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New aspects of dodder–tobacco interactions during haustorium development

Renata Svubova[1] · Zuzana Lukacova[1] · Peter Kastier[1] · Alzbeta Blehova[1]

Abstract

Key message **Peroxidases are highly active enzymes in all the parts of the host tobacco plant which are attacked by dodder, as well as in developing haustorium. Plastids undergo considerable changes during prehaustorium development.**

Abstract New aspects of the parasitic plant (*Cuscuta europaea* L.) and the host (*Nicotiana benthamiana* Domin.) interactions were found in this study. Epidermal, file and digitate cells of developing haustorium began accumulating rather early and also secrete de-esterified pectins, which serve as a cement material that enables parasites to stick to the host. Tobacco plants achieved a dramatic increase of peroxidases (POD) content, activities and changes in isoenzymes composition in the stems attacked by the dodder when compared to untreated controls. The highest POD activities were observed on both sides of the functional connection—haustorium. This is probably an effective mechanism of dodder which makes the host's surface cell walls more flexible, and in the case of the host plant, attempts to eliminate penetration by parasites.

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Introduction

The genus *Cuscuta* is the most widespread agronomical and economically important group of parasitic plants. Most of them are holoparasites with a lacking or low amount of chlorophylls (van der Kooij et al. 2000; McNeal et al. 2007; Těšitel 2016), but the presence of photosynthesis-related genes indicates that some of them can be cryptically photosynthetic (Krause 2008; Braukmann et al. 2013). For example, *C. reflexa* (subgenus *Monogynella*) possesses chlorophylls and is photosynthetically active, even at a reduced rate (Machado and Zetsche 1990; Hibberd et al. 1998). Whereas *C. odorata* and *C. grandiflora* (subgenus *Grammica*) were found to lack not only chlorophyll and thylakoids, but also the *rbcL* gene (van der Kooij et al. 2000). It has been reported that *C. europaea* (subgenus *Cuscuta*) also lacks chlorophyll, grana and thylakoids, and appears incapable of fixing CO_2 (Machado and Zetsche 1990). However, chloroplasts with significant amounts of chlorophylls and the presence of several photosynthesis-related proteins (e.g., RbcL, THF1, D1 and others) in the tips of young seedlings have been previously detected (Švubova et al. 2013). THYLAKOID FORMATION1 (THF1) can ultimately affect thylakoid formation and plastid development which is a crucial step in the survival of photosynthetic eukaryotes (Sakamoto et al. 2003; Wang et al. 2004; Yu et al. 2007). Plastids of holoparasitic angiosperms, including *C. europaea*, during the transition

from autotrophic to heterotrophic feeding strategies, undergo changes, from chloroplasts in shoot tips of young seedlings (7-day-old) to amyloplasts full of starch grains in haustorial cells. Immunofluorescence localization studies and analyses of haustorial plasma membrane fractions revealed that in addition to plastids, the THF1 protein localizes also to plasma membrane and plasmodesmata in developing *C. europaea* haustorium that indicates its important role in complex host–parasite relationship (Švubova et al. 2013).

To develop strategies for the control of parasitic growth and restrict the spread of *Cuscuta* spp. in crop fields, greater knowledge about this pest, study of its life cycle and its molecular mechanisms of infection are required. The initial contact with the host plant induces the formation and subsequent penetration of a specialized absorptive organ called haustorium into the host stem. The adhesive substances secreted by the epidermis (Vaughn 2002), which anchor the parasite to the host and allow the growth of haustorium in the host tissue, use a combination of mechanical pressure and enzymatic digestion (Nagar et al. 1984; Johnsen et al. 2015; Kaiser et al. 2015). Early stage of haustorium differentiation is facilitated by the action of cell wall-modifying enzymes and the recruitment of stress-responsive and defense genes (Srivastava et al. 1994; Ranjan et al. 2014; Kaiser et al. 2015). Cell wall polymer profiling in infecting and non-infecting tissues of *Cuscuta* spp. recently revealed substantial differences in cell wall composition, which appear to be pivotal for haustorium differentiation (Johnsen et al. 2015; Olsen et al. 2016). For example, the presence of cell wall degrading enzymes, e.g., de-esterified homogalacturonans in susceptible *Pelargonium zonale* cell walls and high expression levels of pectate lyase genes combined with high pectinolytic activity, suggests that the parasite contributes directly to host cell wall remodeling (Johnsen et al. 2015).

Plant peroxidases, which are present in all land plants, animals and microorganisms, are involved in a broad range of physiological and biological processes, especially in biotic or abiotic stressful conditions (Passardi et al. 2005). As it was mentioned previously, haustorial cells elongate and their cell walls become more malleable. Growth and elongation are related to cell extensibility. Peroxidases, through their catalytic and hydrolytic cycles, directly or indirectly regulate the cell wall architecture. They can generate reactive oxygen species, regulate H_2O_2 levels and, directly or indirectly regulate the cell wall architecture by polymerizing cell wall compounds during lignin and suberin formation. They are also involved in auxin oxidation and oxidation of cinnamyl alcohols prior to their polymerization, and therefore several roles have been attributed to them in a complex of host–pathogen interactions (Normanly et al. 1995; Kawano 2003; Lopez-Curto et al. 2006).

In mature haustorium, the searching hyphae connect to the host's vascular tissue and differentiate into xylem or phloem hyphae (Vaughn 2002, 2003; Kaiser et al. 2015). The xylem connections between the host and parasite form an open conduit, whereas the phloem connections involve the membrane and cell wall barriers. Therefore, mature haustorium can serve as nutrition as well as for regulatory purposes (Kim and Westwood 2015).

The aim of the present study was to describe the early stages of *C. europaea* prehaustorium development in terms of histochemical and anatomical aspects. The periclinal division of file cells and the expansion of digitate cells generate growth direction toward and inside the host tissues. The peroxidases are very active enzymes in host tobacco plants attacked by dodder as well as in developing haustorium. The potential role of PODs in host plant interaction and in *C. europaea* haustorium development shall be discussed in the next section.

