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ORIGINAL ARTICLE

Agrobacterium rhizogenes transformed calli of the holoparasitic plant Phelipanche ramosa maintain parasitic competence

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Abstract

Phelipanche and *Orobanche* spp. (broomrapes) are economically important parasitic weeds, causing severe damage to many agricultural crops. However, conventional methods to control these parasitic weeds are often not effective. Targeting molecular and biochemical processes involved in the establishment of the connection between the parasite and the host may offer a new perspective for control. However, progress in the understanding of these processes is hampered by the fact that genetic transformation and regeneration of these parasites is difficult if not impossible due to their specific lifecycle. *Phelipanche* and *Orobanche* spp. are holoparasites that need to attach to the roots of a host plant to get their assimilates, nutrients and water to develop and reproduce. The present study describes a highly efficient genetic transformation and regeneration protocol for the root holoparasitic *Phelipanche ramosa*. We present a new transformation system for *P. ramosa* using *Agrobacterium rhizogenes MSU440* carrying a non-destructive selection marker gene coding for a red fluorescent protein (DsRed1). Using this protocol up to 90% transformation efficiency was obtained. We transformed 4 weeks old *P. ramosa* calli and transgenic calli expressing DsRed1 were then cultured on host plants. For the first time, we present shoot and flower development of the transgenic parasitic plant *P. ramosa* after successful connection of transgenic calli with the host plant roots. Moreover, we also present, for the first time, growth and development of *P. ramosa* shoots and flowers in vitro in the absence of a host plant.

Keywords Holoparasitic plant · *Phelipanche ramosa* · Genetic transformation · Regeneration · In vitro

Introduction

Parasitic plants belonging to the genera *Phelipanche* and *Orobanche* (broomrapes) have lost their autotrophic way of life. They lack the capacity for photosynthesis and need to gain their resources by parasitizing other plants. *Orobanche* and *Phelipanche spp*. absolutely rely on a host for all their nutritional needs, and are therefore classified as obligate

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holoparasitic plants (Heide-Jørgensen 2013). *Phelipanche ramosa* and *Phelipanche aegyptiaca* belong to the most widespread root parasites. Many agricultural crops are attacked by these species, such as tomato, tobacco, potato, rapeseed and carrot. Their habitat covers North Africa, Asia, Central and Southern Europe, Central America and Australia (Parker 2009). Field experiments with *P. aegyptiaca* in Israel demonstrated that an infestation of 100 seeds per kg of soil resulted in over 30% yield loss in carrot (Bernhard et al. 1998). The effect on the host and host range is similar in *P. aegyptiaca* and *P. ramosa*. In Central Europe, *P. ramosa* is infecting mainly tobacco and tomato. Cagáň and Tóth (2003) noticed a 40–50% decrease in tomato yield due to the infection of 10–20 *P. ramosa* shoots per tomato plant in southwest Slovakia. Much higher losses, due to *P. ramosa* infection, reaching up to 80% were reported in France in oilseed rape, tobacco and hemp (Gibot-Leclerc et al. 2012).

Parasitic plants have a unique life cycle which makes their management in the field very complicated. Their tiny seeds contain only very few resources and after their germination can only survive for a few days without connection with

a host root (Cardoso et al. 2011). This is the reason, why these parasites evolved a safety mechanism and they germinate only if special requirements are met. At first they must undergo a conditioning period at suitable moisture and temperature. Then the seeds become receptive to the chemical compounds—called germination stimulants—that are exuded from the roots of their host and induce their germination (Matusova et al. 2004). Upon contact with host-produced haustorium inducing factor (Riopel and Timko 1995), the radicle of the germinated seed forms a haustorium, a special organ formed by all parasitic plant species that assists in host attachment, penetration into the host tissue (the root system for *Phelipanche* spp.) and subsequent water and nutrients uptake from the host vasculature (Joel and Losner-Goshen 1994; Joel 2013). Subsequently, the parasite develops a shoot and flower and produces a large amount of seeds that contribute to further soil infestation (Sun et al. 2007). *P. ramosa* can produce exceptionally high numbers of seeds per plant of very small size (0.2–0.3 mm) and they can remain viable in the soil for a long period of time (up to 20 years) in the absence of host plants (Linke and Saxena 1989; Joel et al. 2006). Several chemical compounds were identified as germination stimulants. Among them, the strigolactones (SLs) are the most studied host-derived germination stimulants. SLs are apocarotenoids (Matusova et al. 2005), are, in addition to germination stimulants, also signalling molecules for arbuscular mycorrhizal fungi (Akiyama et al. 2005) and also seem to influence the interaction with other beneficial and pathogenic microorganisms in soil (reviewed in López-Ráez et al. 2017). In addition, SLs were classified as a new class of plant hormones (Gomez-Roldan et al. 2008; Umehara et al. 2008) and they have endogenous roles in many different biological processes in plants (reviewed in Al-Babili and Bouwmeester 2015; Wang et al. 2017).

