

Agrobacterium rhizogenes-mediated transformation of *Prunus* as an alternative for gene functional analysis in hairy-roots and composite plants

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Abstract Resistant rootstocks offer an alternative to pesticides for the control of soil pests. In *Prunus* spp., resistance loci to root-knot nematodes (RKN) have been mapped and a transformation method is needed to validate candidate genes. Our efforts have focused on the generation of transformed hairy-roots and composite plants appropriate for nematode infection assays. An efficient and reliable method using the A4R strain of *Agrobacterium rhizogenes* for the transformation of *Prunus* roots with an *Egfp* reporter gene is given. The rooting efficiency, depending on the genotypes, was maximal for the interspecific hybrid 253 (Myrobalan plum × almond-peach), susceptible to RKN, that was retained for subsequent studies. From the agro-inoculated cuttings, 72% produced roots, mainly at the basal section of the stem. Transformed roots were screened by microscope detection of *Egfp*

fluorescence and molecular analyses of the integration of the transgene. The absence of residual agrobacteria in the plants was checked by the non-amplification of the chromosomal gene *chvH*. *Egfp* was expressed visually in 76% of the rooted plants. Isolated hairy roots in Petri dishes and composite plants (transformed roots and non-transformed aerial part) in soil containers were inoculated with the RKN *Meloidogyne incognita*. In both cases, root transformation did not affect the ability of the nematodes to develop in the root tissues. Our results showed that isolated hairy-roots can be used to validate candidate genes and the conditions in which composite plants offer a complementary system for studying the function of root genes in physiological conditions of whole plants are discussed.

Keywords *Prunus* · *Agrobacterium rhizogenes* · Transgenic roots · Genetic engineering · Acclimation · Nematode

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Introduction

The genus *Prunus* comprises more than 400 species, including fruit producing species, e.g. peach, almond and plum as well as several rootstocks and ornamental species (Rehder 1954). Most of the cultivated species are grown in Mediterranean-like climate regions where root-knot nematodes (RKN) *Meloidogyne* spp. are widely distributed and where the polyphagous and parthenogenetic species *M. arenaria*, *M. incognita* and *M. javanica* are predominant (Sasser 1977; Lamberti 1979). Severe RKN damage in *Prunus* nurseries and orchards (Layne 1987; Nyczepir 1991) have been mainly controlled up-to-now by nematocidal treatments. However, because of the ban of nematocides for their negative environmental impact (Cook and

Evans 1987), an environmentally sound method is urgently needed and the RKN-resistant rootstocks are a promising alternative (Nyczepir and Halbrendt 1993). Several sources of resistance to RKN have been identified in the subgenus *Amygdalus* among the wild (*P. davidiana* Carrière Franch.) or cultivated (*P. persica* L. Batsh) peaches or the almond (*P. dulcis* Mill.) (Esmenjaud et al. 1994, 1997) and in the subgenus *Prunophora* among Myrobalan (*P. cerasifera* Ehrh.) and Japanese (*P. salicina* Lindl.) plums (Esmenjaud et al. 1997; Lecouls et al. 1997; Rubio-Cabetas et al. 1999). Current studies on these sources aim to characterize RKN-resistance genes and to elucidate their mode of action (Lecouls et al. 2004; Dirlewanger et al. 2004; Claverie et al. 2004a). Several major resistance genes have been identified and mapped (Esmenjaud et al. 1996; Lecouls et al. 1997; Claverie et al. 2004a). In particular the *Ma* gene from Myrobalan plum is the focus of a cloning strategy and three candidate resistance genes clustered in the same locus have been identified (Claverie et al. 2004b; Esmenjaud and Dirlewanger 2007). To determine which of these genes confers the resistance, each candidate gene must be transferred into susceptible genotypes and the transformed roots were tested for nematode resistance. Validations of the *HsI^{pro1}* gene for resistance to the cyst nematode *Heterodera schachtii* (Cai et al. 1997) and the *Mil* gene for resistance to the RKN *M. incognita* (Milligan et al. 1998) have already been performed using isolated hairy-roots from sugar beet and tomato, respectively.

Agrobacterium tumefaciens-mediated genetic transformation (Hooykaas 1989) has already been successfully established for some fruit trees, such as apricot (*P. armeniaca* L., Petri et al. 2008a), Japanese (*P. salicina*, Urtubia et al. 2008) and European (*P. domestica* L., Petri et al. 2008b, Petri and Scorza 2010) plums. However, the use of this bacterium is limited in woody plants due to the fact that many species are recalcitrant to regeneration from undifferentiated tissues (Haapala et al. 1994). An alternative to the limiting step of regeneration is the use of *A. rhizogenes*-mediated transformation leading to isolated hairy roots or composite plants (transformed roots and untransformed aerial part). This technique is particularly valuable for genes acting naturally in the root system such as RKN resistance genes. For example, this strategy has been initiated to validate the *Mex-1* RKN resistance in *C. arabica* L. (Alpizar et al. 2006). To reach our objective in *Prunus*, a key step is to develop a resistance test adapted to the pathogen studied and to show that the transformation by agrobacteria does not affect the ability of the pathogen to develop in the root tissues.

This study describes (1) a method for *Prunus* root transformation using *A. rhizogenes* to produce transformed hairy-roots and composite plants; (2) the screening and molecular analysis of putative hairy-roots using a reporter

gene to determine the roots effectively transformed and untransformed; and (3) the successful infection of transgenic isolated hairy-roots and composite plants with the RKN *M. incognita*.

Materials and methods

Plant material and establishment of in vitro cultures

Five clonal INRA accessions were used for this study. These included the three Myrobalan plum genotypes P.2175, P.2032 (both natural selections), C163.31 (intra-specific controlled hybrid) and the genotypes 229 and 253 that are interspecific three-way hybrids [P.2175 × ('Garfi' almond × 'Nemared' peach)₂₂]. From mother plants grown at INRA-UREF in Bordeaux (France), nodal explants of young shoots were collected, briefly rinsed in water, surface-sterilized in 70% EtOH for 1 min and subsequently in 15% calcium hypochlorite for 20 min. Finally, they were washed three times in sterile distilled water. Buds were collected and placed on agar culture medium in Pyrex tubes (150 × 22 mm). The clonal cuttings were cultured in MS basal propagation medium (Murashige and Skoog 1962) with some modifications [50 ml/l Macro Skoog elements 800 (NH₄NO₃/2), 1 ml/l Micro Skoog elements, 37.25 mg/l EDTA-Fe, 1 mg/l vitamin B, 100 mg/l inositol, 30 g/l sucrose, pH = 5.3] supplemented with 0.05 mg/l indole-3-butyric acid, 1 mg/l N6-benzyladenine, 0.15 mg/l gibberelic acid and 7 g/l agar. The cuttings were grown at 21°C under a 16/8-h (light/dark) photoperiod with light supplied by coolwhite fluorescent lightning at an intensity of 67 mmol m⁻² s⁻¹ and sub-cultured on fresh medium every month.

