Hairy root culture as a valuable tool for allelopathic studies in apple

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Abstract

Allelopathic plants exploit their chemical "weapons" to prevail the competition, suppress neighboring plants and consequently use the available resources more efficiently. However, the investigation of plant allelopathic interactions in rhizosphere is difficult to perform because of its high complexity due to interactions of biotic and abiotic factors. Thus, autonomous, aseptic roots cultures of apple (*Malus × domestica* Borkh.) could facilitate allelopathic studies. We report on the successful genetic transformation of apple cultivars Melrose, Golden Delicious, Čadel and Gloster using *A. rhizogenes* strain 15834 and for the first time the establishment of apple autonomous and permanent in vitro hairy root cultures that could be used as a new tool for apple allelopathic assays. Molecular characterization of transgenic hairy root lines was conducted to elucidate possible relationship between expression of T-DNA genes and root growth characteristics that include branching. Similar content of phenolic acids (chlorogenic, caffeic, syringic, *p*-coumaric and ferulic), glycosilated flavonoids (phloridzin, rutin, quercitrin, isoquercitrin, kaempferol-3-glucoside) and flavonoid aglycons (quercetin and naringenin) was detected in untransformed and transgenic apple root tissue by UHPLC/DAD/(+/-)HESI–MS/MS analyses, confirming that genetic transformation did not disturb secondary metabolite production in apple. Chlorogenic and caffeic acids and dihydrochalcones phloridzin and phloretin were detected as putative allelochemicals exuded into the growth medium in which transgenic roots were maintained for 4 weeks. Apple hairy root exudates significantly affected shoot and root development and growth of test plant *Arabidopsis thaliana* seedlings after 5 or 10 days of treatment. Additionally, core cell-cycle genes *CDKA1;1, CDKB2;1, CYCA3;1* and *CYCB2;4* were down regulated in Arabidopsis shoots suggesting, in part, their role in inhibition of shoot growth. Present work highlighted autonomous and permanent in vitro hairy root culture system as a valuable tool for studying allelopathic potential of apple, offering new perspective for allelopathy background elucidation in this important fruit species.

Introduction

Apple (*Malus × domestica* Borkh) is a widely grown fruit tree with considerable economic importance, which recently has rapidly increased due to a growing demand for nutrient-rich dietary food. Traditional breeding has produced more than 7500 apple cultivars with a range of desirable characteristics. Some of the varieties like Melrose (Red Delicious × Jonagold), Gloster (Glockenäpfel × Ričared) and Čadel (Golden Delicious × Jonathan) are grown preferably for their good-quality fruits. Golden Delicious (Grimes Golden x Golden Reinette) is one of the oldest and most popular commercial and breeding cultivar in the world. Because of its economic importance, Golden Delicious was chosen for an apple genome sequencing project (Velasco et al. 2010) as a model cultivar to gain new knowledge or better understanding of traits important for breeding programs, improving fruit quality or studying other relevant apple traits.

Considering current eco-friendly trends in pest and weed management, the understanding the allelopathic capacity of herbaceous and woody crops is currently an important agricultural objective (Amb and Ahluwalia 2016). Allelopathy is known as an occasion where a plant species chemically interferes with the germination and growth of neighboring plants (Molisch 1937), or with the same plant (autoallelopathy). Although a considerable number of plants display allelopathic potential, allelopathy is not a common phenomenon in the plant kingdom. Plants that were characterized as allelopathic include black walnut (*Juglans nigra*) (Willis 2000), tree-of-heaven (*Ailanthus altissima*) (Heisey 1990), eucalyptus (Niakan and Saberi 2009) and Australian blackwood (*Acacia melanoxylon*) (Souto et al. 1994) trees. The dominant position of these tree species in forests significantly influenced plant biodiversity, e.g. vegetation diversity and composition (Bratton 1976; Palik and Engstrom 1999).

Allelopathic plants can exploit their metabolites to suppress neighboring plants, thus affecting vegetation diversity and lead to more efficient use of available resources (Zhang et al.

2008; Jung et al. 2010). Apple allelochemical potential and identification of metabolites responsible for such properties could be of great importance for gaining new, scientific understanding of plant to plant interactions and for improving strategies to potentially control weeds in apple orchards or other agroecosystems. Allelopathic properties of apple trees are not well studied. Apple allelopathy has primarily been investigated as an effort to explain the phenomenon of „*specific apple replant disease*" (SARD), characterized by stunted growth of apple trees that occurs after replanting on a land that previously supported the apple orchards (Börner 1959). Results of Zhang et al. (2007a) and Bai et al. (2009) indicated that root exudates of *M. pumila* and *M. prunifolia* can exhibit inhibitory influence on germination and seedling development of its own species. Despite very questionable etiology of SARD (Weiß et al. 2017; Mahnkopp et al. 2018), the phenolic compounds, including dihydrochalcones phloridzin and phloretin, exuded from apple roots into the soil or arisen by decomposition of the bark, leaves, fruits, roots or other tree parts, could be considered as the key factors involved in manifestation of SARD (Yin et al. 2016; Nicola et al. 2017; Yin et al. 2018).

Most allelochemicals classified as secondary metabolites are produced as offshoots of the primary metabolic pathways, and can be present in several parts of the plants. Apart from allelopathic interactions that occur in the aboveground plant organs such as leaves and stems, root to root interactions play an important role in the establishment and maintenance of plant communities (Bais et al. 2006). However, isolation and characterization of metabolites exuded from roots into the soil, as well as the exclusion of effects of other factors that are present in the soil during assessment of allelopathic properties is very complex. Thus, due to inevitable influence of microorganisms in the soil on chemicals secreted by plants, it is unclear whether allelopathic effects were induced by allelochemicals directly produced in plants, or after conversion of root exudates by microorganisms (Inderjit 2005). Hence, procurement of an aseptic bioassay system, where the examined materials are free from microbial activities, is important to evaluate the allelopathic effects.

Hairy root cultures could be highly beneficial and effective for production and characterization of root secondary metabolites, evaluation of their phytotoxic effects, as well as for functional characterization and genetic manipulation of genes involved in the metabolism of root allelochemicals (Triplett et al. 2008; Kim et al. 2009; Mitić et al. 2012). Since Hoffmann et al. (2011) identified the Rosaceae roots as sites of expression of genes involved in flavonoid biosynthesis, establishment of apple hairy root cultures, capable of autonomous growth separately from shoots and without addition of plant growth regulators (PGR), could serve as a highly valuable system for studying allelopathy in apple.

Although hairy roots have been induced in more than 100 plant species, the proportion of woody species transformed using this system is very low (Christey, 2001). Susceptibility of *Malus* species to *A. rhizogenes* has been previously reported for apple rootstocks (Patena et al. 1988; Rugini and Mariotti 1992; Sutter and Luza 1993; Lambert et al. 1998; Pawlicki-Jullian et al. 2002; Yamashita et al. 2004; Wu et al. 2012). Therein, genetic transformation was used primarily to produce transgenic plants with dwarf attributes or improved rooting abilities.

The objective of the present work was to establish an in vitro assay system for studying allelochemicals in apple roots. For this purpose, domesticated apple cultivars Melrose, Golden Delicious, Čadel and Gloster were transformed using *A. rhizogenes* strain 15834. Transgenic hairy root lines were used to elucidate the relationship between T-DNA gene expression and root growth characteristics such as branching. We also report on the influence of mineral solutions and auxins on hairy root growth and biomass production. To estimate utility of in vitro hairy root culture for apple allelopathic studies, the phytotoxic effects of apple hairy root exudates on test plant *Arabidopsis thaliana*, including effects on seed germination, seedling growth and expression of core cell cycle genes, cyclin-dependent kinases and cyclins, were investigated.

Material and methods

Agrobacterium rhizogenes strain

Agrobacterium rhizogenes agropine strain 15834 harboring the pCAMBIA1301 binary vector (CAMBIA, Canberra, Australia) with the serine proteinase inhibitor gene from *Beta vulgaris* L. (*BvSTI*) (Smigocki et al. 2013) was used for transformation of apple. The full length *BvSTI* coding sequence was cloned behind the constitutive cauliflower mosaic virus 35S promoter (CaMV35S) (see Supplementary Figure 1). pCAMBIA1301 carries the *hpt* marker gene for selection of hygromycin (Hg) resistant transformed plant cells with the nos polyadenylation signal. Bacterial cultures were maintained on solid YEB medium (Vervliet et al. 1975) supplemented with 50 mg L^{-1} kanamycin (Sigma-Aldrich St. Louis, MO, USA). To obtain bacterial suspension for explant inoculation, one full loop (Ø 3 mm) of cold stored bacterial culture were resuspended into 5 mL of sterile liquid YEB medium containing 50 mg L^{-1} kanamycin in 30 mL glass tubes (18 x 150 mm) and incubated on the orbital shaker at 95 rpm for 24 h in the dark at 25±2 °C, to an optical density of $OD_{600} \approx 1.00$.

Apple transformation and establishment of hairy root cultures

Shoot cultures of apple cvs. Melrose, Golden Delicious, Gloster and Čadel, established from vegetative buds of one-year-old branches (Mitić et al. 2012) were used for genetic transformation. The cultires were grown on basal medium (BM) containing Murashige and Skoog (MS; 1962) mineral salts and Linsmaier and Skoog (LS; 1965) vitamins, 3% (w/v) sucrose, 100 mg L^{-1} myo-inositol and 0.7% (w/v) agar, with addition of 0.5 mg L^{-1} N^6-benzylaminopurine (BA) and 0.05 mg L^{-1} α-naphthaleneacetic acid (NAA) (both from Sigma-Aldrich). Cultures were grown under the light (16-h photoperiod; 45 µmol m^{-2} s^{-1}) in a growth room at 25±2 °C and subcultured at four-week intervals.

Three-week old shoots, 2-3 cm long, were inoculated with *A. rhizogenes* either by: (i) wounding the stem with a sterile needle immersed in bacterial suspension at 1.5 cm from the basal end or by (ii) dipping the cut-edge of the shoot into the bacterial suspension for a few seconds. Transformation was performed on total of 313 apple shoots. Shoots used as a negative controls for transformation process itself were processed in the same way as inoculated explants, but they were inoculated with sterile liquid YEB medium instead *Agrobacterium* suspension.

