L.H. conceived the project, designed the experiments, analysed the data and wrote the article; J.E.G., L.H., A.J.F., J.K.H. and R.S. supervised the experiments; L.H. performed most of the experiments with input from S.A., A.B., M.M., A.M., R.S, K.S and T.D.

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Abstract

Stomata are formed by a pair of guard cells which have thickened, elastic cell walls to withstand the large increases in turgor pressure that have to be generated to open the pore that they surround. We have characterised FOCL1, a guard cell-expressed, secreted protein with homology to hydroxyproline-rich cell wall proteins. FOCL1-GFP localises to the guard cell outer cuticular ledge and plants lacking FOCL1 produce stomata without a cuticular ledge. Instead the majority of stomatal pores are entirely covered over by a continuous fusion of the cuticle, and consequently plants have decreased levels of transpiration and display drought tolerance. The *focl1* guard cells are larger and less able to reduce the aperture of their stomatal pore in response to closure signals suggesting that the flexibility of guard cell walls is impaired. *FOCL1* is also expressed in lateral root initials where it aids lateral root emergence. We propose that FOCL1 acts in these highly specialised cells of the stomata and root to impart cell wall strength at high turgor and/or to facilitate interactions between the cell wall and the cuticle.

Introduction

Plant cell walls typically consist of a network of cellulose, hemicellulose, pectin and lignin, but also contain many structural proteins of unknown function such as hydroxyproline rich glycoproteins (HRGPs) (Lamport et al., 2011). This group of proteins include proline-rich proteins (PRPs), arabinogalactan proteins (AGPs) and extensins. HRGPs are sequentially post-translationally modified by proline 4-hydroxylases (P4Hs) converting proline residues to hydroxyproline, and then by O-glycosyltransfeases (GTs) adding sugar moieties to hydroxyproline residues. These post-translational modifications are thought to contribute to the structural and possibly to the intercellular communication properties of the cell wall. Extensins are the best characterised of the plant HRGPs and these are commonly arabinosylated by the HPAT family of GTs before being arabinogalactosylated (Velasquez et al., 2011; Ogawa-Ohnishi et al., 2013). Extensins are cross-linked at tyrosine residues by peroxidases and processed by proteases which insolubilise and lock the extensins into the cell wall structure (Helm et al., 2008; Lamport et al., 2011). Extensins were originally isolated from elongating coleoptiles over 50 years ago (Lamport, 1963) and proposed to be involved in cell wall extensibility, but this has never been functionally confirmed (Lamport et al, 2011). Nonetheless, roles for extensins have been observed in Arabidopsis embryo and root development (Cannon et al., 2008; Velasquez et al., 2011); embryos lacking EXT4 are defective with irregular cell size and shape and root hairs lacking EXT6-7, EXT10, and EXT12 show reduced root hair elongation. Similar root hair phenotypes are seen in plants lacking P4H activity due to reduced proline hydroxylation and O-arabinosylation of extensins, suggesting that these post-translationally modified proteins influence root hair growth (Velasquez et al., 2011). There are 51 genes annotated as encoding extensins or extensin-like proteins in the Arabidopsis genome (Showalter et al., 2010) and it appears likely from their specific expression patterns that they are involved in a range of growth, developmental and stress responses (Merkouropoulos and Shirsat, 2003), although plants manipulated to produce abnormally high levels of EXT1 appear to develop normally with the exception of having thicker stems (Roberts and Shirsat, 2006). Physiological roles in aerial tissues remain elusive and the failure to identify a function for extensins and indeed other HRGPs in shoots is likely to be due to redundancy within this large gene family, a common problem in plant cell wall protein studies.

The studies of extensins in root hairs described above indicate that it is possible to gain information about their function in a specialised and well-studied cell type. We therefore decided that because of the unique properties of guard cell walls and the tractability of measuring stomatal development and function, it might be possible to identify the function of a cell wall protein that is predominantly expressed in guard cells. Pairs of guard cells

surround and adjust the aperture of stomatal pores in response to environmental signals which trigger changes to the turgor pressure of the cells (Kollist et al., 2014). Large turgor changes within guard cells occur over short time scales (typically minutes), with turgor increases causing stomatal opening, and decreases causing closure. Thus in comparison to other cell types, guard cells require particularly strong and elastic cell walls. However, there is currently no genetic evidence of a role for cell wall HRGPs in stomatal function, although individual polysaccharide moieties of the mature guard cell wall are known to be important for pore aperture control as removal of the arabinan component of the guard cell wall or modifying pectin methyl esterification impairs stomatal opening and closing (Jones et al., 2003; Amsbury et al., 2016).

During leaf epidermal development the division of guard mother cells forms pairs of guard cells. Stomatal pores subsequently form between each guard cell pair but little is known of the processes regulating guard cell wall maturation and stomatal pore formation. Microscopic observations show that the cell walls between adjacent guard cells (which are destined to line each stomatal pore) thicken and separate. The exterior surface of the leaf becomes coated with a waterproof layer of cuticle and an extended ledge or lip forms around each stomatal pore which is known as the outer cuticular ledge (OCL). The exact function(s) of this cuticular ledge are unknown, but it has been proposed to prevent water loss by sealing the pore when the stomate is closed; to prevent water droplets entering when the pore is open; and to tilt its orientation to help open and close the stomatal pore (Fricker and Wilmer, 1996; Zhao and Sack, 1999; Kozma and Jenks, 2007). No specific proteins have yet been localised to the OCL. We report here that *Arabidopsis thaliana* plants lacking an OCL-localised gene product annotated (by TAIR www.arabidopsis.org) as an 'extensin-like protein', have larger stomata, show defects in stomatal closure, and most notably possess a malformed outer cuticular ledge that forms a fused cuticular layer over the stomatal pores. Hence we have named this protein Fused Outer Cuticular Ledge or FOCL1. In addition to its roles in stomata, we also report that FOCL1 influences lateral root emergence. Our results therefore provide a link between a secreted proline-rich protein and its function in the cell walls of specific plant cell types.

Results

FOCL1 has features of hydroxyproline rich cell wall glycoproteins

The predicted amino acid sequence encoded by Arabidopsis gene *At2g16630,* named here as *FOCL1,* contains a putative signal sequence suggesting that it is secreted, and a proline-rich domain with several motifs typical of HPRGs; including eight proline-valine motifs which

are normally hydroxylated and four repeated triple proline residues that are most likely post-translationally modified (Kieliszewski and Lamport,1994; Menke et al., 2000) (Fig. 1A and Supplemental Fig. 1). Nonetheless, the FOCL1 protein is not a classical extensin as it lacks the characteristic conserved serine-polyproline repeats and the YXY or VYX domains required for tyrosine intermolecular cross-linking (Kieliszewski and Lamport, 1994). However, FOCL1 may be inter-molecularly bonded by tyrosine residues in a different sequence context, as seen for HRGP, PRP10 (Chen et al., 2015). *FOCL1* orthologues occur across a wide range of plant species but the closest homologue of *FOCL1* in Arabidopsis, encodes a protein of unknown function with only 24% identity. At2g20515 has similarity with the C-terminus of FOCL1 but lacks the N-terminal and central proline-rich regions of FOCL1 (Supplemental Fig. 1).

FOCL1 also shows conservation with an atypical AGP known as AGP31; both possessing distinctive tandem proline-rich PKVPVISPDPPA/TTLPP domains (Showalter et al., 2010; Liu and Mehdy, 2007) (Supplemental Fig. 2). As proline residues of the AGP31 proline-rich domain are known to be hydroxylated and glycosylated (Hijazi et al., 2012), it is likely that this is also the case for the conserved domain in FOCL1. However, FOCL1 and AGP31 have lower proline content than many HRGPs and are therefore unlikely to have very high levels of post-translational glycosylation. Thus FOCL1 resembles a hydroxylated proline-rich, structural cell wall protein but it is neither a classical extensin nor a typical AGP.

BLAST analysis revealed homology of the FOCL1 N-terminus with ‘Pollen Ole e 1 allergen and extensin family’ proteins but these lack the C-terminal domain and proline rich region present in FOCL1 (not shown), suggesting that FOCL1 might be a chimeric protein - with a Pollen Ole e 1 extensin-like domain at the N-terminus, a proline-rich AGP31-like tandem repeat in the middle of its sequence, and an At2g20515-like domain at the C-terminus.