Despite extensive research on the interaction of *Phelipanche* spp. with their hosts, mechanisms to control germination and development of these parasites have not been identified yet (reviewed in Yoder and Scholes 2010; Fernández-Aparicio et al. 2016). One major reason for this is the tight interaction of the parasite with the hosts. The most damage to the host plant occurs during underground development of the parasite after establishment of the physiological connection with the roots of the host. This underground development is difficult to monitor and control. Therefore, several strategies focus on key steps of underground development of the parasite—to prevent germination of their seeds and/or attachment to the host root.

The development of effective transformation and regeneration protocols for parasitic plants is essential for functional studies of parasite genes that are considered to be involved in the parasite-host interaction. However, parasitic plants are difficult to transform and cannot as easily be grown as normal non-parasitic plants, not in the least because they need a host (Westwood and Kim 2017). Although transformation for *P. aegyptiaca*, using *Agrobacterium rhizogenes*, has been described (Fernández-Aparicio et al. 2011) there is no transformation protocol available for *P. ramosa*. Here we present a transformation method for the holoparasitic *P. ramosa* mediated by *Agrobacterium rhizogenes*, the selection of transgenic tissue and subsequent regeneration of shoots and flowers on a host plant. Moreover, in this paper, for the first time, we describe shoot and flower development of *P. ramosa* in the absence of a host plant.

Materials and methods

Plant material

Seeds of *Phelipanche ramosa* (L.) Pomel were collected from mature plants that parasitized tomatoes (collected in 2000, Apricena, Italy and re-grown on tomato in a greenhouse in 2008; and seeds collected in 2012, Branovo, Slovakia); and rapeseed (collected in 2003, France). Seeds of tomato (*Solanum lycopersicum*) cv. MoneyMaker were purchased from the local market. All seeds were stored in the dark at a room temperature until use.

Seed sterilization

Surface sterilization of seeds was achieved by immersion in 2% (v/v) sodium hypochlorite solution containing 0.02% (v/v) Tween-20 for 5 min for *P. ramosa* and for 10 min for tomato with constant agitation. Subsequently, seeds were rinsed several times with sterile demineralised water. After this treatment, no contamination occurred in vitro.

Seed conditioning and germination

Phelipanche ramosa seeds were sown on 8 mm discs of glass fibre filter paper, approximately 100 on each disc. The 10 discs were placed in a sterile 9 cm Petri dish lined with two layers of filter paper wetted with 2.4 mL of distilled water. The Petri dishes were sealed with Parafilm and wrapped with aluminium foil to provide absolute darkness. Then the dishes were placed in a growth chamber with a constant temperature of 22 °C for seed conditioning. After 12 days all discs were transferred to a new sterile Petri dish containing a ring of wetted filter paper to prevent drying of the seeds (6 discs per Petri dish). To each disc we applied 40 µL of a solution of 1 µM GR24 (a synthetic strigolactone analog) in water to induce seed germination. The Petri dishes were incubated under the same conditions as for conditioning.

Growing *P. ramosa* in vitro

Three days later, germinated seeds were placed on culture medium in a plastic Petri dish (6 cm in diameter) at 22 °C in the dark. Gamborg B5 medium (Duchefa) (Gamborg et al. 1968) was supplemented with vitamins (100 mg L^{-1} myo-inositol, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine HCl, 10 mg L^{-1} thiamine HCl), 600 mg L^{-1} casein hydrolysate, 3% (w/v) sucrose and was solidified with 0.8% (w/v) plant agar. The pH of the medium was adjusted to 5.8 before autoclaving. After autoclaving, sterile 5% (v/v) coconut water (Sigma-Aldrich), 1 mg L^{-1} indole-3-acetic acid (IAA) and 8 mg L^{-1} gibberellic acid (GA$_3$) were added to the medium. To promote shoot development of *P. ramosa* without contact with the host, calli were grown in dark for 4 weeks. Then calli were transferred onto fresh medium described above, except without GA$_3$ and supplemented with 0.5 mg L^{-1} 6-benzylaminopurine (BAP). The calli were cultivated in Combiness vessels (Belgium) in the dark for additional 4 weeks with regular subculturing every 2 weeks onto fresh medium. Then calli with developing shoots were transferred onto medium with concentration of BAP increased to 1 mg L^{-1} for 12 weeks in dark, and finally cultivated on the light with regular subculturing for additional 8 weeks.