The *A. rhizogenes* - inoculated shoots were placed on PGR-free BM medium, each shoot in separate 30 mL glass tube (18 x 150 mm) under 16 h light photoperiod at 23 °C. After three days of co-cultivation, shoots were transferred onto PGR-free BM medium supplemented with 500 mg L^{-1} cefotaxime (Tolycar; Jugomedija AD, Zrenjanin, Serbia) to eliminate *Agrobacteria*. Shoots with at least one hairy root at seven weeks after inoculation were considered putative transformants. Transformation efficiency was calculated as follows: (total number of hairy root lines/number of inoculated shoots) $\times$ 100.

Each putative hairy root was established as an independently derived transformant. Twelve Golden Delicious, nine Čadel, three Gloster and 12 Melrose hairy roots were excised and propagated further as separate individual lines in 100 mL Erlenmeyer flasks in 50 mL of liquid BM media supplemented with 300 mg cefotaxime L^{-1} on an orbital shaker at 95 rpm. Cefotaxime concentration was gradually reduced and finally omitted after 4 months of cultivation. Autonomous hairy root lines were maintained under the light (16-h photoperiod) provided by cool-white fluorescent tubes (2 μmol m^{-2} s^{-1}) in a growth room at 25±2 °C and subcultured into cefotaxime-free BM at 3-week intervals.

Untransformed control roots were obtained from rooted shoots, as described in Mitić et al. (2012). Three-week old shoots, 3-4 cm long, were placed on BM medium with 0.5 mg L^{-1} IBA (indole-3-butyric acid). Shoots were grown five days in the dark and two days in the light (16-h

photoperiod), and then transferred to PGR-free BM media where they were grown for six weeks until roots reached approximately 3-7 cm in length. Roots were excised from shoots and propagated in liquid half-strength BM (½BM) supplemented with 0.5 mg L^{-1} IBA on an orbital shaker under the same conditions as the hairy root cultures.

Analysis of transgenes expression in putatively transformed hairy roots by RT-PCR

Expression of genes from T$_L$-DNA (*rolA*, *rolB*, *rolC*, *rolD*) and T$_R$-DNA (*aux1*, *aux2*) region of the Ri plasmid was analyzed in 28 hairy root lines and untransformed (control) roots by RT-PCR. Total RNA from 300 mg of ground tissue was extracted in 100 mM TRIS-HCl, pH 8 25 mM EDTA, 2% CTAB (w/v), 2% PVP (w/v), 2 M NaCl, 0.5 g L^{-1} spermidine and 2% β-mercaptoethanol (v/v) (Gašic et al. 2004). Integrity and size distribution of purified total RNA were checked by agarose gel electrophoresis. DNase I (Thermo Scientific, Waltham, MA USA) was used to remove traces of DNA according to the manufacturer's protocol.

Reverse transcription (RT) of 400 ng of DNase-free total RNA was carried out using RevertAid^{TM} Reverse Transcription Kit (Thermo Scientific) at 42 °C in a 20 µL reactions. cDNA amplification was further performed in a volume of 25 µL PCR mixture containing: 1 µL of template cDNA (corresponding to 20 ng total RNA), 1.25 µL of 5 µM forward and reverse gene specific primers (Table 1), 1 µL of 10 mM dNTPs, 0.25 µL of 5 U µL^{-1}AmpliTaq Gold$^{®}$ polymerase (Applied Biosystems), 0.75 µL of 25 mM MgCl$_2$ with 1.5 µl 10 × PCR buffer. To confirm the absence of residual *A. rhizogenes* in hairy root tissues, PCR using primers specific to the *virD1* gene (present in Ri plasmid outside the T-DNA region) was performed. Gene coding for *M. domestica* actin (*MdActin*) was used as internal control. RT and all PCR reactions were performed in Mastercycler$^{®}$ nexus Gradient (Ependorf AG, Hamburg, Germany) PCR thermal cycler. The PCR program included initial denaturation (95 °C for 5 min), 40 cycles of denaturation (95 °C for 30 s),

annealing (58 °C or 60 °C for *aux2* or all other genes, respectively, for 1 min) and extension (72 °C for 1 min), finally followed by extension (72 ºC for 10 min). RT-PCR amplified gene products were analyzed by electrophoresis on 1.2-1.8% (w/v) agarose (Sigma, St. Louis, MO) gels, depending on PCR product size, visualized with 0.5 μg mL^{-1} ethidium bromide (Valeant Pharmaceuticals, Montreal, Canada) staining and scanned by UltraLum Inc, Gel Exlorer, Exton, PA.

Effect of mineral solutions and auxins on hairy root growth

To increase the biomass production of apple hairy root cultures, the effects of 3liquid mineral solutions at full and half-strength were evaluated. Golden Delicious hairy root line GD12 was chosen for the experiment because it was able to produce sufficient amount of roots for the experimental design. All media were enriched with 3% sucrose, 100 mg L^{-1} myo-inositol and the same composition of vitamins according to Linsmaier and Skoog (1965), but with different composition of macro- and micro-salts. Thus, MS medium consisted of Murashige and Skoog (1962) salts, B5 of Gamborg et al. (1968) salts, while WPM consisted of woody plant medium (Lloyd and McCown 1981) macrosalts and MS microsalts. Approximately 500 mg fresh weight (FW) of hairy root line GD12 harvested from three-week-old cultures was transferred into the 100-mL Erlenmeyer flasks containing 50 mL of liquid MS, B5 or WPM media.

To evaluate the effect of auxins on hairy root growth, approximately 1 g of three-week-old hairy root line GD12 was harvested and transferred to 50 mL of MS liquid medium supplemented with either IBA or NAA at increasing concentrations (0.01, 0.025, 0.05, 0.1, and 0.2 mg L^{-1}). Hairy root line GD12 grown in MS liquid medium without addition of auxins were used as a control. All cultures were maintained on a rotary shaker (95 rpm) in a growth room under the same conditions as mentioned before. Media were replaced with fresh media at three weeks. At six weeks, roots were removed and blotted dry on filter paper and weighted under sterile conditions. The biomass

increment was calculated as follows: biomass increment (%) = [fresh weight after six weeks of cultivation (g) – fresh weight at the beginning of cultivation (g)] / fresh weight at the beginning of cultivation ×100. This experiment was repeated two times with three replicates (Erlenmeyer flasks) per treatment (n=6).

Secondary metabolite analyses in hairy roots and their exudates by UHPLC/(+/-)HESI–MS/MS

Four week old untransformed and Golden Delicious GD12 hairy root grown in ½MS media were analysed for phenolic compounds by Ultra High Performance Liquid Chromatography with Mass Spectrometry (UHPLC/(+/-)HESI–MS/MS). Weighed roots were air-dried at room temperature (23±2 °C) for seven days and weighted (DW). Roots were ground to fine powder in liquid nitrogen and 500 mg was re-suspended in 30 mL of 80% methanol and placed in an ultrasonic bath for 20 minutes. After sonication, extracts were filtered and evaporated to dryness in vacuum rotary evaporator (Buchi R-210, Flawil, Switzerland) at 50 °C. Dry extracts were stored at 4 °C until further analysis. The growth medium (40 mL) in which GD12 hairy root were grown for four weeks was collected for analysis and lyophilized (LH Leybold, Lyovac GT2/045, Frenkendorf, Switzerland) for five days. Samples were stored at -80 °C for further analyses.

Identification and quantification of phenolic compounds were performed on Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Bremen, Germany) configured with a triple-quadrupole mass spectrometer (TSQ Quantum Access Max, Thermo Fisher Scientific, Basel, Switzerland). Phenolics were separated on Hypersil gold C18 (Thermo Fisher Scientific) column (50mm × 2.1 mm i.d., 1.9 μm particle size), thermostated at 30 °C. Mobile phase, consisting of 0.02% acetic acid in water (A) and acetonitrile (B), was eluted according to Mišić et al. (2015). Flow rate was set to 0.4 mL min^{-1}. The injection volume was 10 μL. Heated electrospray ionization (HESI) source of mass spectrometry was operated with vaporizer temperature of 300 °C, spray

voltage 4500 V, sheet gas (N_2) pressure 35 AU, ion sweep gas pressure 1.0 AU and auxiliary gas pressure of 10 AU, capillary temperature at 275 °C, and skimmer offset 0 V. Mass spectrometry data were acquired in a negative mode. Collision-induced fragmentation was performed using argon as the collision gas, and collision energy was set to 30 eV for all the targeted phenolics. Quantification of targeted compounds in the samples was performed using Single Reaction Monitoring (SRM) mode of the instrument.

Identification of phenolic compounds in plant extracts was performed by comparison of their MS and MS^2 spectra and retention times with those of the authentic standards, as well as by literature data search. Compounds were quantified by the external standard method. Standard mixture of pure compounds in methanol was prepared in concentration of 100 μg mL^{-1}. Calibration levels (100 μg mL^{-1} to 0.001 μg mL^{-1}) were obtained by diluting the standard mixture with methanol. Regressions of the calibration curves showed good linearity with correlation coefficients between r = 0.990 and 0.999, p<0.001. Total amount of each targeted compound in the samples was determined by the calculation of peak area. Phenolics concentrations in root tissue were expressed as ng per mg of root dry weight (ng mg^{-1} DW), while their content in hairy root growth medium were expressed as ng exuded in mL of growth medium (ng mL^{-1}) or exuded per mg of dry hairy roots (ng mg^{-1} DW). Standards of chlorogenic, caffeic, syringic, p-coumaric and ferulic, acid, and of phloridzin, phloretin, rutin, isoquercitrin, kaempferol-3-glucoside, quercitrin, quercetin and naringenin were purchased from Sigma-Aldrich (Steinheim, Germany).

Analysis of apple hairy root exudates on A. thaliana seedling germination, growth and development

Liquid medium with ½MS mineral composition in which apple cv. Golden Delicious hairy root line GD12 was grown for four weeks was used to assess potential allelophatic effect on *A. thaliana* L. seedlings.

About 200 *A. thaliana* (L.) Heynh. ecotype Columbia (Col-0) seeds were surface sterilized with 70% (v/v) ethanol and commercial bleach (4-6% NaOCl) in 1:1 ratio for 60 s with shaking, washed four times in sterile distilled water for 60 s, then immersed in sterile liquid ½MS medium and placed in the dark at 4ºC. After three days, seeds were transferred into 100-mL glass Erlenmeyer flasks with 50 mL of liquid ½MS media containing hairy root line GD12 exudates or ½MS as a control. Seeds were cultivated on an orbital shaker (95 rpm) under light (45 ± 2 μmol m^{-2} s^{-1}, 16-h photoperiod) in a growth chamber at 25 ± 2 ºC. At 5 and 10 days, 10 seedlings were randomly selected and the number of lateral roots, shoots and the longest root length was measured. The bioassay was repeated two times using randomized design with three biological replicates (Erlenmeyers) for each treatment (n = 60). The treatment effect (inhibition/stimulation) was calculated using the following formula: Effect (%) = [(treatment/control) – 1] × 100. Negative (-)/positive (+) values represent percentage of inhibition/stimulation compared to the control.

