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Cell remodeling and subtilase gene expression in the actinorhizal plant *Discaria trinervis* highlight host orchestration of intercellular *Frankia* colonization

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Summary

- Nitrogen-fixing filamentous *Frankia* colonize the root tissues of its actinorhizal host *Discaria trinervis* via an exclusively intercellular pathway. Here we present studies aimed at uncovering mechanisms associated with this little-researched mode of root entry, and in particular the extent to which the host plant is an active partner during this process.
- Detailed characterization of the expression patterns of infection-associated actinorhizal host genes has provided valuable tools to identify intercellular infection sites, thus allowing *in vivo* confocal microscopic studies of the early stages of *Frankia* colonization.
- The subtilisin-like serine protease gene *Dt12*, as well as its *Casuarina glauca* homolog *Cg12*, are specifically expressed at sites of *Frankia* intercellular colonization of *D. trinervis* outer root tissues. This is accompanied by nucleo-cytoplasmic reorganization in the adjacent host cells and major remodeling of the intercellular apoplastic compartment.
- These findings lead us to propose that the actinorhizal host plays a major role in modifying both the size and composition of the intercellular apoplast in order to accommodate the filamentous microsymbiont. The implications of these findings are discussed in the light of the analogies that can be made with the orchestrating role of host legumes during intracellular root hair colonization by nitrogen-fixing rhizobia.

Introduction

Nitrogen-fixing root nodule symbioses occur exclusively in plants belonging to the monophyletic Eurosid clade formed by four closely-related orders of flowering plants, comprising legume (Fabales) and actinorhizal hosts (Fagales, Cucurbitales and Rosales). Despite anatomical differences between legume and actinorhizal root nodules and the different nature of the bacterial microsymbiont (either rhizobial proteobacteria or filamentous *Frankia* actinobacteria) there are nevertheless a number of important shared features relating to the establishment of these two types of nitrogen-fixing symbiotic associations. These include a central role in microbe–host communication for the highly conserved host symbiotic signaling pathway known as the CSSP (which also is shared with the ancient and widespread arbuscular mycorrhizal association), as well as similarities in the various strategies which allow the respective microsymbionts to initially enter and colonize the outer root tissues without eliciting host defense responses (reviewed in Svistoonoff *et al.*, 2014; Ibáñez *et al.*, 2016).

Different processes associated with symbiotic microbe root entry have been described for both legume and actinorhizal host plants. The most common and the best studied of these in model legumes is known as the root hair 'intracellular' pathway. This mode of root colonization initiates with the re-orientation of root hair tip growth leading to curling and the physical enclosure of the rhizobia between host cell walls. Host cell wall remodeling and exocytosis then creates a specialized compartment around the enclosed bacterial microcolony (Fournier *et al.*, 2015). Finally,

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progressive polarized plasma membrane invagination elaborates the tubular conduit known as the infection thread (IT), through which the bacteria gain access to the outer root tissues and subsequently to the developing nodule (Fournier *et al.*, 2008). This highly regulated transcellular invasion process is apoplastic because the microsymbiont is maintained within a specialized cell wall/plasma membrane interface throughout. For actinorhizal hosts, intracellular root hair-mediated colonization of filamentous *Frankia via* infection threads also has been described for various Fagales genera including *Casuarina* and *Alnus* (Callaham *et al.*, 1979; Berry *et al.*, 1986; Berg, 1999).

However, initial root colonization of both legume and actinorhizal hosts also can occur in the absence of root hair curling and associated infection thread formation (reviewed in Pawlowski & Demchenko, 2012; Svistoonoff *et al.*, 2014; Ibáñez *et al.*, 2016). In the case of certain legumes, the microsymbiont can exploit either natural openings in outer root tissues – resulting, for example, from lateral root emergence (generally referred to as 'crack entry') – or penetrate the intact epidermis *via* the apoplastic interface between adjacent host cells (referred to as 'intercellular' colonization). In certain cases, infection threads can be formed at a later stage during subsequent cortical cell colonization. Although initially thought to be restricted to several atypical plants adapted to flooded habitats, evidence now suggests that these alternative nonroot hair modes of colonization exist for at least 25% of legume genera. For those actinorhizal hosts studied so far, apoplastic intercellular penetration of the root outer tissues was first described for Rosales genera, notably *Elaeagnus* (Miller & Baker, 1985) and *Ceanothus* (Liu & Berry, 1991a,b). In neither case were infection threads observed during later stages of cortical colonization. Interestingly, the only nonlegume *Rhizobium* host *Parasponia* is also a member of the Rosales order and is colonized *via* a mixture of intercellular and crack entry modes of root entry (Lancelle & Torrey, 1984).

The current lack of knowledge about the molecular and cellular mechanisms associated with nonroot hair microsymbiont colonization incited us to focus on the symbiotic association between *Frankia* and the Rosales species *Discaria trinervis*, where it has been shown that the initial stages of *Frankia* root entry also are exclusively intercellular (Valverde & Wall, 1999). As for *Elaeagnus* and *Ceanothus,* intracellular infection in *D. trinervis* is only observed later during nodule development when the microbial filaments colonize the nodular lobe parenchyma and elaborate vesicle clusters for nitrogen fixation (Valverde & Wall, 1999). The recent development of *Agrobacterium rhizogenes*-mediated root transformation for *D. trinervis* (Imanishi *et al.*, 2011) now makes this actinorhizal host an attractive model for studying the mechanisms of apoplastic intercellular root colonization. However, because reporter-expressing *Frankia* are not currently available and there are no morphological indicators which permit the identification of sites of intercellular *Frankia* root entry, host molecular markers specific for *Frankia* infection represent highly valuable tools in such studies.

Although molecular analyses of the *Frankia*/*D. trinervis* association are relatively recent (Svistoonoff *et al.*, 2013; Imanishi *et al.*, 2014), a number of actinorhizal genes associated with intracellular infection have been identified for the model host *Casuarina glauca*. In particular, it has been shown that the *Cg12* gene, encoding a putative apoplastic subtilisin-like serine protease, is strongly and specifically expressed in host tissues undergoing intracellular *Frankia* infection as well as in infected cells of the *C. glauca* prenodule and nodule (Laplaze *et al.*, 2000; Svistoonoff *et al.*, 2003). In this article we demonstrate that the promoter sequences of both *Cg12* and the orthologous *D. trinervis* gene (*Dt12*) are transcriptionally activated in *D. trinervis* root tissues associated with the earliest stages of *Frankia* colonization. In addition, SYTO 9-staining of *Frankia* filaments within root sections expressing fluorescent reporters under the control of the *Cg12* promoter confirm the correlation between reporter expression and intercellular *Frankia* progression within the *D. trinervis* outer root cortex. Furthermore, confocal microscopy of intact roots undergoing *Frankia* colonization has revealed major intracellular reorganization of the host cells immediately adjacent to sites of *Frankia* colonization of the root cortex, associated with structural modifications to the cell–cell interface. Together, these observations provide strong evidence that the host plant plays a determining role during initial colonization of the root intercellular apoplast by symbiotic filamentous nitrogen-fixing *Frankia*, and we discuss to what extent parallels can be drawn with the well-characterized legume root hair intracellular entry mechanisms.

Materials and Methods

Plant material, growth conditions and *Agrobacterium* transformation

Seeds of *Discaria trinervis* (Hooker *et* Arnot) Reiche (Valverde & Wall, 1999) were chemically scarified for 12 min in 95% H_2SO_4, washed several times in water, surface-sterilized for 2 min in 3.2-% sodium hypochlorite followed by several washes in sterile water, and finally placed on Fåhraeus agar plates supplemented with 1 mM NH_4NO_3 at 4°C for 3–5 d. Plates were then transferred to 24°C (shaded from light) and 4–7 d later young plants with 0.5–1 cm-long roots were transferred to Evans 1/10 agar plates (Valverde & Wall, 1999) supplemented with 360 μM NH_4NO_3. Plantlets were then grown for an additional 10–13 d in a growth chamber at 24°C with 16 h : 8 h, day : night photoperiod and 70 μmol m^{-2} s^{-1} light intensity.

