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RAPID COMMUNICATION

Hypericum perforatum plant cells reduce *Agrobacterium* viability during co-cultivation

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Abstract Plant recalcitrance is the major barrier in developing *Agrobacterium*-mediated transformation protocols for several important plant species. Despite the substantial knowledge of T-DNA transfer process, very little is known about the factors leading to the plant recalcitrance. Here, we analyzed the basis of *Hypericum perforatum* L. (HP) recalcitrance to *Agrobacterium*-mediated transformation using cell suspension culture. When challenged with *Agrobacterium*, HP cells swiftly produced an intense oxidative burst, a typical reaction of plant defense. *Agrobacterium* viability started to decline and reached 99% mortality within 12 h, while the plant cells did not suffer apoptotic process. This is the first evidence showing that the reduction of *Agrobacterium* viability during co-cultivation with recalcitrant plant cells can affect transformation.

Keywords *Agrobacterium* · *Hypericum* · Plant defense response · Plant recalcitrance

Abbreviations

CFU	Colony forming unit
FDA	Fluorescein diacetate
H_2DCFDA	2′,7′-Dichlorodihydrofluorescein diacetate
HP	*Hypericum perforatum*
PI	Propidium iodide
ROS	Reactive oxygen species

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Introduction

Agrobacterium-mediated plant transformation is an indispensable tool in modern plant biology. Regardless of the availability of alternative plant transformation tools, *Agrobacterium* is the most preferred vehicle of gene delivery for its simplicity, cost-effectiveness and frequent single copy gene integration into the host plant genome. Having access to a wide array of useful genes in the post-genomic era, the interest of applying this system to all crops has been intensified. Amenability of different plant species, varieties, tissues and cells to *Agrobacterium* infection greatly varies, which makes transformation either inefficient or impossible in recalcitrant plants. Hence, there is a need to study the basis of plant recalcitrance towards this bacterium in the view of plant–pathogen interaction.

Agrobacterium has been generally recognized as a unique pathogen, which does not induce plant defense response (Robinette and Matthysse 1990; Felix et al. 1999). However, there are evidences for the induction of necrosis (Perl et al. 1996) and programmed cell death (Hansen 2000; Parrot et al. 2002) in plant cells after co-cultivation with *Agrobacterium*. Ditt et al. (2001, 2005, 2006) have shown that plants can modulate their gene expression pattern and trigger defense machinery in response to *Agrobacterium*. They also proposed that the plant defense system has an important role in controlling infection and transformation by *Agrobacterium* (Ditt et al. 2005). Most recently, Yuan et al. (2007) demonstrated that the plant signaling compound salicylic acid (SA) can shut down the expression of *Agrobacterium vir* regulon and directly affect the infection process. Taken these reports into account, the existence of plant resistance response against *Agrobacterium* seems evident.

Exploring the basis of plant recalcitrance to *Agrobacterium*-mediated transformation would be useful to improve

the transformation efficiency of recalcitrant plant species. Such attempt has so for not been made in any of the recalcitrant plant species to the best of our knowledge. Hence, we analyzed the basis of *Hypericum perforatum* L. (HP) plant recalcitrance to *Agrobacterium*-mediated transformation in the current investigation. Our results show that HP plant defense response quickly reduces *Agrobacterium* viability and affects the transformation process during co-cultivation.

Materials and methods

Bacteria, culture conditions and inoculum preparation

Agrobacterium tumefaciens EHA105, *A. rhizogenes* A4 and *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA, USA) were used in the present study. All the strains were transformed with the plasmid pCAMBIA1301 to obtain kanamycin resistance. *A. tumefaciens* and *E. coli* were cultured in Luria–Bertani (LB) medium and *A. rhizogenes* was cultured in YMA medium (0.4 gl^{-1} yeast extract, 10 gl^{-1} mannitol, 0.5 gl^{-1} K_2HPO_4, 0.2 gl^{-1} $MgSO_4$ and 0.1 gl^{-1} NaCl, pH 7.0). Both LB and YMA media were augmented with 50 mgl^{-1} kanamycin. Broth cultures were initiated by inoculating single colonies of these bacteria into flasks (25 ml) containing 5 ml of liquid media. Cultures were kept at 200 rpm on a rotary shaker at specific temperatures (28°C for *A. tumefaciens* and *A. rhizogenes*; 37°C for *E. coli*) for 1 day. Subsequently, 2 ml of grown bacterial cultures were transferred to flasks (500 ml) containing 200 ml of the respective media and incubated as mentioned above. When the optical density (OD) of *A. tumefaciens* and *A. rhizogenes* cultures reached around 0.6 at 660 nm, 200 μM acetosyringone (AS) was added. Bacteria were spun down using a tabletop centrifuge (Sigma, St Louis, MO, USA) at 2,415 *g*, when the cultures reached approximately 1.0 OD at 660 nm. The pellets were gently suspended in 2 ml MS medium (Murashige and Skoog 1962) and used as bacterial stock.