Materials and methods

Plant material cultivation and growth conditions

In vivo cultivated plants of parasitic dodder (*Cuscuta europaea* L.) and tobacco (*Nicotiana benthamiana* Domin.) as a host plant were used in our study. Seeds of *C. europaea* originated from the locality Ivanka pri Dunaji (2014, Slovakia, latitude 48°19′, longitude 17°22′), the Slovak Republic, and the *N. benthamiana* seeds were obtained from the Gene Bank in Gatersleben, Germany. Dodder seeds were scarified by soaking in concentrated H_2SO_4 for 15 min, then washed with distilled water and planted in soil around the base of 8-week-old tobacco plants. The plant material (tobacco plants with dodder) was cultivated in soil in a growth chamber (16/8 h photoperiod) at 23 ± 2 °C and 100 µmol m^{-2} s^{-1} PAR. All experiments in this work were carried out three times.

An anatomical and cytological study of prehaustorium/haustorium development and THF1 protein in situ immunolocalization

Approximately, 0.5 cm-long dodder stem segments (24, 48 h after dodder stems were wrapped around the host plant stems), tobacco stems overgrown with haustorium (72–96 h after dodder stems were wrapped around the host plant stems) and tobacco stems with functional haustorium (10 days after attachment) were harvested and fixed in 2% freshly prepared formaldehyde in a stabilizing buffer (SB buffer: 50 mM PIPES, 5 mM MgSO4, 5 mM EGTA, pH = 6.9) under a vacuum infiltration in desiccators.

Afterward, the dehydrated segments (alcohol series from 10 to 100% EtOH p.a. in PBS buffer: 0.14 M NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH = 7.3) were embedded in Steedman's wax. Unlabeled sections (10 μm thick) were used for anatomical and cytological studies and in situ immunostaining was performed with some modifications of the protocol described by Vitha et al. (2000). The prehaustorium and haustorium sections were incubated for 1 h at room temperature in the polyclonal Psb29 (THF1) primary antibody (1:200 dilution in PBS buffer), washed two times for 10 min in the stabilizing buffer, and incubated overnight at room temperature in the dark in a secondary antibody (1:100 dilution in PBS). The primary antibody against THF1 protein was purchased from Agrisera. A secondary antibody, goat anti-rabbit IgG (whole molecule) F (ab′) fragment-FITC was purchased from Sigma-Aldrich. Negative controls were performed using only the secondary antibody. Positive reactions for this staining were the photosynthetically active chloroplasts in the dodder shoot tips (Švubova et al. 2013).

Assay of POD enzyme activity and POD isoenzymes

Approximately, 0.5 g of *Cuscuta* stems (segments 1–3 cm above and 1–3 cm under the haustorium), the haustorium itself, *N. benthamiana*, the second and the fourth internodes from the control plants (unaffected by dodder) and the tobacco stem segments attacked by parasites above and under the haustorium were homogenized with 1 ml of 50 mM Na-phosphate buffer with 1 mM EDTA (pH 7.8) and complete protease inhibitor cocktail tablets (purchased from Roche). The concentration of proteins was quantified with bovine serum albumin as the calibration standard according to the method of Bradford (1976). Isolated proteins were used for analysis of POD enzyme activity according to Frič and Fuchs (1970). A brownish-red product was measured spectrophotometrically at 440 nm. One unit of activity represents the amount of enzyme dissolved in 1 ml of solution which catalyzes the change in absorbance (λ 0.1) in 60 s.

Isolated proteins were also separated under non-denaturing conditions in 7% polyacrylamide gels using the cathodic system. The POD isoenzymes activities were detected after incubation of gels in 50 mM guaiacol and 10 mM H_2O_2. The method is based on the oxidation rate of guaiacol.

POD histochemical assay and Western blot analysis

The in situ POD activity was detected after the incubation of hand cross sections ($\sim$0.5 mm thick) of dodder stems and their host plant in 100 mM Na-acetate buffer (pH 5.2) with 5 mM 4-methoxy-1-naphthol (4-MN) in ethanol for 15 min at 30 °C (Ferrer et al. 1990; Ďurčeková et al. 2007). Young internodes (the 2nd and the 4th) of tobacco plants not attacked by the dodder were used as controls and the same sections were compared in tobacco stems attacked by the dodder. Also, older host plant parts and parasite stems (segments 0.2–0.5 cm above and under the fully developed haustorium) were analyzed analogously to POD activities analysis. Xylem lignification connected to increase POD activity was considered as a positive reaction for the staining. Three unaffected tobacco plants, as well as plants attacked by dodder and dodder stems themselves, were used for each experiment.

The tobacco and dodder samples (100 mg) were ground using liquid nitrogen and suspended in a protein extraction buffer (28 mM dithiothreitol, 5% SDS, 175 mM sucrose, 28 mM Na_2CO_3, 10 mM EDTA). After 30 min of incubation at 70 °C and 15 min of centrifugation (12,100×g), a supernatant was used for determination of protein concentration using a Bichinonic Acid Kit for Protein Determination (Sigma-Aldrich, St. Louis, MO, USA). The protein samples (25 μg) were separated on a 12% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). For protein immunodetection, a specific primary anti-rabbit IgG (whole molecule)-peroxidase antibody (Sigma-Aldrich, cat. number A0545) and secondary antibody goat anti-rabbit IgG (H + L)–HRP Conjugate (Bio-Rad, Hercules, CA, USA) were used. The signal was revealed using the chemiluminescent kit "Immobilon Western" (Millipore, Bedford, MA, USA).

Determination of pectins and lignin in various stages of prehaustorium and haustorium differentiation

Unesterified (acidic) pectins staining: 10 μm-thick, Steedman's wax-embedded sections of developing prehaustorium were placed in a 0.02% aqueous ruthenium red solution $[(NH_3)_5Ru–O–Ru(NH_3)_4–O–Ru(NH_3)_5]Cl_6.4H_2O$ for 30 min, and afterward mounted in glycerol. As a positive controls, young tobacco internodes (the 2nd and the 4th, without dodder) were used.