FOCL1 **is expressed in guard cells and lateral root primordia**

Published transcriptome data indicate that *FOCL1* is strongly expressed in guard cell protoplasts and in roots, and that expression levels are lower in root than in shoot tissue (Zimmerman et al., 2005; Winter et al., 2007; Yang et al., 2008). We examined *FOCL1* expression patterns using plants expressing the β-glucuronidase gene under the control of the DNA region upstream of the *FOCL1* coding sequence (*pFOCL1:GUS*). GUS expression was predominantly observed in immature and mature guard cells (Fig. 1B and C). Staining was not present in guard cell precursors (such as guard mother cells) suggesting that FOCL1 is not directly involved in the formation or patterning of stomata during shoot development. Staining was also seen in emerged lateral roots (Fig. 1D) and developing primordia. Together these results suggested that FOCL1 is an HRGP which could potentially

function in the cell walls during guard cell maturation and function, and during lateral root development.

Plants lacking FOCL1 have large stomata

Two independent Arabidopsis lines with T-DNA insertions 200bp apart in the third exon of the *FOCL1* gene were isolated and named *focl1-1* and *focl1-2* (Supplemental Fig. 3A). Expression of the *FOCL1* transcript was not detectable by RT-PCR of homozygous *focl1-1* plants with primers spanning the insertion site (Supplemental Fig. 3B) but a product was seen in *focl1-2* with primers upstream of the insertion site, suggesting a truncated protein could be produced. *focl1-1* and *focl1*-2 plants were both smaller than wild-type, with reduced rosette width at bolting. Growth of *focl1-1* plants was more severely affected than *focl1-2* plants, and these were smaller and paler than *focl1-2* (Supplemental Fig. 4). As we had observed strong expression of *FOCL1* in guard cells we examined the leaf surfaces of these plants using epidermal imprints. Both *focl1-1* and *focl1-2* showed significant increases in abaxial stomatal index (SI) in the experiment shown in Fig 1E due to a significant decrease in the number of pavement cells. However we observed no consistent alteration in stomatal density in replicated experiments, no clustering of stomata, or arrested precursor cells as often seen in stomatal developmental mutants (e.g. Hunt and Gray, 2009). Instead, we observed an unusual phenotype; in both imprints and in cleared images of whole leaves *focl1* stomata were obviously larger than normal, and had a pore that appeared to be different to wild-type (Fig. 1, F-I). Measurement of stomatal dimensions confirmed significant increases in width and length of *focl1-1* guard cell pairs; on the abaxial and adaxial leaf surfaces *focl1-1* stomata were 31% and 34% larger than wild-type stomata when their area was calculated as an ellipse (Fig. 1J). To confirm that both the reduced rosette growth and larger stomata were caused by lack of FOCL1, *focl1-1* and *focl1-2* mutations were complemented by transformation with a genomic fragment containing the wild-type *FOCL* gene with an N-terminally fused GFP (*focl1-1pFOCL1:GFP-FOCL1*) or C terminally fused MYC tag (*focl1-2pFOCL1:FOCL-MYC1*). This GFP-FOCL1 protein rescued rosette growth and returned stomatal complex sizes to wild-type values in both mutant backgrounds (Supplemental Fig. 4, A-B, 5A-B).

FOCL1 is involved in the formation of stomatal pore outer cuticular ledges

To investigate *focl1* stomatal morphology in detail we examined leaf surfaces using cryoSEM on 3 week old plants. This revealed that in immature ‘rounded’ stomates the pore is covered by a cuticular layer, which appears to tear to form the outer cuticular ledge and to reveal and surround the pore as the guard cells lengthen and mature. In contrast, *focl1* stomatal pores remained covered-over, or occluded by what appears to be an extension of the cuticle and

do not form an outer cuticular ledge around the pore (Figs. 2A-F). Further SEM analysis showed that even after fixation and dehydration the majority (approx. 90%) of *focl1* pores on mature leaves and stems remain occluded (Supplemental Fig. 6) with a minority of stomata forming a slit-like opening (Fig. 2, and Supplemental Fig. 7). To confirm that the numerous occluded stomatal pores were not an artefact of electron microscopy we imaged the epidermal surface topography of several stomates from fresh leaf tissue using both vertical scanning interferometry (VSI; Fig. 3, A and B) and atomic force microscopy (AFM; Fig. 3, C and D*;* Supplemental Fig. 8). These two techniques which physically probe the surface of an object to measure height differences both confirmed that *focl1* stomatal pores are covered by what appears to be a continuous layer of cuticle. Furthermore light microscopy of stained cross-sections of stomata also revealed a continuous 'fused' cuticular ledge formed between the edges of the two guard cells surrounding the pore (Fig. 3, E-H).

Staining with the lipophilic stain Nile red, revealed a sharp discrete cuticular ledge surrounding the outer edge of wild-type stomatal pores, attached to the guard cells (Fig. 4A). In *focl1* stomates this staining was more diffuse and spread across the whole pore area (Fig. 4B). To further investigate the chemical nature of this lipophilic material covering the stomatal pores, we used Raman microscopy. The wild-type and *focl1-1* guard cells produced similar Raman spectra when central regions of the cells distant from the ledge were analysed (Fig. 4, C and F). Peaks of wavelength 2840 and 2880, indicative of epicuticular waxes (Greene and Bain, 2005), were observed in the cuticular ledge region of wild-type guard cells (Fig. 4D). Similar peaks in the spectra were observed after analysis of spots in the middle of the occluded *focl1-1* pore (Fig. 4G) whereas the spectrum over the wild-type pore aperture area did not show peaks at these wavelengths (Fig. 4E). Thus it appears that guard cells lacking FOCL1 are able to produce epicuticular wax material but are unable to properly form a cuticular ledge around their stomatal pores, and consequently the cuticle forms a continuous layer across the pore.

FOCL1 protein localises to the outer cuticular ledge of guard cells

To investigate whether FOCL1 is a secreted cell wall protein as predicted by its sequence, and whether it could act in the formation of the guard cell cuticular ledge, we examined the subcellular localisation of the FOCL1 protein. To do this we analysed the expression of a FOCL1-GFP fusion protein *in vivo* (in *focl1-2* plants transformed with the promoter and coding region of *FOCL1* in frame with a C-terminal GFP tag). The results shown in Figs. 5A and B indicate that the fluorescent fusion protein accumulates specifically in the cuticular ledge of guard cells, further indicating that FOCL1 is secreted from guard cells and acts directly in the formation of the cuticular ledge.

225

226 **Lack of FOCL1 impairs stomatal aperture control and transpiration**

227 We tested whether the fused stomatal cuticle phenotype of *focl1* mutants would affect the
228 ability of plants to carry out gas exchange. To assess transpiration, plants were grown at
229 high humidity and kept well-watered (in a propagator with a lid). Their leaf surface
230 temperatures were monitored by infrared thermography, which is a proxy measure of
231 transpiration rate. On average mature leaves of *FOCL1* mutants were approximately 1°C
232 warmer than control plants and remained hotter for at least 2.5 hours after humidity was
233 reduced (by removal of the propagator lid) suggesting a reduced level of transpiration and
234 evaporative cooling in the *focl1* plants (Fig. 6, A and B). The *focl1* plants retained their
235 warmer temperature throughout the experiment whilst the wild-type plants slowly adjusted to
236 the less humid environment by reducing their level of transpiration and eventually increasing
237 their temperature to a similar level to that of the mutants (presumably by closing their
238 stomatal pores). Leaf porometry measurements on well-watered unperturbed plants also
239 confirmed a substantially reduced level of stomatal conductance from *focl1* leaves (Fig. 6C)
240 which is consistent with the observation that *focl1* stomata are partially or completely
241 occluded by a covering of cuticle (Fig. 2 and Fig. 3). We confirmed that the reduced
242 transpiration phenotype was due to loss of FOCL1 by showing that leaf temperatures were
243 returned to wild-type levels when *focl1-1* or *focl1-2* were complemented with the wild-type
244 gene (in *focl1-1pFOCL1:FOCL-MYC1 or focl1-1pFOCL1:GFP-FOCL1* or *focl1-*
245 *2pFOCL1:FOCL-MYC1*; Supplemental Fig. 9).