Co-cultivation of *Agrobacterium* with HP cells

HP cell suspension culture was established from the variety "Helos" (Richters seeds, ON, Canada) as described earlier (Dias et al. 2001). After 5 days of subculture, HP suspended cells were aseptically collected in a sterile beaker. The cell density was estimated in an improved Neubauer haemocytometer (Neubauer, Wertheim, Germany) and adjusted to a final concentration of about 5,000 HP cells ml^{-1} using MS medium. A final concentration of 100 μM AS was added to the suspended cells. Flasks containing 80 ml of this HP cell suspension were inoculated with the stock of *Agrobacteria* (*A. tumefaciens* or *A. rhizogenes*) in a way to reach 250×10^7, 125×10^7 and 25×10^7 bacterial cells ml^{-1}, concentrations generally used in plant transformation protocols. Flasks containing just HP cells served as HP control. Flasks containing 80 ml MS medium inoculated just with bacteria to a final concentration of 250×10^7 cells ml^{-1} served as the bacterial control. All the experiments were done in triplicate.

To examine the effect of HP cells on a non-pathogenic strain, *E. coli* DH5α was co-cultivated with HP cells. The effect of a non-recalcitrant plant on *A. tumefaciens* viability was scored by co-cultivating this bacterium with tobacco BY2 cells (PC-1181, DSMZ, Braunschweig, Germany).

All the treatment and control cultures were incubated under photoperiod at 25°C in a growth chamber during co-cultivation.

Intracellular reactive oxygen species (ROS) production

Intracellular reactive oxygen species (ROS) production in the control and *Agrobacterium*-inoculated HP cells was measured using 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) probe (Molecular Probes, Invitrogen). Briefly, 1 ml of HP cell suspension taken at different post inoculation timings (0, 15, 30, 60, 120, 180, 240, 300 and 360 min) were transferred to Eppendorf tubes containing 10 μl of 200 μM H_2DCFDA and mixed in a vortex for 20 s. After 15 min incubation in dark, the cells were spun down and 0.5 ml of the supernatant was transferred to a cuvette containing 2.5 ml sterile distilled water. Fluorescence of the samples was read in a Perkin Elmer LS50 spectrofluorimeter (Perkin Elmer, Buckinghamshire, UK) at 488 nm excitation and 525 nm emission with 1 s integration.

Measurement of cell viability

Control and treated plant cultures were checked for their cell viability after bacterial inoculation, for five consecutive days, by fluorescein diacetate (FDA) and propidium iodide (PI) double staining as described by Conde et al. (2007). Briefly, after thorough mixing using cut pipette tips, 1.0 ml cell suspension from each cultures were taken in Eppendorf tubes. To these samples, 10 μl FDA (500 μg μl^{-1}, Sigma) and 1 μl PI, (500 μg μl^{-1} Sigma) were added, thoroughly mixed and incubated in dark at room temperature (±25°C). After 10 min of incubation, 100 μl of cell suspension was spread on a glass slide and observed under a Leica DM 5000B microscope (Leica Microsystems, Wetzlar, Germany) equipped with AF6000 fluorescent lamp (Leica Microsystems) programmed for excitation at 490 nm and emission at 510 nm, for FDA, and excitation at 543 nm and emission at 570 nm, for PI. Light microscopic and fluorescent images were acquired using a DFC350 camera (Leica Microsystems) attached to the microscope.

Additionally, 1 ml samples of HP cell suspension co-cultivated with *Agrobacterium* as described above were transferred to solid MS medium containing 0.5 mgl^{-1} NAA, and cultured to check if they grow normally.

The bacteria viability (*A. tumefaciens*, *A. rhizogenes* and *E. coli*) was also monitored during co-cultivation with HP cells. Briefly, 100 µl samples were taken from plant cell cultures at different time intervals (0, 6, 12, 24 and 48 h) after bacterial inoculation and spread on plates containing semi-solid LB (for *A. tumefaciens* and *E. coli*) or YMA (for *A. rhizogenes*) medium augmented with 50 mgl^{-1} kanamycin. In order to count the colony forming units (cfu), 10^{-6} dilution of these samples were also plated in the same manner. Plates were incubated in the dark at specific temperature mentioned before for each bacterial strain. *A. tumefaciens* and *A. rhizogenes* colonies were counted, respectively, after 2 and 4 days of incubation.