Lignification: For analysis of the lignification of xylem vessels in functional haustorium (10 days after attachment), Steedman's wax-embedded cross sections (10 μm thick) were stained with phloroglucinol and hydrochloric acid (20%).

Microscopy

Hand ($\sim$0.5 mm thick) and Steedman's wax-embedded (10 μm thick) sections were examined under a fluorescence microscope (Axioskop 2 plus, Zeiss), equipped with 485/20 nm excitation filter, 510 nm beam splitter and

515 nm LP barrier filter (filter Zeiss set 16, 25) using a confocal microscope (CLSM Fluoview FV1000, Olympus, Japan) with an excitation laser line of 488 nm and BA505-550 barrier emission filter.

Statistical analysis

One-way ANOVA was performed using Statgraphics Centurion XV.I software for statistical significance at $P < 0.05$. All the data were tested for normality and outliers (extreme values) were identified and defaulted. All the results were expressed as mean ± SE for defined replications. The means were separated using the least significant difference (LSD) test at a 5% level of significance.

Results

Anatomical and cytological study of prehaustorium/ haustorium development

After finding a susceptible tobacco plant, the first physical contact initiates the attachment stage in which the haustorial initials (Fig. 1a) develop from dodder cortical cells. Important to note is that epidermal cells, especially at the site of future contact, have a dense inner content (Fig. 1a, arrows). Anatomical studies showed that *C. europaea* prehaustorium has an endogenous origin and develops from a disk-like meristem (Fig. 1b). In developing prehaustorium, the zone of the vascular proximal meristematic cells was followed by a layer of mitotically active file cells. The distal-most zone of elongated digitate cells with prominent nuclei was evident in the central part of developing prehaustorium (Fig. 1c). Interestingly enough, the cells above the digitate cells were also mitotically active (data not shown).

The formation of the functional connection to the host vascular tissues is the most important step in the life cycle of *C. europaea*. Upon contact with the host tissues (about 10 days after attachment), the xylem vessels are formed in the haustorium in the direction toward the haustorial tip (Fig. 1d). During tracheary element differentiation, the secondary wall material is deposited as discrete annular rings, or in a spiral pattern, with the thickening parts separated by bands of primary wall (Fig. 1e, arrowheads). Immediately after the functional connection between the host and parasite, the secondary walls of the tracheary elements are impregnated with lignin (Fig. 1f, arrowhead), which is not usually present in the primary walls.

In an effort to complete the information dealing with prehaustorium development, THF1 in situ immunolocalization technique was used. While no THF1 signal was detected in dodder cells before attachment (Fig. 2a), a strong THF1 fluorescence was characteristic for dodder prehaustorial cells soon after attachment to the host tobacco plant (Fig. 2b). In elongated digitate cells, plastids with three to five large starch grains that depressed the THF-positive stroma to the central part were observed (Fig. 2c, e, arrows). However, in dividing file cells, the presence of THF1-positive chloroamyloplasts with one to two small starch grains was recorded (Fig. 2d, f, arrows).

As mentioned above, the dodder epidermal cells contain a dense material (Fig. 1a, arrows) and staining with Ruthenium red confirmed the presence of de-esterified pectins in these cells (Fig. 3a, b, asterisks). Later, secretion of pectins onto the surface of epidermal cells serving as cement for the contact with the host was observed (Fig. 3c, d, asterisks). It also appears that pectins are secreted not only at the sites of contact with the host, but also in the inner tangential and radial cell walls (Fig. 3d, arrows). Surprisingly, the presence of pectin in the digitate cells precursors has been unnoticed until now (Fig. 3a, arrow). These precursors later changed from small and isodiametric to elongate in a perpendicular position to the parasite surface. Massive elongation of digitate cells and the accumulation of pectins suggests their high synthetic activity (Fig. 3c, d).

Activity of peroxidases during the prehaustorium/ haustorium formation

Peroxidases activity and in situ histochemical assay of POD (Fig. 4) was also established. To avoid the effect of tobacco stem aging, two different internodes (the 2nd and the 4th) of unaffected plants were stained at first (Fig. 4a, c). This staining revealed POD activity of control tobaccos only in the area of the developing secondary xylem (Fig. 4a, c, arrows). In comparison with the same tobacco plant parts (the 2nd and the 4th internode) attacked by dodder, a striking change in terms of higher POD activities in the epidermal, cortical and central cylinder cells was achieved (Fig. 4b, d, arrows). Epidermal cells were detected as places of higher POD activities in the older tobacco internodes, but, again, only in the plants attacked by dodder (Fig. 4d, black arrow). Cross sections of tobacco stem under (Fig. 4e) and above (Fig. 4f) the fully developed haustorium has shown higher POD activity in epidermal cells and in the area of the developing secondary xylem.