Agrobacterium culture

The *A. rhizogenes* strain A4R (Tepfer 1990) is an agropine mannopine strain derived from the wild strain A4 and modified for resistance to the antibiotic rifampicin (Jouanin et al. 1986). The binary vector pK7WGF2,0 (Karimi et al. 2005) was introduced into the agrobacteria by electroporation (Sambrook et al. 1989). The T-DNA contained the reporter gene *Egfp* that codes for a synthetic enhanced green fluorescent protein (Egfp) under the control of the CAMV 35S promoter (Franck et al. 1980). In addition, the T-DNA contained the neomycin phosphotransferase II (NPTII) selection marker for resistance to kanamycin (Kan) under the control of the nopaline synthase promoter (Shaw et al. 1984; Pridmore 1987). Because *Prunus* spp. are sensitive to kanamycin (Petri and Burgos 2005), in our study this antibiotic was not used as a selection marker but as a second reporter gene. The binary vector pKGW

(Karimi et al. 2005) containing the NPTII marker but not the Egfp reporter gene was also used in the same conditions as the vector pK7WGF2,0 to have transformed plants without Egfp expression (transformed control plants).

From a single bacterial colony a preculture was grown in 3 ml MYA medium (0.25 g/l casamino acid, 2.5 g/l bacto yeast extract, 2.5 g/l NaCl, 4 g/l mannitol, and 1 g/l MgSO₄, pH = 6.6) supplemented with rifampicin (50 mg/l) and spectinomycin (50 mg/l), on a shaker at 220 rpm, 30°C, for 12 h. The culture was diluted (1:10) in fresh medium and grown to an OD600 comprised between 0.6 and 0.9. Bacteria were pelleted (4,000 rpm for 10 min, at 4°C) and resuspended in inoculation hormone-free MS basal propagation medium to an OD600 comprised between 0.8 and 1.0. Acetosyringone (50 µmol/l) was added and the suspension was let at room temperature for 2–3 h before transformation.

Transformation and production of hairy-roots and composite plants

Transformation was globally performed on more than 1,000 cuttings during all the experiments reported hereafter. Cuttings, 1-month old, approximately 2-cm long with 2–3 leaves and no roots, were inoculated with *A. rhizogenes* by injecting the agrobacteria solution (approximately 10 µl) into the stem 5–10 mm above the basis of the micro-plant with a sterile syringe. The infected cuttings were placed on hormone-free MS basal propagation medium with 7 g/l agar, in 500 ml plastic containers (Duchefa) (6 cuttings per container) and kept in dark. Different temperatures and times for co-cultivation of the cuttings and the agrobacteria were tested. After this co-cultivation experiment, the best combination was retained to evaluate the ability of the different genotypes to produce roots. In this experiment and in further ones, non-transformed control cuttings inoculated with hormone-free MS basal propagation medium without agrobacteria were also processed in the same conditions. Agrobacterium-infected cuttings, from the different genotypes, that produced roots were submitted to two different ways. (1) From a part of them, roots were separated from the stem, transferred into Petri dishes by gently pushing the roots into the hormone-free MS basal propagation medium (supplemented with 500 mg/l cefotaxime to eliminate agrobacteria) and kept dark by wrapping the dishes in aluminium foil. Roots that developed autonomously were considered as putatively transformed, designated as hairy roots and grown for 1–1.5 months until an eventual transfer onto a fresh medium. (2) From the rest of the cuttings, putative composite plants (non-transformed aerial part and putative hairy roots) were produced. In this objective, rooted cuttings were transferred individually into 300 ml plastic containers (Duchefa) on the same MS basal

propagation medium, also by gently pushing the roots into the agar. Composite plants were incubated at 25°C under a 16/8-h (light/dark) photoperiod at a light intensity of 67 mmol m⁻² s⁻¹ and also grown for 1–1.5 months until an eventual transfer onto a fresh medium.

The genotype that produced the highest percentage of rooted cuttings was then selected for subsequent experiments. For this genotype, rooted cuttings were designated as putative composite plants and grown in the same conditions as previously described. The transformed status of the roots of the composite plants was studied in details using first non-destructive microscopic observations of Egfp and then molecular analyses. Root samplings for molecular analyses were performed by cutting out and keeping at –20°C lateral branchings (up-to 3 cm long) of all longest axes of roots when these latter had the best development, i. e. before their transfer within new fresh medium. After transplantation into new plastic containers (composite plants) and new Petri dishes (isolated hairy roots), sections of roots resumed their development from the breaking points by emitting new secondary rootlets. For studies of nematode infection (see further), a sample of composite plants validated by Egfp expression and PCR was then used partly for the production of acclimated composite plants and partly for the production of hairy roots in Petri dishes.

Microscopic observations of Egfp expression

Composite plants with newly generated roots were examined for Egfp expression under a fluorescence stereo microscope (MZ FLIII Leica Microsystem) with a GFP Plus filter system (excitation filter 480/40 nm, emission filter 510 nm). Pictures of Egfp expression were taken using a digital camera (Coolpix 995 Nikon).

Molecular analyses of transformed roots

Total genomic DNA was isolated, for each plant separately, from samples of putatively transformed roots (lateral branchings of the 2–5 axes of roots) and of non-transformed control roots. An automated shaker (Retsch MM300, Haan, Germany) was used to crush the roots into 400 µl extraction buffer (0.35 M Sorbitol, 0.1 M Tris, 5 mM EDTA, pH = 7.5), 400 µl lysis buffer (0.2 M Tris, 5 mM EDTA, 2 M NaCl, 2% CTAB) and 160 µl 5% *N*-laurylsarcosine with stainless steel beads (3 mm diameter), for 3–4 min at 30 Hz. DNA was extracted using the Genomix Cells Small Scale DNA Extraction Kit (Talent srl, Trieste, Italy). DNA concentration and quality were evaluated using a biophotometer (Eppendorf).

The integration of the T-DNA binary plasmid was analyzed by PCR using the *nptII* specific primers NPTFo

(5'-TCAGAAGAACTCGTCAAGAA-3') and NPTBa (5'-AACAAAGATGGATTGCACGCA-3') and the *Egfp* specific primers GFPFo (5'-ATGGTGAGCAAGGGCGAG GAG-3') and GFPBa (5'-GTACAGCTCGTCCATGCC GAG-3'). The absence of residual *A. rhizogenes* was checked by PCR on the chromosomal virulence gene *chvH* of *A. rhizogenes*. For this purpose, a *chvH* amplification product from *A. rhizogenes* was obtained using the *chvH* primers previously designed by Susuki et al. (2001) for *A. tumefaciens*. The sequence of the amplified product was used to design the *A. rhizogenes*-specific primers *chvHRhFo* (5'-TTCGGTCCGCAAGGGCAACG-3') and *chvHRhBa* (5'-TAGGAAACGTCTTCCGTGGCG-3').

The PCR mixture (25 μ l) contained 30 ng of DNA, 1 unit of *Taq* DNA polymerase (Invitrogen), 1 \times PCR buffer and 1.5 mM MgCl₂ provided with the enzyme, 0.2 mM of dNTPs (Eurogentec) and 0.2 μ M of each primer (Eurogentec). The amplification reaction was performed on a PTC 200 thermal cycler (MJ Research, San Francisco, CA, USA) as follows: 95°C for 4 min; followed by 35 cycles at 95°C for 45 s, and then 45 s at 68°C for *Egfp*, 56°C for *nptII*, 60°C for *chvH*, 72°C for 1 min 30 s and additional 72°C for 4 min. The amplified products were separated on 1% agarose gel, stained with ethidium bromide and observed under UV light.