Moreover, media from control and HP cultures co-cultivated with *Agrobacterium* for 24 h were filtered through 0.45 µm sterile membrane filter (Schleicher and Schuell, Dassel, Germany). Each filtrate was inoculated with fresh *Agrobacterium* to a final concentration of 250×10^7 cells ml^{-1} and 100 µl aliquots were plated after 24 h incubation, and bacterial growth was checked after 2 days.

Effect of *Agrobacterium* co-cultivation on the stability of HP DNA

To observe whether *Agrobacterium* can induce DNA fragmentation of HP cells, DNA was isolated using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), from control and treatments after 1, 2 and 3 days of co-cultivation and 0.5 µg of DNA/lane were resolved in 0.8% agarose gel with ethidium bromide.

Northern-blot analysis of PAL gene expression in HP cells

Total RNA was isolated from control and *A. tumefaciens* treated HP cells after 0, 4, 12 and 24 h of inoculation following the procedure of Çakir et al. (2003). From each sample, 20 µg RNA was taken and separated in formaldehyde–agarose gel before transferring them to Hybond-N® membrane (Amersham Biosciences, Buckingham, UK) via capillary transfer. A *PAL* gene specific fragment amplified from HP cDNA library was labeled with α-[^{32}P] dCTP (Amersham) using Prime-a-Gene® labeling kit (Promega, Madison, WI, USA) and used as probe for hybridization. Hybridized blots were washed twice with 2× SSC + 0.1% SDS (2 × 15 min) and with 0.1× SSC + 0.1% SDS (5 min) at 65°C. The blots were exposed to the imaging screen for 12 h and scanned in a Personal Molecular Imager (Bio-Rad, Hercules, CA, USA).

Assay for T-DNA transfer

The presence of T-DNA in the HP and tobacco BY2 cells after co-cultivation with *A. tumefaciens* was analyzed by GUS assay (Jefferson et al. 1987). Samples (1.0 ml) taken from plant cultures in successive days (1, 2, 3, 4 and 5 days) were centrifuged at 600 *g*. The supernatant was discarded and the pellet was thoroughly washed in sterile distilled water to get rid of the bacteria. Then the plant cells were re-suspended in 1.0 ml GUS solution and incubated at 37°C for 24 h. Cells were viewed under a light microscope for the presence of blue staining.

Statistical analysis

For all the treatments, three independent experiments (each with three replica) were done. Statistical analysis was performed using GraphPad Prism, version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

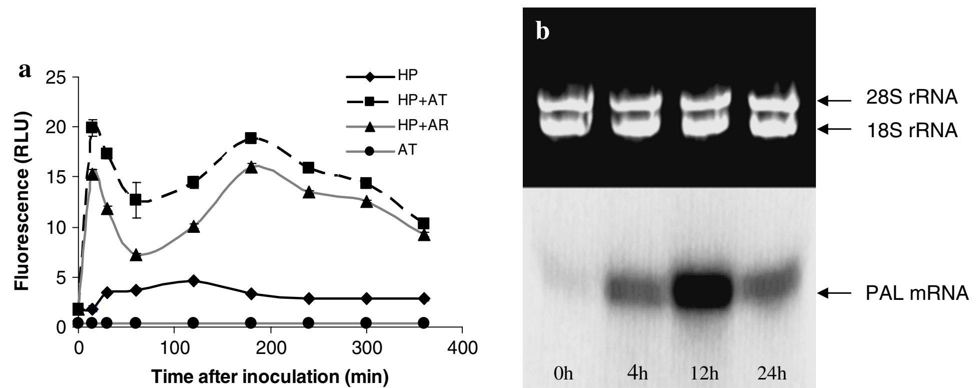
Agrobacterium inoculation rapidly evokes HP defense response

HP cells co-cultivated with *A. tumefaciens* or *A. rhizogenes* produced an intense biphasic ROS burst when compared to control cells (Fig. 1a). A first ROS peak was observed within 15 min and a second one between 2 and 5 h of co-cultivation (Fig. 1a). Moreover, *PAL* gene expression was up regulated as early as in 4 h after challenging with *Agrobacterium* and reached the peak in 12 h, declining thereafter (Fig. 1b).