Transformation of *P. ramosa* calli

Agrobacterium rhizogenes strain MSU440 containing the plasmid pRiA4 (Sonti et al. 1995) and binary vector pRed-Root (Limpens et al. 2004) was used for genetic transformation of *P. ramosa*. pRedRoot contains DsRED1 as visual marker under the control of the constitutive *Arabidopsis* Ubiquitin10 promoter, *nptII* as selection marker and the NOS terminator (Limpens et al. 2004). *A. rhizogenes* cells were grown in liquid Luria–Bertani (LB) broth with 100 mg L^{-1} spectinomycin, 50 mg L^{-1} kanamycin and 400 µM acetosyringone at 28 °C overnight with shaking, in dark. The liquid culture was concentrated by centrifugation (1500 g/4 min) and the pellet was used for transformation. Four weeks old *P. ramosa* calli were inoculated with the *Agrobacterium* pellet with a syringe needle dipped in the pellet and co-cultivated for 4 days on solid Gamborg B5 basal salts medium supplemented with 400 µM acetosyringone, at 22 °C in darkness. The calli were then carefully rinsed in sterile water and transferred onto Gamborg B5 medium including vitamins, supplemented with 600 mg L^{-1} casein hydrolysate, 3% (w/v) sucrose, 1 mg L^{-1} IAA, 8 mg L^{-1} GA$_3$, 5% (v/v) coconut water, 300 mg L^{-1} cefotaxime and 0.8% (w/v) plant agar. Calli were subcultured every 2–3 weeks onto fresh medium. Some of the *P. ramosa* calli were fragile and disintegrated into smaller parts during manipulation. To calculate transformation efficiency, each callus developed from a single germinated seed was therefore wounded only once by a needle carrying *A. rhizogenes* and then rinsed and cultivated separately.

Selection of transformed lines

Distinction between transformed calli and untransformed calli was based on expression of DsRED1, detected by a Leica MZ10 F Fluorescence stereomicroscope. Transgenic tissues were identified visually, separated from non-transgenic by forceps and sub-cultured. The identification of transgenic parts and careful separation was repeated before each subculture until the DsRED signal was present in all cells of the callus.

In vitro infection

The development of a connection between the *P. ramosa* calli and a host plant was examined in rectangle sterile Petri dishes (2 × 15 × 15 cm). Sterile seeds of host plants were sown on sterile filter paper in the Petri dish and they were watered with a modified half-strength Hoagland nutrient solution (2.4 mM KNO$_3$, 1.6 mM Ca(NO$_3$)$_2$, 0.2 mM KH$_2$PO$_4$, 0.8 mM MgSO$_4$, 0.18 mM FeSO$_4$, 0.1 mM Na$_2$EDTA, 4.5 µM MnCl$_2$, 23 µM H$_3$BO$_3$, 0.3 µM CuSO$_4$, 1.5 µM ZnCl$_2$ and 0.1 µM Na$_2$MoO$_4$). The nutrient solution was regularly added as needed. Approximately 2 weeks after sowing the host plants had developed a healthy, sterile, root system, while the shoots were protruding from the Petri dish through a small hole that was otherwise closed by sterile cotton wool. Calli of *P. ramosa* were placed on the elongation zone of the roots. The Petri dishes were sealed with Parafilm and wrapped in aluminium foil to allow roots to grow in darkness. Dishes were kept vertically in a climate room at 25 °C with 16/8 h light/dark cycle. Petri dishes were regularly checked for establishment of parasite–host root connection and development.

Results and discussion

Phelipanche ramosa callus initiation and growth

Upon transformation of *P. ramosa* callus derived from different seed batches (different locations and parasitizing different hosts) we observed different callus morphologies. The *P. ramosa* seeds obtained from rapeseed developed short radicles and subsequently calli of characteristic small globular shape. *P. ramosa* seeds obtained from a tomato host developed longer radicles and subsequently fast growing calli with many adventitious roots. Tomato was infected with wildtype calli that were later transferred into soil and grown until seed production (data not shown). Calli generated from *P. ramosa* seeds collected from a tomato field

(locality Branovo, Slovakia) developed a shoot meristem in vitro growing on a medium without any host plant present. This interesting phenomenon was also observed by Zhou et al. (2004), but they did not achieve further development of shoots. We regularly subcultured calli with developing shoots (Fig. 1a–d) to fresh media and kept them in the dark to imitate the underground stage of shoot growth. Since the shoot developed very slowly on this medium, we changed the plant hormone composition. A medium without GA$_3$ but with cytokinin BAP in a concentration of 0.5 mg L^{-1} enhanced shoot development. After 4 weeks the concentration of BAP was increased to 1 mg L^{-1} and shoots were grown in the dark for additional 12 weeks. Then shoots were transferred to the light for 8 weeks. Developed shoots were quite long, and reached about 12 cm in height, although they were thin and fragile compared to *P. ramosa* shoots developing on a host root. Nevertheless, flowers of pale violet colour developed (Fig. 1c, d). The original callus from which these shoots emerged was hard and white, but turned brown and necrotic during cultivation. To our knowledge, this is the first report on the development of *P. ramosa* shoots and flowers in vitro in the absence of any host. Considering the fact, that *P. ramosa* is self-fertile (Kreutz 1995), this provides the interesting opportunity to regenerate *P. ramosa* plants fully in in vitro conditions without any host plant present.