For the experimental data presented in Fig. 1, *Agrobacterium rhizogenes*-mediated transformation of *D. trinervis* was performed using the protocol described in Imanishi *et al.* (2011). For the remaining experiments the following modified *in vitro* protocol was used. The ARqua1 strain of *A. rhizogenes* (Quandt *et al.*, 1993) was grown for 24 h at 28°C in liquid tryptone-yeast extract-$CaCl_2$ with appropriate antibiotics. The culture was centrifuged, washed and concentrated to a thick paste. Inoculation was carried out by stabbing the hypocotyls two or three times with a thin needle (0.4 mm/27G) dipped in the ARqua1 paste. Plants were then grown for a further 2–3 wk on fresh Evans 1/10 agar plates supplemented with 360 μM NH_4NO_3 and, when applicable, emerging transgenic roots screened for either YFP

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(yellow fluorescent protein), mCherry or DsRed fluorescence under a stereomicroscope (Axio Zoom.V16; Zeiss) equipped with adequate filters. Once the fluorescent roots were 2–3 cm in length, the main root was excised and the composite plants transferred to hydroponic *in vitro* growth on a modified Broughton and Dilworth (mBD) liquid nutrient solution (pH 6.7) supplemented with 5 mM KNO_3 as nitrogen source (Broughton & Dilworth, 1971; Svistoonoff *et al.*, 2010). When fluorescent roots were *c.* 8–10 cm long, plants were transferred to *ex vitro* growth conditions in 1-l pots filled with liquid mBD solution as described in Svistoonoff *et al.* (2010) and grown in chambers at 25°C with a 16 h : 8 h, day : night photoperiod and 380 μmol m^{-2} s^{-1} light intensity.

Frankia culture conditions and host inoculation protocols

Frankia BCU110501 (Chaia, 1998) was cultivated at 28°C in the dark in a modified BAP medium (Valverde & Wall, 1999) supplemented with 20 mM glucose. To prepare the inoculum, *Frankia* filaments were pelleted by centrifugation of a 7 d-old culture (5000 ***g*** for 5 min), washed in sterile water and then fragmented by repeated passages through 0.8 mm/21G and 0.5 mm/26G needles. For liquid inoculation of composite *D. trinervis* plants, the suspension of fragmented filaments was pipetted directly onto the root system in a 15 cm Petri dish. After 3–4 h the plants were returned to hydroponic culture pots. Alternatively, for localized root inoculation, we used a modified version of the technique described in Obertello & Wall (2015) where plants are grown in pouches (Valverde & Wall, 1999) and inoculation performed using *Frankia* embedded in agar strips (0.7% agar with 1.5 μl ml^{-1} of fragmented *Frankia* suspension).

Cloning of *Dt12* and construction of Pro*Dt12*/*Cg12* fluorescent reporters

The *D. trinervis Dt12* gene was identified using PCR primers based on a sequence alignment of the *Cg12* and *Ag12* genes from *Casuarina glauca* (Laplaze *et al.*, 2000) and *Alnus glutinosa* (Ribeiro *et al.*, 1995), respectively. PCR amplification using as template *D. trinervis* genomic DNA and the primers Cg12Fwd2 (5-GGAACAGCAAGAGGCATTGC-3) and Cg12Rev (5-CATAGCAGATTGATGTAGTCTT-3) led to the isolation of a 1185-bp PCR product showing sequence similarity to the *Cg12*/*Ag12* genes. The full-length DNA sequence of *Dt12* (GeneBank accession no. MG920845) was obtained using the Universal Genome Walker kit (TBUSA, Mountain View, CA, USA) and the following pairs of forward and reverse primers:

Dt12_GSP1_5′ (5-CTTTGGGCATAATGGACTTGTCTG TGTG-3), Dt12_GSP2_5′ (5-CTACTGGTGTTGCTGAA TTAGCATGGAG-3),

Dt12_GSP1b_5′ (5-CTGTCCGAACTAAAGAAGCAACT TCTCC-3), Dt12_GSP2b_5′ (5-CAGTGAAACGATATGG GCAATTCCATAGG-3),

Dt12_GSP1_3′ (5- ATCCCGGTTTGATCTACGATGC TACTCC-3), Dt12_GSP2_ 3′ (5-CAAGCATTGTTCAT TGGGACATCAGAACG-3).

A MUSCLE alignment and a maximum-likelihood phylogeny were performed using protein sequences corresponding to Dt12 and other characterized plant subtilases described in Taylor & Qiu (2017) using the phylogeny.fr pipeline (Dereeper *et al.*, 2008) with default parameters (see Supporting Information Fig. S1). For constructing the Pro*Dt12:GFP* reporter a 1500 bp genomic DNA fragment upstream of the *Dt12* start codon was amplified by PCR using primers ProDt12_Fwd (5-CTCGGTCTCGaaatTACCAGATCAGTTTCTCCATGT-3) and ProDt12_Rev (5-CTCGGTCTCAtgatCCTGTAGCTAA ACTTTTGCTT-3) using a high-fidelity polymerase (Phusion; NEB, Ipswich, MA, USA). This promoter fragment, together with the *GFP* (GFP, green fluorescent protein) reporter, was introduced into the pDISC binary vector (Fliegmann *et al.*, 2013) *via* Golden Gate cloning (Engler *et al.*, 2008). The *GFP* coding sequence was amplified with the pair of primers GFP-ProDt12_Fwd (5-CTCGGTCTCGatcaATGGTGAGCAAGGGCGAGGA-3) and GFP_Rev_GG (5-CTCGGTCTCGcgtaTTACTTGTACAGCT CGTCCATGCC-3) using the pHKN29 binary vector (Kumagai & Kouchi, 2003) as template.

The Pro*Ubi:mCherry-ER*/Pro*Cg12:NUP-YC2.1* double construct was generated by multiple cloning steps. The Pro*Ubi:mCherry-ER* sequence was first introduced into the pDISC binary vector using the Golden Gate cloning system. The Pro*Cg12:NUP-YC2.1* sequence was then synthesized by GeneCust (Ellange, Luxembourg) and cloned into the *Xho*I site. The synthetic cassette Pro*Ubi:NUP-YC2.1*/Pro*Cg12:mCherry* was synthesized by GeneCust and cloned into the *Sal*I site of the pBinPlus binary vector (van Engelen *et al.*, 1995).

Histochemical GUS staining in *Discaria* roots and root sections and visualization in EM sections

For the detection of β-Glucuronidase (GUS) activity, roots and nodules from plants expressing Pro*Cg12:GUS* (Svistoonoff *et al.*, 2003) were stained in a 50 mM phosphate buffer (pH 8.0) containing 1 mM X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide), 1 mM Na_2 EDTA, 0.5% Triton X-100 and 2 mM of both $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$. Explants were incubated at 37°C for 12 h and then fixed for 12 h in a 50 mM phosphate buffer (pH 7.0) containing 4% paraformaldehyde, 0.25% glutaraldehyde. After fixation, explants were progressively dehydrated with ethanol (10%, 30-%, 50%, 75%, 90% and 100%, 30 min each), embedded in Technovit 7100 (Heraus-Kulser, Wehrheim, Germany) and sliced into 4–6 μm thick sections using a Leica HM355S microtome. Whole root segments and thin sections were viewed with a DMI6000B (Leica Microsystems, Nanterre, France) or DMRB (Leica) microscope, and images were acquired using either a DFC295 (Leica) or a MP5 (Qimaging, Surrey, BC, Canada) camera. The *A. rhizogenes*-transformed roots from *c.* 25 *D. trinervis* plants expressing Pro*Cg12:GUS* were analysed for spatiotemporal transgene expression patterns during different stages of infection/nodulation following *Frankia* inoculation. This overall analysis was repeated at least three times and representative images are presented in Fig. 1(a,