Agrobacterium co-cultivation does not reduce HP cell viability

Control HP cultures remained characteristic yellowish throughout the experiment (Fig. 2a). Meanwhile, all the HP cultures inoculated with *Agrobacterium* and *E. coli* darkened within a day (Fig. 2b, c) irrespective of the bacterial concentration. Nevertheless, the intensity of darkness was directly proportional to bacterial concentration (data not shown). For example, the darkening was more intense and developed faster in the cultures inoculated with 250×10^7 bacterial cells ml^{-1} than in the lower bacterial densities. In the tobacco BY2 cultures co-cultivated with *A. tumefaciens*, no darkening was noticed (Fig. 2d). HP cells have remained viable after bacterial co-cultivation (Fig. 2f–f1, g–g1) similar to the control HP (Fig. 2e–e1) and tobacco BY2 cells (Fig. 2h–h1). These HP cells grew normally on solid MS medium as the control cells (Fig. 3a, b). Genomic

Fig. 1 Intracellular ROS production (**a**) in HP cells during co-cultivation with *Agrobacterium*. Control HP cells without bacteria (*HP*), HP cells co-cultivated with *A. tumefaciens* (*HP + AT*), HP cells co-cultivated with *A. rhizogenes* (*HP + AR*) and control *A. tumefaciens* without HP cells (*AT*). Northern-blot analysis (**b**) of *PAL* gene expression in HP cells after *A. tumefaciens* inoculation