Real time PCR (qPCR) analyses of cell cycle genes in A. thaliana

Total RNA was isolated, as described above, from shoots and roots of *A. thaliana* seedlings grown for 10 days in liquid ½MS medium in which hairy roots of line GD12 were previously maintained for four weeks or ½MS medium (control). Each sample consisted of pooled shoots or roots germinated from 400 seeds. Samples were collected in three biological replicates. The subsequent DNase I treatment and reverse transcription of RNA were performed using a RevertAid^{TM} Reverse Transcription Kit (Thermo Scientific), as described above. Derived cDNA was used for qPCR with QuantStudio 3 Real-Time PCR System (Applied Biosystems) and Maxima

SYBR Green/ROX Kit (Thermo Scientific), per manufacturer's instruction. Each 10 µL-PCR reaction was comprised of 5 µL of the qPCR Master Mix, 1 µL cDNA (corresponding to 10 ng total RNA) and 0.6 µL of 5 µM forward and reverse primers each for specific gene amplification. The primers used for *CDKA1;1, CDKB2;1, CYCA3;1,* and *CYCB2;4* gene amplification are presented in Table 2. *A. thaliana* actin gene (*ACTIN7*) (Table 2) was used as reference gene. All primers correspond to a particular *A. thaliana* gene in GenBank™. Specificity of all primers used was confirmed by BLAST, RT-PCR products, and analysis of melting curves during qPCR.

qPCR reaction conditions were 95 ℃ for 5 min; followed by 40 cycles of denaturation at 95 ℃ for 30 s, annealing at 60 ℃ for 1 min, and extension at 72 ℃ for 1 min, with final extension at 72 ℃ for 10 min. For each sample, qPCR reactions were carried out in technical triplicate. The results were analyzed using QuantStudio Design & Analysis Software v1.4.2 (Applied Biosystems).

The expression level for *CDKA1;1, CDKB2;1, CYCA3;1* and *CYCB2;4* genes under the influence of apple hairy root exudates were calculated relative to an expression of non-treated control according to the ΔΔCt method (Livak and Schmittgen 2001), and presented as a $\log_2$ transformation of fold changes.

Data analyses

All experiments were set up in a randomized design. The percentage data were subjected to angular transformation and the lateral roots number data to square root transformation before statistical analysis, followed by inverse transformation for presentation. The data for hairy root biomass increase influenced by type of medium or auxins were subjected to one-way analysis of variance (ANOVA). The means were subjected to *post hoc* Fisher's least significant difference (LSD) test. Differences between means of phenolic compound content in untransformed roots and hairy roots, as well as between means of morphological parameters of control Arabidopsis seedlings

and seedlings treated with apple hairy root exudates, were evaluated by the *t*-test for dependent samples. For all analyses the confidence level was $P<0.05$. All data were analyzed by SAS software (SAS Institute, 2002; SAS/STAT, ver. 9.00. SAS Institute Inc., Cary, NC, USA).

Results

Induction, culture establishment and growth pattern of transgenic apple hairy roots

Hairy roots of apple cvs. Melrose, Golden Delicious, Čadel and Gloster were initiated by inoculation of in vitro grown shoots using *A. rhizogenes* strain 15834. Two inoculation procedures were applied: shoot stem wounding by infected needle or dipping the basal cut end of the stem into bacterial suspension. Appearance of hairy roots on shoots was scored two weeks after infection regardless of the inoculation method. Inoculation by stem wounding incited emergence of hairy roots directly from the wound sites (Fig. 1), while basal cut inoculation induced root growth from the cut-edges and surrounding tissues, as high as 10-20 mm upstream from the basal end of the stem (Fig. 2A). In a few cases, roots appeared on the leaf surface (Fig. 2A). Pale, loose calli were often present at the sites of agrobacterial inoculation on shoots that did not produce roots. New hairy roots emerged over a five week period while existing ones continued to grow, reaching up to 12 cm in length.

Most roots displayed apparent agravitropic response, with some of them growing upward, forming loops (Fig. 2B). Larger lateral roots were occasionally fused together at their base forming irregular shape structures that frequently exhibited callus growth at the surface (Fig 2C). Most roots were thick and white, sometimes with light pink coloration (Fig. 2D) or with yellow tinge (Fig. 2E). Lateral roots without root hairs were sparsely emerged along the roots. A few hairy root lines were observed without or only with 1-2 lateral roots spreading along the axial root. Often, callus was present on the whole surface of the regenerated roots (Fig. 2C; 2F).

Unlike the hairy root morphology that was cultivar independent, transformation efficiencies were dependent on both the cultivar and the inoculation method (Table 3). Melrose cultivar gave the highest transformation efficiency of 70.2% on wounded stems and 3.3%, when the basal end was inoculated by dipping. In contrast, the dipping method proved to be more efficient in cv. Golden Delicious (39.3%), and the only one that enabled successful transformation of cvs. Čadel and Gloster at 30% and 10%, respectively.

Seven weeks after inoculation, hairy roots were excised from the shoot explants and cultured as individual lines. Characteristic hairy root phenotypes were observed with varying degrees of branching, growth rate and biomass production. A few hairy root lines were fast growing and highly branched (Fig. 3A and B), while majority of them were characterized by slower growth of axial roots, poor branching and low biomass accumulation (Fig. 3C and D). Eight lines turned brown and died shortly after excision from the shoots or during the first four months of individual cultivation. Thus, from total of 36 hairy root lines, 28 survived and were subjected to molecular analyses to confirm successful integration and expression of T-DNA transgenes. No roots regenerated from control apple shoots inoculated with agrobacteria-free sterile liquid YEB. Control root lines were established by rooting apple shoots on medium supplemented with the auxin IBA (Fig. 3E). Roots that developed and were excised and propagated in PGR-free medium turned brown and died soon after. Only untransformed roots grown on IBA produced large number of lateral roots. Few weeks later, lateral root formation declined considerably and biomass increased only from the apical growth of existing roots. Those in vitro cultures had long, thin and not branched roots that were visually distinct from the transgenic hairy root phenotypes (Fig. 3F). After several months of cultivation, roots became yellow, started to callus and did not survive.

Gene expression in transgenic hairy roots

Molecular analyses revealed that all 28 analyzed hairy root lines were not contaminated with *A. rhizogenes* since RT-PCR analysis detected no *virD1* gene transcripts (Table 4). Analysis for expression of genes from T_L-DNA (*rolA*, *rolB*, *rolC*, *rolD*) in the transgenic hairy roots revealed that the complete T_L-DNA region was integrated and the *rol* gene transcripts present, confirming the transgenic nature of the hairy root lines (Table 4 and Supplementary Figures 2, 3, 4 and 5). On the other hand, the *aux1* and *aux2* genes from T_R-DNA were sporadically expressed (Table 4 and Supplementary Figures 2, 3, 4 and 5). The expression of both *aux1* and *aux2* genes was detected in only three lines (M2, M6 and GD3), while 10 hairy root lines expressed neither of these two genes (Table 4). Untransformed roots (control) had very low and hardly detectable background signals of the *rolA* gene in cultivar Melrose, *rolC* in Golden Delicious and Gloster, and *rolD* in Čadel and Gloster (Table 4 and Supplementary Figures 2, 3, 4 and 5).

The effect of mineral solutions and auxins on apple hairy root growth

In general, majority of the autonomously growing hairy root lines were characterized by poor yield of biomass following one subculture period thus necessitating optimization of the cultivation protocol. Since Golden Delicious line GD12 displayed constant growth in PGR-free BM medium to provide enough plant material, it was selected for experiments to assess the effect of different mineral solutions and auxins on root growth. GD12 root growth in MS, ½MS, WPM, ½WPM, B5 and ½B5 was slow during the first two weeks of cultivation followed by more rapid growth especially in ½MS and ½B5 media. After six weeks of cultivation in ½MS, GD12 roots displayed the most vigorous growth and branching, producing the highest fresh weight increase (231%), compared to other mineral solutions (165% and below, Fig. 4A). No statistically significant differences ($P \le 0.05$) in root fresh weight increase were noted between ½MS and ½B5 treatment,

however, GD12 hairy roots turned brown after second subculture in ½B5 unlike those in ½MS. Thus, ½MS mineral solution was chosen for micropropagation of the apple hairy root lines.

To further accelerate the growth of apple hairy roots, the effect of IBA and NAA at increasing concentrations was tested (Fig. 4B). Auxins influenced branching and the growth rate. Relative to the auxin-free control (93% biomass increase), the highest increase in biomass was observed in ½MS media supplemented with NAA at 0.1 mg L^{-1} (455%) and IBA at 0.025 mg L^{-1} (435%). Despite the initial intense growth stimulated by 0.1 mg L^{-1} NAA, prolonged cultivation in this medium induced callus formation that arrested root elongation and branching, causing root tissue death. Overall, NAA supplementation induced more root callus formation than IBA. Thus, lower concentrations of IBA (0.01 to 0.025 mg L^{-1}) were more appropriate options for root growth and branching. However, prolonged root cultivation with IBA also induced some degree of callus formation with an effect on root growth. Thus, for continued cultivation and maintenance of slow-growing lines, roots should be grown by shifting between IBA-containing and IBA-free media.

UHPLC/(+/-)HESI–MS/MS analysis of phenolic compounds in apple hairy root tissue and growth medium

Phenolic acids (chlorogenic, caffeic, syringic, *p*-coumaric and ferulic), glycosilated flavonoids (phloridzin, rutin, quercitrin, isoquercitrin, kaempferol-3-glucoside) and flavonoid aglycons (floretin, quercetin and naringenin) were detected by UHPLC/(+/-)HESI–MS/MS analyses in untransformed apple cultivar Golden Delicious roots, GD12 hairy root line and growth medium in which GD12 roots were cultivated for four weeks (Table 5).