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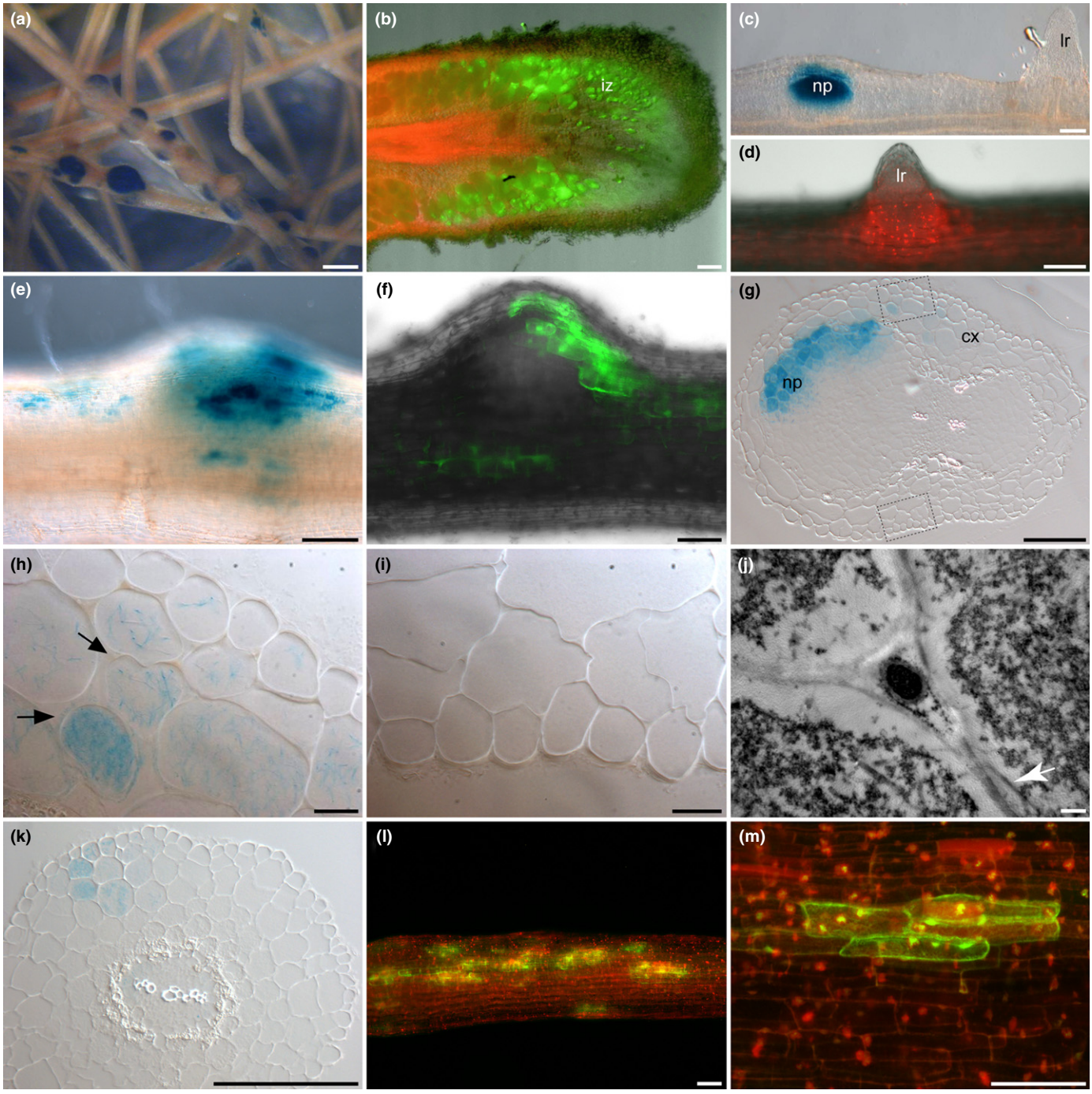

Fig. 1 *Cg12* and *Dt12* promoters are activated in *Discaria trinervis* roots after inoculation with *Frankia* and during nodule development. *D. trinervis* composite plants expressing either Pro*Cg12*:*GUS* (a, c, e, g–k) or Pro*Dt12*:*GFP*/Pro*Ubi10*:*DsRed* (b, d, f, l, m) were inoculated with *Frankia* and observed at different time points either as intact roots or sections of fixed material. (a) Roots expressing Pro*Cg12*:*GUS* in emerging nodules 2 wk after *Frankia* inoculation. (b) Pro*Dt12*:*GFP* expression in a longitudinal section of a mature *D. trinervis* nodule lobe. GFP fluorescence is restricted to cortical cells of the infection zone as described by Valverde & Wall (1999). (c, d) Pro*Cg12*:*GUS* (c) or Pro*Dt12*:*GFP* (d) expression is detectable only in developing nodules, not in lateral roots. (e, f) Early nodule development (10–15 d post-inoculation) showing patchwork expression of either Pro*Cg12*:*GUS* (e) or Pro*Dt12*:*GFP* (f) primarily located on one side of the emerging nodule. (g) Semi-thin transversal section of an inoculated Pro*Cg12*:*GUS* root with typical bipolar nodule development. GUS expression is lower in the root cortex (upper dashed box) compared to the developing nodule parenchyma. (h) Magnification of upper boxed region in (g) showing GUS expression correlated with enlarged intercellular apoplastic spaces (arrows) in a putative zone of intercellular infection. (i) Magnified area of root cortex (lower boxed region in g) without GUS expression and without intercellular apoplastic space enlargement. (j) Transmission electron microscopy image of a sectioned *Frankia* filament within the intercellular space between Pro*Cg12*:*GUS*-expressing cells. Putative GUS crystals are indicated in the host cortical cells (arrow). (k) Transverse root section of *D. trinervis* showing early Pro*Cg12*:*GUS* expression in the epidermis and outer root cortex without visible nodule development. (l, m) Early expression of Pro*Dt12*:*GFP* in *D. trinervis* roots (5–7 d after inoculation) observed in small patches comprising several cells in outer root tissues at two different magnifications. cx, cortex; lr, lateral root; np, nodule primordia. Bars: (a) 500 μm; (b–g, k–m) 100 μm; (h, i) 10 μm; (j) 0.5 μm. GUS, β-Glucuronidase; GFP, green fluorescent protein.

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c,e). For different developmental stages, thin sectioning was performed using at least five independent roots for each experiment (Fig. 1g–i,k).

Based on the above analyses, GUS-expressing regions from three independent roots were selected for more detailed ultrastructural TEM studies (Fig. 1j) as described previously by Valverde & Wall (1999). Samples were post-fixed with osmium tetroxide (20 g l^{-1}), progressively dehydrated with ethanol (50%, 70%, 80%, 95% and 100%) and embedded in Epon-Araldite resin (3 d at 70°C). Ultrathin sections were stained with 20 g l^{-1} uranyl acetate and 20 g l^{-1} lead citrate for 2–3 min each, and then analyzed in a Jeol (Tokyo, Japan) JEM 1200 EX II transmission electron microscope.

Fluorescence reporter imaging in intact roots and in root sections

Intact roots and nodules of *Frankia*-inoculated composite *D. trinervis* plants expressing Pro*Dt12:GFP* were imaged using a Leica DMI6000B microscope equipped with the GFP (470–40 nm excitation/525–50 nm emission) and N2.1 (515–570 nm excitation/590 nm LP emission) filter cubes, and a Leica DFC365FX camera. Roots expressing Pro*Dt12:GFP* were also imaged using a C1 confocal microscope (Nikon, Melville, NY, USA) with a PlanApo ×20 vc 0.75 objective. Excitation of GFP and DsRed were performed at 488 and 543 nm and the emitted light was captured at 515/530 and 605/675 nm, respectively. Transformed roots from at least 20 plants were analysed in detail for Pro*Dt12:GFP* expression following *Frankia* inoculation (Fig. 1b,d,f,l,m).

Roots of *Frankia*-inoculated composite *D. trinervis* plants expressing Pro*Ubi:mCherry-ER*/Pro*Cg12:NUP-YC2.1* and Pro*Ubi:NUP-YC2.1*/Pro*Cg12:mCherry* were monitored and imaged using a Zeiss AxioZoom V16 stereomicroscope (Fig. S2). For SYTO 9-staining of *D. trinervis* root sections, 0.4–0.5 cm-long root segments were excised from either control or inoculated plants, embedded in 4% low gelling temperature agarose, and then sectioned (100–200 µm-thick) using a vibrating-blade microtome (Leica VT1000 S). Sections collected on glass slides were stained with 5 µM SYTO 9 (Molecular Probes, Eugene, OR, USA) and immediately imaged (Figs 2, S3).