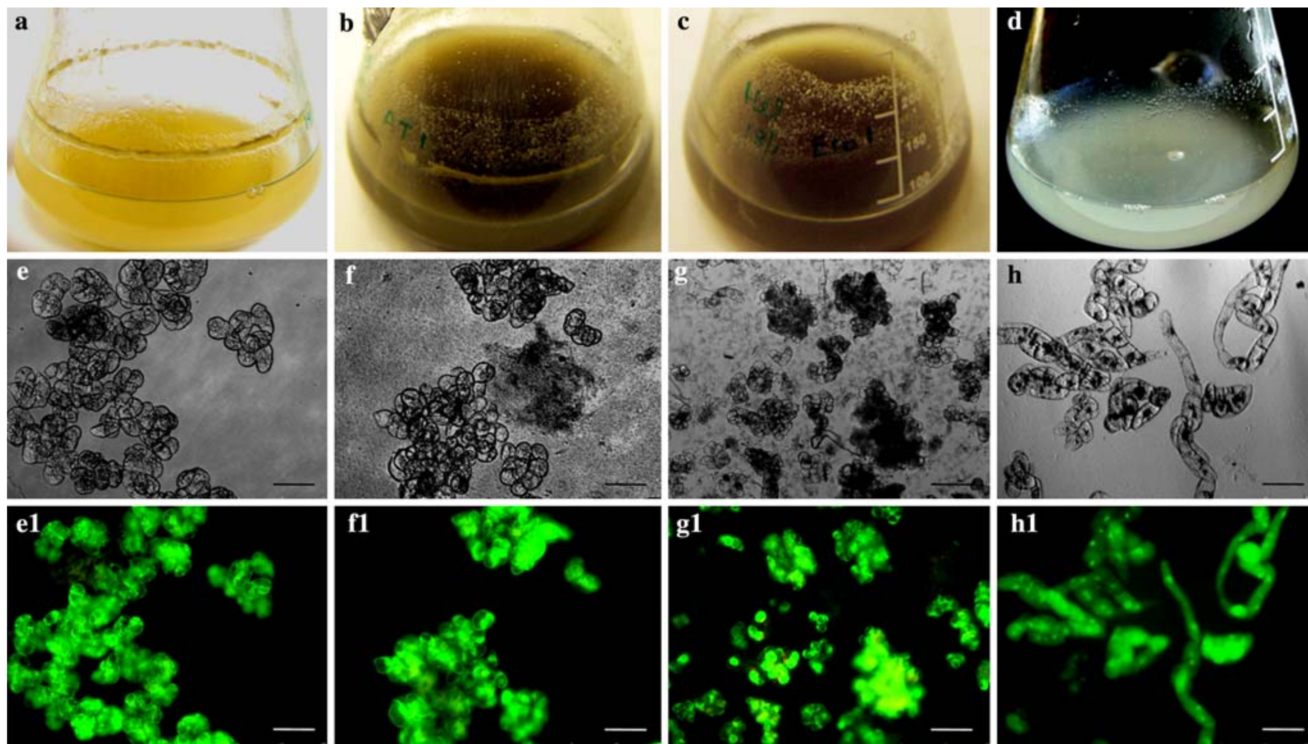


Fig. 2 Changes in HP cells in response to *Agrobacterium* co-cultivation. Control HP cell culture remain characteristic *yellowish* (**a**), HP cultures inoculated with *A. tumefaciens* (**b**) and with *E. coli* (**c**) became dark, while tobacco cells inoculated with *A. tumefaciens* (**d**) remain *white*. Light microscopic view of control HP cells (**e**), cells inoculated with *A. tumefaciens* (**f**) cells inoculated with *E. coli* (**g**), and tobacco cells inoculated with *A. tumefaciens* (**h**) and the corresponding epifluorescent view of the frames (**e1**–**h1**). HP cells remains viable after 3 days of co-cultivation with *Agrobacterium*, as shown by FDA-PI double staining (*green* fluorescence indicating viable cells and *red* fluorescence indicating dead cells). *Bar* = 125 μm

DNA isolated from the HP cells co-cultivated with these bacteria did not show DNA laddering (Fig. 3c, lanes 3–5) similar to the control cells (Fig. 3c, lane 2).

HP cells reduces *Agrobacterium* viability during co-cultivation

Agrobacterium viability declined quickly and reached complete mortality, when co-cultivated with HP cells (Fig. 4a–j). However, *E. coli* viability was not affected (Fig. 4k–o). Fluorescent staining showed that *Agrobacterium* retained 100% viability, when co-cultivated with tobacco BY2 cells (Fig. 5c–c1), similar to the control bacteria (Fig. 5a–a1). However, when co-cultivated with HP cells, they were completely killed in 24 h (Fig. 5b–b1). The number of colony forming units of *A. tumefaciens* and *A. rhizogenes* started to decline within 3 h and suffered 99% of viability reduction within 12 h of co-cultivation (Fig. 6).

The cell-free liquid media filtered from *Agrobacterium*-treated HP cultures inhibited the growth of *Agrobacterium*,

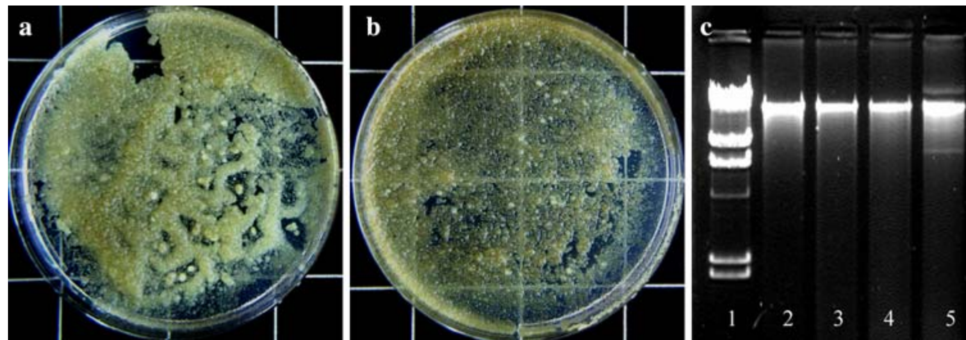


Fig. 3 Growth of HP cells on solid MS medium established from control (**a**) and from cells co-cultivated for 5 days with *A. tumefaciens* (**b**) (pictures taken after 10 days of culture). **c** Agarose gel electrophorogram of HP genomic DNA isolated from control and cultures inoculated with bacteria after 3 days, *lane 1 Hind*III digest of λ phage DNA, *lane 2* control cells, *lane 3* culture inoculated with *A. tumefaciens*, *lane 4* culture inoculated with *A. rhizogenes*, *lane 5* culture inoculated with *E. coli*