In both untransformed and hairy roots, phloridzin was the predominant phenolic compound with 1763.83 and 2643.60 ng mg^{-1} DW, respectively, being about 100- to 1000-fold higher than the

other phenolics (Table 5). Phloridzin aglycon - phloretin was not detected, while other two aglycons, quercetin and naringenin were present in trace amounts and could not be quantified.

Seven compounds were detected in the growth media of line GD12, but only four (chlorogenic and caffeic acids, phloridzin and phloretin) in concentration that could be quantified (Table 5). Phloridzin concentration was about 500 times lower than in roots, but in the same range as phloretin and phenolic acids.

Allelopathic effect of apple hairy root exudates on A. thaliana seedlings

Apple hairy root exudates did not have an effect on *A. thaliana* seed germination. After five and 10 days of treatment, however, root and shoot growth was significantly affected as compared to untreated controls (Fig. 5 and 6).

In control seedlings, white, thin and short lateral roots were observed five days after exposure to light, with an average 1.94 lateral roots per seedling (Fig. 6A). Lateral roots formed mainly on the upper third of the axial root with no root hairs. In contrast, exudate treated seedlings rarely formed lateral roots (0.18 lateral root/seedling). Over the next five days, thin lateral roots did emerge (9.6 lateral root/seedling), but they were much shorter, not branched and with less root hairs than on controls (36.8 lateral roots/seedling). Apple root exudates not only inhibited lateral root formation (74%), but also significantly inhibited elongation of axilar roots (35.15%) (Fig. 6B).

Arabidopsis shoot morphology was affected by the hairy root exudates. Shoot length was reduced by almost 50% at five days of treatment, increasing to 62.1% at 10 days (Fig. 6C). Only cotyledons were visible in treated seedlings compared to control seedlings that had, in addition to cotyledons, two completely developed leaves and a second pair emerging from the bud. In addition, leaves were smaller and pale as compared to the controls (Fig. 5).

Exudate effect on A. thaliana cell cycle related genes

Expression levels of cyclin-dependent kinases (*CDKA1;1,* and *CDKB2;1*) and cyclins (*CYCA3;1* and *CYCB2;4*) genes were analysed in *A. thaliana* shoots and roots treated for 10 days with apple hairy root exudates.

Apple hairy root exudates did not affect expression levels of analysed genes in roots, but gene transcript levels were reduced in shoots, with the highest descent in expression observed for *CDKA1;1* and *CYCB2;4* (Fig.7).

Discussion

Transformation of members of *Malus* genus by *A. rhizogenes* has been applied mainly to improve the rooting capacity of apple rootstocks (Pawlicki-Jullian et al. 2002; Yamashita et al. 2004; Wu et al. 2012). Accordingly, there have been no attempts to isolate hairy roots from mother plants and to cultivate them as individual root cultures. In this study, we report on transformation of apple cultivars Melrose, Golden Delicious, Čadel and Gloster using *A. rhizogenes* 15834 that, for the first time, establishes in vitro propagated hairy root cultures of apple.

Several factors have been found to affect transformation efficiency of fruit trees (Petri and Burgos 2005). Among them, genotype remains the most serious limiting factor for gene transfer to fruit crops. Similarly, we expected that the transformation efficiency in this study would be apple cultivar dependent, but as it turns out the *Agrobacterum* inoculation method proved to be equally important. Wounding of Melrose stems with *A. rhizogenes* gave the best transformation efficiency (70.2%) compared to 3.3% when basal cut end of the shoots was dipped into bacterial suspension. Although inoculation by wounding with infected needle or scalpel is a widely used method (Gutièrrez-Pesce et al. 1998; Vinterhalter et al. 2006; Ninković et al. 2010; Bogdanović et al. 2014),

the dipping method proved to be more efficient in cv. Golden Delicious (39.3%), as well as the only way that cvs. Čadel and Gloster (30% and 10%, respectively) were successfully transformed. This method was successfully used to transform *Malus baccata* (Wu et al. 2012) and Jork 9 (Pawlicki-Jullian et al. 2002) apple rootstocks. In Jork 9, transformation frequencies of 16.7 to 77.8% were dependent on the strain of *A. rhizogenes* used, strain 15834 giving the lowest efficiency (Pawlicki-Jullian et al. 2002). Using the same strain and dipping method in our study, we obtained efficiencies of up to 39.3% in Golden Delicious. Application of this method favored not only hairy root formation from the stem cut-edges, but also upstream from the basal end of the shoot, on the stem and leaf. This could be the consequence of agrobacteria infection of leaf cells that were wounded accidentally during manipulation, or by penetration of xylem and phloem elements on the cut-edges, reaching damaged cells at a distance.

Although possessed an apparent agravitropic response, majority of the apple hairy root lines were slow growing, with sparse lateral roots distributed along the axial root and lacking root hairs that commonly characterize hairy root phenotypes (Tepfer and Casse-Delbart 1987). This morphology could be the specificity of the *Malus* genotype since hairy roots with similar phenotypes were also obtained with *Malus pumila*, Jork 9 and *Malus baccata* rootstocks (Sutter and Luza 1993; Pawlicki-Jullian et al. 2002; Wu et al. 2012). The degree of hairy root branching and biomass increase was more notable after roots were excised from the shoots and cultivated as individual lines. This phenomenon was reported for hairy root cultures derived from a wide range of plant species (Chaudhuri et al. 2005; Tiwari et al. 2007; Taneja et al. 2010; Mitić et al. 2012). These differences appear to be specific to the hairy root line itself, since the different characteristics were found in lines that originated from the same shoot. Some authors suggested that these alterations are a consequence of the variable integration of genes from T_L- and T_R-DNA into the plant genome (Batra et al. 2004; Tiwari et al. 2007; Taneja et al. 2010). Nevertheless, in our work molecular

analysis did not reveal any correlation between expression of *rol* and *aux* genes and growth characteristics of apple hairy root lines. Lines that did not express one or both of *aux* genes were incapable to produce enzymes involved in auxin biosynthetic pathway (tryptophan monooxygenase or/and indole-3-acetamide hydrolase), thus to synthesize IAA, but differed greatly in growth characteristics. Similar phenomenon was observed in hairy root cultures of *Catharanthus roseus* (Batra et al. 2004; Taneja et al. 2010). Based on the presented results it could be concluded that genes from T_R-DNA were not responsible for growth characteristics of hairy roots in apple. Since all four *rol* genes (*rolA, B, C* and *D* gene) were expressed in all of the 28 analyzed hairy root lines, additional analysis using Real time PCR (qPCR) would be necessary for determination of precise *rol* genes expression level, and elucidation of its possible relation with hairy root branching and growth. Additionally, explanation could be sought in other factors such as transgene copy number, position effects or epigenetic control. Since it is known that *rol*B expression is largely auxin-inducible, and in turn, fate of *rol*B induced meristem depends upon the local hormonal balance of the cell (Baumann et al. 1999), difference among hairy root lines could be the consequence of their origin from different initially infected cells, characterized by the different hormone level and/or sensitivity. Additionally, Alpizar et al. (2008) and Taneja et al. (2010) reported sporadic lack of integration of individual *rol* genes from T_L-DNA in coffee and *Catharanthus roseus*, while T_R-DNA was always completely integrated or completely missing. In contrast, in our study, all *rol* genes from T_L-DNA were always integrated together, while integration of *aux* genes was sporadic.

The low intensity signals detected by RT-PCR for *rolA, rolC*, and *rolD* gene in our untransformed control roots of all four apple cultivars could be explained by possible ancient transfer between *A. rhizogenes* and apple ancestor. This phenomenon was suggested by hybridization of probes specific to T_L- and T_R-regions of T-DNA to untransformed apple rootstock M26 DNA restriction fragments (Lambert and Tepfer 1992). Similar phenomenon was also

observed in genus *Nicotiana* (White et al. 1983; Meyer et al. 1995; Frŭndt et al. 1998), as well as in *Convolvulus arvensis* (Tepfer 1983) and *Ajuga reptans* (Tanaka et al. 1998).

Half-strength MS (½MS) medium was found to induce the highest level of growth (231%) in apple transgenic line GD12. The same mineral solution proved to be the most suitable for hairy root cultures of *Fagopyrum esculentum* (Lee et al. 2007), while maximum biomass increase of *Panax hibride* (Washida et al. 1998) and *Platycodon grandiflorum* (Tada et al. 1995) hairy roots was achieved on B5 media, suggesting that the content and concentration of macro and micro nutrients is species specific.

Transgenic hairy roots are characterized by high branching and intense growth due to their increased sensitivity to auxins caused by activity of the *rolB* gene (Shen et al. 1988; Nilsson and Olsson 1997; Baumann et al. 1999). Thus, addition of exogenous auxins to culture media might have positive or adverse effects on hairy root growth, depending on the concentration and type of auxin applied (Liu et al. 2002; Alpizar et al. 2008). We found that supplementing the growth medium with lower concentration of IBA (0.01mg L^{-1} or 0.025 mg L^{-1}) stimulated most branching and elongation of lateral roots with increasing biomass accumulation, while higher concentrations (0.1 mgL^{-1} or 0.2 mg L^{-1}) did not have a positive effect on root growth. On the contrary, higher concentration of IBA, 0.1 mg L^{-1} (Alpizar et al. 2008) and 0.5 mg L^{-1} (Washida et al. 1998), were the most effective in stimulating hairy root branching and growth in coffee and Panax hybrid, respectively. Liu et al. (2002) reported that *Pueraria lobata* hairy root growth and lateral root elongation was stimulated at 0.02 mg L^{-1}, not effected at 0.2 mg L^{-1}, and inhibited at 0.5 mg L^{-1} IBA. It appears that the endogenous content of auxin, characteristic for every species, dictates the extent to which exogenous auxins could stimulate the lateral root branching and growth. Additionally, inhibitory effect of higher auxin concentrations could be the result of altered signaling responses of other endogenous hormones involved in the regulation of lateral root development and growth, like

cytokinins (as reviewed by Su et al. 2011) and gibberellins (Fu and Harberd 2003; Gou et al. 2010). The results also revealed that hairy root callusing in apple was more pronounced in the case of NAA than at the same concentrations of IBA. A similar observation was reported in hairy root cultures of opium poppy and *Karwinskia humboldtiana*, (Park and Facchini 2000; Kollárová et al. 2004). Although in apple hairy roots IBA at lower concentrations 0.01 mg L^{-1} and 0.025 mg L^{-1} stimulated initiation and growth of lateral roots, longer period of exposure led to increased callusing and consequently reduced hairy root growth. Therefore, the liquid ½MS medium with the addition of IBA at 0.025 mg L^{-1} was chosen for initial stimulation of growth of hairy roots after their excision from primary explants, while prolonged cultivation was carried out in the PGR-free liquid ½MS medium. For prolonged cultivation and maintenance of slow growing lines, the altering of IBA-containing and IBA-free media is recommended.