Root sections and selected infection sites on intact roots were imaged with a Leica SP8 confocal laser scanning microscope equipped with a long-distance 25× HC Fluotar L NA 0.95 water-immersion objective. The argon laser bands of 488 and 514 nm were used to excite SYTO 9 and YFP, respectively, and a 561 nm diode to excite mCherry. Specific emission windows used for SYTO 9, YFP and mCherry were 500–530, 525–550 and 600–630 nm, respectively, and emitted fluorescence was false-colored in green (SYTO 9 or YFP) or red (mCherry). The confocal images shown are either single sections, maximal projections of selected planes of a z-stack, orthogonal sections of a z-stack or 3D-reconstructions of confocal image stacks. Images were acquired using ZEISS Efficient Navigation (ZEN; Carl Zeiss microscopy, GmbH) for epifluorescence/stereomicroscope images or Leica LAS AF (Leica Microsystems CMS GmbH) for confocal images and processed using ZEN, Leica LAS AF LITE, FIJI (https://fiji.sc, Schindelin *et al.*, 2012) or VOLOCITY v.6.0.1 (Perkin-Elmer, Waltham, MA, USA) software.

The analyses of the SYTO 9-stained sections presented in Figs 2 and S3 are based on a total of 64 sections (including the negative controls). Intercellular *Frankia* filaments were identified in 23 sections from six independent *Frankia*-inoculated plants, and, amongst these, four sections (three independent plants) revealed filaments crossing the epidermis and four sections (two independent plants) provided information directly linking Pro*Cg12*-expression with the presence of adjacent colonizing *Frankia* (Fig. 2a). It should be noted that Pro*Cg12:mCherry* expression will only be detected if the cell is intact and close to the sectioned surface. Results shown in Fig. 2(c–f) relating to *Frankia* intercellular colonization of nodule tissues are representative of a total of three sections (two independent plants). This limited analysis was performed primarily to confirm that the stained *Frankia* filaments could be visualized during all stages of intercellular colonization, and confirm previously published findings in *Elaeagnus* (Miller & Baker, 1985).

Concerning the *in vivo* confocal experiments illustrating host cell responses occurring at putative *Frankia* colonization sites within *D. trinervis* root outer tissues (Figs 3, S2, S4, S5), out of 40 Pro*Cg12*-labeled sites analysed (10 independent plants for each construct), 31 showed one or more putative epidermal entry sites (Figs S4, S5) and 21 showed major cortical cell remodeling associated with enlarged apoplastic interfaces (Fig. 3).

Results

Cg12 and *Dt12* promoter expression correlates with *Frankia* root colonization sites in *D. trinervis*

Exploratory experiments introducing the Pro*Cg12:GUS* reporter into *D. trinervis* roots *via A. rhizogenes*-mediated transformation had revealed strong GUS expression in root nodules following *Frankia* inoculation (Fig. 1a). This prompted us to look for the ortholog of *Cg12* encoding the equivalent subtilase in *D. trinervis*. PCR amplification and the use of primers based on conserved regions of both *Cg12* and the *A. glutinosa* ortholog *Ag12*, led to the isolation of the *Dt12* gene (see the Materials and Methods section). The corresponding Dt12 protein shares an overall similarity of 77% with both Cg12 and Ag12 (Fig. S1a). A maximum-likelihood phylogeny performed with previously characterized plant subtilases (Taylor & Qiu, 2017) further showed that Dt12 and other actinorhizal symbiosis-related subtilases cluster together in a well-supported group which also includes two *Medicago truncatula* proteins (Fig. S1b). A transcriptional fusion between the *Dt12* promoter and the fluorescent GFP reporter was then constructed (see the Materials and Methods section) in order to study spatiotemporal expression of the *Dt12* gene throughout *Frankia* infection/nodulation and to compare this with Pro*Cg12:GUS* expression in *D. trinervis*. The Pro*Dt12:GFP* construct includes a *DsRed* gene driven by the *Ubiquitin10* constitutive promoter to facilitate the identification of the genetically-transformed roots.

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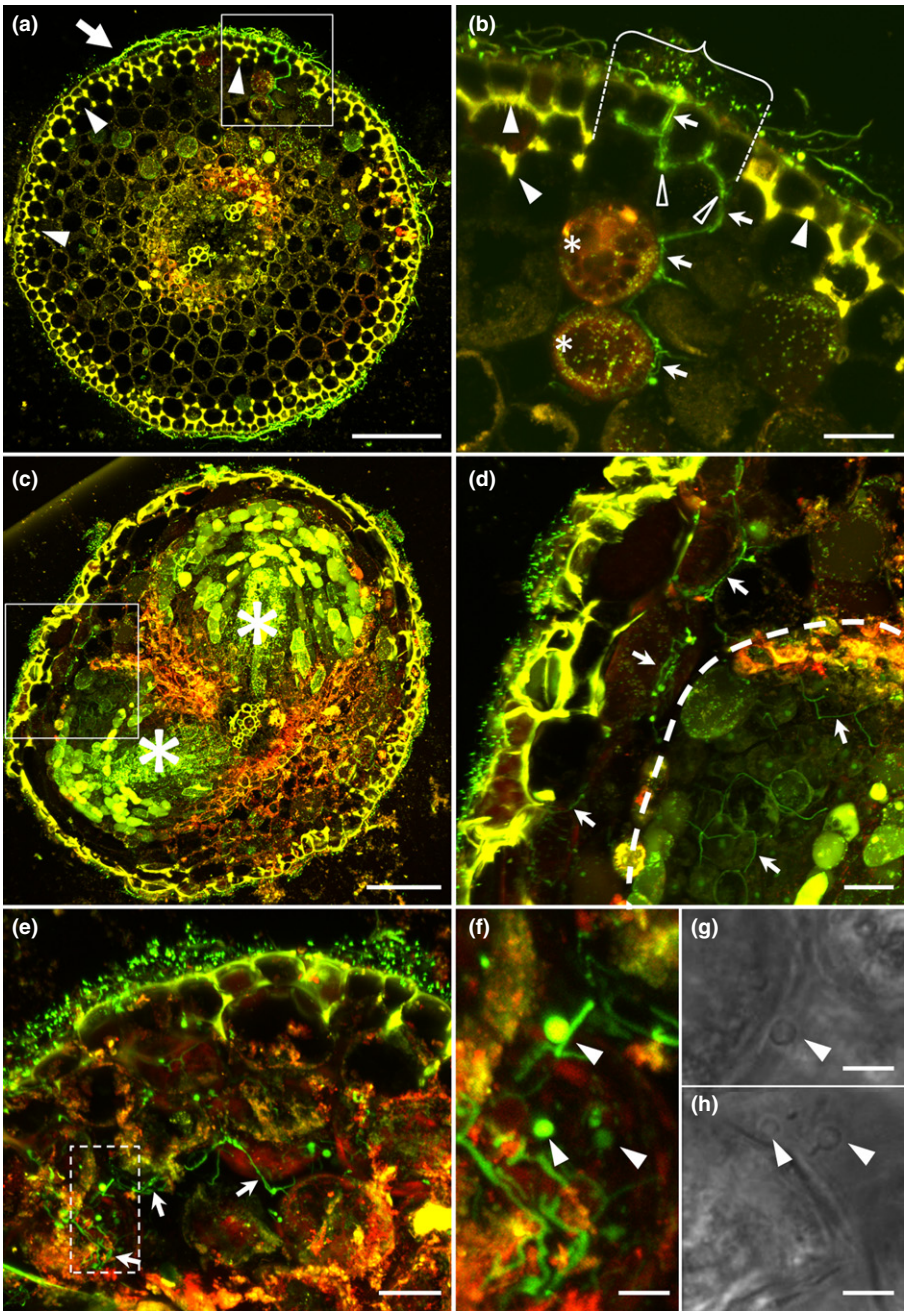

Fig. 2 Detection of intercellular *Frankia* filaments in colonized roots of *Discaria trinervis*. *D. trinervis* composite plants expressing either Pro*Ubi*:*NUP-YC2.1*/Pro*Cg12*:*mCherry* (a, b) or Pro*Ubi*:*mCherry-ER*/Pro*Cg12*:*NUP-YC2.1* (c–h) were inoculated with *Frankia*. SYTO 9 staining was used to visualize *Frankia* filaments within 100–200 μm transverse sections obtained from root zones with either small groups of Pro*Cg12*-expressing cells (a, b) or young developing nodules (c–h). (a, b) Images of a whole root section (a) and the framed magnified region (b) show extensive aggregates of extraradicular *Frankia* filaments (in green; arrow in a). The bracketed region in (b) highlights a zone where *Frankia* filaments have colonized the apoplast of both epidermal and outer cortical cell layers (arrows in b). Two of the cortical cells in close proximity to *Frankia* filaments are expressing mCherry (asterisks in b). In addition, the intense yellow fluorescence located at epidermal/cortical cell junctions (arrowheads in a, b) is absent from those junctions colonized by *Frankia* filaments (open arrowheads in b). (c, d) A transverse section across two developing nodules (asterisks in c) reveals intercellular *Frankia* filaments both in the outer cortical tissue and the developing nodule cortex (arrows on both sides of the dashed line in the magnified image d). (e–h) Small spherical SYTO 9-stained structures are visible in the magnified image (arrowheads in f) corresponding to the framed area of the *Frankia*-colonized section depicted in (e). Bright-field imagery shows that these structures resemble typical *Frankia* vesicles (arrowheads in g, h). Images in (a–f) are z-projections of confocal image stacks, combining the SYTO 9 fluorescence (in green) and mCherry fluorescence (in red). In these samples, bright yellow/orange corresponds to SYTO 9-stained apoplastic material. Bars: (a, c) 100 μm; (b, d, e) 20 μm; (f–h) 5 μm.