Of note is that POD activity was achieved also in dodder stems as isolated areas with one or more cells in the cortex. The sections were done on the dodder stems under (Fig. 4g) and above (Fig. 4h) the fully developed haustorium. Peroxidases were also very active during prehaustorium development in the peripheral cells (Fig. 4i) as well
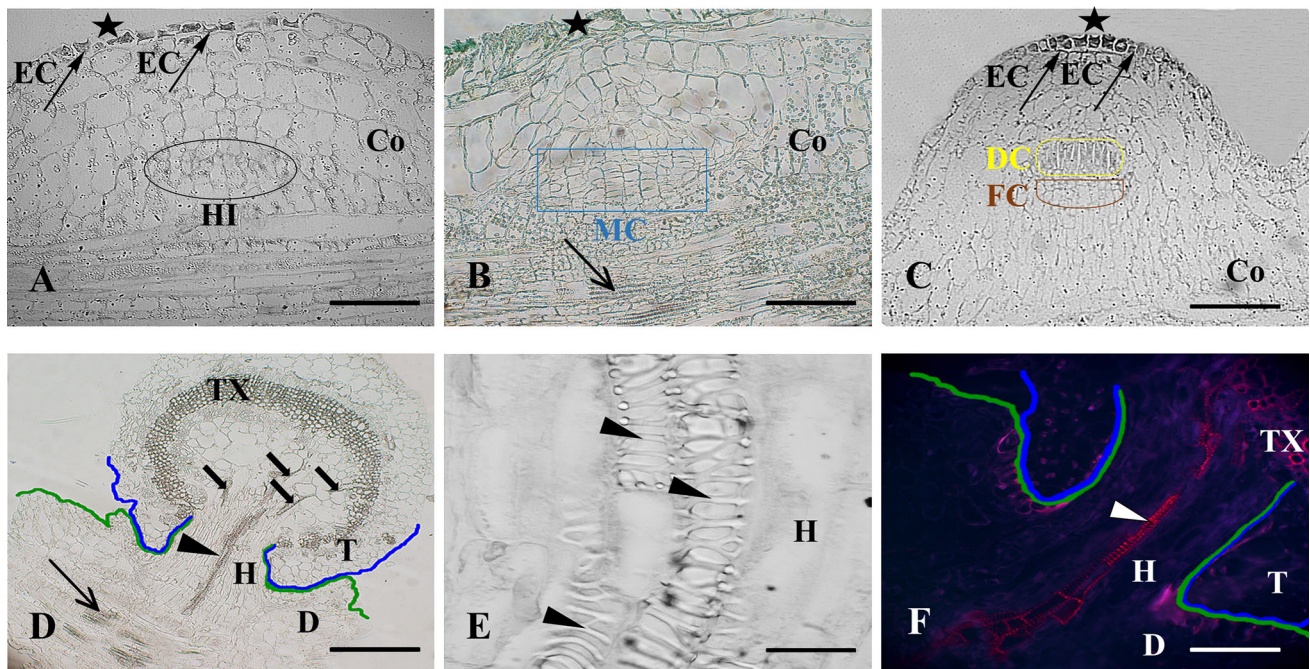
Fig. 1 *Cuscuta europaea* haustorium in various developmental stages. **a**, **b** before the attachment stage, **c** emerging dodder prehaustorium, **d–f** mature dodder haustorium. **a** Longitudinal section of a dodder stem. After finding a susceptible tobacco plants, the first physical contact initiates the attachment stage in which the haustorial initials (*HI*) develop from dodder cortical cells (*Co*). *Arrows* mark dodder epidermal cells (*EC*) with dense cytoplasm. **b** Longitudinal section of dodder prehaustorium. A group of meristematic cells (*MC*), the initiation of which is manifested by the division of haustorial initials (*HI*) at sites adjacent to the dodder vascular tissue (*arrow*). **c** Longitudinal section of dodder prehaustorium. Emerging dodder prehaustorium consists of dividing file cells (*FC*) and elongated digitate cells (*DC*). Dodder cortical cells (Co), *asterisks* indicate site of the contact with host tobacco stem. **d** Transverse section of a tobacco stem (*T*) with an attached dodder (*D*). Mature haustorium (*H*) of dodder (demarcated with a *green line*) is embedded into the tobacco stem (demarcated with a *blue line*). H austorial xylem elements (*arrowhead*) and xylem serching hyphae (*arrows*) that make contact with tobacco xylem (*TX*). **e** Dodder haustorial xylem elements, *arrowheads* mark annular pattern of cell wall thickening. **f** Transverse section of a tobacco stem (T demarcated with a *blue line*) with an attached dodder (D demarcated with a *green line*) stained with phloroglucinol–hydrochloric acid. Mature haustorium (*H*) shows xylem elements (*arrowhead*) with lignified cell walls. *Scale bars* 100 μm (**a**, **b**, **c**, **f**); 200 μm (**d**); 25 μm (**e**)

as in functional haustorium, especially in xylem elements (Fig. 4j).

Dramatic changes in POD activities in the host and parasitic plants, observed using histochemical staining, were confirmed by the measurements of guaiacol POD activities (Fig. 5) and by separation of the isoenzymes (Fig. 6) in the different plant parts. Young internodes of tobacco control plants showed low activity of this antioxidant enzyme (Fig. 5, lines 1 and 2). Significantly higher POD activities were achieved in all tobacco stems attacked by the parasite (Fig. 5, lines 3 and 5). A significant increase was achieved in the tobacco stems which bordered with fully developed haustorium (Fig. 5, line 4); however, the activity was the highest in the isolated haustorium (Fig. 5, line 7). Also important was that the dodder stems (Fig. 5, lines 6 and 8) had significantly higher POD activities when compared with the tobacco stems (Fig. 5, lines 1–5), which points to the importance of this enzyme in the host–parasite interactions.

Four isoenzymes with sizes of approximately 250, 130, 54 and 13 kDa were detected in the dodder and tobacco samples (Fig. 6). In the control tobacco internodes where the POD activity was very low (Fig. 5, lines 1 and 2), no POD isoforms were detected (Fig. 6, lines 1 and 2). However, the most apparent isoforms were observed in the dodder stems as well as in differentiated haustorium (Fig. 6, lines 6–8). In the tobacco segments that were isolated under the developed haustorium, only two POD isoforms were confirmed (Fig. 6, line 5).