Genetic transformation and calli development

One of the requirements for successful plant transformation is the effective selection of transgenic tissue. The frequently used kanamycin resistance as a selectable marker for transformation of plants seems to be not effective for broomrapes. Previously, we tested the effect of kanamycin in concentrations ranging from 0 to 250 mg L^{-1} on in vitro growing

P. ramosa calli in solid and liquid media (Kullačová and Matúšová 2015). After 4 weeks of cultivation at the highest concentration of kanamycin (250 mg L^{-1}) many calli were smaller, darker and brownish with 69% necrotic calli compared to the control. However, this relatively high kanamycin concentration did not cause death of all cells, neither in solid nor liquid cultures and some calli regenerated after transfer onto medium without kanamycin. The same effect was observed by Fernández-Aparicio et al. (2011) for *P. aegyptiaca*. The authors observed slower growth, but not death of *P. aegyptiaca* calli at 100 mg L^{-1} kanamycin in liquid culture. It is also known from literature, that some plants are able to tolerate higher concentration of kanamycin in the environment. Chauvin et al. (1997) reported that gladiolus can tolerate more than 500 mg L^{-1} and tulip more than 750 mg L^{-1} kanamycin in the medium.

An alternative approach to using antibiotic resistance genes is the use of fluorescent markers. *Agrobacterium rhizogenes* strain MSU440 containing the binary vector pRedRoot (Limpens et al. 2004) with the red fluorescent marker gene, DsRED1, was chosen for selection of transgenic tissue of *P. ramosa*. Four weeks old calli were infected by *A. rhizogenes* and transgenic tissue emerged directly from the inoculated site of the calli. The transgenic tissues expressing DsRED1 were clearly visible under the stereomicroscope with an appropriate filter settings 3 weeks after transformation (Fig. 2a, b) and based on expression of DsRED1 protein, 90% transformation frequency was obtained. Uninfected control calli did not show any fluorescence. Similarly, Fernandez-Aparicio et al. (2011) reported transformation of 3 month-old calli of *P. aegyptiaca* by *A. rhizogenes* with subsequent 4 months selection and growth of calli to be able to parasite host. In our system, we transformed 1 month-old calli of *P. ramosa* and applied 3 months old transgenic calli
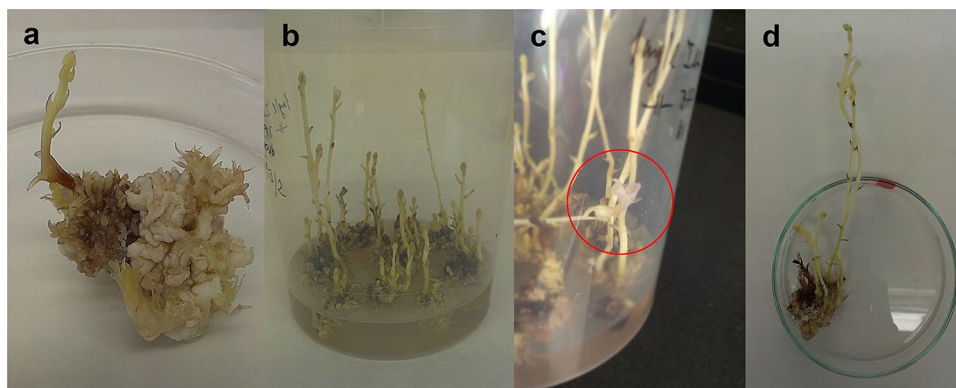

Fig. 1 Development of *Phelipanche ramosa* shoots in vitro in absence of a host. **a** *P. ramosa* calli were grown and regularly subcultured on Gamborg B5 medium supplemented with 600 mg L^{-1} casein hydrolysate, 3% (w/v) sucrose, 5% (v/v) coconut water (Sigma-Aldrich), 1 mg L^{-1} IAA, 8 mg L^{-1} gibberellic acid (GA$_3$) solidified with 0.8% (w/v) plant agar and developed small shoots in 6 weeks. **b** Developing shoots growing on the same medium but with 0.5 mg L^{-1} BAP and without GA$_3$ for 3 months. **c, d** Shoots elongated and developed flowers after another 2 months growing on the latter medium but with 1 mg L^{-1} BAP