Both *Cg12* and *Dt12* promoters were found to be strongly activated in mature nodule tissues, especially in the nodule infection zone (Fig. 1b) whereas GUS/GFP reporters were undetectable in developing lateral roots (Fig. 1c,d). In addition to a standard *Frankia* liquid inoculation procedure, we also made use of an agar-strip protocol that allows *Frankia* targeting to

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specific *D. trinervis* root zones (Obertello & Wall, 2015) in order to facilitate the study of the early stages of symbiotic root colonization/nodulation. Pro*Cg12*:*GUS* and Pro*Dt12*:*GFP* expression were both detected exclusively in root zones in contact with *Frankia* inoculum and most strongly in developing nodules. At early stages of nodule emergence, reporter expression was often quite patchy and restricted to one side of the developing organ (Fig. 1e,f). In a section from a young nodule expressing Pro*Cg12*:*GUS*, weaker reporter activity also could be observed within the root cortex immediately adjacent to the emerging nodule (Fig. 1g), suggesting that colonizing *Frankia* may be entering from one side of the nodule. Detailed observation of this region further revealed that GUS expression in the cortex is associated with enlarged intercellular spaces (Fig. 1h), which were never found in GUS-negative areas of the root cortex (Fig. 1i). This is in line with earlier *Frankia* infection studies performed on *D. trinervis*, interpreted as evidence for *Frankia* intercellular colonization (Valverde & Wall, 1999). Transmission electron microscopic analysis (see the Materials and Methods section) also has provided evidence that *Frankia* filaments within the intercellular junction are associated with cells expressing Pro*Cg12*:*GUS* (Fig. 1j). Finally, the observation of small scattered groups of GUS/GFP-expressing cells in the outer root tissues of inoculated roots at early time points following inoculation provided evidence that subtilase gene activation also was triggered during the initial stages of *Frankia* root entry (Fig. 1k–m).

Together, these results indicate that the *Cg12* and *Dt12* promoters have remarkably similar spatiotemporal expression patterns throughout the early stages of *Frankia* infection/nodulation of *D. trinervis* roots, characterized by the transcriptional activation of the subtilase gene at sites of putative apoplastic intercellular colonization. Not only does this provide valuable gene markers for identifying potential *Frankia* infection sites, but also the first evidence for the active participation of the host during the earliest stages of *D. trinervis* root colonization.

SYTO 9-labeling to track intercellular *Frankia* colonization of the *D. trinervis* root

In the light of these findings, and with the objective of monitoring the earliest stages of *D. trinervis* colonization in living tissues, two reporters were constructed based on the *Cg12* promoter driving either red fluorescent mCherry or the yellow fluorescent nuclear-localized cameleon NUP-YC2.1. The Pro*Cg12*:*mCherry* reporter was most useful for detecting *Cg12* promoter activation in root sections following SYTO 9 staining, and the Pro*Cg12*:*NUP-YC2.1* reporter combined with Pro*Ubi*-*mCherry-ER* for monitoring both nuclear position and host cell remodeling in Pro*Cg12*-expressing cells during early *Frankia* root colonization (see following Results section). To facilitate identification of the genetically transformed roots, the two Pro*Cg12* reporters were coupled to the complementary fluorescent proteins driven by the *Ubiquitin10* constitutive promoter (see the Materials and Methods section). These two constructs (Pro*Ubi*:*NUP-YC2.1*/Pro*Cg12*:*mCherry* and Pro*Ubi*:*mCherry-ER*/Pro*Cg12*:*NUP-YC2.1*) were introduced into *D. trinervis* roots *via A. rhizogenes*-mediated transformation (see the Materials and Methods section). Composite plants were then cultured in liquid medium, inoculated with *Frankia*, and roots regularly monitored for the activation of the *Cg12* promoter. Reporter gene expression was observed for both constructs from 4 to 5 d post-inoculation (dpi) onwards. In line with the earlier experiments with Pro*Dt12*:*GFP* (Fig. 1l,m), small isolated groups of fluorescence-labeled outer root cells were identified close to the growing tip of the *D. trinervis* root (Fig. S2a,b). In these experiments *Cg12* promoter activity can be visualized as either red nucleo-cytoplasmic fluorescence (mCherry) (Fig. S2c,d) or yellow nuclear fluorescence (NUP-YC2.1) (Fig. S2e,f). However, before using these transformed roots to study the early stages of intercellular *Frankia* infection at the cellular and subcellular levels, it was first necessary to confirm the correlation between subtilase gene promoter activation and the localization of the colonizing *Frankia* within the outer root tissues.

In order to identify the microsymbiont in relation to the potential *Frankia* infection sites illustrated in Fig. S2, nonfixed transverse sections (100–200 μm) from the corresponding zone of a *D. trinervis* root expressing the Pro*Ubi*:*NUP-YC2.1*/Pro*Cg12*:*mCherry* construct were treated with the nucleic acid stain SYTO 9 and immediately observed in the confocal microscope. In the root section shown in Fig. 2(a), extraradical aggregates of green fluorescing SYTO 9-stained filamentous *Frankia* can be observed at the root periphery, and close inspection reveals fluorescent intercellular filaments which have penetrated the epidermal/outer cortical root tissues (Fig. 2b). Importantly, the intercellular progression of the SYTO 9-stained *Frankia* filaments within the outer root cortex is associated with the expression of the red fluorescent Pro*Cg12*:*mCherry* reporter.

In order to examine later stages of *Frankia* colonization within *D. trinervis* root tissues, SYTO 9-stained sections were prepared from more mature root regions where nodule emergence is visible (root expressing Pro*Ubi*:*mCherry-ER*/Pro*Cg12*:*NUP-YC2.1*). One of these sections, in which two characteristic nodular lobes can be identified (Valverde & Wall, 1999), is shown in Fig. 2(c). Detailed examination of the root cortex/nodule boundary region (Fig. 2d) reveals intercellular *Frankia* filaments present in both tissues. This observation is in line with data presented in Fig. 1(g) suggesting that *Frankia* enters the developing *D. trinervis* nodule *via* peripheral tissues as the nodule extends across the already colonized root outer cortex (see also Valverde & Wall, 1999). Similar findings also were reported for intercellular *Frankia* colonization of *Elaeagnus* (Miller & Baker, 1985, 1986). Examination of additional sections from similar root zones also revealed a small number of SYTO 9-labeled spherical structures associated with intercellular colonization of the outer root cortex by *Frankia* filaments (Fig. 2e,f). When examined in bright-field (Fig. 2g,h), these structures closely resemble the vesicles observed in axenic *Frankia* cultures (Wall, 2000), suggesting that *Frankia* morphology is little altered during the initial phase of intercellular root colonization. In conclusion, SYTO 9 staining of nonfixed root sections is a powerful technique to observe the earliest stages of *Frankia* root entry and colonization, and confirms the close

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association between Pro*Cg12* activation and the progression of *Frankia* within the *D. trinervis* intercellular apoplast.