246 We next explored whether the alterations in the morphology of *focl1* stomata and their
247 cuticles affected their ability to close their pores in response to environmental stimuli. To
248 investigate the effect of the lack of FOCL1 on stomatal aperture control we measured
249 stomatal pores from isolated epidermal strips following incubation with 10μM ABA (a plant
250 stress hormone that triggers stomatal closure). All pores in the field of view were measured
251 as it was not possible to tell under light microscopy whether they were covered-over or not.
252 Although the *focl1* stomata closed to some extent in response to ABA, they were unable to
253 close as fully as wild-type stomata and the width and areas of their pore apertures remained
254 significantly larger (Fig. 6, D-F). To take account of the increased stomatal complex size in
255 *focl1* in these experiments, we calculated the relative reductions in pore width and area; in
256 the presence of ABA wild-type stomatal pore width and area decreased by 90% and 91% but
257 *focl1-1* stomatal pore width and area decreased by only 54% and 42% respectively. Thus, it
258 appears that loss of FOCL1 leads to impaired guard cell movement. However, despite their
259 impaired ABA-inducible stomatal closure, *focl1* plants wilted less readily than wild-type when
260 water was withheld for 7 days, presumably because of their occluded stomata and reduced

level of transpiration. In these experiments both *focl1* lines displayed drought tolerance, showing no visible signs of water stress whereas the wild-type plants were unable to recover when re-watered (Fig. 6G).

FOCL1 acts during lateral root emergence and influences root architecture

A detailed study of *pFOCL1:GUS* roots indicated that *FOCL1* is expressed at a very early stage of lateral root development. Lateral roots originate from lateral root founder cells located opposite xylem pole pericycle cells. *FOCL1* is expressed soon after division of the founder cells (Fig. 7A). *GUS* expression is first seen in stage II primordia (Peret et al., 2009) and then continues throughout the further stages of lateral root primordia development (stages III to VII) and emergence (Fig. 7A). *FOCL1* expression appeared to be specifically associated with the developing and emerging lateral root primordia and no staining was observed in the surrounding or the overlying cells of the parent root prior to emergence.

As *FOCL1* is expressed in early root development we explored whether *focl1-1* and *focl1-2* mutants had defects in lateral root primordia development and emergence. Lateral root numbers, density, primary root lengths and lateral root stages were measured in 11 day old seedlings. As shown in Fig. 7, C-E, there was a significant reduction in primary root length, lateral root number and lateral root density in *focl1* seedlings compared to wild-type. To further explore if this defect was due to defects in lateral root growth rate or in lateral root initiation and/or emergence, roots were cleared and all stages of lateral root primordia scored. The results shown in Fig. 7F indicate that lateral root development was significantly delayed in *focl1-1* at stages IV and V. These are the stages when a series of anticlinal and periclinal divisions produce a dome shape structure that protrudes through the cortex towards the epidermal layer prior to emergence. These data indicate that the FOCL1 protein is required for the growth of early lateral root primordia through the parent root.

Discussion

FOCL1 is a putative cell wall structural protein

Plants produce many non-enzymatic proteins that are believed to influence the structure and mechanical properties of their cell walls. However, despite extensive study, the function of most of these proteins remains elusive. We have characterised a putative Arabidopsis cell wall structural protein which is required for the correct functioning of guard cells and lateral root initials. The expression of *FOCL1* in these discrete cell types of the epidermis and root suggests that this protein is required to create the particular cell wall properties associated with their specific functions. The FOCL1 protein has a predicted signal sequence and proline-rich region typical of cell wall HRGPs (Kieliszewski and Lamport, 1994). The deduced protein sequence bears limited similarity to extensins except for several potentially hydroxylated proline residues which are conserved with the proline rich domain of AGP31 (Supplemental Fig. 2). Thus, FOCL1 is not an extensin and appears to be the only member of a distinct subgroup of Arabidopsis HRGPs. The proline-rich sequence suggests that FOCL1 most likely interacts with other cell wall components through its primary structure or through specific post-translational modifications of hydroxyproline residues. Through these interactions it may guide the assembly of new cell wall material, or it may be involved in maintaining the structure and rigidity of the cell wall.

Role and structure of the stomatal outer cuticular ledge

The guard cell wall and its extracellular matrix have an important and specialised role in the functioning of stomata and in preventing plant desiccation (Jones et al., 2003). We show that FOCL1 is localised in the guard cell outer cuticular ledge and that plants lacking FOCL1 have their stomata occluded by a continuous layer of cuticle formed from a fused outer cuticular ledge. The retarded growth of these plants is most likely explained by reduced CO_2 entry and carbon assimilation, although it is possible that the delayed development of their root initials may also contribute to poor seedling establishment. The timing of *FOCL1* expression during guard cell maturation (Fig. 1) and the relatively normal structure of stomates beneath the *focl1* cuticle suggest that OCL formation occurs after guard mother cell division and pore formation. This indicates that the *focl1* guard cells may have a defect in the framework or assembly of the cell wall which normally sculpts the cuticular ledge into a distinct elliptical shape (Fig. 2). This defective cell wall is also likely to be the reason for the increased size of *focl1* stomata; turgor pressure is probably exerting a force to inflate the guard cells that is normally restrained in wild-type guard cells by their more rigid cell wall framework. It is possible, but less likely, that larger stomata could be due to reduced intercellular CO_2 concentration (Ci) resulting from abrogated stomatal function. Low C_i has

been associated with an increase in stomatal complex size but this is normally linked to a decrease in stomatal density (Franks and Beerling, 2009) and *focl1* showed an increase in stomatal index and no difference in density, suggesting that it is most likely due to an impairment in guard cell wall function. In line with this proposal, we also found that the *focl1* stomata were impaired in their ability to close (Fig. 6). This is most likely due to a defect in the guard cell walls and may be indicative of a lack of elasticity in the rather large *focl1* guard cells.

The stomatal OCL has been little studied and FOCL1 is the only protein known to be localised to this structure. Mutant plants that are unable to synthesise cutin, such as *lacs2,* have diminished cuticular ledges and increased transpiration rates, indicating a probable role in preventing water loss (Li et al., 2007; Macgregor et al., 2008). In contrast, plants lacking FOCL1 have the opposite phenotype: an overgrowth of the cuticular ledges associated with reduced transpiration, suggesting that FOCL1 defines the extent of the OCL in guard cells. The OCL is an extension of the guard cell wall derived from the middle lamella which contains unesterified pectins and glycans (Majewska-Sawka et al., 2002; Merced and Renaglia, 2014; Wilson et al., 2015; Amsbury et al., 2016). Plant cuticles are anchored to cell walls by extended pectic lamellae, and can be released by pectinase or cellulase treatment (Jeffree, 2006). As the proline-rich region of FOCL1 is likely to be decorated with pectic sidechains containing galactose and arabinose (Hijazi et al., 2012) it is possible that the post-translationally modified FOCL1 protein normally interacts with pectin or cutin in the OCL where it is located (Fig. 5B). Thus FOCL1 could be required to facilitate interactions between the guard cell wall and the cuticle that are necessary for OCL formation (Jeffree, 2006).

FOCL1 is involved in lateral root development.

Plants lacking FOCL1 show defects in primary root and lateral root development. However, in our experiments *pFOCL1:GUS* staining was not consistently observed in the primary root (Fig. 7) and it is possible that reduced primary root growth is related to the smaller size of *focl1* plants due to their covered-over stomata, or that additional *FOCL1* promoter regions are required for primary root expression. Nonetheless, the specific *GUS* expression pattern in developing and emerged lateral roots and lateral root defects in *focl1* plants indicate that FOCL1 has a direct effect on lateral root development. The lateral root emergence process is thought to involve a separation of overlying cortex and epidermal cells along their middle lamella. Indeed, cell wall modifications have previously been shown to play a role in lateral root development (Swarup et al., 2008). Several genes encoding cell wall remodelling enzymes show specific expression in the cells overlaying new lateral root primordia and are induced by auxin, which plays a key role in initiation, emergence, and elongation of lateral

roots (Swarup et al., 2008, Voss et al., 2015). It is unlikely that FOCL1 is directly involved in this cell separation process though as its expression is restricted to developing lateral root primordia and is never detected in the outer tissues. Interestingly the reduced cutin levels in the *lacs2* mutant cause both a defective OCL and increased lateral root formation (Macgregor et al., 2008) which may be related to an altered root cuticle, or indirectly related to the increased transpiration in these mutants. Thus the *focl1* root phenotype, like the *focl1* occluded stomata phenotype, might also result from a defective relationship between the cell wall and the cuticle.

Our experimental results indicate that FOCL1 is not required for lateral root initiation but is required for the development of lateral root primordia prior to emergence (Peret et al., 2009). During this period the lateral root initial cells of the pericyle divide periclinally and expand radially, whilst the endodermal cell layer overlaying the primordium separates to allow the lateral root to expand and protrude through into the cortical layer. The process by which the lateral root passes through these cell layers is poorly understood but is believed to involve both biomechanical forces and cell wall modifications (Geldner, 2013). Indeed it has recently been suggested that a build-up in turgor pressure within the cells of the primordium through the regulation of water flux by aquaporin activity and auxin, enables the lateral root to extend and force itself through the overlying cell layers (Peret et al., 2012). Thus it appears possible that in lateral root primordia, and in guard cells, FOCL1 could provide the cell wall strength that allows cells to withstand the high turgor pressures required to expand and to fulfil their function. Alternatively FOCL1 could be involved in guiding and directing newly synthesised components into the cell wall that are required for cellular expansion and function.