Fig. 2 Selection of transgenic lines. Selection of transgenic calli of *P. ramosa* was based on the expression of the red fluorescent marker DsRED1. **a, b** Three weeks after transformation; **c, d** continuous sub-culturing of selected transgenic tissue for 2 months—calli with small parts of non-transgenic tissue; **e, f** transgenic calli ready to be placed on host roots. **a, c, e** observed under white light; **b, d, f** observed under UV light with appropriate filter settings
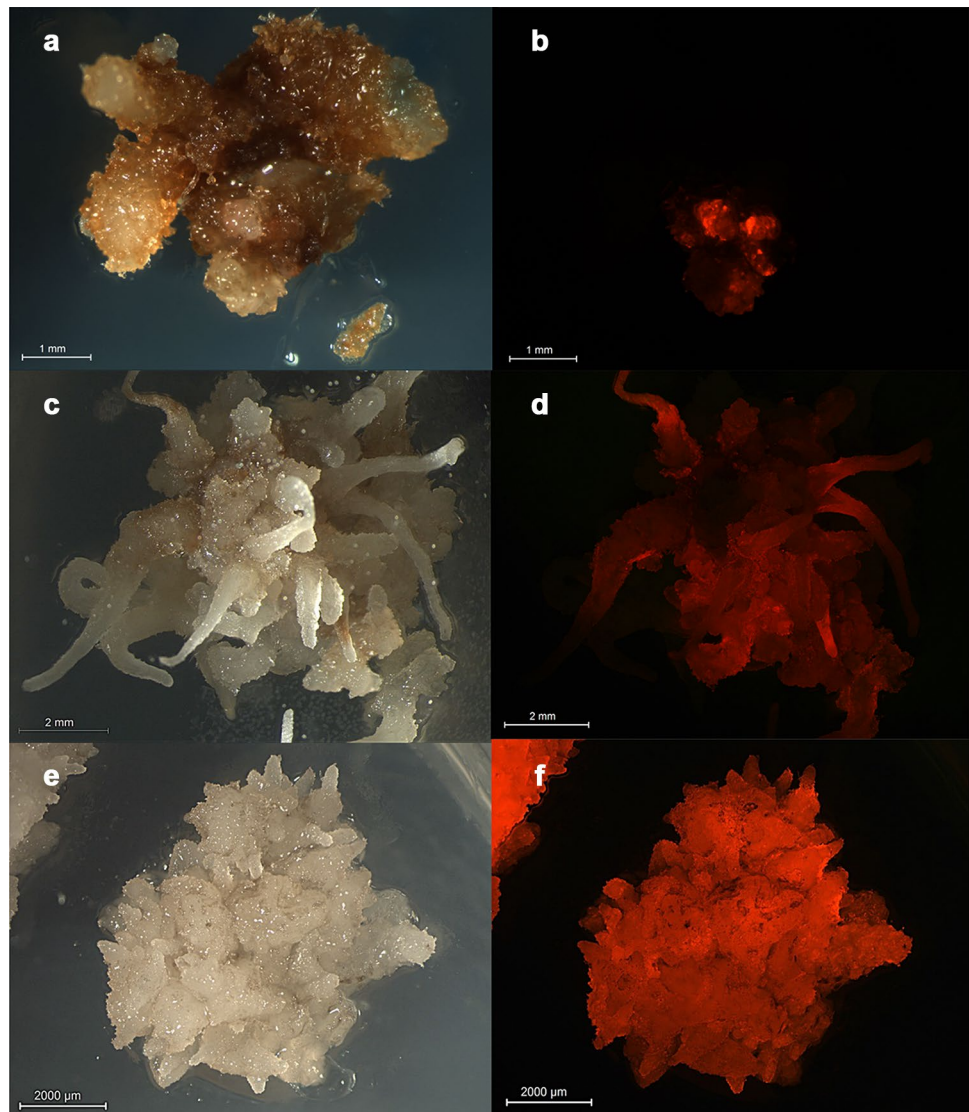

to host roots, which is quite a bit faster. The transformation efficiency of 90% is substantially higher than reported for *P. aegyptiaca* (15%; Fernández-Aparicio et al. 2011) and the facultative parasite, *Triphysaria versicolor*, for which root transformation was used, also with *A. rhizogenes* (33%; Bandaranayake et al. 2010).

The calli were regularly inspected and transgenic tissues expressing fluorescent DsRED1 selected under the stereomicroscope and subsequently sub-cultured. Three months later, sufficient amount of transgenic lines expressing DsRED1 protein were obtained and used to infect host roots to monitor their ability to regenerate into a plant. The transgenic calli were morphologically indistinguishable from untransformed controls. We observed calli with multiple adventitious roots (Fig. 2c, d), most of the transgenic calli were beige, some transgenic calli were pink. Similar morphologically normal transgenic roots emerging from shoot tissue infected by *A. rhizogenes* were observed by Limpens et al.