Finally, a striking feature of the SYTO 9-stained section shown in Fig. 2(a) is the intense fluorescence associated with the intercellular junctions between epidermal and adjacent outer cortical cells. Intriguingly, this fluorescence is systematically weaker and often totally absent from the epidermal/cortical cell junctions at sites of *Frankia* entry (Fig. 2a,b). Although control sections from fluorescence-negative roots following *A. rhizogenes* co-culture and without *Frankia* inoculation unexpectedly showed that this intense fluorescence is SYTO 9-dependent (Fig. S3a,b), signal enhancement did reveal a low-intensity autofluorescence associated with the identical epidermal–cortical junctions in the absence of SYTO 9 (Fig. S3c,d). Together, this suggests that certain cell wall/matrix components specific to these particular cell junctions are labeled by the SYTO 9 dye. Thus, although the precise nature of the SYTO 9-stained material remains to be determined, this observation provides indirect evidence for the remodeling of the host cell matrix interface associated with *Frankia* intercellular colonization, addressed in more detail in the following section.

Major host cell remodeling accompanies early intercellular *Frankia* root colonization

In order to perform confocal imaging of intact *D. trinervis* roots during the earliest stages of *Frankia* colonization, we primarily made use of composite plantlets with root systems expressing the Pro*Ubi*:*mCherry-ER*/Pro*Cg12*:*NUP-YC2.1* construct. This combination facilitated the localization of *Frankia* infection sites both along the root epidermis and within outer cortical tissues *via* nuclear expression of the Pro*Cg12*:*NUP-YC2.1* reporter. Once such sites had been identified, the Pro*Ubi*:*mCherry-ER* reporter was then used to identify potential host cell reorganization in cells expressing the *Cg12* promoter.

Figure 3(a) illustrates a putative *Frankia* infection zone comprising a group of epidermal cells expressing Pro*Cg12*:*NUP-YC2.1* (green fluorescent nuclei), which have been labeled e1–e6 in the adjacent magnified image (Fig. 3b). If we first focus on the lower group of Pro*Cg12*-expressing cells, and extend the confocal image stack below the epidermis, two additional fluorescent nuclei (c1 and c2) can be identified in the outer cortical layer directly underlying the e1–e4 epidermal cells (Fig. 3c and associated cartoon in Fig. 3d). Detailed examination of individual confocal sections moving from the epidermal layer (notably cell e3) down to the cortical layer (cells c1, c2) reveals a striking cellular reorganization associated with these three Pro*Cg12*-expressing cells (Fig. 3e–j). Both of the XY images (Fig. 3e,g,i) and the corresponding orthogonal XZ reconstructed images (Fig. 3f,h,j) reveal that the apoplastic interface located between c1 and c2 (arrow in Fig. 3g) is significantly enlarged compared to normal cortical interfaces (asterisks in the same image). Such enlarged intercellular apoplastic spaces were never observed in noninoculated roots. Furthermore, the c1 and c2 nuclei are embedded within mCherry-ER-labeled cytoplasmic accumulations positioned on either side of the apoplastic domain (Fig. 3i). This association of ER-rich cytoplasmic accumulations and a remodeled cell interface was observed at other putative *Frankia* infection sites (see the Materials and Methods section) and provides strong evidence for localized plant exocytosis leading to enlarged apoplastic interfaces.

Unfortunately, it has proved more difficult to study cell remodeling during initial intercellular *Frankia* entry through the epidermis using confocal imagery in living root tissues. This may be due both to the limited duration and number of epidermal entry events by comparison with the very extensive outer cortical colonization by the filamentous microsymbiont. Nevertheless, several potential epidermal entry sites were identified in close proximity to outer cortical colonization sites. One example, visible in the upper portion of Fig. 3(b) and analyzed in more detail in Fig. S4, appears as a localized widening of the cell-to-cell interface between epidermal cells e3 and e5, both expressing the Pro*Cg12* reporter (Fig. S4a). Significantly, *Frankia* filaments could be identified on the root outer surface around this location (Fig. S4b, arrowheads), reminiscent of the SYTO 9-labeled *Frankia* infection site described earlier (Fig. 2b). Furthermore, several Pro*Cg12*-expressing cortical cells (c3, c5) are present directly underneath this potential root entry site (Fig. S4c,d). The combination of the confocal XY images and corresponding orthogonal XZ images (Fig. S4e–j) emphasize the enlarged cell–cell interface between e3 and e5 immediately adjacent to the e5 nucleus, as well as mCherry-ER-labeled cytoplasm associated with the c3–c5 nuclei. The fact that the e3 nucleus is now associated with the cortical colonization site described in the previous paragraph (Fig. 3c–j) suggests that this nucleus may have been originally facing the e5 nucleus before migration following initial *Frankia* epidermal entry. Further examples of putative *Frankia* root entry sites associated with Pro*Cg12* activation are presented in Fig. S5. In all cases, similar localized widenings of the epidermal cell-to-cell interface can be clearly seen (Fig. S5a–d), and for one of these sites (Fig. S5a) a virtual transverse section also reveals associated enlarged apoplastic interfaces for the underlying cortical cells. In conclusion, confocal imaging of intact *D. trinervis* roots undergoing *Frankia* colonization has revealed major host cell reorganization and interface remodeling associated with intercellular infection of outer root tissues.

Discussion

Evidence for an active role of the plant host during intercellular symbiotic colonization

During the establishment of bacterial and fungal root endosymbiotic associations involving intracellular colonization strategies, the plant host orchestrates microbial entry and progression across the root outer tissues *via* the *de novo* construction of transcellular infection compartments typified by the rhizobial-containing legume infection threads (ITs) (reviewed in Parniske, 2008; Harrison & Ivanov, 2017; Martin *et al.*, 2017). In this article we have addressed the role of the actinorhizal host plant *Discaria trinervis* during the initial stages of intercellular colonization by nitrogen-fixing *Frankia*, where the filamentous bacterium gains access to

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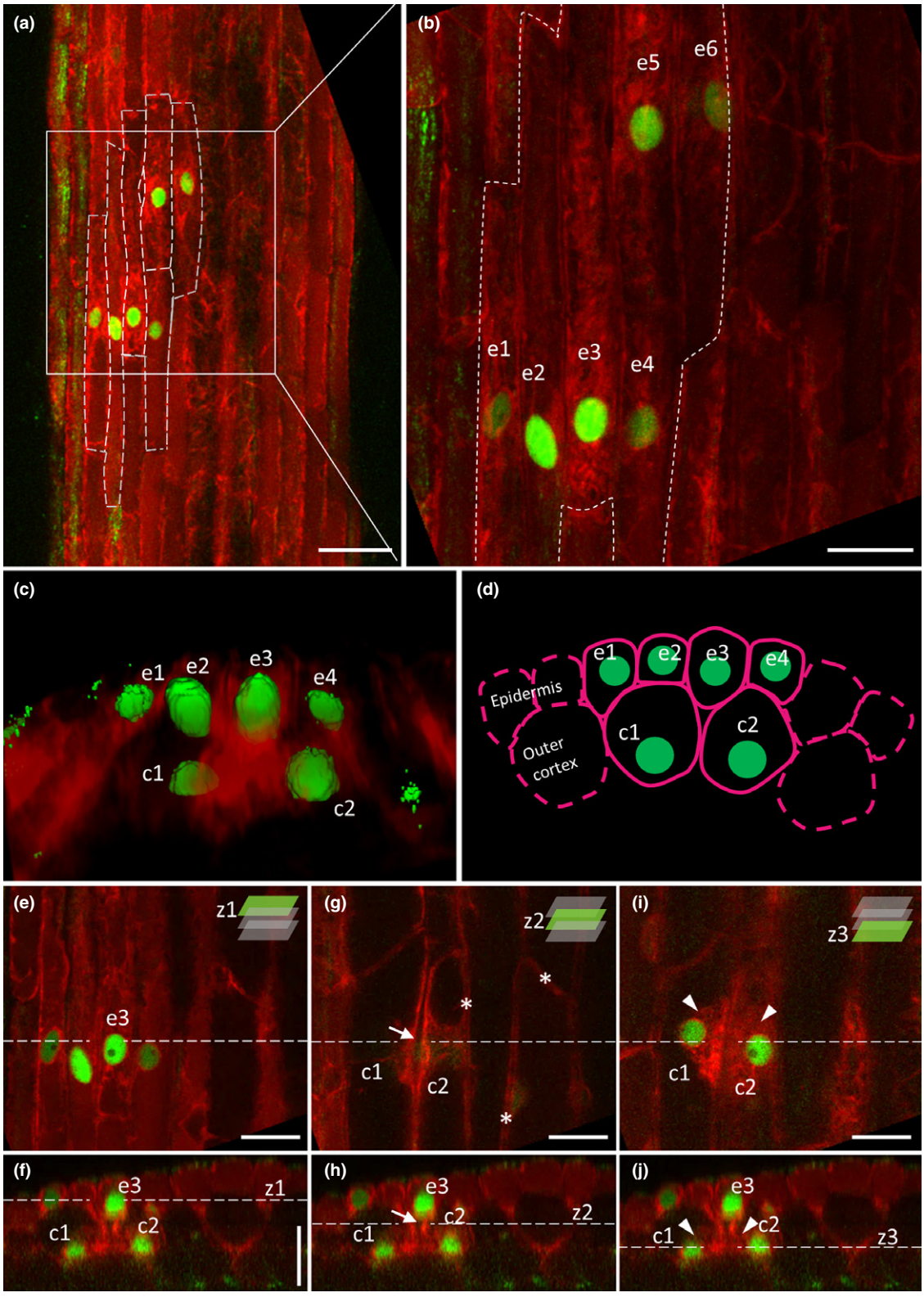