These data correlate with the Western blot analysis. The level of POD with a size of approximately 36 kDa was the highest in the dodder stem above the haustorium (Fig. 7, line 1) as well as in functional haustorium (Fig. 7, line 3), while in the tobacco and dodder stems under the haustorium the accumulation was seemingly lower (Fig. 7, lines 2 and 6). The control tobacco plants showed the lowest accumulation of this antioxidant enzyme (Fig. 7, lines 7 and 8).
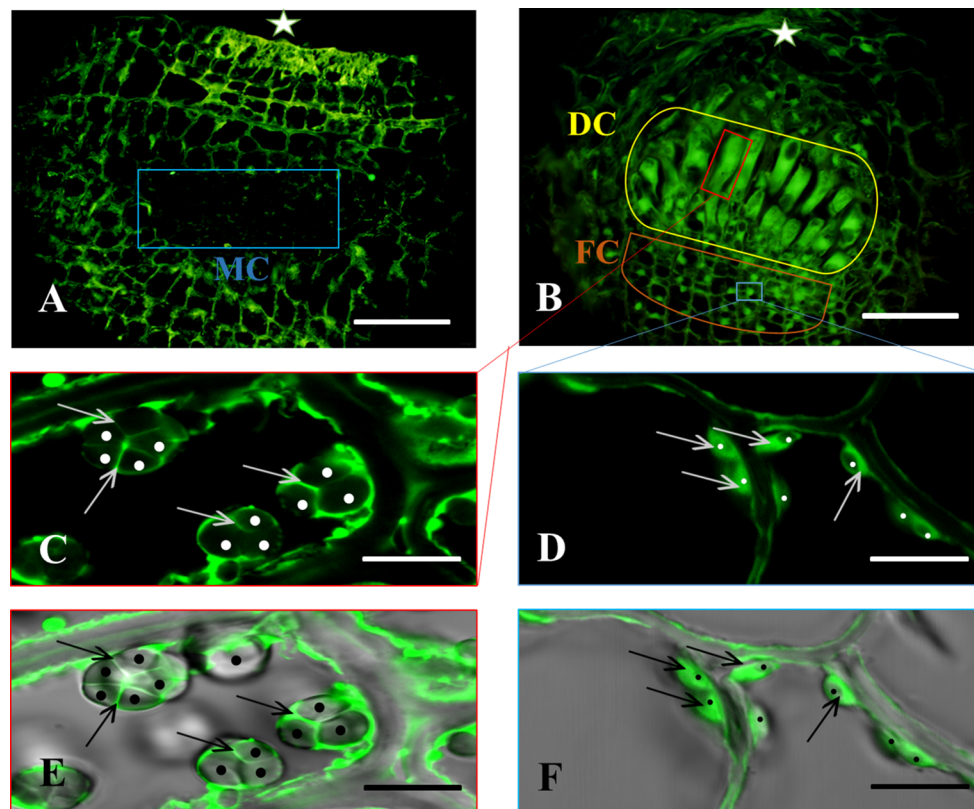
Fig. 2 In situ immunolocalization of THF1 protein at early prehaustorial stages. **a** Mitotically active meristematic cells (*MC*) without THF1-positive signal. **b** Emerging dodder prehaustorium with THF1-positive digitate cells (*DC*) and file cells (*FC*). (**c**, **e**) Detailed view on amyloplasts in elongated digitate cells; *dots* mark starch grains and the *arrows* mark the THF1-positive stroma. **d**, **f** Chloroamyloplasts in file cells; *dots* marks small starch grains and the *arrows* mark THF1 positive stroma. *Scale bars* 100 μm (**a**, **c–f**); 50 μm (**b**)

Discussion

The stem parasite *Cuscuta europaea* has only a root-like structure which disappears by the seventh day after germination and is replaced by haustorium (data not shown). This special stem-derived organ is shared among all parasitic plants, whose functions include the attachment and invasion to a host's tissues and the physiological redirection of host resources into the parasite (Westwood et al. 2010; Kim and Westwood 2015). Previous anatomical studies have shown that this structure has an endogenous origin and develops from a disk-like meristem (Lee and Lee 1989; Vaughn 2003). In developing *C. europaea* prehaustorium, we noticed various zones of cells similarly described by Lee (2007). The dodder epidermal cells in contact with the host became elongated. We propose that these cells might be precursors as well for searching hyphae previously suggested for digitate cells in *C. pentagona* (Alakonya et al. 2012). Also important is that the cells at the tip of *C. europaea* prehaustorium, observed previously in *C. australis* and assigned as compressed cells (Lee and Lee 1989), were also mitotically active.

The first physical contact between the host and parasite is established through the secretion of sticky substances produced by the parasite (Vaughn 2002, 2003) as well as by the host plant (Albert et al. 2006). Subsequently, secretion of these substances onto the surface of epidermal cells can serve as a cementing material for close and really tight contact with the host plant during the attachment stage. Pectins can also act as an anchor for POD, which would cross-link extensions to create a dense and solid network in the host plant cell wall with the aim of limiting pathogen colonization (Passardi et al. 2004). On the other hand, such interaction has a defense function in this relationship since it can make the host plant cell wall harder to penetrate (Almagro et al. 2009; Johnsen et al. 2015). Also important is that the accumulation of de-esterified pectins in the inner tangential and radial cell walls indicates that it may have a crucial role during the penetration stage as well (Fig. 3d).