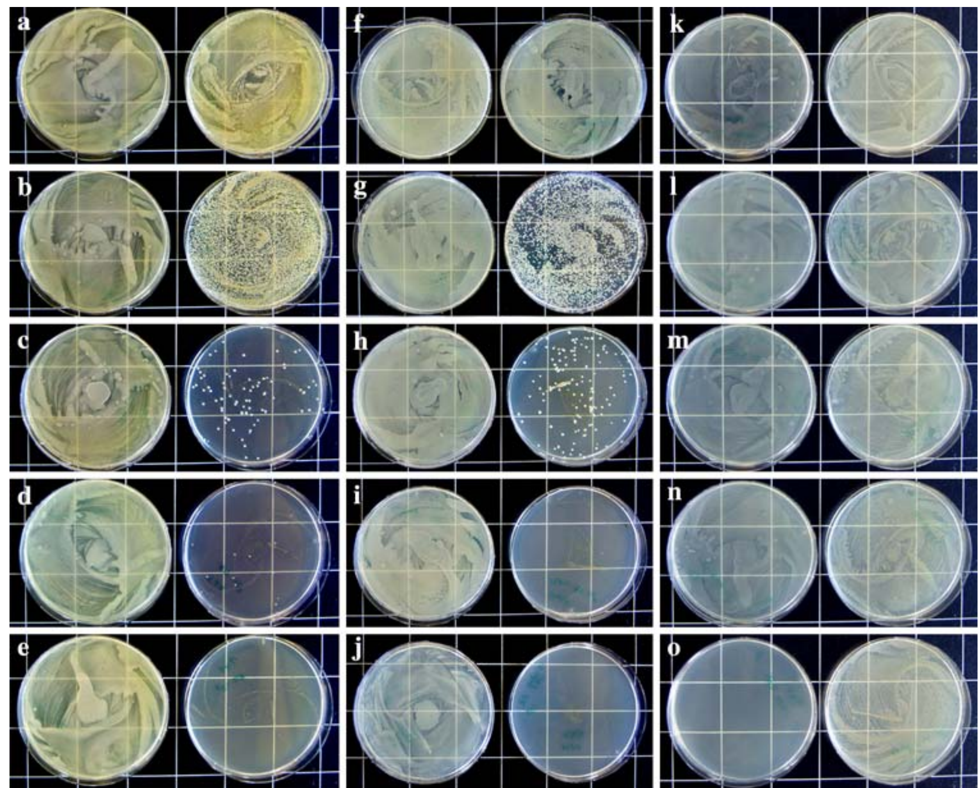


Fig. 4 Growth of bacteria after co-cultivation with HP cells. Plates spread with 100 µl suspension containing bacterial cells plus plant cells taken from HP cultures after 0, 6, 12, 24 and 48 h of contact with *A. tumefaciens* (**a**–**e**), *A. Rhizogenes* (**f**–**j**) and *E. coli* (**k**–**o**). Corresponding controls on the *left* side

whereas, the corresponding filtrate from control HP cultures did not show any negative effect.

Agrobacterium viability reduction affects HP transformation

Irrespective of the bacterial concentration and period of co-cultivation, HP cultures co-cultivated with *A. tumefaciens* did not show any transformed cells. Whereas, tobacco BY2 cell culture co-cultivated with *A. tumefaciens* had many transformed cells.

Discussion

Agrobacterium possess the unique ability of inter-kingdom gene transfer by introducing a defined piece of DNA, known as T-DNA (transferred DNA), from its tumour inducing (Ti) or root inducing (Ri) plasmid to the host plant cells by conjugal transfer. Integration of T-DNA into the plant genome and the expression of encoded genes lead to the development of crown gall or hairy roots in plants. This natural gene transfer phenomena has been exploited for the modern plant genetic engineering. Even though this