In conclusion we propose that FOCL1 is specifically required for the function of lateral root tip cells and guard cells by playing a role in assembling or strengthening the cell wall, and in anchoring it to the developing cuticle. As it appears that the same protein has been recruited to fulfil a function in the walls of cell types with two very different functions, *focl1* mutants provide a new tool for the study of HRGPs. We hope that future studies of *focl1* roots and stomata may reveal the precise role of a plant proline-rich cell wall protein.

Materials and Methods

Plant Materials

Arabidopsis thaliana plants were grown on a 9hr day (200 µmol $m^{-2}s^{-1}$ light, 22°C), 15 hr night (16°C) cycle at 60% humidity. T-DNA insertion lines WiscDsLoxHs053_08G (*focl1-1;* Woody et al., 2007) and SK5131 (*focl1-2;* Robinson et al., 2009) were obtained from NASC, Nottingham UK. Plants were confirmed as homozygous for the insertion by PCR verification

with primers WiscDsLoxHs053_08G ,5'-gagccatcagcttgttctcac-3', 5'-tgttcatgtccctctggaatg-3 or SK5131 5'-gcttccaccattgcctcaaa-3', 5'-tgttcatgtccctctggaatg-3'. To confirm lack of, or truncated, *FOCL1* transcript RT-PCR was carried out. RNA was extracted with Spectrum RNA kit (Sigma-Aldrich) and 2µg converted to cDNA with Maxima H Minus reverse transcriptase (Thermo Fisher Scientific). cDNAs were diluted five-fold and *FOCL1* transcript amplified using primers foclf1 5'-gcttcaggtcctgcacagaaa-3', foclr1 5'- tctgcaggtcccggaattag-3' and foclr2 5'-acaaaaagaacttggctgaactgg-3'. *ACT3* was amplified as loading control using 5'-ctccggcgacttgacagagaag-3' and 5'-ggaggatggcatgaggaagaga-3'.

Histochemical GUS staining

pFOCL1:GUS gene construct was produced by PCR amplifying 2kb upstream from the *FOCL1* translation start site with primers 5'-tgtatgataattcgagctacgattctaggcgcaaaag-3', 5'-agaaagctgggtcggagcaataaagaagaagaagagaaac-3' and combined by Gibson cloning (Gibson et al., 2009) into *pBGWFS7* (Karimi et al., 2002) containing the upstream region of *EPF2* (Hunt and Gray, 2009) which was then removed by digestion with SacI and AscI. The plasmid was transformed into Agrobacterium GV3101 by freeze/thaw, and plants transformed by the floral dip method (Clough and Bent, 1999). Transformants were selected by spraying with Basta (Liberty, Agrevo). Histochemical staining for GUS activity was carried out on leaves of T1 seedlings in 50 mM potassium phosphate, 1mM potassium ferrocyanide, 1 mM potassium ferricyanide, 0.2% Triton X-100, 2 mM 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid, and 10 mM EDTA after vacuum infiltration at 37°C. Leaves were decolorized in 70% ethanol, cleared in 80% chloral hydrate and images captured with an Olympus BX51 microscope connected to a DP51 digital camera using Cell B software. Expression pattern shown was typical of several independently transformed lines. GUS staining in the roots was performed on 11 day old roots as described previously (Lucas et al., 2012).

Genetic Complementation

pFOCL1:FOCL1-GFP was generated by amplifying genomic DNA with primers 5'-tgtatgataattcgagctacgattctaggcgcaaaag-3', 5'-agaaagctgggtcggagcaataaagaagaagaagagaaac-3' and combined via Gibson cloning into *pMDC107* previously cut with XbaI and AscI. *pFOCL1:FOCL1-MYC* was generated by cutting *pFOCL1:FOCL1-GFP* with KpnI and SacI. The MYC tag from *pCTAPa* (Rubio et al, 2005) was amplified using the primers 5'-tggtacctaacagcgggttaattaac-3' and 5'-tgaacgatcggggaaattcg-3'and the product digested with KpnI and SacI and ligated into a similarly cut *pFOCL1:FOCL-GFP* to create *pFOCL1:FOCL-MYC*. The plasmid was

transformed into Agrobacterium strain GV3101 by freeze thaw by floral dip method and selected on 0.5 x MS plates containing 5mg/L hygromycin.

pFOCL1:GFP-FOCL1 was generated by overlapping PCR using primers 5'-taaaacgacggccagtgccaacgattctaggcgcaaaag-3', 5'-ctttactcatggtggctaagcagagaac-3', 5'-ttacaccatttttgtatagttctaccatgcc-3', 5'-actatacaaaaatggtgtaaccggatatg-3' and 5'-cgatcggggaaattcgagctttgctgagcgttgatgtg-3. The products were ligated into pJET1.2 by blunt ended cloning then excised using XhoI and XbaI. The digested product was ligated into pMDC99 cut with SalI and SpeI and transformed into *focl1-1* as above.

Stomatal density, size and aperture measurements

Stomatal density was taken from fully mature leaf surfaces (3 areas per leaf) using nail varnish imprints from dental resin impressions (Impression plus, TryCare) and mounted directly onto slides. Images were recorded using an Olympus DX51 light microscope. To analyse stomatal complex size, images from imprints (3 areas per leaf, at least 10 stomata per plant) were measured using Line tool in Image J. Stomatal complex size was calculated using the formula $area = \pi ab$ where a is the guard cell pair short radius and b the long radius.

The control of stomatal apertures was analysed using leaf abaxial epidermis (Webb and Hetherington, 1997). Strips of epidermis were taken from leaves of five to six week-old plants (3–5 leaves of each genotype) using tweezers and then floated on resting buffer (10 mM MES, pH 6.2) for 10 minutes. Strips were transferred to opening buffer (10 mM MES, 50 mM KCL, pH 6.2) in the light (300 µmol m^{-2} s^{-1}), aerated with CO_2-free air and maintained at 20°C for 2 hours. To investigate the effect of ABA on stomatal aperture, opening buffer was supplemented with 10µM ABA. Pore widths and lengths were recorded from at least 100 stomata for each treatment. Pore area was calculated as above.

Microscopy and cell surface analyses

For cryo-scanning electron microscopy (cryo-SEM), excised leaves were placed flat on a brass stub, stuck down with cryo glue consisting of a 3:1 mixture of Tissue-Tec (Scigen Scientific, USA) and Aquadag colloidal graphite (Agar Scientific, Stansted, UK) and plunge frozen in liquid nitrogen with vacuum applied. Cryo fracture leaf samples were placed vertically in recessed stubs held by cryo glue. Frozen samples were transferred under vacuum to the prep chamber of a PT3010T cryo-apparatus (Quorum Technologies, Lewes, UK) maintained at -145°C. Surface ice was removed using a sublimation protocol consisting of -90°C for 3 min. For cryo fracture, no sublimation was carried out and instead a level semi-rotary cryo knife was used to randomly fracture the leaf. All samples were sputter coated with platinum to a thickness of 5 nm. Samples were then transferred and maintained

cold, under vacuum into the chamber of a Zeiss EVO HD15 SEM fitted with a cryo-stage. SEM images were captured using a gun voltage of 6 kV, I probe size of 460 pA, a SE detector and a working distance of 5 to 6mm.

Scanning electron microscopy (SEM) specimens were fixed overnight in 3% glutaraldehyde, 0.1M sodium cacodylate buffer, washed in 0.1M sodium cacodylate buffer and secondary fixed in 2% aqueous osmium tetroxide 1 hr before dehydrating through 50-100% ethanol series 30 mins each and drying over anhydrous copper sulphate. Specimens were critically point dried using CO_2 as the transitional fluid then mounted with sticky tabs on 12.5mm diameter stubs, and coated in an Edwards S150B sputter coater with approximately 25 – 30 nm of gold. Specimens were viewed using a Philips SEM XL-20 at accelerating voltage of 20kv. For atomic force microscopy (AFM) 28 day old leaves were excised and fixed to glass slides using Provil Novo before submerging under a drop of water and imaging with an Asylum MFP-3D (Oxford Instruments Co., Santa Barbara, California) using contact mode. Height and deflection images were obtained with triangular silicon nitride probes (Bruker SNL10, nominal spring constant 0.35N/m) using Asylum instrumentation software by scanning at 2Hz on contact mode with set point 1V.