During haustorium development, the continuity of cortical and endoplasmic cytoskeleton elements in both dividing and elongating cells may be associated with a cell signaling system, but they also may contribute to division and elongation (Kaštier et al. 2015). High POD activity
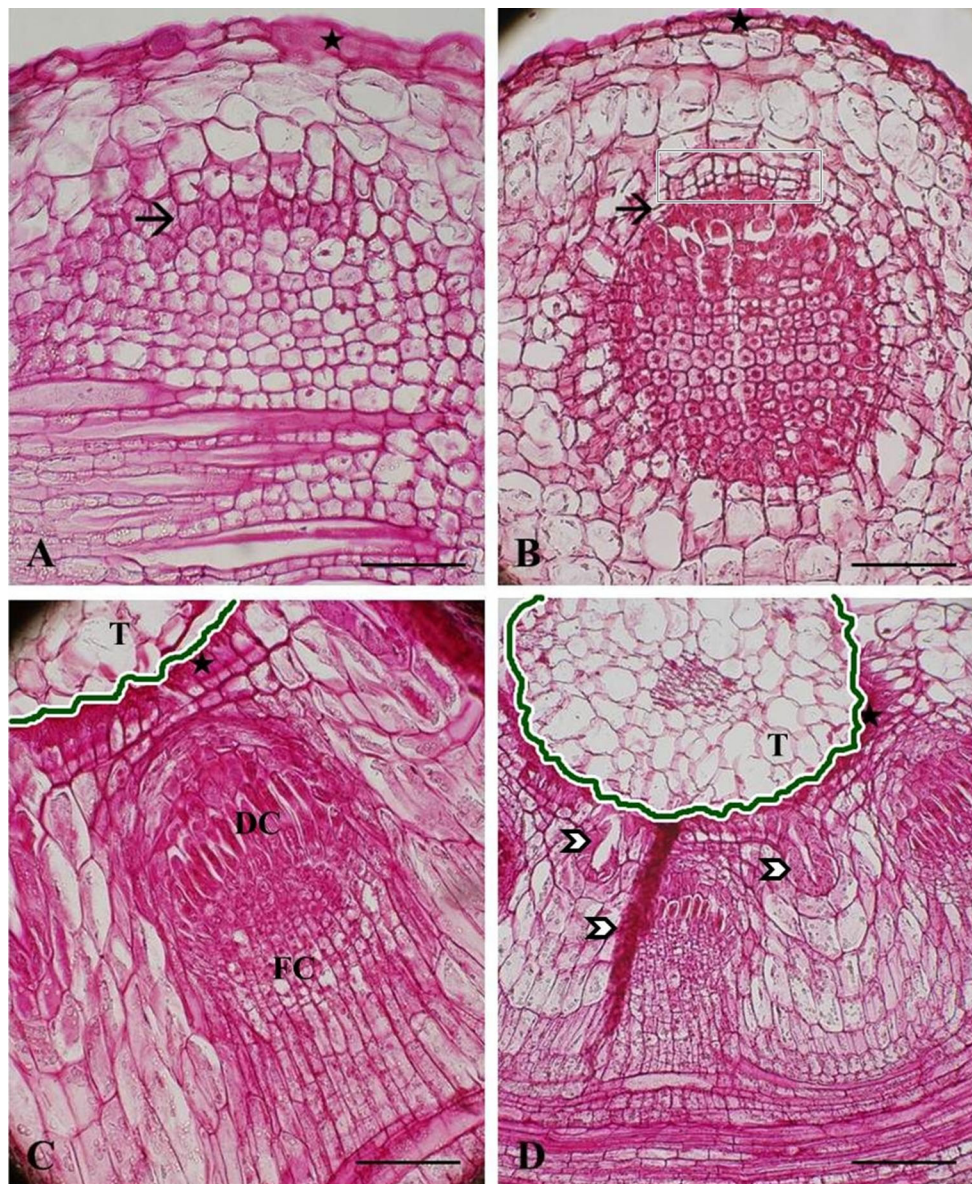
Fig. 3 Ruthenium red staining of 10 µm-thick longitudinal sections of dodder stems during prehaustorium development. Very early accumulation of pectins in the epidermal cells (*star*) and precursors of digitate cells (*arrows*, **a**, **b**). Mitotic active cells above the digitate cells precursors (*rectangle*, **b**). Massive elongation of digitate cell precursors and accumulation of pectins suggesting their high synthetic activity (**c**). Secretion of de-esterified pectins onto the surface of epidermal cells (*star*) serving as cement for the contact with the host (**c**). De-esterified pectins in the inner tangential and radial cell walls (*arrowheads*, **d**). The *green line* represents the interface between host plant (*Tobacco*) and parasite. *Scale bars* 100 µm

(Fig. 5, line 4) likely controls H_2O_2 levels, which can cause proliferation and elongation of prehaustorial cells, possibly through the modulation of the cytoskeleton (Gupta et al. 2015). In parasitic plants, peroxidases could be associated with cell wall modifications and their high abundance may indicate its important role during penetration (Lopez-Curto et al., 2006; Olsen et al. 2016). Despite the obvious differences concerning host invasion, it is reasonable to believe that parasitic plants have some similar strategies compared to the single-celled fungal hyphae and the needle-like stylet of nematodes, but the size and origin of the multicellular penetrating haustorium of parasitic plants is strikingly different (Mayer 2006; Kaiser et al. 2015). In addition to enzymes involved in modification of its own cell walls, parasitic plants secrete enzymes (e.g., pectinases, cellulases and proteases) that modulate the lignified and unlignified host plant tissue to withdraw nutrients (Nagar et al. 1984; Srivastava et al. 1994; Lopez-Curto et al. 2006; Ranjan et al. 2014; Johnsen et al. 2015; Kaiser et al. 2015). The formation of a functional connection to