the root *via* intercellular junctions of the epidermal/cortical cell layers. A variety of approaches have revealed that the intercellular apoplast at sites of *Frankia* root colonization is significantly remodeled, leading to both physical enlargement and a likely modification in the composition/structure of the intercellular matrix, as suggested by the loss of SYTO 9 staining (schematic illustration in Fig. 4a). These conclusions are in line with the earlier findings of Valverde & Wall (1999) for *D. trinervis*, Liu &

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Fig. 3 Major cell remodeling accompanies *Frankia* colonization in the *Discaria trinervis* root outer cortex. Host cell re-organization associated with *Frankia* intercellular colonization was identified *via Cg12* promoter activation in a *D. trinervis* root expressing the Pro*Ubi*:*mCherry-ER*/ Pro*Cg12*:*NUP-YC2.1* construct. (a, b) Overview of a group of six root epidermal Pro*Cg12*-expressing cells (YFP-labeled nuclei, in green) with the cell contours delineated (dashed line). The framed area is shown at a higher magnification in b with individual numbering (e1–e6) for the Pro*Cg12*-expressing epidermal cells. (c, d) Anticlinal view of the 3D reconstruction of the e1–e4 epidermal cells and two underlying outer cortical cells (c1 and c2). Surface rendering of the fluorescent nuclei was used to facilitate visualization (c), accompanied by a schematic representation of the relative positions of the cells (d). (e–j) Successive confocal imaging (z1–z3) reveal details of the major cell remodeling associated with an intercellular *Frankia* infection site. A significantly enlarged apoplastic cell interface can be observed in the z2 section (g, h, arrow) located between the two outer cortical cells (c1, c2) and the overlying epidermal cell (e3). Furthermore, when visualized using the deeper z3 section (i, j) the c1 and c2 cells both display a striking intracellular reorganization with intense mCherry-ER labeling of cytoplasmic accumulations surrounding the two nuclei (i, j, arrowheads) and flanking the shared enlarged apoplastic domain (g, h, arrow). All images are composites of the mCherry-ER fluorescence (in red) and the YFP fluorescence of NUP-YC2.1 (in green). Images in (a, b) are maximal z-projections of confocal sections encompassing the Pro*Cg12*-expressing epidermal cells. Images in (e, g, i) are selected single XY optical sections, and images in (f, h, j) are the corresponding orthogonal XZ views (horizontal dashed lines indicate the respective Z and X positions). In addition, the corresponding Z positions are indicated (in green) in the upper right corners of (e, g, i). Bars: (a) 40 μm; (b, e–j) 20 μm. ER, endoplasmic reticulum; YFP, yellow fluorescent protein.

Berry (1991a,b) for *Ceanothus* and Miller & Baker (1985, 1986) for *Elaeagnus*, who identified electron dense material present within the enlarged apoplast associated with intercellular *Frankia* colonization. In addition, Liu & Berry (1991b) went on to show that, in the case of *Ceanothus*, a major component of the modified apoplastic interface consists of pectic polysaccharides.

The confocal studies presented in this article further show that, in *D. trinervis*, this remodeling is accompanied by a striking intracellular reorganization within the adjacent cortical cells that involves nuclear migration associated with an accumulation of endoplasmic reticulum-rich cytoplasm on either side of the modified cell junction (Fig. 3). The most likely interpretation of this nucleo-cytoplasmic reorganization is the concerted secretion of extracellular components into the shared apoplastic space that contributes to the observed modifications. Furthermore, because

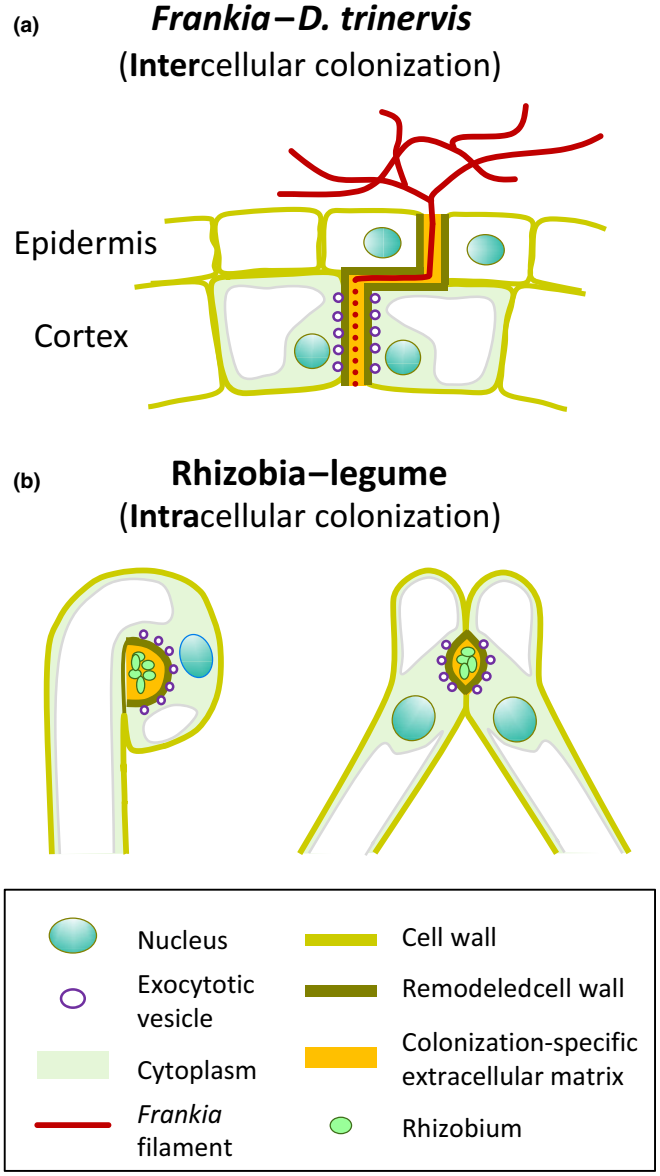

Fig. 4 Comparison of the cellular mechanisms associated with the early stages of inter- and intracellular root colonization by symbiotic nitrogen-fixing bacteria. (a) Schematic illustration of intercellular colonization of *Discaria trinervis* outer root tissues by filamentous *Frankia*. Based on the results presented in this article and the earlier studies on actinorhizal intercellular infection discussed in the text, we propose that *Frankia* root entry is accompanied by major modifications both to the intercellular apoplast and the adjacent host cell walls. The striking nucleo-cytoplasmic reorganization observed in these adjacent cells argues that these modifications are the consequence of concerted exocytosis directed towards the intercellular spaces through which the *Frankia* transit. Of course, the existence of this exocytotic activity remains to be demonstrated. The *Frankia* filaments have been represented by dotted lines within root tissues because the respective kinetics of *Frankia* intercellular progression and apoplastic remodeling are not yet known. Because our results have come primarily from the observation of outer cortical cell layer colonization, the illustration focuses on this particular stage of root entry (Fig. 3). Nevertheless, the more limited data available for epidermal entry (Supporting Information Figs S4, S5) suggest that a similar process accompanies initial intercellular *Frankia* colonization. (b) Schematic illustrations of the initial stage of rhizobial intracellular colonization *via* legume host root hairs based on findings presented in Fournier *et al.* (2015). Whether for individual curled root hairs (left-hand side) or two contacted root hairs (right-hand side) this stage precedes the initiation of infection thread (IT) formation and involves the creation of a new compartment within the so-called infection chamber enclosed between host cell walls and containing the rhizobial micro-colony. Exocytosis and symbiotic cell-wall markers have shown that the progressive creation of this compartment surrounded by remodeled host cell walls results from exocytotic activity associated with characteristic nucleo-cytoplasmic reorganization. We propose that mechanistic parallels exist between the creation of this novel apoplastic compartment before IT initiation and the analogous cellular processes we have observed for intercellular *Frankia* colonization of actinorhizal hosts presented in (a).