For Southern blotting obtained from PCR, the amplified products were transferred to a nylon membrane after separation on agarose gel (Sambrook et al. 1989; Gartland et al. 2000). ³²P-labelled *nptII* or *chvH* DNA probes were synthesized with the specific primers described above and labelled using Amersham Megaprime DNA Labelling Systems (GE Healthcare, UK). Hybridizations were performed over night at 60°C, then filters were washed at 60°C for 1 h in 2 \times SSC + 0.2% SDS and 1 h in 1 \times SSC + 0.2% SDS. Filters were analyzed using a fluorescent image analyzer FLA-3000 with IP-stage (Fujifilm, Japan). For Southern blots obtained from enzyme-digested products, the amounts of root DNA needed were obtained as follows. Hairy roots putatively transformed with the plasmid pKGW,0 were cut, isolated from their mother cutting during 1 month after bacterial inoculation and grown individually in Petri dishes. Every 3–4 weeks, roots that grew vigorously from this initial sampling were divided into several pieces and subcultured into separate Petri dishes to produce high amounts of plant tissue. After several rounds of multiplication, material that had produced the higher amount of roots from the same initial putative transformation event was retained to perform a Southern blot. DNA was digested separately with *EcoRV* and *HindIII*, separated on agarose gel (Sambrook et al. 1989; Gartland et al. 2000) and transferred to a nylon membrane. Subsequent steps were the same as previously described for Southern blotting from PCR.

Acclimation of transformed composite plants

Composite plants [approx. 4–5 cm long shoots with 3–4 well-developed roots (3.0–4.0 cm) and 5–6 expanding leaves] were transplanted into trays filled with a perlite substrate. Trays were covered by a transparent screen to maintain high humidity, and kept for 1 month at 25°C in a growth chamber under a 16/8-h (light/dark) photoperiod at a light intensity of 67 mmol m⁻² s⁻¹. The plants were transferred into pots (1 l) containing a mix of sterile silt-clay soil (3/8), siliceous sand (1/2) and peat moss (1/8) and maintained in the growth chamber under the same conditions as before.

Nematode infection of hairy roots and transformed composite plants

Hairy-roots were tested for their ability to be infected by *M. incognita* second-stage juveniles (J2s) 5–6 weeks after being isolated in Petri dishes. The roots were inoculated with 2,000 sterile J2s (Sijmons et al. 1991) distributed on the apices. One month after transfer into soil, composite plants were inoculated with 20,000 J2s homogeneously applied around the collar. Successful nematode development was ascertained by the presence of galls. Galls were dissected to confirm the presence of characteristic inflated nematode stages on isolated roots or composite plants 2 and 4 weeks after infestation, respectively.

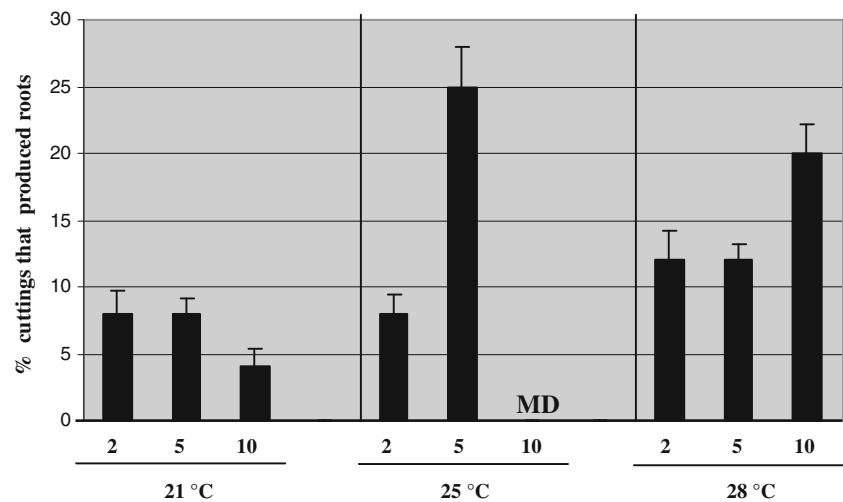
Results

Generation of hairy-root and composite plant

We generated roots from *Prunus* cuttings by injection of *A. rhizogenes* strain A4R harbouring the vector pKGWF2,0 (with *Egfp*) into the stems.

In a first experiment satisfactory plant and bacteria co-cultivation conditions were searched for using different co-cultivation times and temperatures on five *Prunus* genotypes, P.2032, P.2175, C163.31, 229 and 253 (354 total cuttings). Three different temperatures were tested: 21°C, used in our laboratory for in vitro culture of *Prunus*; 28°C, that allows rapid growth of *A. rhizogenes* (Haggman and Aronen 2000) and 25°C as an intermediate temperature. Three co-cultivation times (2, 5 and 10 days) were compared. Although numbers of the different genotypes in this experiment were quite different, the genotypes followed a similar trend (data not given) and were thus pooled. Among the combinations studied, 5 days co-cultivation at 25°C appeared as a good compromise (Fig. 1) and this combination was retained for subsequent experiments.

Fig. 1 Root generation efficiency for different temperatures (21, 25 and 28°C) and co-cultivation times (2, 5 and 10 days) with a pool of five *Prunus* genotypes). Values are percentages (\pm SD) of agro-inoculated cuttings that produced roots. MD missing data



In a second experiment, the ability of the five genotypes to generate roots was compared, under the conditions described above, using a total of 620 cuttings inoculated with *A. rhizogenes*. One hundred twenty-five additional control cuttings without agrobacteria, representative of all genotypes (including 50 cuttings from genotype 253), did not generate roots in these conditions. The root generation efficiency, calculated for each genotype as the percentage of agro-inoculated cuttings that produced roots, varied considerably and ranged from 15 (P.2032) to 72% (253) (Fig. 2). The 21% efficiency observed for P.2175 should be considered with caution due to the low number of plants analyzed. The newly generated roots appeared 3–4 weeks after *Agrobacterium* inoculation. A total of 50 roots representative of the five genotypes were isolated on hormone-free medium in Petri dishes and used to describe the dynamics of root growth. The isolated roots exhibited a hairy-root phenotype similar to that described in other tree species: vigorous growth, high branching and loss of

gravity response (Fig. 3a). After 1 week, 22% of the roots stopped their growth and 8–12% stopped growing each week up to 5 weeks. After 5 weeks, 40% of the roots were still growing and among these the hairy-roots had covered the plates after 12 weeks (Fig. 3b, c).

The genotype 253, which had shown the highest root generation capacity was used for all subsequent experiments. The outline of all experiments conducted with this genotype is schematized in Fig. 4. All rooted material (72%) out of the 286 cuttings of genotype 253 showed normal growth of the aerial part associated with vigorous growth and high branching of the roots after 5 weeks. The composite plants were conserved in vitro up to 3 months with transfer onto a fresh medium after 30–45 days (Fig. 3d).