(2004) and Crane et al. (2006) for *Arabidopsis* and *Medicago truncatula* as well as for the facultative parasitic plant *Triphysaria versicolor* (Tomilov et al. 2006).

Selected transgenic calli were placed on the roots of tomato (Fig. 3) or tobacco host plants. Some calli attached to the hosts, however, the percentage of attachments was low (5%) similar to the percentage of attachments of non-transgenic calli. Following vascular connection with the host, a thickening of the host root at the site of successful attachment occurred, characteristic for radicle attachment of broomrapes. Attached calli subsequently developed tubercles with 90% of frequency about 4 weeks after inoculation. We noticed formation of many tubercles, which differed in size between several millimetres to several centimetres, some of which developed lateral haustoria. The similar large diversity in success of germinated seeds to attach, diversity in tubercule size and morphology occurs in *P. ramosa* calli grown in vitro on tomato seedlings (not shown).

Fig. 3 Development of parasitic plant *Phelipanche ramosa* from transgenic calli transformed by *A. rhizogenes* MSU440. **a** Transgenic calli placed on tomato roots (cv. MoneyMaker). Necrotic calli did not invade the tomato root. Transgenic callus that successfully penetrated tomato roots (arrow) developed further. **b** Detail of successfully connected callus; **c** 1 week later we noticed further growth of callus; **d** in 3 weeks shoot buds started to develop (arrow); **e** elongation of first *P. ramosa* shoot; **f** in 6 weeks more shoots developed; **g** shoots elongated and flowers of typical pale violet colour appeared
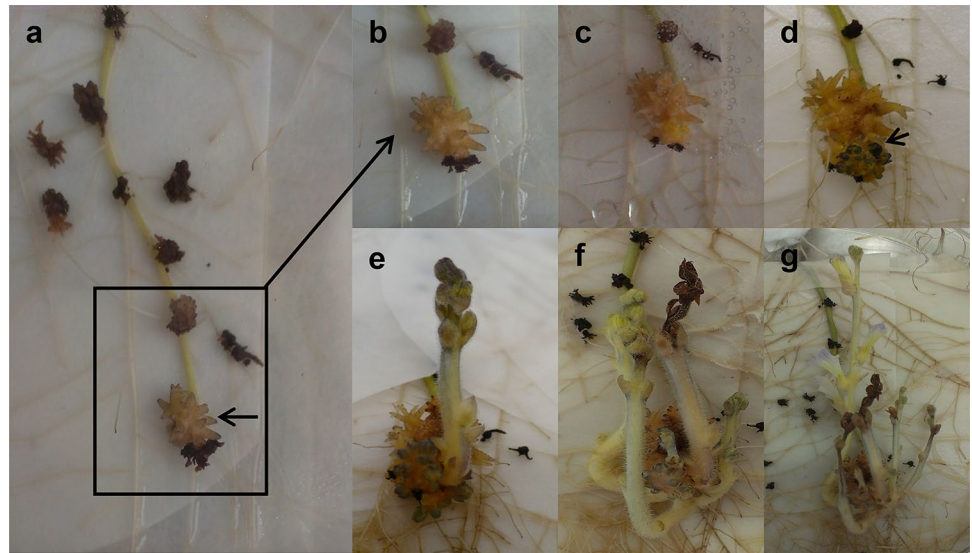
The development of transgenic parasitic plants from the tubercles was challenging. We managed to obtain regeneration of transgenic parasitic plants up to the flowering stage in vitro (Fig. 4). The first shoots appeared about 6 weeks after inoculation of the host roots with the transgenic calli. Subsequent growth then proceeded quite quickly including the emergence of additional shoots from the tubercle. The shoots elongated and blossomed into typical pale violet flowers. We examined the plants under the fluorescence microscope and noticed the red fluorescent