In light of the crucial impact of auxins on plant physiology (Teale et al. 2006; Péret et al. 2009; Kieffer et al. 2010), absence of auxins in apple root culture medium would be important for investigating allelopathic potential of apple trees. Since untransformed apple roots would not survive and grow without IBA supplementation, satisfactory constant growth of apple hairy roots in the absence of PGRs, like hairy root line GD12, should be of particular importance for chemical characterization of apple exudates and for application in bioassays.

Phenolic compounds are considered potent allelochemicals in most plant species (Li et al. 2010). Many polyphenolic compounds have been detected in roots, leaves, bark, fruits and seeds of domesticated apple (Hunter and Hull 1993; Awad et al. 2000; Treutter 2001; Pontais et al. 2008). Some of them were also found in the soil in which apple trees were grown, or in the close distance of the trees as a result of decomposition of apple tissues or the apple tree root exudation (Hofmann et al. 2009; Yin et al. 2016).

UHPLC/(+/-)HESI–MS/MS analyses showed the presence of phenolic acids, chlorogenic and caffeic, as well as dihydrochalcones phloridzin and phloretin as putative allelochemicals exuded by hairy root line GD12 into the growth medium. In hairy root GD12 and GD untransformed control roots, a larger number of phenolic compounds were detected, indicating that not all of synthesized compounds were being secreted. Even though *rol* genes are capable of altering intensity of secondary metabolite production in transformed plants (Bonhomme et al. 2000; Kiselev et al. 2007; Shkryl et al. 2008), similar content of phenolics detected in untransformed and transgenic apple root tissue indicated that *Agrobacterium*-mediated genetic transformation did not disturb phenolic secondary metabolite production in apple hairy root. Phloridzin was recognized as the most abundant of all phenolics in apple root tissues, with concentration that was from 100- to 1000-fold higher than content of other detected compounds. Predominance of phloridzin was commonly found in other apple tissues (Hunter and Hull 1993; Treutter 2001; Pontais et al. 2008). Since the dry weight of apple hairy roots is approximately 10% of the FW, phloridzin content in hairy root GD12 is approximately 0.3 mg g^{-1} FW, similar to untransformed roots of in vitro grown cv. Liberty and McIntosh apple shoots (0.4 to 1.2 mg g^{-1} FW) (Hrazdina 2003). Despite the high amounts of phloridzin detected in the hairy root tissue, less than one percent (~ 0.13%) of its content was released into the growth medium. Nevertheless, the content of phloridzin secreted from hairy roots into the growth medium is similar to that released from roots of apple plants grown in the soil (Hofmann et al. 2009).

Interestingly, phloretin, a phloridzin precursor which is glycosylated in apple tissues by phloretin 2'-O-glycosyltransferase forming phloridzin (phloretin 2'-O-β-D-glucopyranosid) (Jugdé et al. 2008: Gosch et al. 2010), was not detected in roots. Likewise, Hrazdina (2003) did not detect phloretin in stems and roots of McIntosh and Liberty cultivars. No phloretin was detected in leaves of 14 different *M. domestica* cultivars (Mikulic-Petkovšek et al. 2009). Hairy root GD12, in addition

to the phloridzin, contained only glycosylated forms of other flavonoids that included rutin, quercitrin, isoquercitrin and kaempferol-3-glucoside, while aglycons like floretin, quercetin and naringenin were not present or were detected in trace amounts. Taken together, this suggests that plants strive to rapidly convert newly synthesized metabolites, like dehydrochalcones and other flavonoids, into soluble glycosylated forms and stored them in the form of more stable pools (Jones and Vogt 2001). This process enables plants to control the quantity of physiologically active metabolites and the overall dynamics of physiological processes, and for self-protection from toxic endogenous and exogenous substances (Sirikantaramas et al. 2008). Flavonoids like quercetin could act as mutagens or cause fragmentation of some tryptophan-containing proteins (Ahmed et al. 1994; Rueff et al. 1995). It was known that pyrolisis of tryptophan resulted in more mutagenic activity than did any other common amino acid (Matsumoto et al. 1977). Expectedly, plant might convert flavonoids into glycosylated forms and actively transport them into vacuoles (Klein et al. 2006).

It is well documented that phloridzin is prone to degradation by enzymes produced by soil microorganisms (Jayasankar et al. 1969; Chatterjee and Gibbins 1969; Natsume et al. 1982). In the aseptic conditions of hairy root in vitro culture, it is possible that phloridzin was aglycosylated spontaneously, or due to activity of the apoplastic glycoside hydrolases (Günl et al. 2011) or cytosol β-glycosidases (Warzecha et al. 1999; Czjzek et al. 2000), released from root cells after their damage or necrosis.

Within *M. domestica*, the concentration of dihydrochalcones can vary depending on the cultivar (Łata et al. 2009), tissue (reviewed by Gosh et al. 2010), developmental stage (Zhang et al. 2007b) and sampling time (Hunter and Hull 1993; Mikulic-Petkovšek et al. 2009). Environmental factors like seasonal changes and pathogens also may affect phloridzin content in the same type of apple tissue (Mikulic-Petkovšek et al. 2008). In vitro hairy root cultures eliminate environmental factors and pathogens, providing stable growing conditions. In addition, they mimick natural

dynamics in metabolism of dihydrochalcones. Accordingly, this system approved to be suitable for studying the allelopathic potential of dihydrochalcones and other phenolics in apple. Additionally, secretion of phloridzin, chlorogenic and caffeic acid from hairy root cultures growing separately from shoots, confirms that apple roots are not only the sink in which the chemicals accumulate when transported from the shoots but also are the primary site of synthesis of these phenolic substances. This justifies the use of hairy root culture not only as a tool for studying allelopathy, but also for functional characterization of genes involved in biosynthesis of these compounds and for genetic manipulations to enhance apple allelopathic attributes by improving allelochemical production (Bourgaud et al. 2001).

To study the allelopathic potential of apple root exudates, *A. thaliana* was chosen as it previously was shown to be a sensitive test plant suitable for allelopathic assays (Pennacchio et al. 2005) and for examining genomic responses (Bais et al. 2003). In contrast to numerous allelochemicals that inhibited *Arabidopsis* seed germination (Pennacchio et al. 2005; Reigosa and Pazos-Malvido 2007), apple hairy root exudates did not inhibit germination but did significantly affect seedling growth. The strongest inhibitory effect was on the formation of lateral roots (74.0% inhibition). Root elongation and shoot growth were also significantly affected (35.1% and 62.1% reduced, respectively). Moreover, apple root exudates disrupted the morphology of Arabidopsis shoots and leaves. That phenolic compounds like chlorogenic and caffeic acid are plant allelochemicals have already been documented by a number of authors (Patterson 1981; Garg and Garg 1989; Reigosa and Pazos-Malvido 2007; Batish et al. 2008). Aqueous extract of *Delonix regia*, an ornamental tree of Taiwan, containing several phenolic compounds including chlorogenic and caffeic acid, exhibited profound inhibitory effect on growth of lettuce (*Lactuca sativa*), Chinese cabbage (*Brassica chinensis*) and alfalfa (*Medicago sativa*) (Chou and Leu 1992) at various concentrations. Caffeic acid exhibited inhibitory effect on mung bean (*Phaseolus aureus*) seedling

root, shoot elongation and biomass increase (Batish et al. 2008). In *A. thaliana*, 10-1000 µM chlorogenic acid did not influence germination rate but significantly decreased seedling root elongation (Reigosa and Pazos-Malvido 2007), while caffeic acid inhibited Arabidopsis germination by 1.5 to 100% depending on its concentration (1-100 mM) (Pennacchio et al. 2005). Considering that the contribution of phenolic compounds to allelopathy was usually not the result of a single substance (Einhellig et al. 2004), the role and the allelopathic activity of dihidrochalcones phloridzin and phloretin needs to be explored, especially in light of their involvement in the phenomenon known as "*specific apple replant disease*" (Nicola et al. 2017; Yin et al. 2016). Recent overview on this phenomenon is close to the early statement of Börner (1959) who associated SARD with autoallelopathy, implying the chemicals released from apple roots into the rhizosphere, by exudation or decomposition of roots, bark or leaves, interfere with the germination and growth of neighbouring individuals (Zhang et al. 2007a; Bai et al. 2009). The treatment of Fuji apple seedlings with mixture of unaffected soil and grounded roots of rootstock M26 caused significant inhibition of seedling growth and decrease in chlorophyll content (Nicola et al. 2017). This allelopathic effect authors primarily attributed to phloridzin as conceivably dominant phenolic compound detected in soil/roots mixture. The apple transgenic hairy root culture could be convenient method to study prospective autoallelopathy role of apple allelohemicals exuded from roots, by testing their autotoxicity on apple seed germination and seedlings growth in vitro.