Egfp expression and molecular analysis of hairy-roots

To analyze root transformation, 67 composite plants from the set of plants of the genotype 253 transformed with the

Fig. 2 Root generation efficiency for five *Prunus* genotypes. Values are percentages (\pm SD) of agro-inoculated cuttings that produced roots. The numbers of plants analyzed are indicated in brackets. Co-cultivation experiments for transformation were conducted at 25°C during 5 days

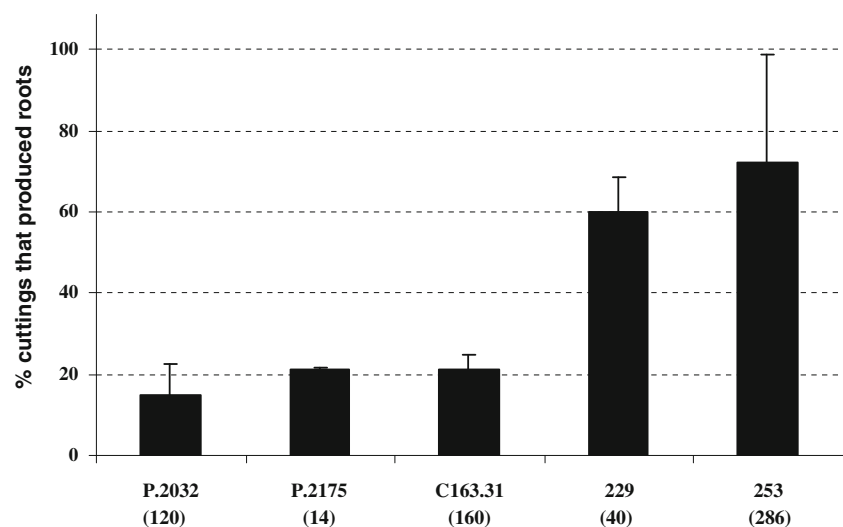
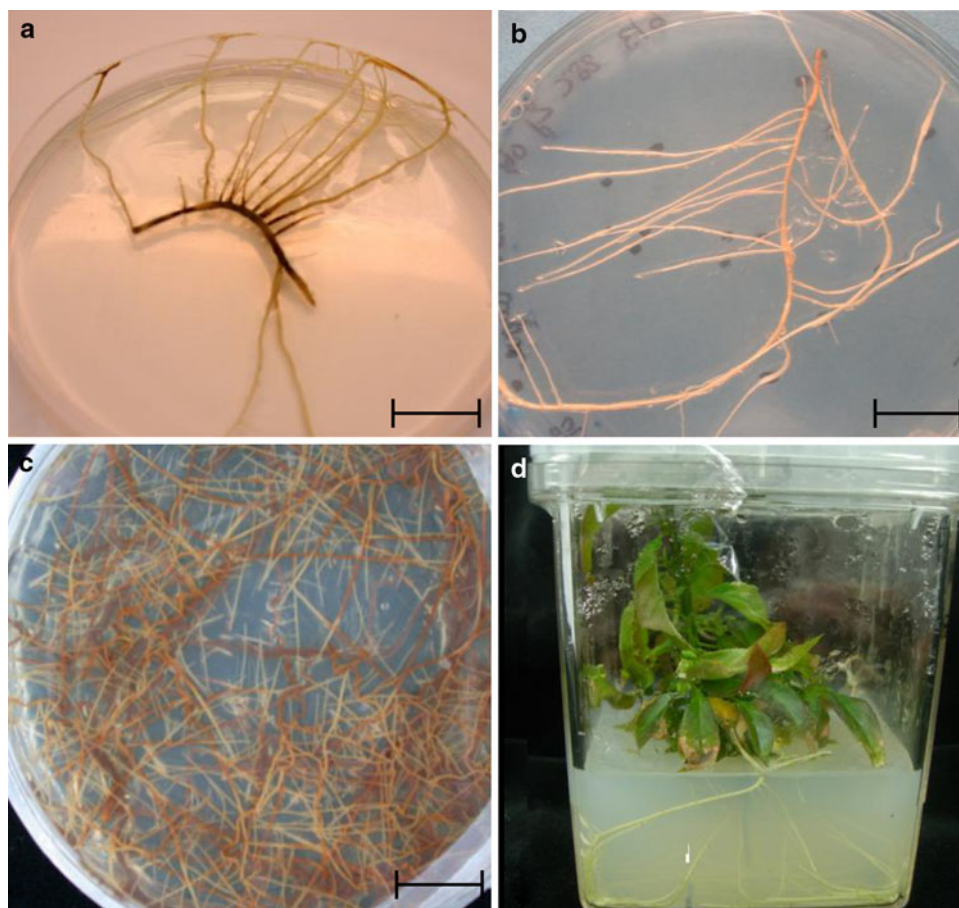


Fig. 3 Development of isolated roots and composite plants, from genotype 253, on solid hormone-free MS medium. Typical phenotype of hairy-roots observed at 4 weeks (a), 5 weeks (b) and 12 weeks (c). d A rooted composite plant 2 months after agro-inoculation. Scale bar 10 mm



vector pK7WGF2.0 were examined for the integration of the *Egfp* reporter gene and the correlative expression of the green fluorescence protein. A faint green auto-fluorescence, presumably emitted from phenolic compounds (Cho et al. 2000), was detected in both the root and aerial parts of transformed control plants (vector pKGW.0, without *Egfp*) (Fig. 5a). Thus, a GFP Plus filter (excitation filter 480/40 nm, emission filter 510 nm) was used to distinguish the *Egfp* fluorescence from the auto-fluorescence. *Egfp* fluorescence was detected on the root system of 76% (51 out of 67) of the composite plants (Fig. 5b).

In each of these root systems we observed heterogeneous *Egfp* expression. To evaluate the rate of transformed roots we randomly selected a sample of 27 composite plants (amongst the 51 that expressed *Egfp* fluorescence) and analyzed in details their whole roots (89 individual roots, i.e. a mean of 3.3 roots per plant) (Table 1). Ten of the composite plants expressed *Egfp* in each of their roots (37%) and had a mean number of roots (2.0) lower than plants which expressed *Egfp* in only part of them (4.1) (Table 1a). Out of the 89 total roots, *Egfp* fluorescence was clearly detected on 63 roots (71%). This value is significantly higher than the number of roots without fluorescence (Chi-square = 15.4; p value < 0.001). In these composite

plants hairy-roots were more abundant at the basal section of the stems (81%) than at the agro-inoculation site (19%) (Table 1b). From our observation on a limited number of roots, no noticeable difference in the proportion of roots that expressed *Egfp* was observed in those generated from the agro-inoculation site as compared to those from the basal section of the stems. The intensity of *Egfp* signal varied, the strongest *Egfp* expression being observed in actively growing tissues such as apices and lateral branchings (Fig. 5c). *Egfp* expression was verified on a longer duration on five composite plants. They still expressed the *Egfp* fluorescence after 2 months in the apices of the roots (data not shown).