Vertical scanning interferometry (VSI) was carried out on abaxial surfaces of fully expanded leaves, with leaf held flat by pressing on to double sided tape using a Wyko NT9100 surface Profiler and images were analysed on Vision 4.10. For light microscopy stem samples (~1cm lengths from the bases of branches of mature plants) were fixed in 4% (w/v) formaldehyde in PEM buffer (0.1M PIPES, 2mM EGTA, 1mM MgSO4, adjusted to pH7) by vacuum infiltration then dehydrated in an ethanol series (30min each at 30%, 50%, 70%, 100% EtOH) and infiltrated with LR White Resin (London Resin Company) diluted in ethanol (45min each at 10%, 20%, 30%, 50%, 70% & 90% resin then 3x8h+ at 100%). Samples were stood vertically in gelatine capsules filled with resin and polymerised > 5 days at 37°C. 3µm sections were cut using a Reichert-Jung Ultracut E ultramicrotome, stained with Toluidine Blue, visualised using an Olympus BX51 microscope, and images captured using Cell B software. Epidermal peels were stained by adding a drop of 1ng/µl Nile red in 50% DMSO and imaged by fluorescence microscopy with an Olympus DX51 microscope using 460-490 excitation, 510-550 emission and 505 dichroic mirror. FOCL-GFP images were captured as above, with a 1s exposure time.

Raman Spectroscopy

Raman microscopy was performed using a Renishaw InVia system fitted with a 532nm laser and a 2400 lines/mm grating. Fresh leaf sample blocks (5x5mm) were attached to aluminium slides using carbon tape and Raman 2D mapping was carried out using a 100x objective

with a 1 s/pixel exposure time, 3x accumulation. Spectral range was set at 2439 to 3324 (centre 2900) Raman shift (cm^{-1}). Data were analysed using Renishaw WiRE software, with scans being obtained across stomatal regions of interest from at least 3 independent biological samples.

Transpiration measurements

Transpiration rates were measured using a porometer (Decagon Devices) with 3 measurements taken per plant from 4 plants of each genotype. Only *focl1-2* was studied as *focl1-1* leaves were too small to insert into the porometer chamber. Infrared thermography was used as a proxy measure of evaporative cooling from transpiration. 8 week old plants were kept under a propagator lid for 24hrs before analysis. The lid was removed 4hrs into the photoperiod and images captured with a FLIR SC660 thermal imaging camera and analysed using ThermaCAM Researcher Professional 2.9. For each image the mean temperature from spot readings from the centre of 3 fully expanded leaves from 6 plants of each genotype was calculated and a mean temperature per plant used for statistical analyses.

Root growth analysis

Seedlings were grown vertically on 0.5 x MS plates and number of emerged lateral roots and primary root lengths were recorded at 9, 10 and 11 days. Roots were then cleared (Peret et al, 2012) and mounted in 50% glycerol and stages of lateral root primordia were determined using a Leica DMRB microscope.

Statistical analysis.

Unpaired t-tests were performed using Microsoft Excel.

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Supplemental Data

Fig. S1. Alignment of FOCL1 amino acid sequence with orthologues from wheat, and *Physcomitrella patens* and with closest Arabidopsis homologue, At2g20515.

Fig. S2. Alignment of deduced amino acid sequences of FOCL1 and AGP31.

Fig. S3. Insertion positions and expression of *focl1-1* and *focl1-2.*

Fig. S4. Rosette widths of *focl1-1* and *focl1-2.*

Fig. S5. Complementation of *focl1* restores stomatal complex size to wild type.

Fig. S6. Wide view of abaxial epidermis of mature leaves of Col-0 and *focl1-2*.

Fig. S7. SEM of *focl1-1* stomate showing partial opening

Fig. S8. Deflection images for two stomata from Col-0 and *focl1-1.*

Fig. S9. Complementation of *focl1-1* and *focl1-2* restores leaf temperature to wild type.

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641

Fig. legends

Fig. 1. *FOCL1* encodes a proline-rich protein, is expressed in guard cells and roots and affects stomatal index and stomatal complex size. (A) Domain structure of the FOCL1 protein to illustrate positions of proline valine and triple proline motifs typical of HPRGs. Potentially hydroxylated prolines are indicated in green (PV context) or red (PPP context). SP = signal peptide (B-D) Histochemical staining of 2 week old Arabidopsis seedlings expressing *pFOCL:GUS*. (B) Immature leaf, (C) developing epidermis, (D) developing lateral root. (E) Stomatal index and pavement cell density of abaxial surfaces of fully expanded leaves. n = 7-9 plants, means of 3 areas from 1 leaf of each plant were compared. Representative experiment of 3 independent experiments is shown. (F and G) Images of epidermal imprints of adaxial leaf surfaces and (H and I) cleared tissue of mature leaves. Scale bars (B) 500µm, (C) 10µm, (D) 250µm, (F and G) 5µm, (H and I) 10µm. (J) Stomatal length and width and area. n = 4-7 plants, means of measurements from at least 10 stomates from one leaf of each plant were compared *= significant statistical difference from Col-0, $p<0.05$, Error bars: SD.

Fig. 2. FOCL1 is required for formation of the stomatal outer cuticular ledge. Cryo-SEM images of wild-type Col-0 (A, C, E) and *focl1-1* (B, D, F) stomata at different stages of development reveal the occlusion of mature *focl1-1* stomata by a membranous cuticle. (A and B) Both WT and focl1-1 developing stomates in have a plug of material in the pore between guard cells. (C) In larger wild-type stomata this material appears to be torn apart to reveal the stomatal pore, whereas in *focl1-1* stomata (D) the pore remains generally occluded, although some tearing to reveal a subtending pore is visible. (E) In mature WT stomata a cuticular ridge bordering the central pore is formed. (F) In mature *focl1-1* stomata the central pore can remain totally blocked by the membranous cuticular material. gc = guard cell. Bars A, B = 4µm; C, D = 5 µm; E, F = 6µm.

Fig. 3. *focl1* mutants have fused outer cuticular ledges. (A and B) Abaxial surfaces of wild-type Col-0 and *focl1* stomates imaged by VSI. Depth is indicated in nm. (C and D) AFM deflection images of stomates. (E and F) Transverse sections of stem epidermis stained with Toluidine blue. Position of outer cuticular ledges (ocl) indicated by arrows. (G and H), Adaxial leaf epidermis. Scale bars A, B, E and F = 5µm; C, D, G & H = 10µm). p = stomatal pore, ocl = outer cuticular ledge; gc = guard cell; op = occluded pore.

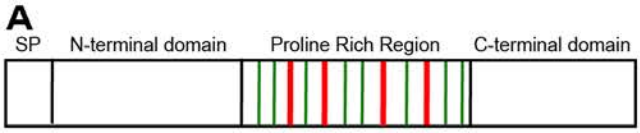
Fig. 4. A lipid rich cuticle extends across the pore of *focl1* stomata. (A) Wild-type Col-0 and (B) *focl1-1* stomatal surfaces imaged by fluorescent microscopy after staining with Nile red (which fluoresces green). (C to G) Col-0 (C, D, E) and *focl1-1* stomatal surfaces (F,G) were imaged using Raman spectroscopy over a range of wavelengths from c. 2400 to 3300nm. Maps were obtained across stomata and point scans (indicated by cross-hairs) shown for different regions. Point scans taken from the guard cell surface (C,F), the wild-type cuticular ledge (D) and from the centre of the pore region (E,H). Maps were taken from at least 3 independent biological samples, with similar results obtained in each case. Scale bar A, B = 5µm; C-G = 6 µm.

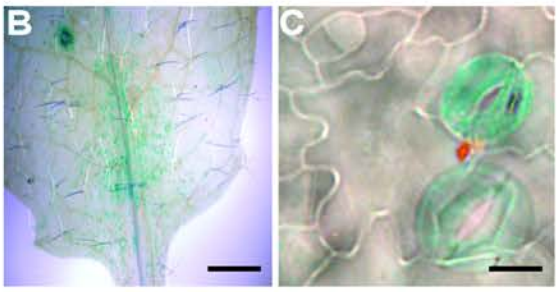
Fig. 5. FOCL1-GFP localises to the cuticular ledge. Seedlings of T2 lines of *focl1-2* expressing *pFOCL1:FOCL1-GFP* were analysed by epifluorescence microscopy. Wild-type Col-0 samples showed weak auto-fluorescence (A) compared to complemented *focl1-2* plants (B) where FOCL1-GFP signal is largely restricted to the OCL in developing (right) and mature guard cell (left). Scale bar = 15 µm.