Retardation of Arabidopsis shoot and root growth and lateral root formation affected by apple hairy root exudates can be the result of disturbance of diverse physiological and biochemical processes (Weir et al. 2004). Thereby, many plant allelochemicals have frequently been reported to retard cell division (Nishida et al. 2005; Sánchez-Moreiras et al. 2008; Zhang et al. 2010; Soltys et al. 2011; Mitić et al. 2012; Dmitrović et al. 2015). Although, nearly 200 genes are involved in regulating the cell cycle in Arabidopsis, central role belongs to core cell cycle genes (cyclins and

cyclin-dependent kinases) (Menges et al. 2005; Van Leene et al. 2010). Two cyclin-dependent kinases CDKA1;1 and CDKB2;1 and cyclins CYCA3;1 and CYCB2;4 were chosen for our experiments. Kinase CDKA1;1 which is constitutively expressed to regulate the entrance to the cell cycle, as well as both the G1-to-S and G2-to-M transitions, is postulated to interact exclusively with type D cyclins (CYCD) and specific S-phase cyclins type A, like CYCA3;1 (Vandepoele et al. 2002; Van Leene et al. 2010). Cyclin-dependent kinase CDKB2;1 and cyclin CYCB2;4 are involved in the control of the G2-to-M checkpoint (Menges et al. 2005). Additionaly, research of Lee et al. (2003) indicated that rice CDKB2;1 interacts exclusively with B2 cyclins. Our treatment of *A. thaliana* shoots with apple hairy root exudates caused down-regulation of the *CDKA1;1* and *CDKB2;1* cyclin-dependent kinases and *CYCA3;1* and *CYCB2;4* cyclins genes. In roots, expression levels of *CDKs* as well as *CYCs* genes stayed unchanged. Therefore, it can be postulated that the observed inhibition of Arabidopsis shoot development and elongation is, at least partially, due to down-regulation of the core cell-cycle genes in shoots. However, the involvement of core cell cycle genes in inhibition of lateral root formation and root elongation could only be elucidated after examining cell cycle gene expression at time points after the onset of the treatment with the root exudates. We cannot rule out that factors different from those included in cell cycle regulation, e.g. giberelin metabolism (Gou et al. 2010) or polar auxin transport (Brown et al. 2001; Peer et al. 2004), are much more engaged in the root growth retardation, which is the object of our further research aimed to elucidate the background of the allelopathy in apple. In addition, allelopathic effect demonstrated on arabidopsis nominated the apple in vitro culture products as putative eco-friendly bio-herbicides for weed control in crop fields (Jabran et al. 2015). Therefore, the apple hairy root cultures could be used as bioreactors for accumulation of secondary metabolites with bio-herbicidal properties, enabling at the same time their enhanced production by elicitation (Ramirez-Estrada et al. 2016).

Conclusion

The present work describes successful genetic transformation of four apple cultivars Melrose, Golden Delicious, Čadel and Gloster by *A. rhizogenes* strain 15834, and the first in vitro establishment of apple hairy root cultures. UHPLC/(+/-)HESI–MS/MS analysis of phenolic compound content in untransformed and hairy root tissues revealed high similarity, confirming that genetic transformation by agrobacterium did not disturb the production and profile of phenolic secondary metabolites in apple. Chlorogenic and caffeic acid, as well as dihydrochalcones phloridzin and phloretin were annotated as putative allelochemicals in hairy root growth medium that caused a significant retardation in the development and growth of *A. thaliana* seedling used as a test plant. Down-regulation of the core cell-cycle genes *CDKA1;1, CDKB2;1, CYCA3;1* and *CYCB2;4* in shoots is, at least partially, responsible for such effect. As an aseptic system free from microbial activities as well as other interactions commonly present in natural habitats, the hairy root culture can serve as a valuable tool for studying the allelopathic potential of apple. Since this system mimicked natural dynamics in metabolism of dihydrochalcones and other phenolics in apple, it could also be suitable for functional characterization of genes involved in biosynthesis of these compounds, as well as genetic manipulations to improve apple allelopathic attributes.

Conflict of interest

We declare that we have no conflict of interest.

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Figure legends

Figure 1. Hairy roots emerging from wound sites on apple stem.

Figure 2. Regenerated apple hairy roots seven weeks after shoot inoculation with *A. rhizogenes* strain 15834. Hairy roots emerging from the cut-edge of apple shoot (red arrow), ~15 mm upstream the cut-edge (black arrow) and on the leaf surface (blue arrow) (A); Agravitopic growth of regenerated hairy root (B); Hairy roots fused together at their bases forming irregular shape structure (arrow) (C); Light pink (D) or yellow (E) coloration of hairy roots; Callusing surface of hairy roots (F).

Figure 3. Morphology of transformed and untransformed apple root lines. Transgenic fast growing line GD12 (A) and GD1 (B); moderately growing line GD11(C); and a poor growing line GD3 (D). Rooted Golden Delicious control shoot (E); Control root line supplemented with the auxin IBA (E).

Figure 4. Influence of mineral solutions (A) and auxins in ½MS (B) on biomass production in hairy root line GD12 at six weeks of cultivation. Values are means of three replicates per each treatment repeated twice (n = 6). Treatments denoted by the same letter are not significantly different (P ≤ 0.05) per Fisher's least significant difference (LSD) test. MS = Murashige and Skoog mineral solution (Murashige and Skoog, 1962), ½MS = half strength MS, B5 = Gamborg mineral solution (Gamborg er al., 1968), ½B5 = half strength B5, WPM = Woody plant medium (Lloyd and McCown, 1980), ½WPM = half strength WPM, IBA = indole-3-butyric acid, NAA = α-naphthaleneacetic acid.

Figure 5. Effect of apple hairy root exudates on *A. thaliana* growth at 10 days of treatment. Control seedlings in ½MS medium (A), Arabidopsis seedlings treated with apple hairy root exudates in ½MS (B); Bar = 5 mm.

Figure 6. Effect of apple hairy root exudates on *A. thaliana* seedling lateral root number (A), root length (B) and shoot length (C) at five and 10 days of treatment as compared to untreated control. Values are presented as means (± SE) of three replicates with 10 explants per each treatment repeated twice (n = 60). Asterisks indicate statistically significant difference at $P < 0.05$ based on *t*-test, while numbers above the bars indicate the percentage of inhibition (−).

Figure 7. Expression levels of *CDKA1;1*, *CDKB2;1*, *CYCA3;1* and *CYCB2;4* genes in *A. thaliana* shoots and roots at 10 days of treatment with apple hairy root exudates. Data are presented as means of three biological replicates with standard error bars. Expression levels were calculated relative to an expression of related non-treated control according to the $\Delta\Delta Ct$ method and presented as log2 transformation of fold changes.
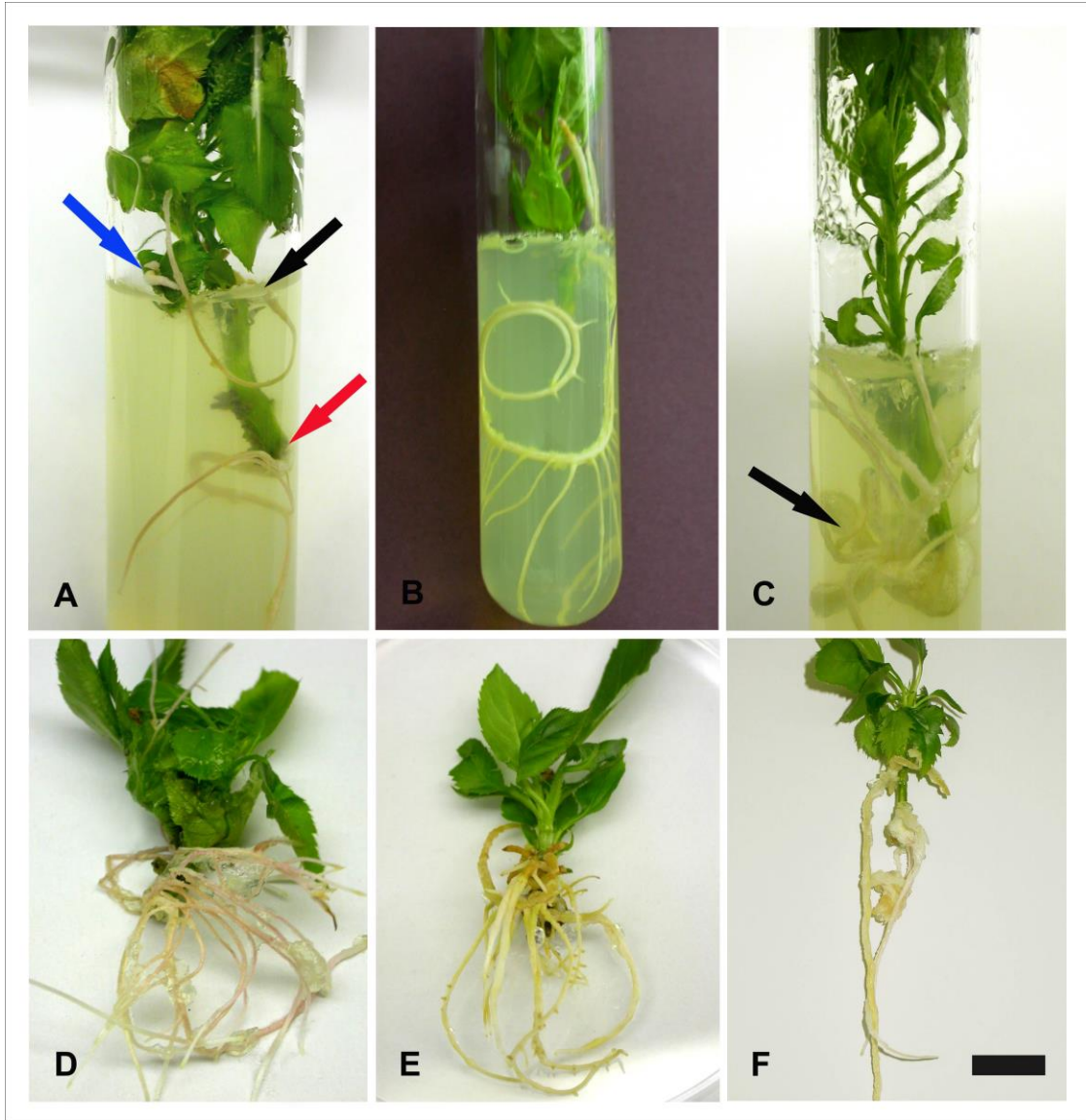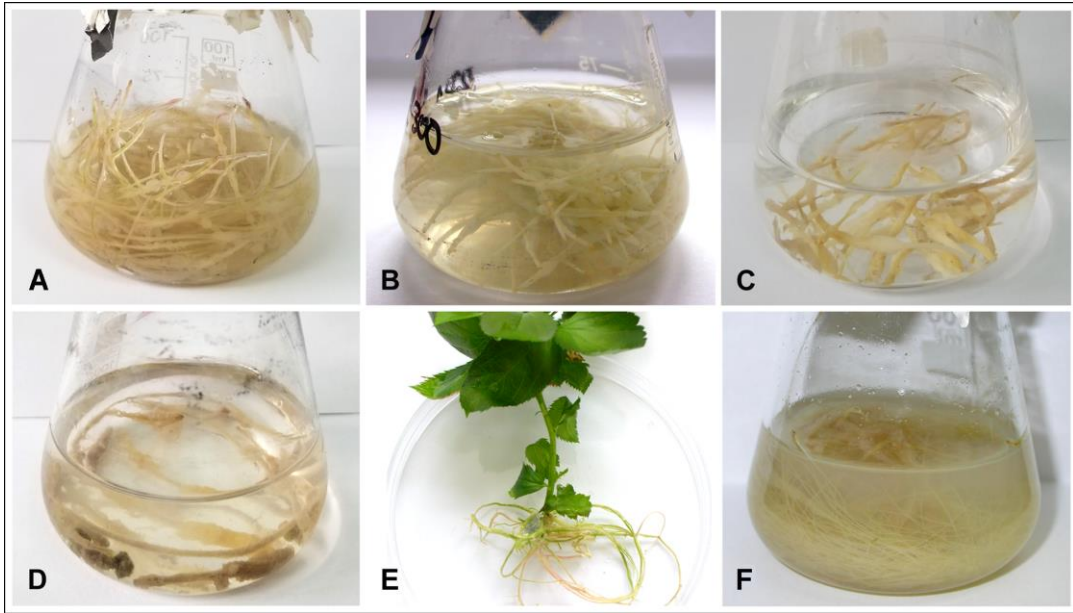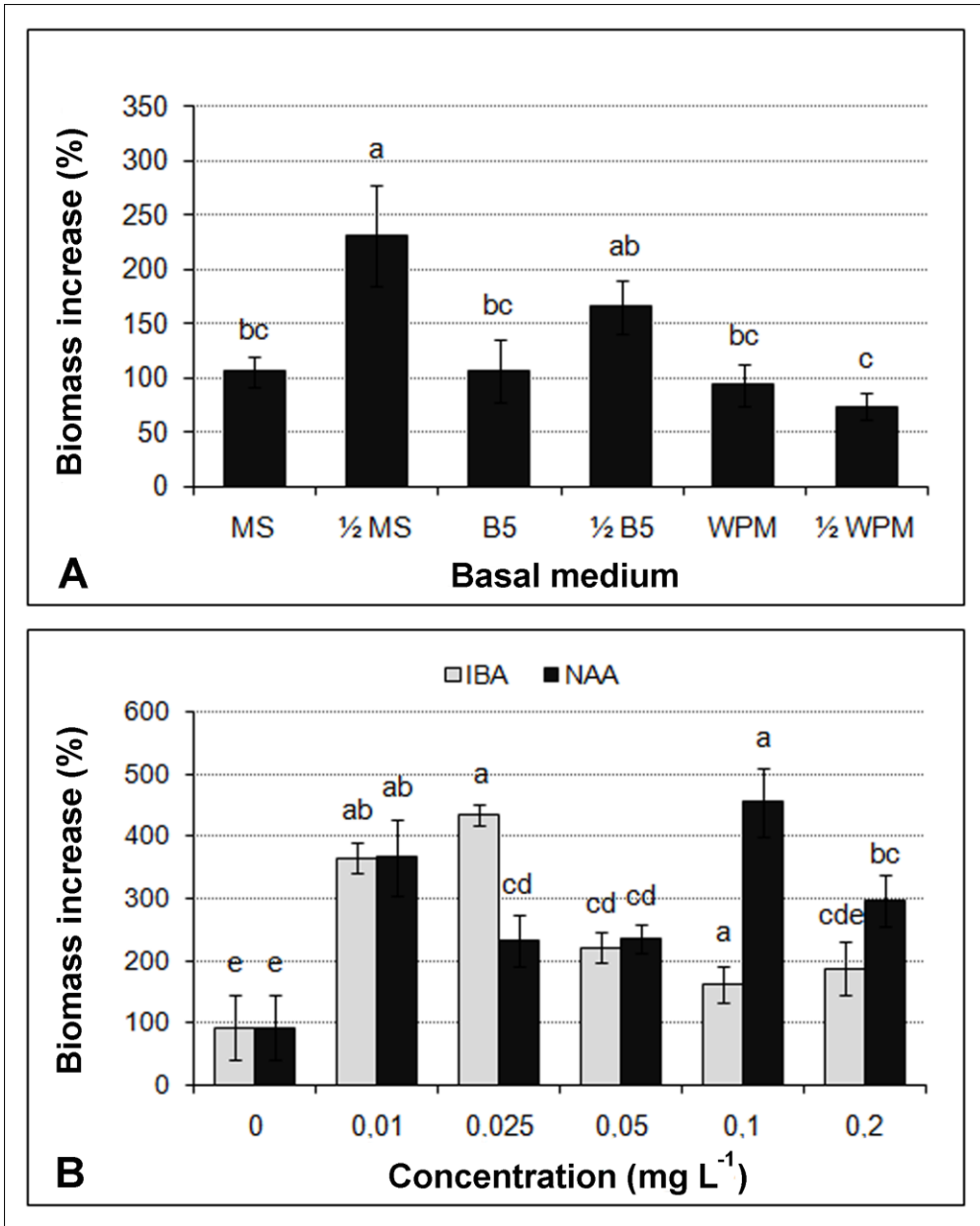