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the spatio-temporal expression pattern of the *Discaria Dt12* gene correlates with sites of *Frankia* colonization, it is likely that the encoded subtilase is one member of the battery of symbiosis-related secreted matrix components. We therefore propose that the host plant is an active partner during the early stages of this mode of intercellular root entry, with the actinorhizal host machinery primarily responsible for the modifications to the intercellular apoplast which accompany *Frankia* colonization of the root epidermis and outer cortex. In line with this, genomic sequencing and secretome analyses of several *Frankia* species have uncovered only a limited number of genes encoding potential plant cell wall-degrading enzymes by comparison with nonsymbiotic actinomycete species (Mastronunzio *et al.*, 2008). The loss of such genes in microbes adapted to plant root symbioses has also been reported for both arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungal species, interpreted as an evolutionary mechanism limiting inappropriate activation of host defenses (Tisserant *et al.*, 2013; Kohler *et al.*, 2015). Further studies will now be required to investigate if similar host remodeling also occurs during certain phases of crack-entry type colonization or endophytic accommodation of other actinobacteria (van der Meij *et al.*, 2018), and whether host-driven apoplastic interface modification is a general process accompanying microsymbiont intercellular colonization (see following section).

Relationship between intercellular and intracellular endosymbiotic colonization

In this article we describe the identification of the *Dt12* gene as the ortholog of the subtilase-encoding *Cg12,* a *Casuarina glauca* gene expressed during IT-dependent intracellular *Frankia* colonization (Laplaze *et al.*, 2000; Svistoonoff *et al.*, 2003). Not only do the corresponding proteins show a high level of sequence similarity, but also the *Cg12* and *Dt12* promoters possess remarkably similar reporter expression patterns during intercellular *Frankia* colonization of *D. trinervis* (Fig. 1). This has proved to be of paramount importance in enabling us to identify potential sites of intercellular colonization in living root tissues and suggests that closely related subtilisin proteases are secreted into both the *C. glauca* IT apoplast and the *D. trinervis* intercellular apoplast during the initial stages of *Frankia* colonization. Although the cellular target(s) for these secreted subtilisins have not yet been identified in either case, these findings point to likely similarities in the composition of the extracellular cell wall/matrix which surrounds the filamentous *Frankia* during either intra- or intercellular colonization of the respective actinorhizal host plant. Interestingly, phylogenetic analysis shows that *Cg12* and *Dt12* are both members of the same subtilisin gene subfamily as *MtCG12a* (Fig. S1), a *Medicago truncatula* gene expressed specifically during rhizobial infection/nodulation according to the *M. truncatula* gene atlas (He *et al.*, 2009). In addition, it has been demonstrated that the *Cg12* promoter is transcriptionally activated in IT-containing root hairs of *M. truncatula* during rhizobial root colonization (Svistoonoff *et al.*, 2004), whereas the *MtENOD11* gene, encoding a rhizobial infection-associated wall protein in *M. truncatula*, is activated during *Frankia* colonization in both *D. trinervis* (Imanishi *et al.*, 2011) and *C. glauca* (Svistoonoff *et al.*, 2010). Together, this suggests that functional analogies, including conservation of *cis* regulatory sequences, related to symbiotic apoplastic components during microsymbiont colonization in actinorhizal hosts probably also extend to legume hosts.

In the case of another model legume, *Lotus japonicus*, the exploitation of various combinations of host and *Mesorhizobium loti* symbiotic mutants which block normal IT formation has revealed the existence of an alternative intercellular colonization pathway leading to inefficient nodulation (Madsen *et al.*, 2010). This was interpreted as evidence for an ancient intercellular default pathway that evolutionarily pre-dates the more sophisticated IT-mediated pathway. Further insight for comprehending such an evolutionary transition may come from our finding that the host plant significantly modifies the intercellular apoplast during *Frankia* colonization of *D. trinervis* roots, thus prefiguring the *de novo* creation of the IT compartment. In this context, it is noteworthy that, before the initiation of polar tip growth of the IT, the earliest stage of root hair intracellular infection in *M. truncatula* is characterized by the formation of an exocytosis-driven apoplastic compartment surrounding the rhizobia (Fournier *et al.*, 2015; see Fig. 4b). These novel compartments are created within chambers resulting either from root hair tip curling or from wall–wall contact between adjacent root hairs and can be considered to be topologically equivalent to the remodeling of the extracellular space between adjacent cell walls during intercellular *Frankia* colonization.

Intercellular modes of root colonization also are a common feature of the more ancient mycorrhizal symbioses. In the case of ECM associations, fungal hyphae penetrate the outer host root tissues uniquely *via* the intercellular apoplast to form the extensive symbiotic interface known as the Hartig net (Martin *et al.*, 2016). Although the molecular and cellular mechanisms of this process are poorly understood, it is well-established that major modifications to the host apoplast accompany ECM fungal colonization. Arum-type AM colonization also involves an important phase of intercellular colonization when longitudinally growing hyphal 'runners' extend the zone of arbuscule formation within the root inner cortex (Genre & Bonfante, 2005). Again, little is known about the mechanisms underlying this process, but it will be important in the future to examine possible mechanistic parallels with intercellular colonization in both legumes and actinorhizal hosts, as already shown for intracellular AM and rhizobial colonization of *M. truncatula* outer root tissues (Genre *et al.*, 2005; Fournier *et al.*, 2008; Sieberer *et al.*, 2012).

Amongst the additional areas of research which now need to be addressed in the light of the findings reported here is the possible role of microbe–host signaling and the associated activation of the host conserved symbiotic signaling pathway (CSSP) during intercellular root colonization. Activation of the CSSP is known to be important for the formation of the specialized infection compartments during both rhizobial and AM intracellular colonization (reviewed in Oldroyd, 2013; Venkateshwaran *et al.*, 2013). Studies over recent years have provided strong evidence that actinorhizal hosts such as *C. glauca* and *D. trinervis* also possess a functional CSSP (Svistoonoff *et al.*, 2013, 2014), and

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furthermore that the *C. glauca*-nodulating *Frankia* strain CcI3 secretes symbiotic signaling molecules which activate the *C. glauca* CSSP (Chabaud *et al.*, 2016). However, a similar approach has so far failed to show CSSP activation in the *Discaria* epidermis in response to *Frankia* BCU110501 exudates (Chabaud *et al.*, unpublished results), although there is good evidence that the CSSP is required for nodulation in *D. trinervis* (Svistoonoff *et al.*, 2013). Nevertheless it is possible that microbe–host signaling leading to CSSP activation in *D. trinervis* before intercellular colonization requires direct physical contact between the two symbiotic partners. If so, this would be consistent with the failure to obtain *trans* complementation for microbe–host signaling between *D. trinervis* and a nonhomologous *Frankia* species (Gabbarini & Wall, 2011).

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Author contributions

J.F., L.I., M.C., H.G., S.S., D.G.B. and L.G.W. designed the research; J.F., L.I., M.C., I.A-P., A.G., L.Brichet, H.R.M., N.M., A.V., E.P., L.Brottier and S.S. performed the experiments; J.F., L.I., M.C., H.G., V.H., S.S., D.G.B. and L.G.W. analyzed the data; and J.F., D.G.B. and L.G.W. wrote the paper with input from M.C., A.G., H.G. and S.S.; J.F. and L.I. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

Fig. S1 Analysis of the symbiotic subtilase Dt12 from *D. trinervis*.

Fig. S2 Pro*Cg12*:*mCherry* and Pro*Cg12*:*NUP-YC2.1*-expressing cells in root outer tissues following *Frankia* inoculation.

Fig. S3 SYTO 9-dependent fluorescence labeling of cell–cell interfaces in outer root tissues of *D. trinervis*.

Fig. S4 Putative *Frankia* epidermal entry site in vicinity of the cortical colonization site illustrated in Fig. 3.

Fig. S5 Further examples of putative *Frankia* epidermal entry sites in roots of *D. trinervis*.

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