Fig. 4 In situ histochemical staining of POD activity (*white* and *black arrows* showing the differences in POD activity): handmade cross sections (~0.5 mm) of dodder stems and its tobacco host plant. Section of young tobacco internode (the 2nd) without dodder (**a**) and attacked by dodder (**b**). The fourth tobacco internode without dodder (**c**) and attacked by dodder (**d**). Cross section of tobacco stem approximately 0.2–0.5 cm below (**e**) and 0.2–0.5 cm above (**f**) the penetrating haustorium. Cross section of dodder stem approximately 0.2–0.5 cm below (**g**) and 0.2–0.5 cm above (**h**) the penetrating haustorium. Host–parasite interactions—vascular connection between dodder and tobacco (**i**, **j**). *EC* epidermal cells, *C* cortical cells, *X* xylem, *P* pith, *V* vessels, *H* haustorium. *Scale bars* 200 μm (**a**, **g–i**); 100 μm (**b–d**, **e–j**)
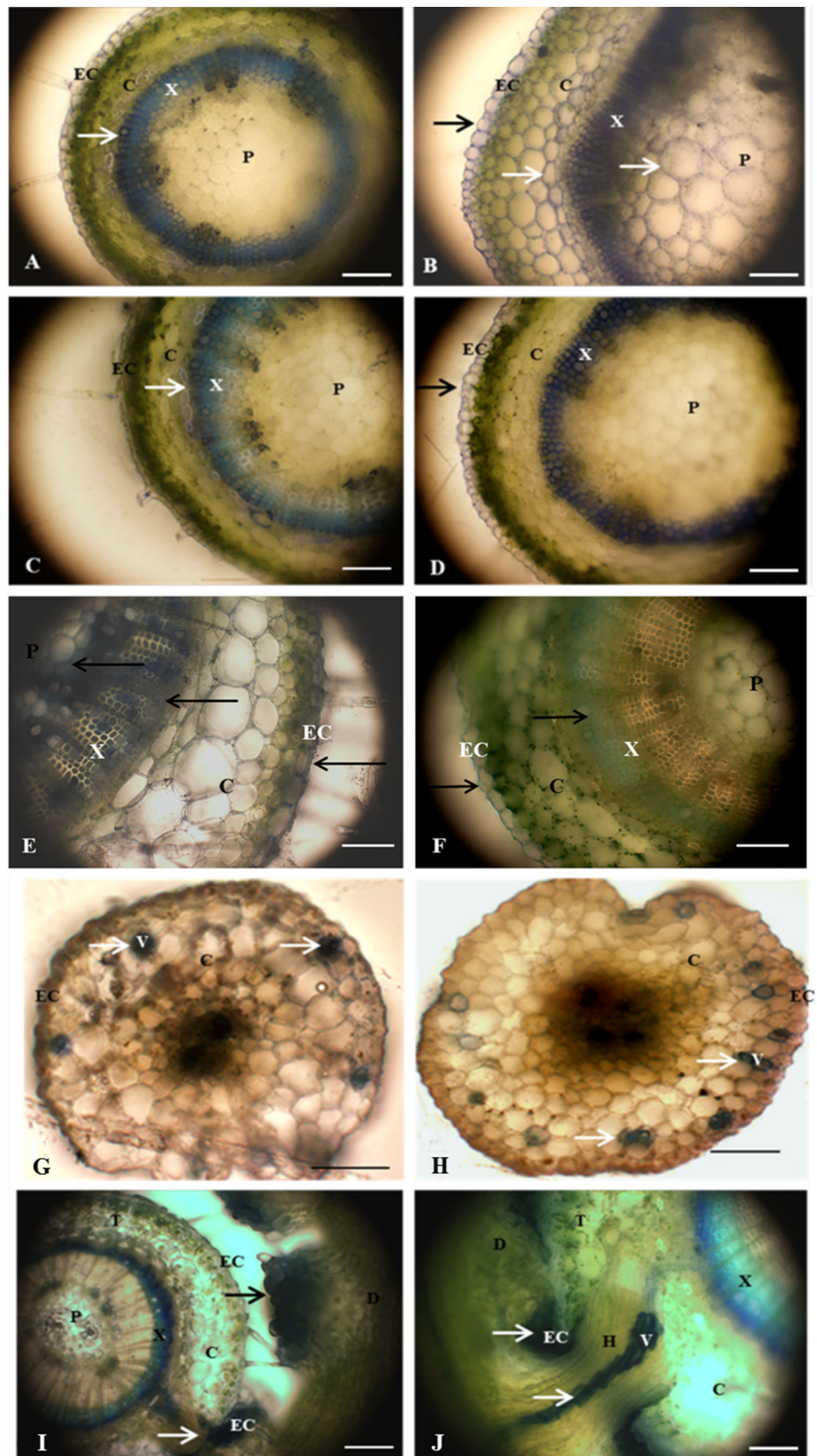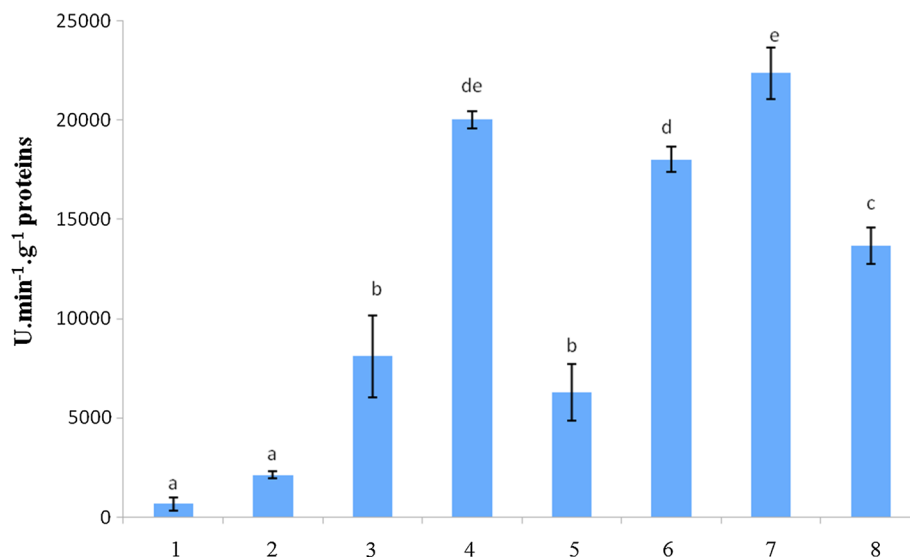
Fig. 5 Enzymatic activity of guaiacol POD in dodder and tobacco stems. Young tobacco internodes (2nd and 4th) were used as controls. T. below haustorium: tobacco stems with haustorium developed 0.2–0.5 cm above this section. T. haustorium: tobacco stems in the places of penetrating haustoria T. above haustorium: tobacco stems with haustorium developed 0.2–0.5 cm below this section. D. below haustorium: a section of dodder stems 0.2–0.5 cm below haustorium. D. haustorium: developed dodder haustoria. D. above haustorium: a section of dodder stems 0.2–0.5 cm above haustorium. Presented data are means (±standard errors) of three independent experiments (ten plants for each experiment were used). The *small letters inside the graphs* (*a*, *b*, *c*, *d*, e) denote significant difference at $P < 0.05$ (LSD test)