Fig. 4 In vitro regenerated transgenic *P. ramosa* on tomato host. **a** 6 shoots developed from a single tubercule and developed flowers of pale violet colour. Some of the shoots turned necrotic; **b–e** details of flowers under light and fluorescence stereomicroscope. Red fluorescent signal was observed in all organs of the plant, the strongest fluorescence was observed in the tubercle and in the stigmas; **f** flower detail. Scale bar for **b–e** pictures represents 2 mm
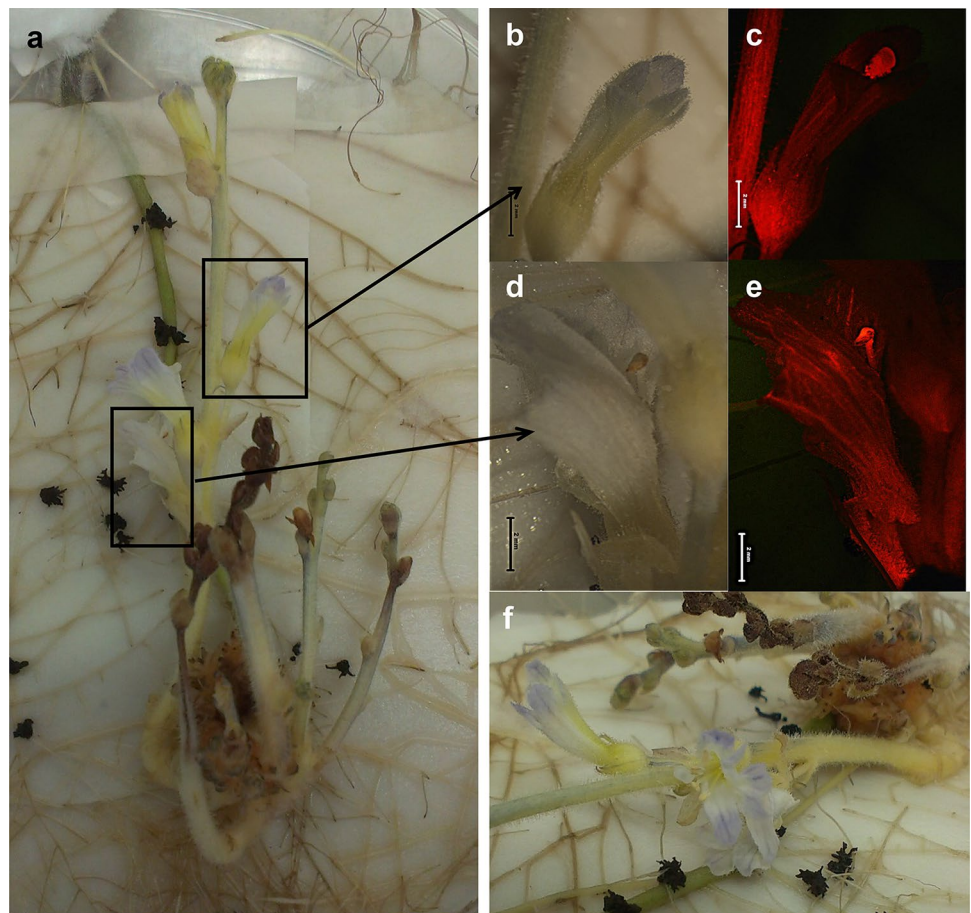
signal in all of the plant organs. The most vivid fluorescence was observed in the tubercle, stamens and stigma (Fig. 5).

Size and development of the transgenic plants seemed to be negatively affected by the restricted growth of the host under in vitro conditions. Therefore, we also carefully transferred tomato plants with developing transgenic *P. ramosa* on the roots to soil. The development and growth of adventitious roots was observed (Fig. 6). Unfortunately, no seeds were obtained because the plants did not survive.

Conclusion and perspectives

The development of an efficient transformation and regeneration protocol for *P. ramosa* presented here is a significant step forward in the study of the functions of genes involved in the interaction between plant parasites and their host. This protocol now allows to study the function of parasitic plant genes, for example in haustorium formation. *P. ramosa* develops a terminal haustorium at the apex of the radicle in response to haustorium-inducing factors released from the host. The recently published excellent