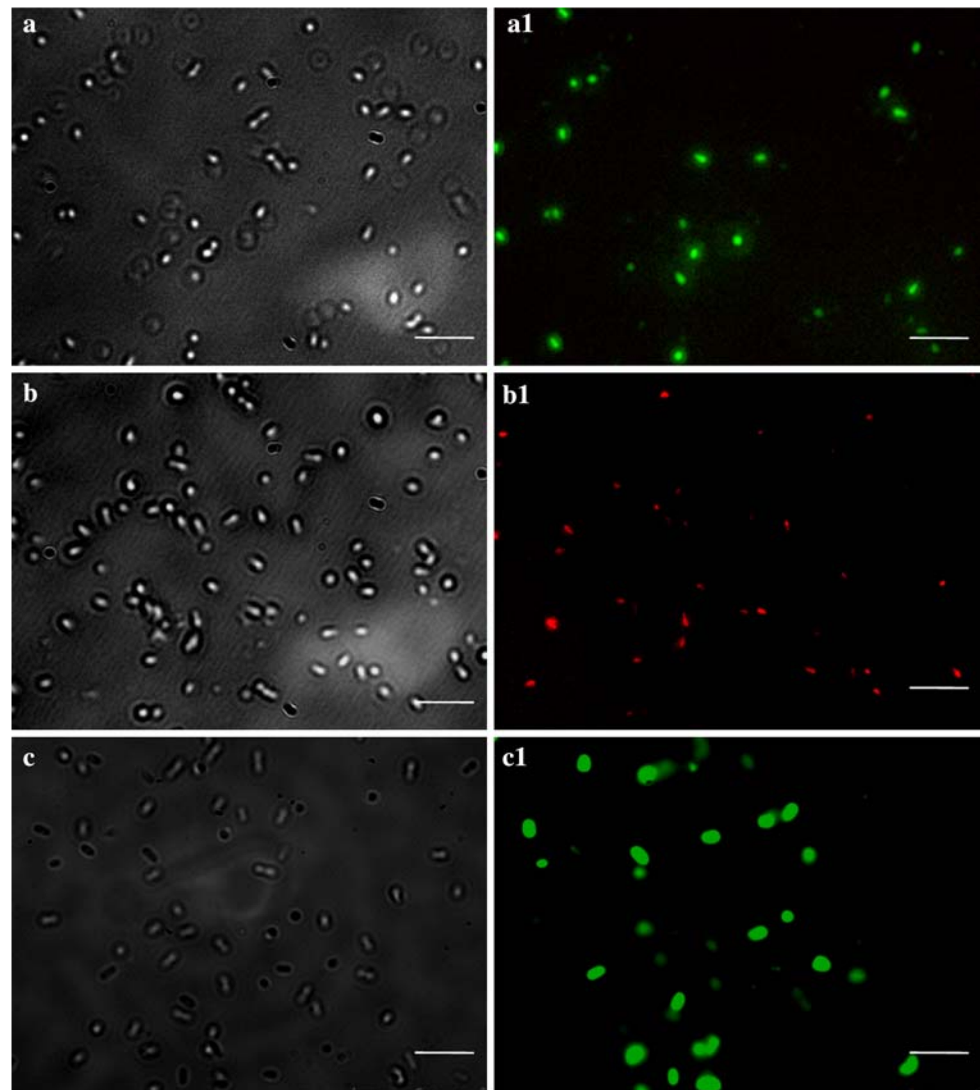


Fig. 5 *A. tumefaciens* viability of after co-cultivation with HP and tobacco cells. *Bar* = 25 μm. Light microscopic view of *A. tumefaciens* control (**a**), after 24 h co-cultivation with HP cells (**b**), and after 24 h co-cultivation with tobacco BY2 cells (**c**); epifluoresent view of the corresponding frames on the *right* (**a1**, **b1** and **c1**)

transformation tool has been extended to several crops, many plant species remain recalcitrant. This might be due to their defence responses against these bacteria.

In any plant–pathogen interaction, one of the earliest events of plant stress response is an intense oxidative burst due to the excess production of ROS in the plant cells. Similar ROS burst was observed in HP cells clearly demonstrating that *Agrobacterium* impose an intense stress to the recalcitrant HP plant cells. Even though there is no report, the existence of a ROS burst in *Agrobacterium*–plant interactions could be the reason for the common utilization of antioxidants (ROS scavengers) for successful transformation (Perl et al. 1996; Frame et al. 2002). Hence, and based on our results, we assume that intense ROS production could be an event of *Agrobacterium*–plant interactions, which may affect transformation process.

The several fold up-regulation of *PAL* gene expression quickly after *Agrobacterium* inoculation indicates that the HP phenolic metabolism was activated immediately after sensing the bacterium. Increase in PAL synthesis and corresponding mRNA accumulation might be connected to the up-regulation of downstream products, which would either protect the plant cells or harm the bacteria. Transcriptome analysis of *Arabidopsis thaliana* challenged with *Agrobacterium* also revealed several fold up-regulation of phenolic pathway genes downstream of *PAL* (Ditt et al. 2006). Increased expression of *PAL* gene in response to microbial or endogenous elicitors in many other plant–pathogen systems (Gomez-Vasqez et al. 2004) is a known plant defense mechanism.

The time-course darkening of HP cells and corresponding *PAL* gene up-regulation clearly demonstrates that these two events are interrelated. Previously, it was shown that HP cells challenged with a fungal pathogen (*Colletotrichum gloeosporioides*), exhibited similar darkening together with change in the phenolic profile (Conceição et al. 2006). Darkening of explants frequently observed