Fig. 6 Different isoforms of POD in dodder and tobacco plants. *Line 1* tobacco—control plant (the 2nd internode); *line 2* tobacco—control plant (4th internode); *line 3* tobacco plant 0.2–0.5 cm above the haustorium; *line 4* tobacco—haustorium; *line 5* tobacco plant 0.2–0.5 cm below the haustorium; *line 6* dodder stem 0.2–0.5 cm above the haustorium; *line 7* dodder–haustorium; line 8 dodder stem 0.2–0.5 cm below the haustorium
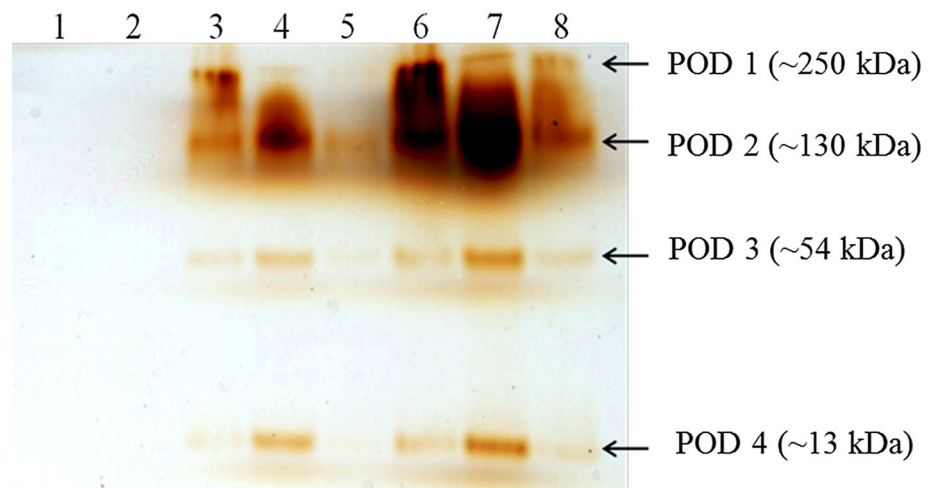
the host's vascular tissue is the most important step in the life cycle of *Cuscuta* spp. Upon contact with the host tissues (about 10 days after attachment), xylem vessels are formed in the haustorium in the direction toward the haustorial tip (Fig. 1d). We further observed that protoxylem is formed with annular or spiral secondary walls (Fig. 1e). It has been suggested that the role of cortical microtubules in this process enables wall deposition in the plasma membrane (Paredez et al. 2008; Gutierrez et al. 2009), since the clustering of microtubules proceeds cell wall thickening and has a parallel orientation with the cellulose microfibrils and patterned secondary walls

(Kaštier et al. 2015). Of note is that the high POD activity achieved in the place of the newly forming central xylem sieve (Fig. 1f) indicates its pivotal role in the H_2O_2-dependent lignification process (Czaninski et al. 1993; Olson and Varner 1993; Ros Barceló 1998; Weir et al. 2005). However, peroxidases were also identified in tobacco and its activities could be coupled with the improving the host plant fitness after dodder infection (data not shown), it is not possible to conclude which plant partner encodes the responsible enzymes.

For the stem parasitic weed dodder, different degrees of plastid functionality, ranging from intact chloroplasts, via
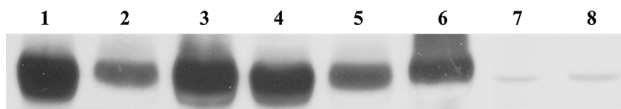
Fig. 7 Western blot analysis of POD in: *line 1* dodder stem 0.2–0.5 cm above the haustorium, *line 2* dodder stem 0.2–0.5 cm below the haustorium, *line 3* dodder haustorium, *line 4* tobacco stem in place of dodder haustorium penetration, *line 5* tobacco stem 0.2–0.5 cm above the haustorium, *line 6* tobacco stem 0.2–0.5 cm below the haustorium, *line 7* young tobacco control plant (2nd internode), *line 8* young tobacco control plant (4th internode)

plastids with impaired protein production and gene expression to plastids with reduced plastome genome are characteristic (van der Kooij et al. 2000; Berg et al. 2004; Ichihashi et al. 2015). In this study, we observed the numerous starch-containing amyloplasts in the cortex of older stems and also in haustorial beginnings, localized near the vascular bundles. This starch is possibly used as a source of energy in the dedifferentiation and reprogramming of these competent cells. In these cases, cycles of cell division are accompanied by the formation of proplastids, which have no internal membranes. Although the cells before penetration typically contain chloroamyloplasts (with 1–2 starch grains), this prehaustorial stage is photosynthetically inactive and thus the residual pigments may be involved in signaling rather than photosynthesis (Haidar 2011). The reduced content of chlorophylls and carotenoids during the formation of absorptive organs and its subsequent penetration into the host stem has been detected by Švubova et al. (2013), indicating that carotenoids can act as precursors for strigolactone biosynthesis (Seto and Yamaguchi 2014). This is in line with recently published findings that the genes for strigolactone biosynthetic enzymes (MAX1, MAX3, and MAX4) were upregulated in dodder prehaustorial cells (Ranjan et al. 2014). In this developmental stage, only low photochemical activity of PSII was previously detected by Švubova et al. (2013). Apparently, after successful parasitism, *C. europaea* plants acquire nutrients from the host, mostly through haustorial transport, and reduce chlorophyll biosynthesis and photosynthesis to a minimal level. These findings are consistent with the overall low expression of photosynthesis-related genes and also a higher expression of the genes for the transport to other holoparasite *C. pentagona* (Ranjan et al. 2014).

Previously, several nuclear- and plastid-encoded genes (e.g., FtsH5/VAR1, FtsH2/VAR2) have been identified which can ultimately affect thylakoid formation and plastid development (Sakamoto et al. 2003; Wang et al. 2004; Yu et al. 2007). The important role of THF1 (THYLAKOID FORMATION1) protein during plastid endomembrane system differentiation has been described in many photosynthetically active plants (Wang et al. 2004; Huang et al. 2006). On the other hand, accumulation of THF1 in *C.*

europaea haustorial cells, notably its specific extra-plastidial localization to plasma membrane and plasmodesmata (Švubova et al. 2013) demonstrates its important role in complex host–parasite relationships, possibly in cell-to-cell communication and signaling. In this context, the information that THF1, as an interaction partner of α-subunit of heterotrimeric G-protein (GPA1) is involved in the D-glucose signaling pathway, seems to be very important (Huang et al. 2006). Therefore, the significance of this protein to regulate haustorium differentiation and thus dodder parasitism will be an interesting question to investigate in the future.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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