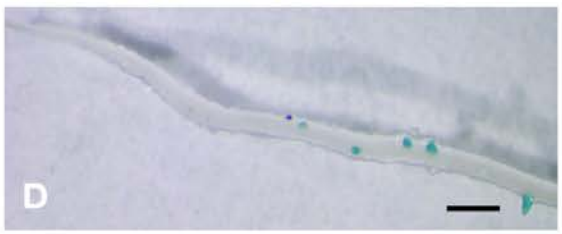
Fig. 6. *focl1* mutants have impaired transpiration and stomatal aperture control. (A) Infrared thermal images of representative mature Col-0 and *focl1* plants taken at start of experiment. (B) Time course of mean leaf temperature recorded by infrared thermography after reduction in humidity. *focl1-1* and *focl1-2* had similar temperatures throughout and are virtually indistinguishable on this graph. n = 6 plants of each genotype with measurements from 3 leaves of each plant. (C), Leaf porometry measurements of Col-0 and *focl1-2* stomatal conductance. n = 4 (1 leaf from 4 plants of each genotype). *= significant statistical difference from Col-0, $p<0.01$. (D and E) measurements of Col-0 and *focl1-1* stomatal pore widths (D), and calculated pore areas (E) following incubation with 10µM ABA. Bars with identical letters are not statistically different, $p<0.05$. n= >100 stomata. Error bars: SD. Data from one independent experiment is shown; a replicated experiment showed similar results (F) Representative images of stomata from (D and E). Scale bar: 10µm. (G) Representative images of 8 week old plants under drought conditions after water was withheld for 7 days then rewatered for 3 days.

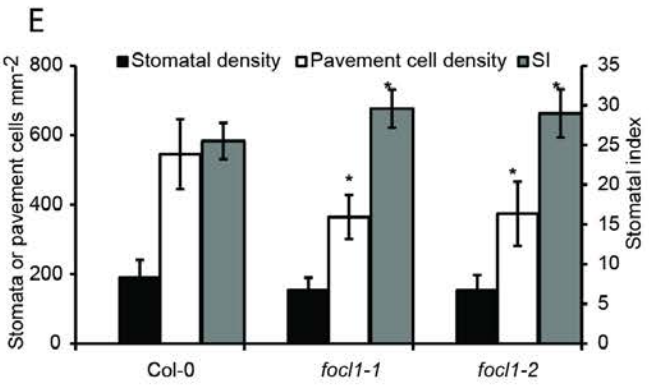
Fig. 7. FOCL1 affects root growth. (A) *GUS* expression pattern in *pFOCL1:GUS* roots. Lateral root emergence stages are indicated with Roman numerals. PR = primary root. Scale bars: 20µm. (B), images of seedlings 8 days after transfer to light for root growth analysis. Scale bar: 1cm. (C-E) Measurements of roots 11 days after light transfer: (C) lateral root

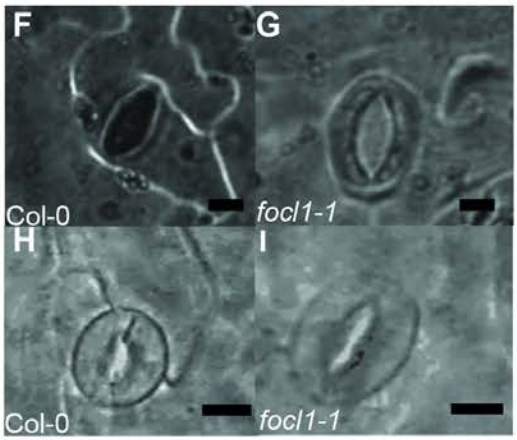
density; (D) number of lateral root branches per seedling; (E) primary root lengths. n=12 seedlings. A typical experiment from 3 independent replicates is shown, each experiment showing a similar result (F) proportion of lateral roots at each stages of primordial development. *= statistically significant from Col-0, $p<0.05$. n= 7 (Col-0) or 8 (*focl1-1*). Error Bars: SE