Out of the 27 composite plants fully analyzed, 21 could be successfully characterized for the insertion of the T-DNA from the binary vector into the genomic DNA. Using PCR, we tested on 6-week-old plants the presence of the *Egfp* and *nptII* (kanamycin resistance) genes. The presence of contaminating *A. rhizogenes* was simultaneously discarded by amplification of the bacterial chromosomal gene *chvH*. The specific *Egfp* (714 bp) and *nptII* (788 bp) fragments were amplified, respectively, in all and 17 of the 21 plants (Table 2). One plant out of the 21 plants gave the 528 bp *chvH* fragment indicating the

Fig. 4 General outline of the transformation experiments conducted with the genotype 253

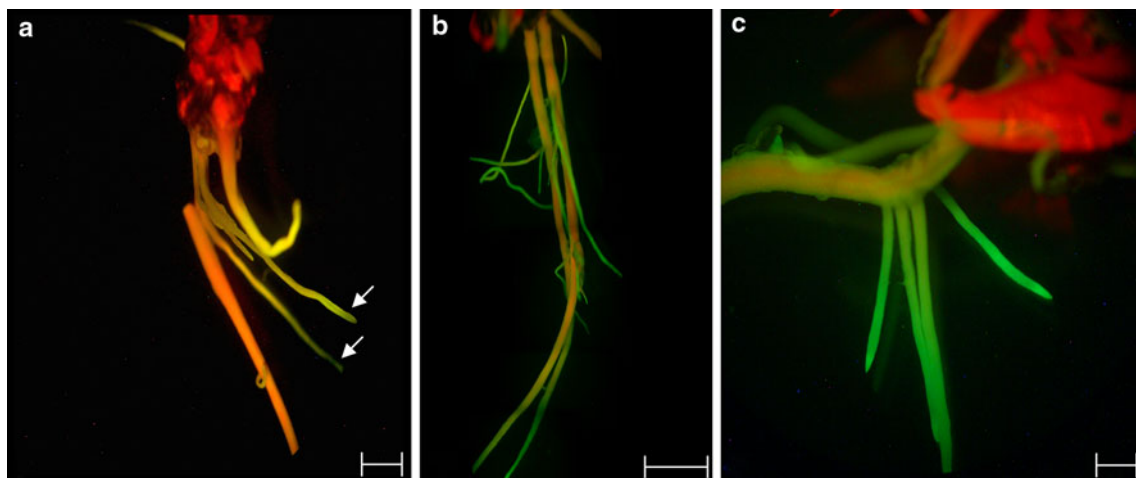
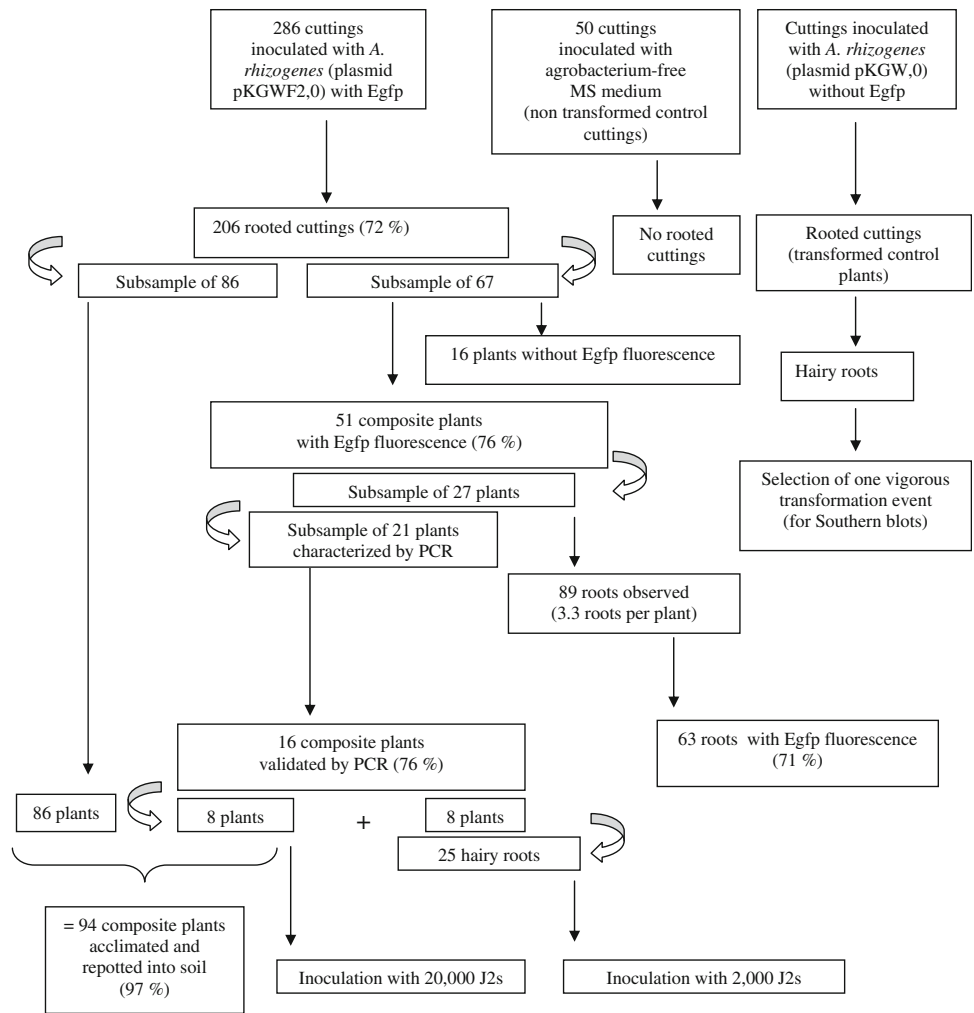


Fig. 5 Egfp expression observed by fluorescence microscopy in roots of genotype 253. **a** Auto-fluorescence observed in root apices (arrows) of a transformed control plant without Egfp (vector pKGF, 0). **b** Egfp

expression in the root system of a composite plant. **c** Details of root apices and lateral branching. Scale bar 10 mm

Table 1 Expression of Egfp on roots from 27 composite plants of the genotype 253

Plants with Egfp expression	Number (%)	Number of roots per plant	
		Mean	Range
(a) Rate of plants with expression in all or part of the roots			
In each of the roots	10 (37)	2.0	1–4
In some of the roots	17 (63)	4.1	2–12
	Total (%)	Roots with fluorescence (%)	Roots without fluorescence (%)
(b) Detailed distribution of roots			
Roots at the basal section of the stems	72 (81)	49 (68) a ^A	23 (32) b
Roots at the agro-inoculation site	17 (19)	14 (82) a ^B	3 (18) b
Total	89	63 (71) a ^C	26 (29) b

26 plants had roots at the basal section of the stems and 5 plants had roots at the agro-inoculation site. Four plants had roots both at the basal section of the stem and at the agro-inoculation site and one plant had roots only at the agro-inoculation site. Values are numbers of roots showing (or not) the Egfp fluorescence. Values within the same row followed by different letters are significantly different

^A $\chi^2 = 14.9$ ($p < 0.0001$)

^B $\chi^2 = 7.1$ ($p < 0.0076$)

^C $\chi^2 = 15.4$ ($p < 0.0001$)

Table 2 PCR characterization of the 21 composite plants from the genotype 253 that showed Egfp expression (column 1) for the amplification of the *Egfp*, *nptIII* and *chvH* genes

Egfp expression	<i>Egfp</i>	<i>NPTIII</i>	<i>chvH</i>	Number of plants (%)
+	+	+	–	16 (76)
+	+	–	–	4 (19)
+	+	+	+	1 (5)
–	–	–	–	UC

(+) and (–) indicate, respectively, the presence and the absence of the PCR fragment

UC untransformed control plant

contamination with external *A. rhizogenes* bacteria. No bands were amplified from a wild untransformed control plant (Fig. 6; Table 2). The presence or absence of contaminating bacteria was confirmed by Southern blotting. In two plants in which *nptIII* was detected, the absence of hybridization with the *chvH* probe in genomic DNA extracted from the root system provided evidence for the integration of the T-DNA in the genome (Fig. 7). Finally, excluding the plant which resulted positive for *chvH*, the rate of transformation confirmed by PCR analysis was 76% (16 out of 21).