Figure 1.

Figure 2.

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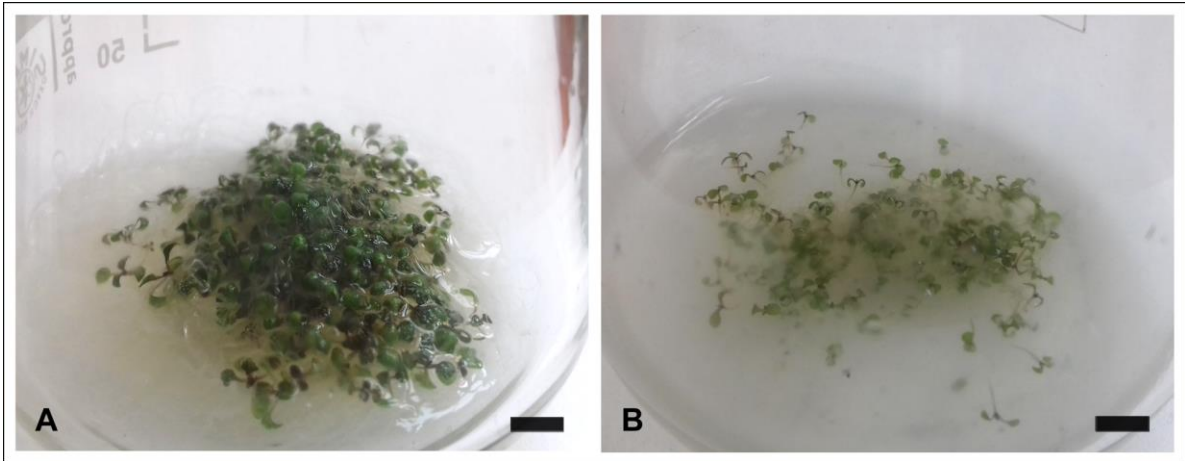
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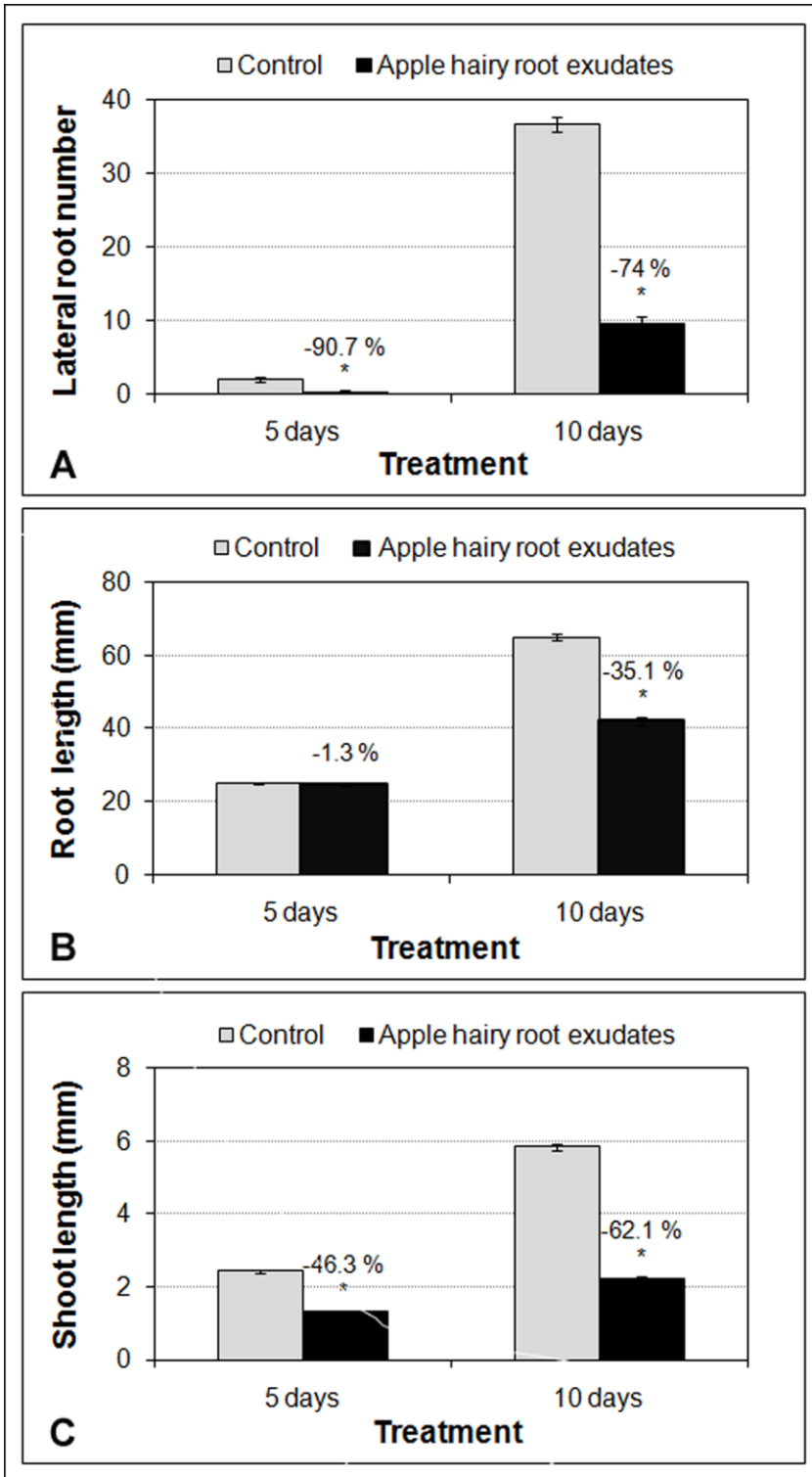
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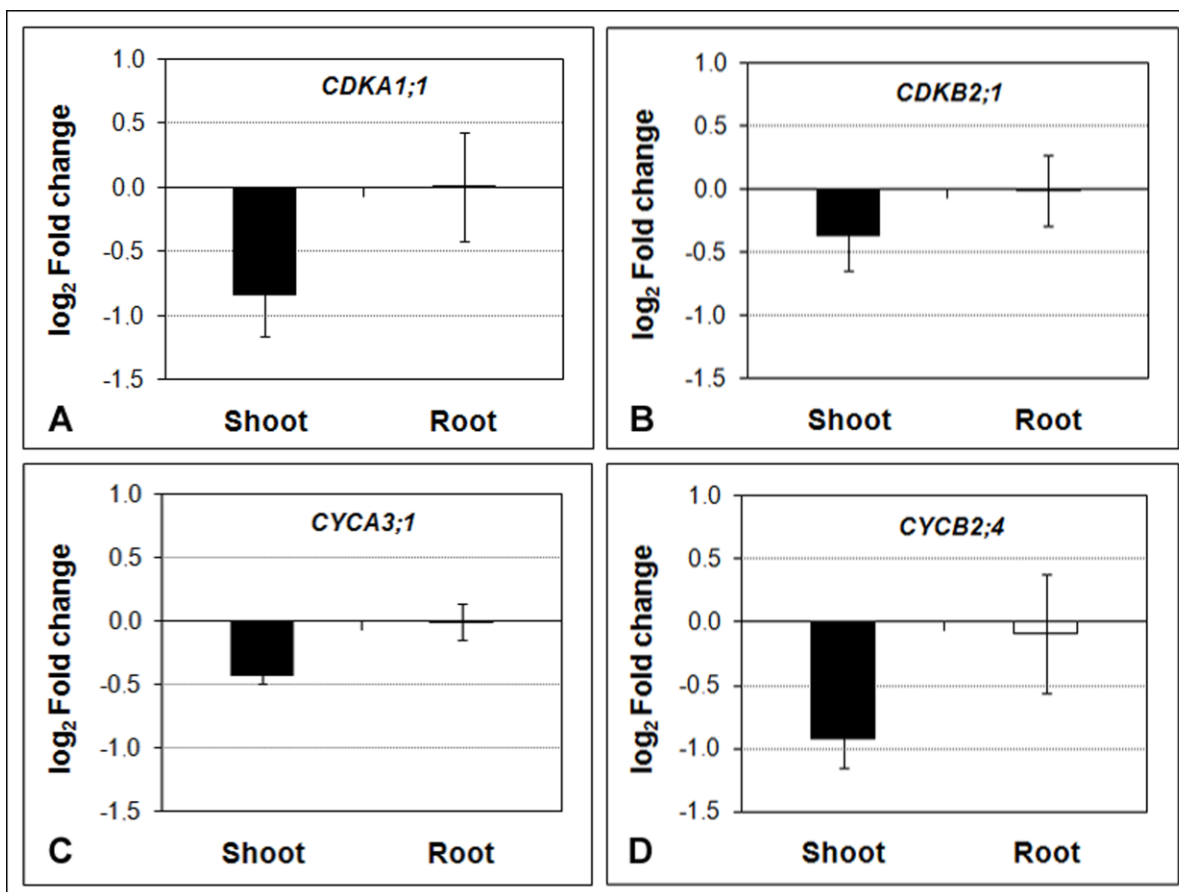
Figure 7.