Fig. 5 Transgenic flowers of *P. ramosa*. **a** Observed under white light; **b** observed under UV light with appropriate filter settings
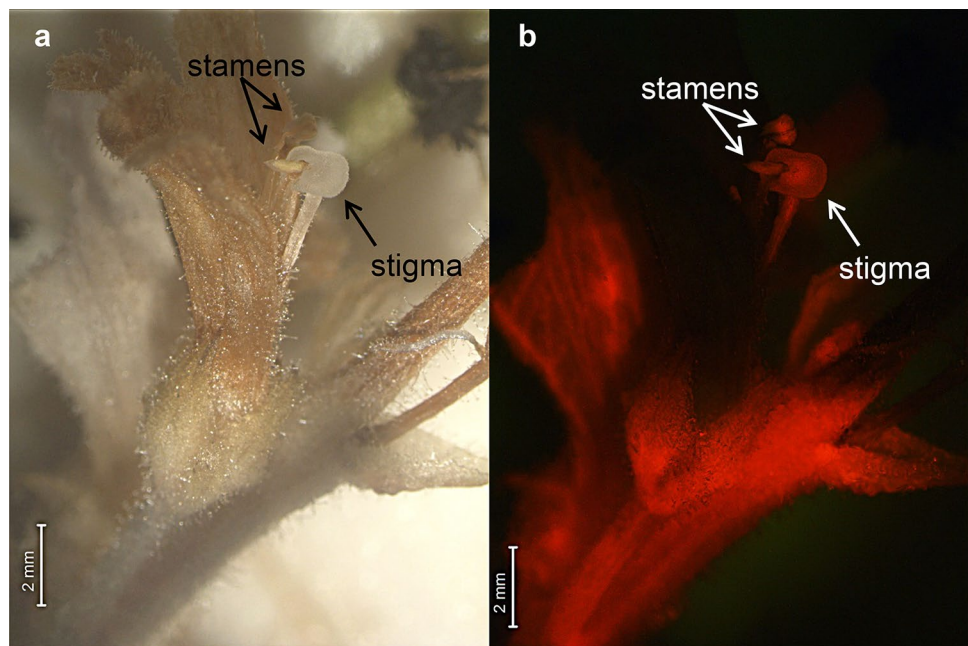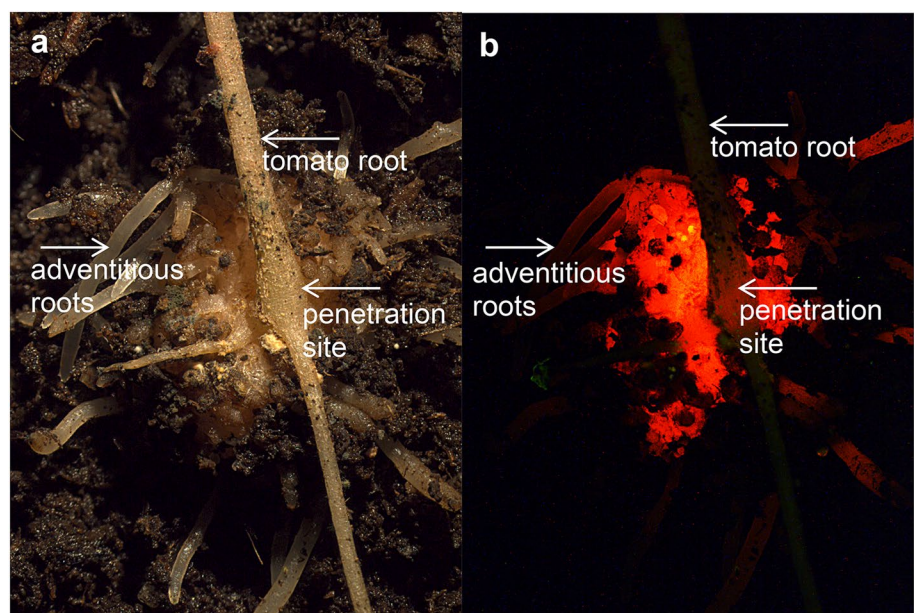

Fig. 6 Developing transgenic *P. ramosa* on tomato root in soil. Transgenic calli successfully penetrated into a tomato root and developed tubercule with adventitious roots. The tomato root exhibits the typical swelling above the parasite penetration site. **a** Observed under white light; **b** observed under UV light with appropriate filter settings

work of Goyet et al. (2017) showed that cytokinins exuded from the host roots are major players in haustorium formation and aggressiveness of *P. ramosa*. They identified several genes differentially expressed during induction of early haustorial structures (swelling of the root apex and epidermal cells to form papillae), which could be candidate genes for further characterisation using the transformation/regeneration protocol described here. Also, genes essential for plant parasitisms identified in comparative transcriptome analysis in parasitic and nonparasitic angiosperms (Yang et al. 2015), including genes upregulated during haustorium development would be worthwhile to investigate. Using the genome editing CRISPR/Cas9 system (clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) system) to knockout these genes could help to functionally characterized these genes. Assuming their key role in haustorium formation and/or attachment to the host tissue, regeneration in vitro without attachment to a host may result in seed production and further functional characterisation. However, it should be noted that although regeneration from callus up to flowering was achieved, with or without host, some problems such as low frequency of callus (transgenic or non-transgenic) attachment to the host root, and robust seed production still remain. Our present work is focused on the improvement of this. This should help us answer the question, how these parasites control their interaction with their hosts and how this may be used in engineering resistant crops to these parasitic weeds (Ishida et al. 2011).

The development of new transformation methods is essential for functional genomics studies in parasitic plants. We have established a highly efficient *Agrobacterium*-mediated transformation protocol for *P. ramosa*. Examination of whole developed transgenic *P. ramosa* plants under the fluorescence microscope proved, that the DsRED signal was present in all parts. We consider this plant as fully transgenic and plant development was not influenced by presence of the DsRed gene.

Author contributions Conceived and designed the experiments: RM, DL. Performed the experiments: RM, DL. Analysed the data: RM, DL, CRS, HJB. Contributed reagents/materials/analysis tools: RM, HJB. Contributed to the writing of the manuscript: DL, CRS, HJB, RM. All authors read and approved the manuscript.

Compliance with ethical standards

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