A rate of 76% of composite plants carries, at least, one transformed root. To confirm integration of T-DNA, hairy roots from the same transformation event, carrying the plasmid pKGW,0 which produced the higher amount of white root tips (after several rounds of multiplication in Petri dishes), were used for Southern blot. Genomic DNA

extracted from the hairy roots was digested with *EcoRV* or *HindIII* and hybridized with a probe for *nptIII* (Fig. 8a). The Southern indicated a single integration of the T-DNA in the event considered (Fig. 8b).

Nematode infection of hairy-roots and composite plants

The 16 out of the 21 above-mentioned composite plants from genotype 253, validated for transformation by visual observation of Egfp expression in the roots and by appropriate pattern of amplification for the *Egfp*, *nptIII* and *chvH* genes (Table 2), were retained for nematode evaluation. Twenty-five hairy-roots, collected from eight of those plants, were grown individually in Petri dishes for 5 weeks and then were infected on their root apices with *M. incognita* nematodes. The other eight composite plants were acclimatized and grown in soil to improve the conditions for proper nematode development and to better reproduce the physiology of in vivo infected roots.

In hairy roots, J2s were still mobile after 2–3 days and some had spread all over the agarose medium. The first galls were visible at the apex of the hairy-roots 9 days after infection (Fig. 9a, b) and extensive galling expressing Egfp was observed after 16 days (Fig. 9c). A browning appeared on the galls after 45 days. New attacks were visible on young rootlets of roots having survived beyond 45 days. Because J2s from the initial inoculum cannot survive up to 45 days in the agar medium and because the life cycle of the nematodes lasts 4–5 weeks under our experimental conditions, the occurrence of new attacks shows that some

Fig. 6 PCR analysis on a subset of composite plants from genotype 253. **a** Detection of the *Egfp* gene. **b** Detection of the *nptII* gene. **c** Detection of the chromosomal *A. rhizogenes chvH* gene. Lanes: *M* molecular ladder, *T*- wild untransformed plant (negative control), *T*+ *A. rhizogenes* transformed with the binary vector pK7WGF2,0 (positive control), 1–9 roots collected from individual plants numbered 1 to 9 and showing Egfp fluorescence in the roots

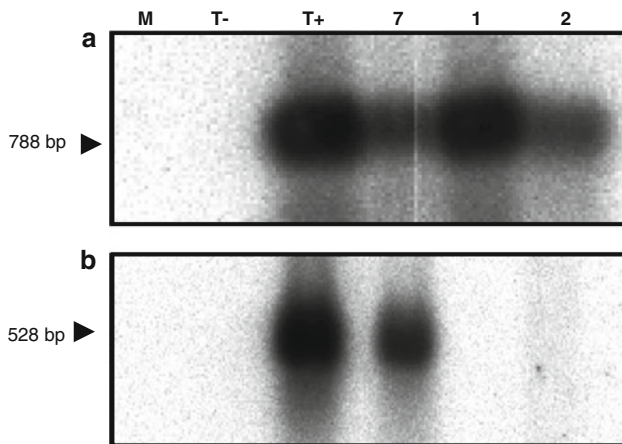
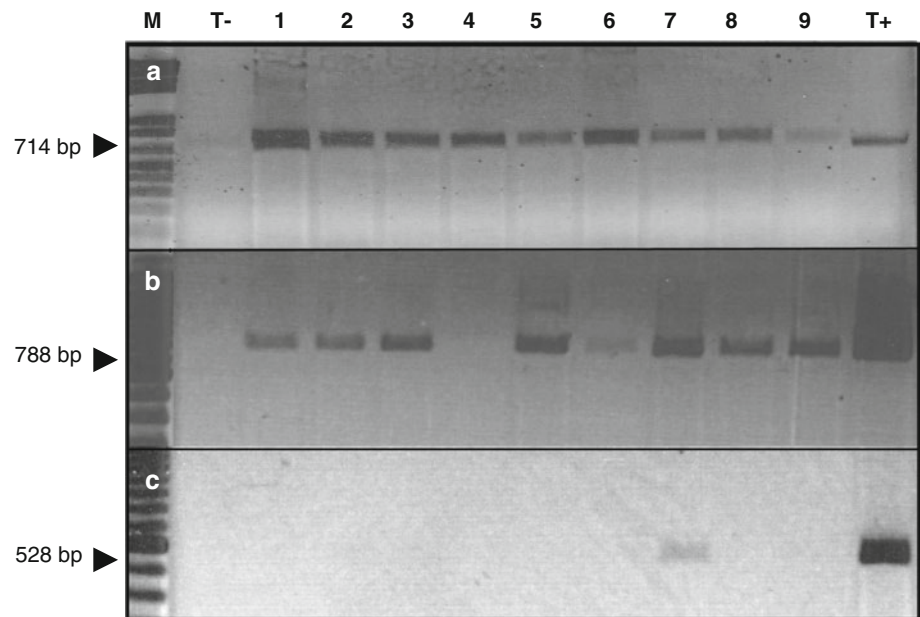


Fig. 7 Southern blot of PCR products from transformed roots of composite plants (genotype 253). **a** Hybridization with ^{32}P -labelled *nptII* DNA probe of PCR products amplified with *nptII*-specific primers. **b** Hybridization with ^{32}P -labelled *chvH* DNA probe of PCR products amplified with *chvH*-specific primers. Lanes: *M* molecular ladder, *T*- wild untransformed plant (negative control), *T*+ binary vector pK7WGF2,0 (positive control), 7, 1 and 2 PCR amplicon of kanamycine gene or *A. rhizogenes* chromosomal gene from roots of composite plants 7, 1 and 2, respectively (see Fig. 6)

nematodes had completed their life cycle. Thus, *in vitro* infections suggested that root transformation did not modify the response of the accession 253 as representative genotype to nematodes.

To test the response of composite plants to nematode infection, the eight composite plants from genotype 253 were transferred to perlite trays (Fig. 10a, b) during 1 month and repotted into soil (Fig. 10c). These plants were then infected with J2s after 1 month. The development of gall symptoms was then assessed 1 month after

infection (Fig. 10d). All plants were attacked and root systems had the same visual gall density as non-transformed control susceptible plants. Galls expressing Egfp (Fig. 10e) were dissected and the different larval (J2, J3 and J4) and adult (males and females) stages were observed showing that the nematodes did develop normally on transformed roots.

Complemental experiments were set up to refine the acclimation rate of composite plants from genotype 253. Finally, from a total of 97 composite plants (89 additional plants of this accession plus the 8 plants previously mentioned) that were acclimated (Fig. 4), 94 (97%) survived the transfer into perlite tray. All plants, repotted after 1 month into soil (1-l pots), grew actively for the 5 months of the assays (Fig. 10c).