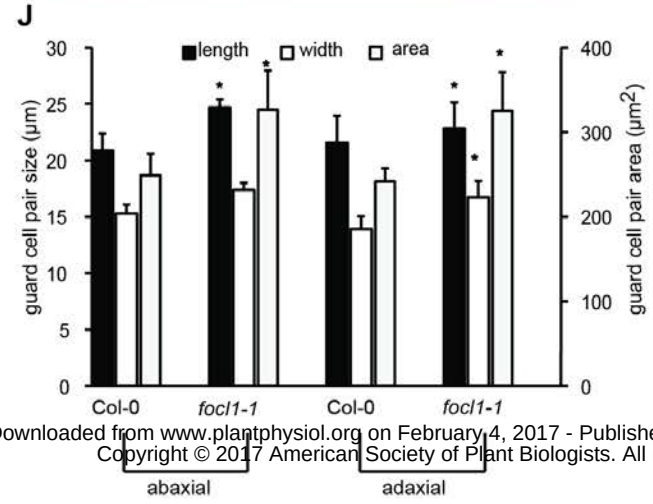


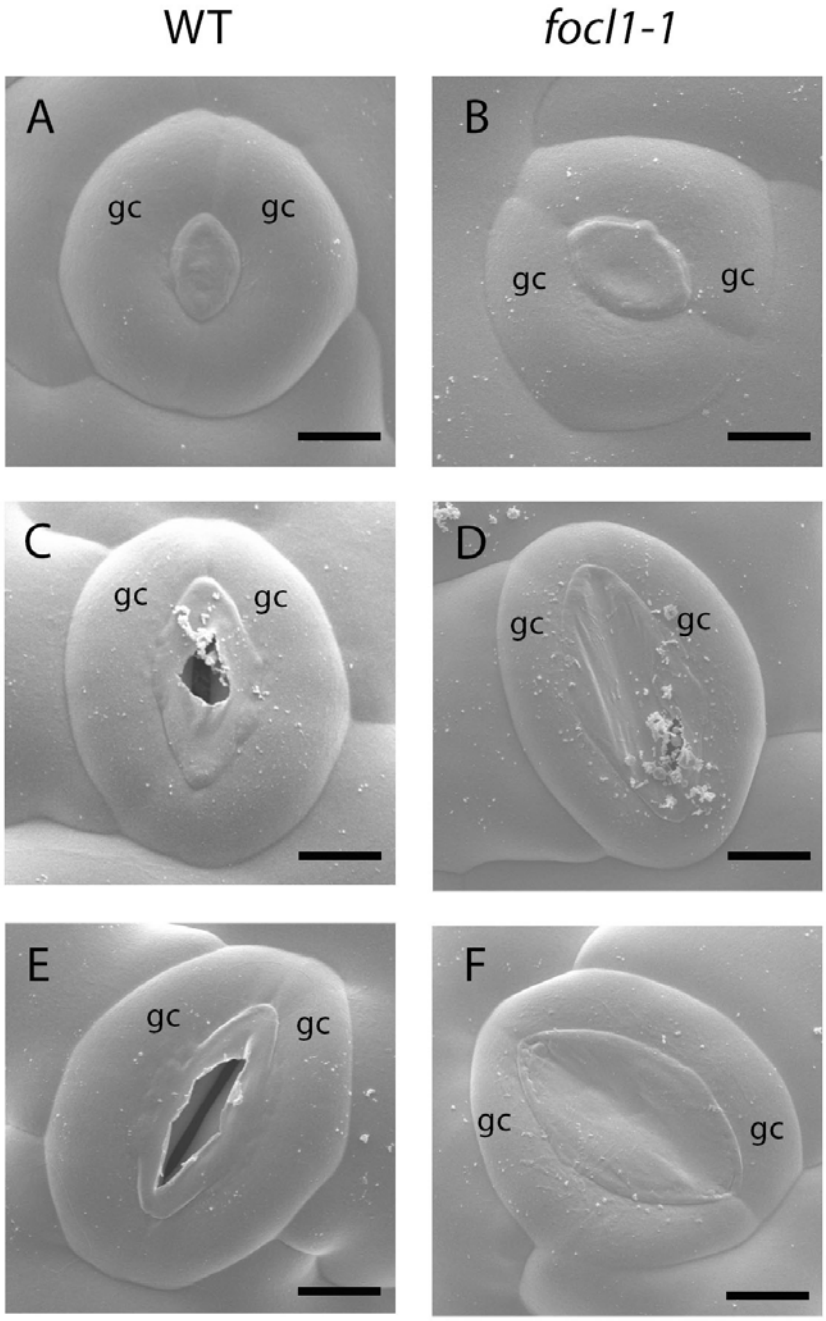


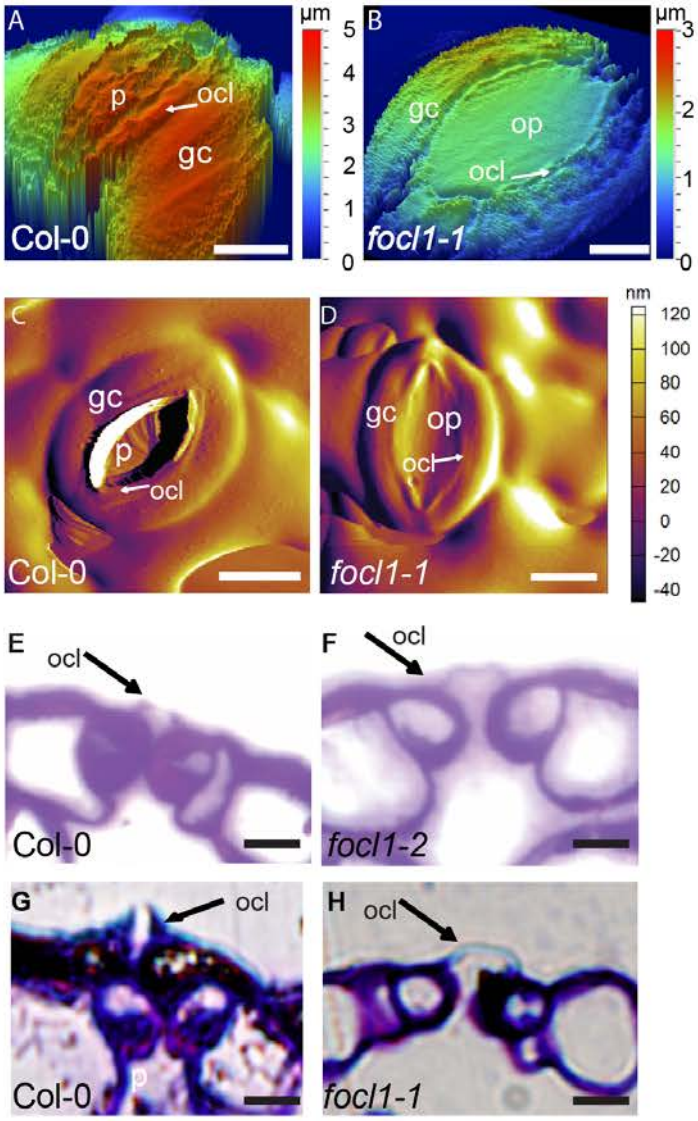


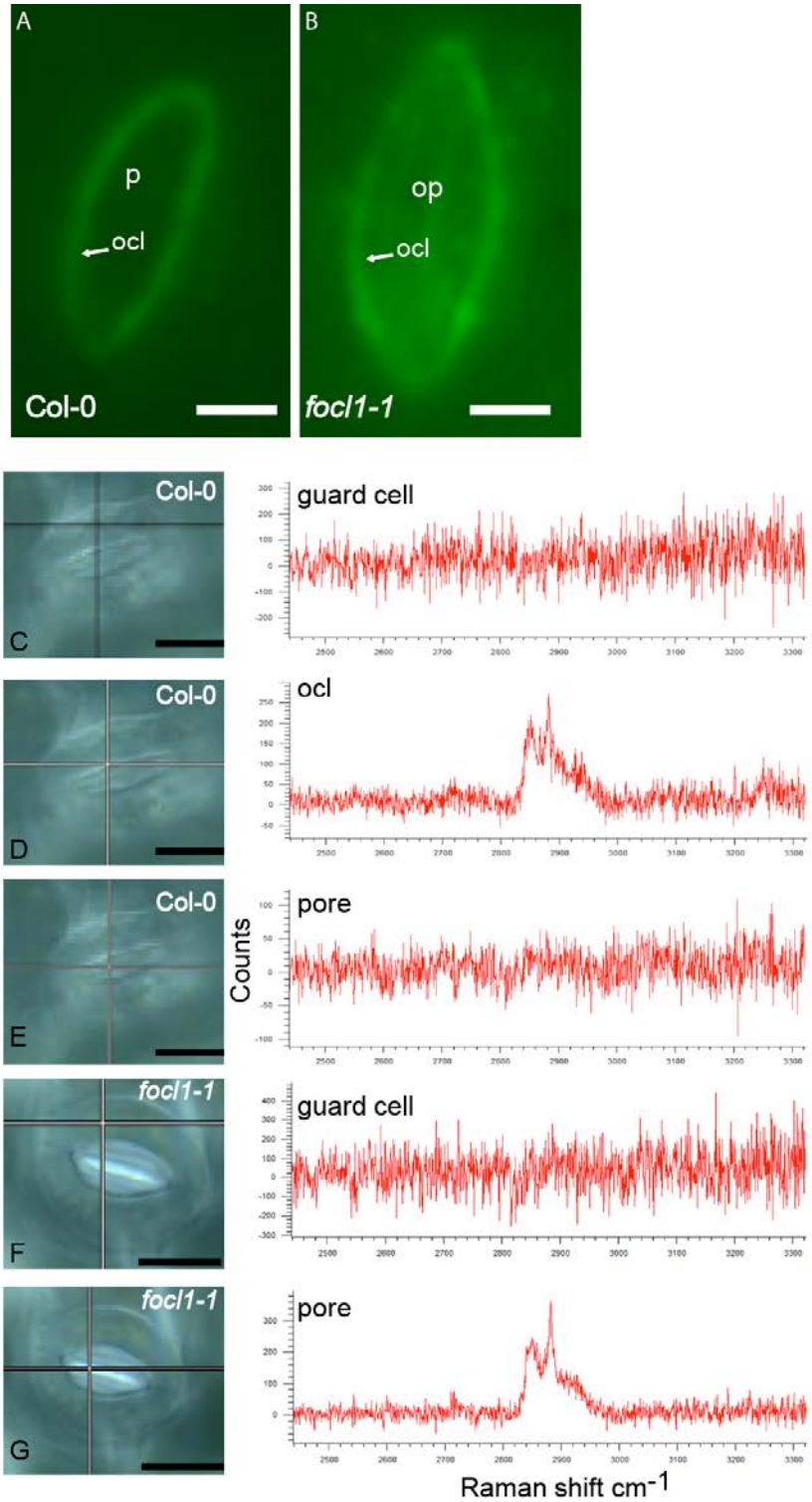


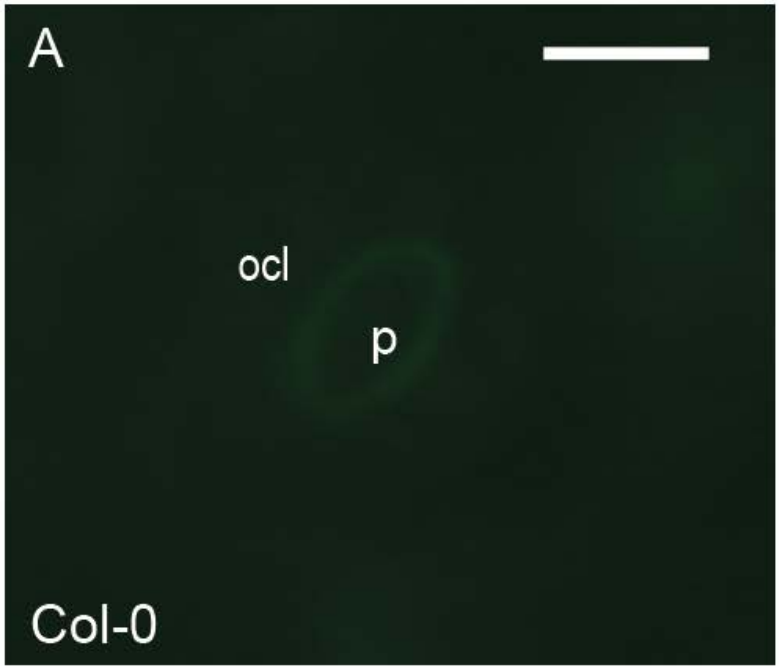