Table 1. List of primers used for RT-PCR analyses of transgenes in *A. rhizogenes* putatively transformed apple hairy roots.

Gene name	Sequence (5'-3')	Amplified product size (bp)
rolA	F: GTTAGGCGTGCAAAGGCCAAG R: CGTATTAATCCCGTAGGTC	203
rolB	F: AAAGTCTGCTATCATCCTCCTATG R: AAAGAAGGTGCAAGCTACCTCTCT	348
rolC	F: TACGTCGACTGCCCGACGATGATG R: AAACTTGCACTCGCCATGCCTCAC	342
rolD	F: CCTTACGAATTCTCTTAGCGGCACC R: GAGGTACACTGGACTGAATCTGCAC	477
aux1	F: CATAGGATCGCCTCACAGGT R: CGTTGCTTGATGTCAGGAGA	198
aux2	F: AACGATAATAGCCCGCTGTG R: CGTCTTGGGTTTGTGGTTCT	217
virD1	F: ATGTCGCAAGGCAGTAAG R: CAAGGAGTCTTTCAGCATG	441
MdActin	F: ATTGGAATGGAAGCTGCTGG R: CAATGGATGGACCTGACTCG'	310

Table 2. List of primers used for qPCR analyses of cell cycle related genes in *A. thaliana.*

Gene name	GenBank accession number	Sequence (5'- 3')	Amplified product size (bp)
CDKA1;1	NM_114734	F: CCGTACGAGGATACATGGCG R: GGAGATCGACTCCATCGGGA	124
CDKB2;1	NM_106304	F: GTACGAGCCAGCGAAACGAA R: GCAGCACACTAGAGATATGCTTGA	170
CYCA3;1	NM_123674.2	F: GCGGGTGATTTGAAAGAATGTGTG R: GGTGATACAGGCATTGTCGCT	134
CYCB2;4	NM_106281	F: GCATCGGCGATCTACACTGC R: CCCTGCCTTGTGATGCAAAC	141
ACTIN7	NM_121018.4	F: ACAGGAAATGCTTCTAAGTGTGTCT R: ACACAAGACTTCTTGACACAACCA	171

All primer sequences were taken from Dmitrović et al. (2015)

Table 3. Hairy root regeneration from in vitro-grown shoots of four apple cultivars using wounding or dipping inoculation methods.

Cultivar	Number of inoculated shoots	Percent of shoots with hairy roots (%)	Total number of hairy root lines	Mean number of hairy root lines per shoot with hairy roots	Transformation efficiency (%)
Inoculation by wounding					
Melrose	94	18.1	66	3.9	70.2
Golden Delicious	36	2.8	1	1.0	2.8
Čadel	34	0.0	0	0.0	0.0
Gloster	31	0.0	0	0.0	0.0
Inoculation by dipping cut stem in bacterial suspension					
Melrose	30	3.3	1	1.0	3.3
Golden Delicious	28	39.3	11	1.0	39.3
Čadel	30	20.0	9	1.5	30.0
Gloster	30	10.0	3	1.0	10.0

Transformation efficiency was calculated as: (total number of hairy root lines/number of inoculated shoots) × 100.

Table 4. RT-PCR analysis of *rol, aux* and *virD1* genes expression in hairy root lines of Melrose (M), Golden Delicious (GD), Gloster (G) and Čadel (Č) apple cultivars.

Hairy root lines	T_L-DNA				T_R-DNA		virD1	MdActin
	rolA	*rolB*	*rolC*	*rolD*	*aux1*	*aux2*		
*A.r.*15834	+	+	+	+	+	+	+	-
M nt root	+	-	-	-	-	-	-	+
GD nt root	-	-	+	-	-	-	-	+
Č nt root	-	-	-	+	-	-	-	+
G nt root	-	-	+	+	-	-	-	+
M1	+	+	+	+	-	-	-	+
M2	+	+	+	+	+	+	-	+
M3	+	+	+	+	+	-	-	+
M4	+	+	+	+	-	-	-	+
M5	+	+	+	+	+	-	-	+
M6	+	+	+	+	+	+	-	+
M7	+	+	+	+	-	+	-	+
GD1	+	+	+	+	-	+	-	+
GD2	+	+	+	+	-	+	-	+
GD3	+	+	+	+	+	+	-	+
GD4	+	+	+	+	-	+	-	+
GD5	+	+	+	+	-	-	-	+
GD6	+	+	+	+	-	-	-	+
GD7	+	+	+	+	-	+	-	+
GD8	+	+	+	+	-	+	-	+
GD9	+	+	+	+	-	+	-	+

GD10	+	+	+	+	-	+	-	+
GD11	+	+	+	+	-	-	-	+
GD12	+	+	+	+	-	+	-	+
Č1	+	+	+	+	-	+	-	+
Č2	+	+	+	+	-	+	-	+
Č3	+	+	+	+	-	-	-	+
Č4	+	+	+	+	-	+	-	+
Č5	+	+	+	+	-	-	-	+
Č6	+	+	+	+	-	+	-	+
G1	+	+	+	+	-	-	-	+
G2	+	+	+	+	-	-	-	+
G3	+	+	+	+	-	-	-	+

Positive control – *A. rhizogenes* strain 15834; Negative control - untransformed roots (nt root).

Table 5. Phenolics in untransformed (control) roots, hairy root line GD12, and growth medium in which GD12 roots were maintained for four weeks.

Phenolic	Phenolic concentration (ng mg^{-1} DW of roots)		Phenolics secreted per mg of dry hairy root (ng mg^{-1} DW)	Phenolics secreted per mL of hairy root growth medium (ng mL^{-1})
	Untransformed root	**Hairy root**		
Chlorogenic acid	22.10 ± 0.27	22.10 ± 1.70	4.08 ± 0.62	8.82 ± 0.68
Caffeic acid	29.99 ± 0.68	29.40 ± 1.31	6.71 ± 0.87	14.58 ± 0.83
Syringic acid	7.17 ± 0.08	6.58 ± 0.11	nd	nd
p-Coumaric acid	38.34 ± 0.18	38.37 ± 0.92	nd	nd
Ferulic acid	6.16 ± 1.51	3.84 ± 0.71	nd	nd
Phloridzin	1753.83 ± 343.65	2643.60 ± 241.93	3.34 ± 0.73	7.49 ± 1.86
Phloretin	nd	nd	3.60 ± 0.29	8.01 ± 1.051
Rutin	10.87 ± 0.21	10.84 ± 0.28	nd	nd
Isoquercitrin	2.03 ± 0.25	1.51 ± 0.59	t	t
Kmf-3-glc	0.64 ± 0.36	1.71 ± 0.40	nd	nd
Quercitrin	8.29 ± 1.98	5.32 ± 0.52	nd	nd
Quercetin	t	t	t	t
Naringenin	t	t	t	t

Values are presented as means (±SE) of three biological replicates. Phenolic compound values for untransformed roots and hairy roots are not significantly different (P ≤ 0.05) according to *t*-test; nd - not detected; t- trace quantity.