Discussion

In genotypes of *Prunus* spp., a successful *A. tumefaciens*-mediated transformation and regeneration have been obtained from leaf explants of cherry (Song and Sink 2006), almond (Ramesh et al. 2006), apricot (Petri et al. 2008a) and domestic plum (Petri and Scorza 2010) but remains so far difficult. Besides this, non-clonal transgenic *Prunus* trees were obtained from seed-derived explants of peach (Perez-Clemente et al. 2004) and of the European (*P. domestica* L.; Mante et al. 1991; Ravelonandro et al. 2000; Padilla et al. 2003; Petri et al. 2008b) and Japanese (*P. salicina*, Urtubia et al. 2008) plums. In comparison with *A. tumefaciens* strategies, root transformation with *A. rhizogenes* does not require the regeneration step. This technique was notably used in the nineties

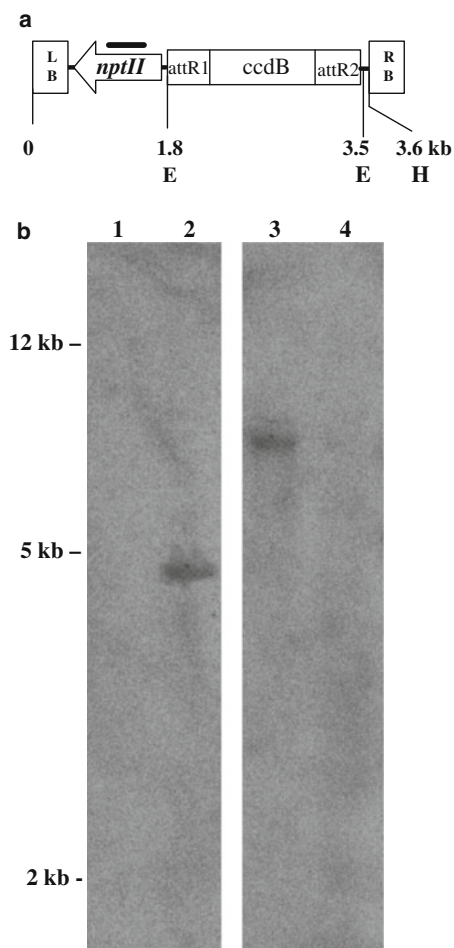


Fig. 8 **a** Schematic representation of T-DNA from pKGW,0 used in the transformation experiment. E, *EcoRV*; H, *HindIII*, LB left border, RB right border. AttR1, *ccdB* and attR2 are components of the Gateway plasmids. Thick bar above *nptII* represent ^{32}P -labelled *nptII* (kanamycin) DNA probe. Values are distances from LB (in kb). **b** Southern blots of DNA from a single isolated hairy root (genotype 253) hybridized with *nptII* (kanamycin) DNA probe on *EcoRV* (lanes 1–2) and *HindIII* (lanes 3–4) digested DNA. 1 and 4 DNA from wild untransformed plant (negative control), 2 and 3 DNA from transformed roots. The presence of a single band in lanes 2 and 3 shows that a single integration of the T-DNA has occurred in this transformation event

to trigger the rooting capacity of almond (*P. dulcis*, Damiano et al. 1995) and cherry (*P. avium* L., Gutierrez-Pesce et al. 1998; Druart et al. 1998). In our laboratory, we are interested in the functional analysis of *Prunus* resistance genes against RKN. We have reported here a method for the transformation of *Prunus* using the *A. rhizogenes* strain A4R that was chosen based on its ability to mediate root transformation in the woody plants *Citrus aurantium* L. (Chavez-Vela et al. 2003), *G. biloba* L. (Ayadi and Trémouillaux-Guiller 2003), *Aesculus hippocastanum* L. (Zravkovic-Korac et al. 2004) and *C. arabica* (Alpizar et al. 2006).

In *Prunus*, as in other fruit trees, the genotype appears as a major determinant of transformation efficiency (reviewed by Petri and Burgos 2005). In our study, genotypes 229 (60%) and 253 (72%) showed the highest ability to produce roots under *A. rhizogenes* induction. These two genotypes are interspecific Myrobalan plum \times almond-peach hybrids and combine three species genomes, contrary to the other tested genotypes which are pure Myrobalan plums. The low rooting ability of these plums needs further investigations on different culture media and hormonal balances to compensate the apparent disequilibrium consecutive to the transformation procedure.

Generally, transformation events increase with plant-bacteria co-cultivation time. In our study it seems to be the case at 25 and 28°C but not at 21°C. However, more than 3–4 days co-cultivation renders difficult the subsequent control of agrobacteria propagation (Petri and Burgos 2005) even though 5 days have been reported to be necessary for *A. tumefaciens* in woody species such as tea (*Camellia sinensis* L., Mondal et al. 2001) and pear (*P. communis* L., Matsuda et al. 2005). Co-cultivation temperature also affects *Agrobacterium*-mediated transformation particularly in trees, as observed on hevea (*Hevea brasiliensis* Willd., Blanc et al. 2006) and coffee (*C. arabica*, Alpizar et al. 2006), and in our experiments transformation rates were the highest at 25°C. A temperature of 25°C was also successfully used in oak (*Quercus suber* L., Alvarez et al. 2004), chesnut (*Castanea sativa* Mill., Corredoira et al. 2004) and pear (Yancheva et al. 2006). In our hands, the rooting efficiency, defined as the percentage of inoculated cuttings that produced roots, reached 72% for genotype 253 after 5 days of co-culture at 25°C. This is comparable to the root generation reported by authors also working on *A. rhizogenes* transformation for *P. avium* (62–80% by Gutierrez-Pesce et al. 1998) and *C. arabica* (70% by Alpizar et al. 2006) and superior to what was obtained on olive (*Olea europaea* L.; 25–60%), apple (*Malus*; 45–48.3%), almond (*P. dulcis*; 55–60%) and pistachio (*Pistacia vera* L.; 46.6–51.6%) (Rugini and Mariotti 1991).

Prunus cuttings produced newly generated roots 3–4 weeks after agro-inoculation, which is rapid in comparison with another tree such as poplar (3–4 months) (Cseke et al. 2007). The roots had the characteristic phenotype of hairy-roots with vigorous growth and high branching. These morphological alterations are due to the integration and expression, in the plant cells, of *rol* and *aux* oncogenes carried by the T-DNA of the Ri plasmid (Grant et al. 1991). To confirm that the T-DNA of the binary vector was also integrated in the genome of the root cells, we used the reporter gene *Egfp* as a non-destructive screening (Haseloff and Siemerling 1998). In *Prunus* species, screenings based on *gfp* have been used in leaves of