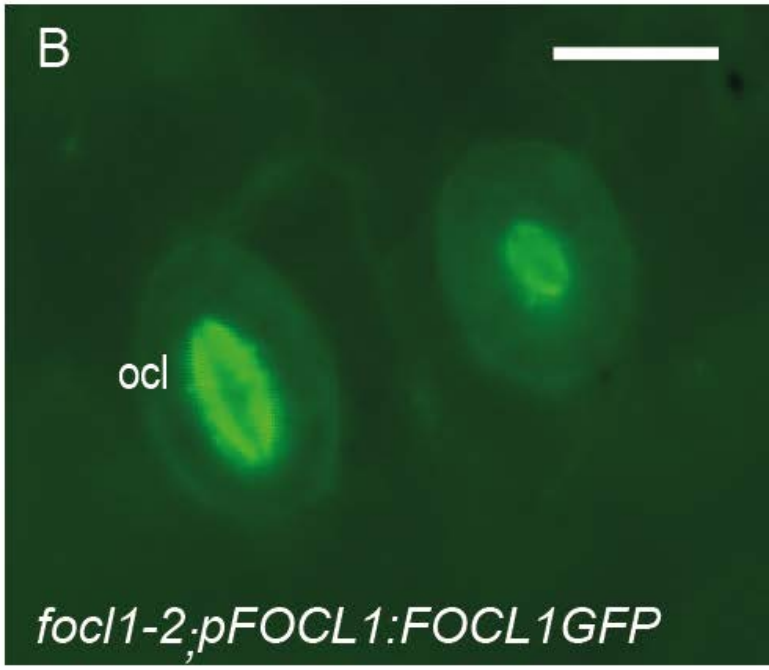


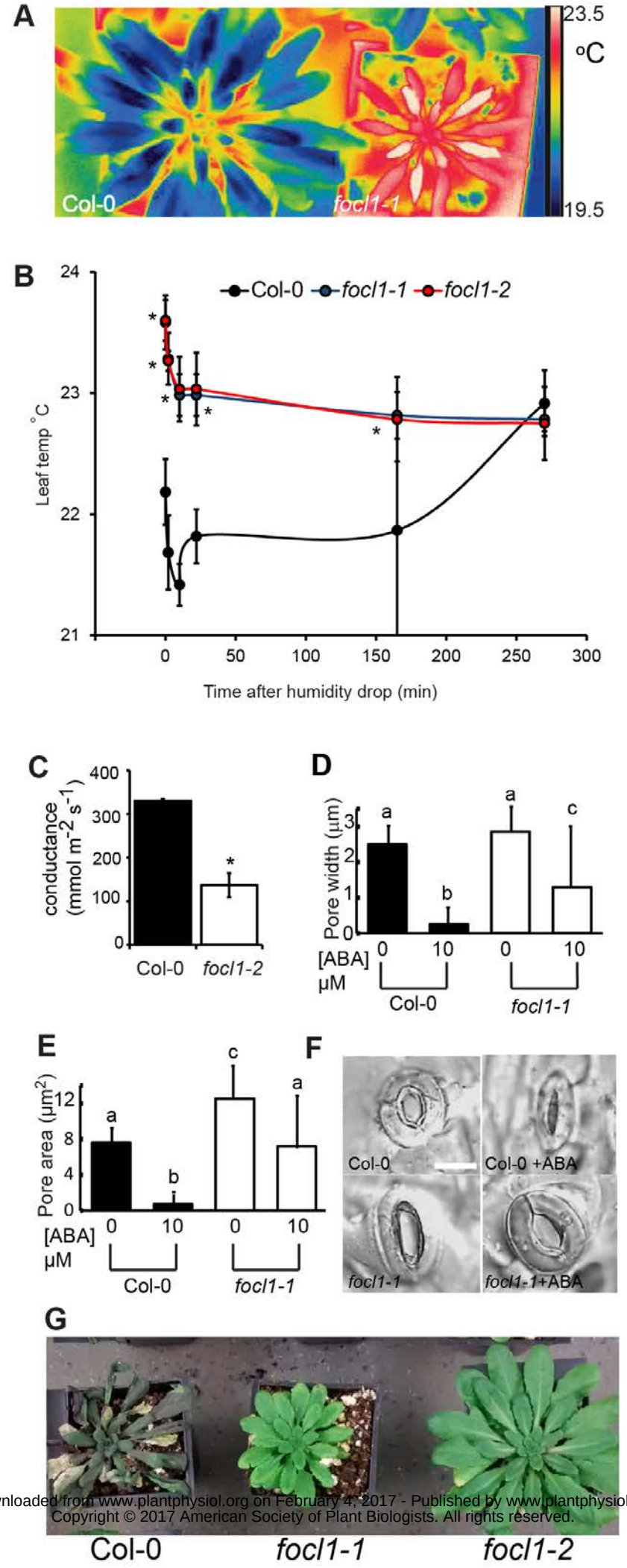


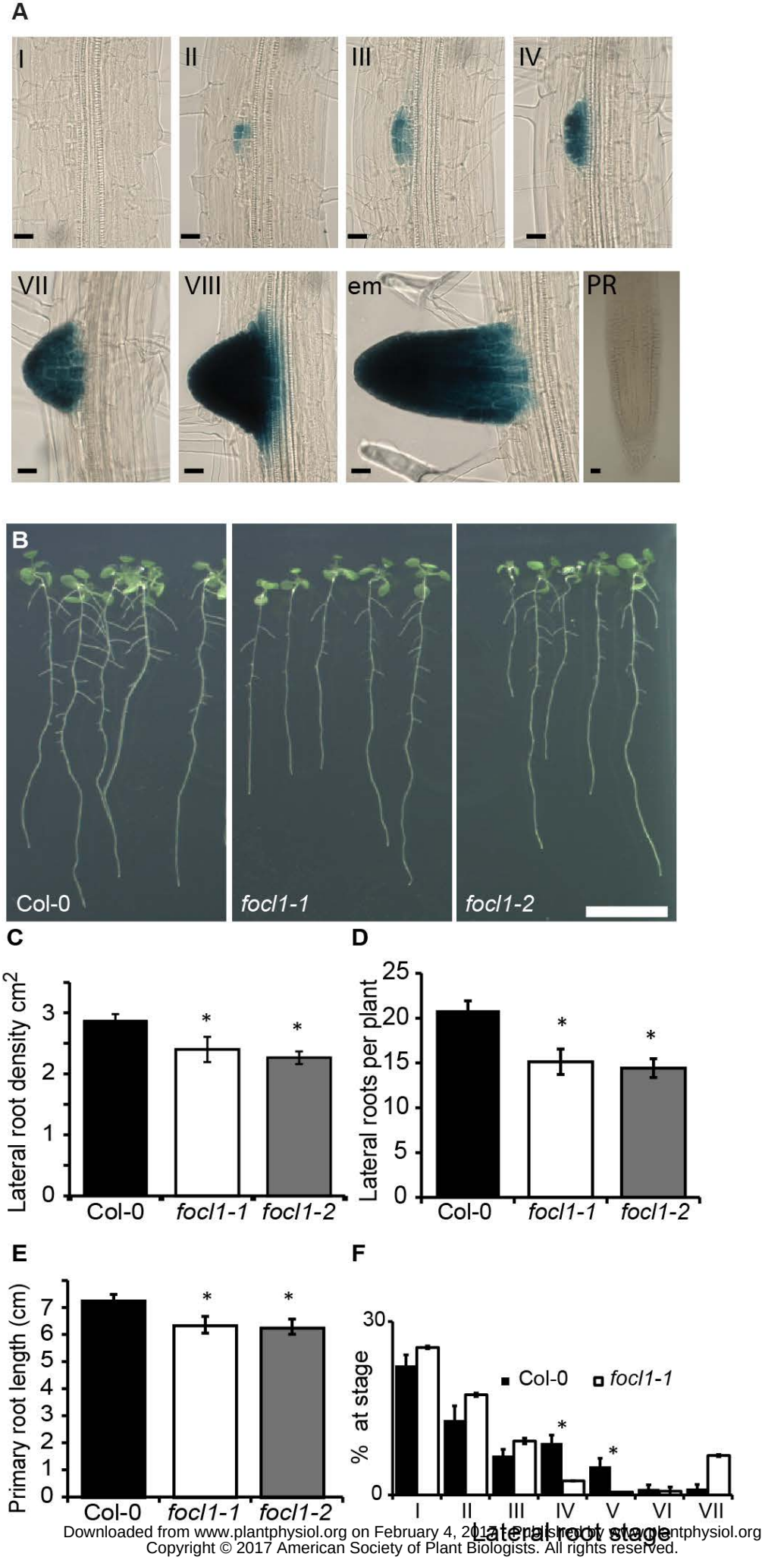












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