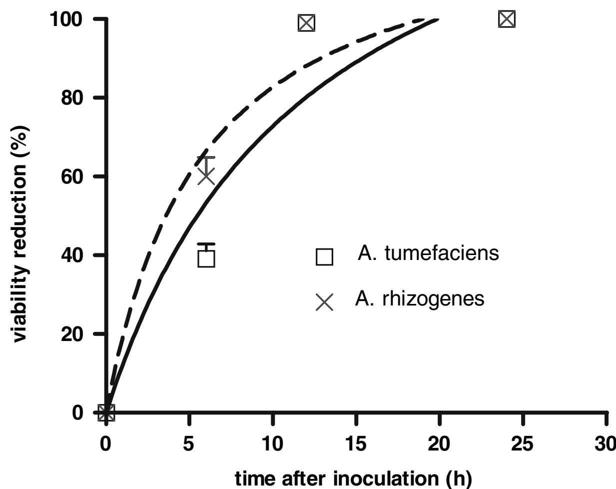


Fig. 6 Reduction of *A. tumefaciens* and *A. rhizogenes* colony forming units after 0, 6, 12 and 24 h of their inoculation into HP cells. To estimate the number of viable bacterial cells, HP culture was diluted until 10^{-6} and 100 μl (which contains approximately 250 bacterial cells) was spread on culture plates and incubated in dark at 28°C

during *Agrobacterium*-mediated plant transformation also has generally been attributed to phenolic production, which may eventually lead to the death of plant cells (Perl et al. 1996; Hansen 2000; Parrot et al. 2002). In our case, HP cells did not lose their viability in spite of their darkening. Furthermore, the absence of DNA fragmentation in the HP cells treated with *Agrobacterium* confirm that the incompatibility of *Agrobacterium*-mediated transformation in HP is not due to apoptosis or programmed cell death, as reported in other species (Hansen 2000; Parrot et al. 2002).

The drastic reduction of *Agrobacterium* viability during co-cultivation with HP cells was not expected, since this bacterium is postulated not to induce plant defense response (Robinette and Matthysse 1990; Felix et al. 1999). There has been no previous evidence that these bacteria could be killed by plant cells during co-cultivation, as we have shown here. Antimicrobial potential of the cell-free liquid medium of *Agrobacterium*- treated HP cultures suggests that the HP cells have released some antimicrobial substance(s) to the media.

Since the *A. rhizogenes* strain A4 has reached 99% mortality within 12 h of co-cultivation with HP cells in the present study, the hairy root induction of HP reported previously (Vinterhalter et al. 2006) is paradoxical.

The high correlation between the time-course mortality rate of *Agrobacterium* and the PAL mRNA accumulation in the HP cells indicates that the activation of phenolic metabolism might play an important role in the killing. It is well known that HP plants produce certain antimicrobial secondary metabolites like hypericin and hyperforin, which could be induced in response to biotic elicitation (Sirvent and Gibson 2002). However, these compounds can accumulate only in specialized tissue glands (Pasqua et al. 2003) but not in suspended cells (Conceição et al. 2006). Moreover, the antimicrobial activity of these compounds is confined to Gram-positive bacteria (Avato et al. 2004). Altogether, these observations imply that the killing of *Agrobacterium* is not due to the antimicrobial activity of hypericin and/or hyperforin.

Histochemical GUS assay of HP cultures co-cultivated with *A. tumefaciens* revealed the absence of transformed cells, while several transformed cells could be seen in tobacco BY2 cultures, which were co-cultivated under similar conditions. T-DNA transfer failure of HP has shown to be independent of the explants types (leaf blade, petiole, stem, root etc.), *Agrobacterium* species (*tumefaciens*, *rhizogenes*), virulence of the bacterial strains (EHA 105, LBA4402, A4, LBA9402) and co-cultivation conditions (Franklin et al. 2007). However, when particle-bombardment was used as the method of gene delivery, HP cell suspension cultures were successfully transformed with the same plasmid (pCAMBIA1301) and transgenic plants could be obtained (Franklin et al. 2007). Taken together, these results strongly suggest that the recalcitrance of HP towards *Agrobacterium*-mediated transformation is not due to a block point at later steps in the transformation process (T-DNA integration or efficient transgene expression), but due to the direct effect of plant defense on *Agrobacterium* viability.

Conclusion

From our study, it is clear that HP recognizes *Agrobacterium* as a potential pathogen and rapidly evokes its defense responses, leading to the drastic reduction of *Agrobacterium* viability. This could be one of the main reasons for the recalcitrance of HP. Although *Agrobacterium* is recognized as a tool for plant transformation, naturally it is a potent pathogen causing neoplastic diseases. As the disease progresses independent of the causative agent following the initial transformation event, strategies to eliminate this pathogen must necessarily be exercised before infection to avoid T-DNA entry. In this context, further exploration of anti-*Agrobacterium* defense mechanism of HP and characterization of related genes would provide effective ways to control the neoplastic diseases.

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