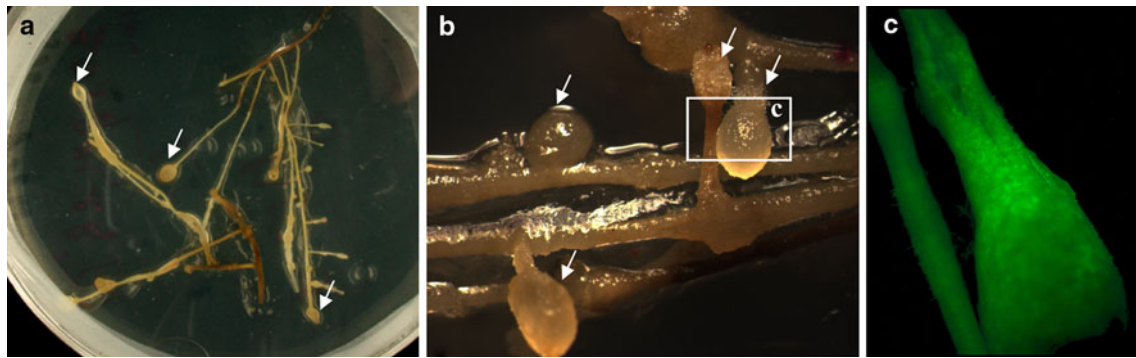
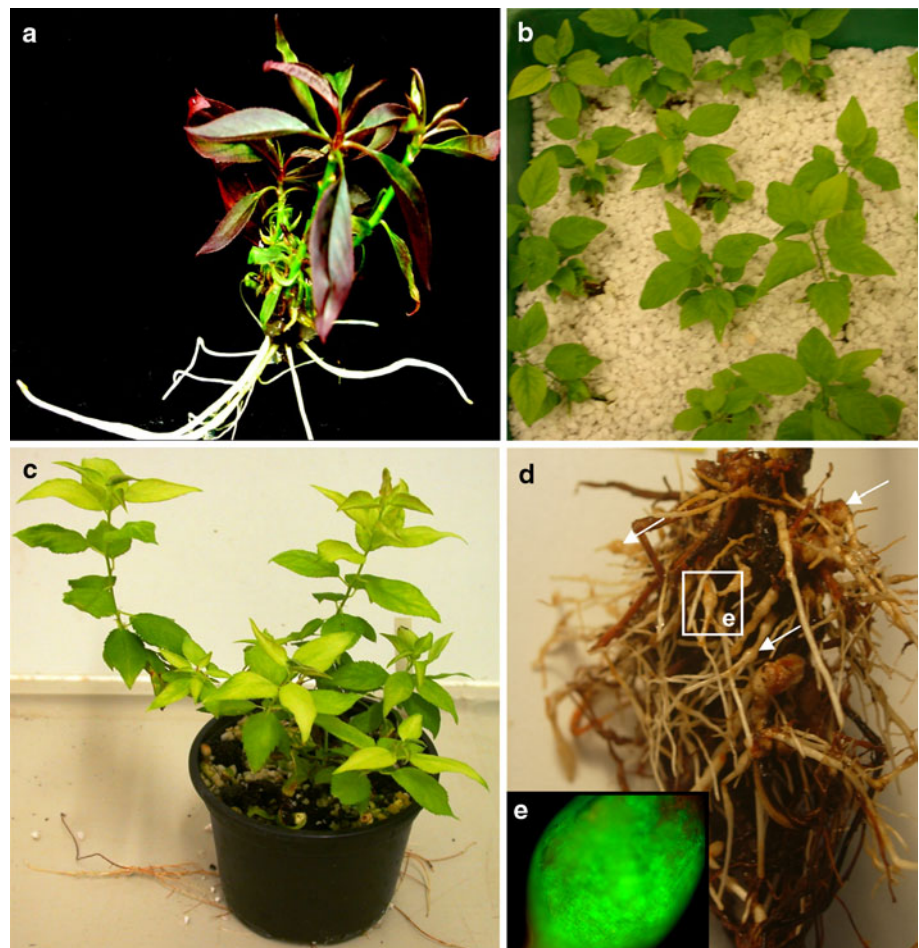


Fig. 9 Gall symptoms due to the infection by *M. incognita* of the susceptible genotype 253 transformed with *A. rhizogenes* (in vitro conditions). **a** General view of the symptoms on isolated hairy-roots

16 days after infection. Scale bar 14 mm. **b** Detail of apical galls. Scale bar 2.5 mm. Arrows indicate some of the galls. **c** Gall showing *Egfp* fluorescence

Fig. 10 Acclimation of transgenic composite plants of the genotype 253. **a** Micro-plant after in vitro culture. **b** Plants repotted into perlite. **c** Plant transferred into the final soil substrate. **d** Gall symptoms (arrows) on the root system of a composite plant, 1 month after infection. **e** Detail of a gall showing *Egfp* fluorescence



apricot (Petri et al. 2008a) and in somatic embryos of Japanese plum (Urtubia et al. 2008). In our experiments, stable *Egfp* expression was detected in the root system of 76% of the composite plants. Moreover, when the root system of a composite plant expressed *Egfp*, green fluorescence was detected in at least 2/3 (71%) of the roots and more than 1/3 (37%) of these plants expressed this reporter gene in all their roots. Because auto-fluorescence was

relatively high in untransformed control roots and fluorescence could not be stated clearly in some parts of the hairy-roots, the integration of the T-DNA was further tested by PCR amplification and Southern blotting. The detection of both *nptII* and *Egfp* genes and the absence of the agrobacterium chromosomal gene *chvH* confirmed the transgenic status of hairy-roots with strong fluorescence. The presence of the transgenes was not systematically

confirmed in hairy-roots with faint Egfp fluorescence, probably due to auto-fluorescence. Nevertheless, the successful integration of the construct (from the plasmid pKGW, without Egfp) could be shown from a single isolated hairy root (selected among other events for its vigorous growth and, thus, producing high amounts of rootlets for DNA extraction) that proved unambiguously to have incorporated one copy of the T-DNA (Fig. 8).

In our hands, 76% of the rooted plants from genotype 253 showed Egfp. Finally the percentage of plants that expressed Egfp is 55% (corresponding to 76% Egfp positive root systems out of the 72% rooted plants) of the initial number of cuttings inoculated with agrobacteria. Considering now the roots sampled from the plants of genotype 253 showing Egfp, we could estimate a transformation efficiency of 76% (16 out of 21) using PCR validation. Thus, we can conclude that about 42% (i. e. 76 out of the previous 55) of the cuttings initially inoculated with the agrobacteria produced plants with validated transformed roots.

With the aim of studying nematode resistance genes that prevent the development of galls, we tested if hairy-roots from susceptible genotypes can be infected by nematodes and produce galls. Isolated hairy-roots cultured on Petri dishes and validated for transformation by PCR were infected with J2s of *M. incognita* and the development of galls was observed. These results are consistent with the previous observation of galls on hairy-roots from sugar beet (*Beta vulgaris* L.; Kifle et al. 1999) and soybean (*Glycine max* L.; Cho et al. 2000) infected with cyst nematodes. However, it is expected that the response of isolated roots cultured in vitro to nematode attack might be different from the response of whole plants infected in soil. To get closer to the natural conditions of nematode infection, composite plants of the RKN susceptible genotype 253, which had been validated for transformation by PCR analysis, were transferred into soil. These plants in soil grew similarly to non-transformed plants and were tested for nematode infection. The galls observed after infection contained all nematode developmental stages, indicating a similar response of transformed and non-transformed roots.

Finally, the dual 'isolated hairy roots' and 'composite plants' system developed in our study should make it possible to carry out functional analysis of transgenes acting in the underground part of *Prunus* species. However, regarding composite plants that may produce non-transformed roots, a fine confirmation of the presence of the transgene by molecular analysis is indispensable before phenotype interpretation. In this objective our results suggest that each of the axes of roots growing from the initial cutting part of the plant has to be tested by PCR before considering the whole composite plant as transformed. Moreover, our work did not consider the putative development and growth of

new roots after transfer of the plants into soil. This late growth of non-transformed roots is not excluded and might also be a limiting factor for functional analysis purposes. Nevertheless, this phenomenon can be limited by keeping above the soil, at planting, the higher part of all roots having emerged from the ancient stem, thus preventing the contact of the non-transformed part of the plant with soil.

In Myrobalan plum, high-resolution genetic and physical maps have been constructed in the region of the major dominant *Ma* gene for RKN resistance (Claverie et al. 2004b) and a cluster of three candidate genes has been identified at the *Ma* locus (Esmenjaud and Dirlwanger 2007). For their functional analysis, isolated hairy roots validated individually for transformation by PCR and completed with composite plants also validated on each of the roots growing from the initial cutting part of the plant are being used.

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