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Transformation of *Mecardonia* (Plantaginaceae) with wild-type *Agrobacterium rhizogenes* efficiently improves compact growth, branching and flower related ornamental traits

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ABSTRACT

Mecardonia constitutes an emergent crop in the floriculture market. In order to develop plants with improved compact growth by molecular breeding, we transformed *Mecardonia* cv. 'Guarani Amarilla INTA' using a wild-type strain of *Agrobacterium rhizogenes*. An advantage of using this approach is that generated plants are stable and considered non-GMO in several countries. Adventitious roots were produced by inoculating *Mecardonia* shoots with agropine ATCC15834 strain. Twenty independent root cultures regenerated spontaneously into shoots in hormone-free medium and presence of T_L-DNA oncogenes were confirmed in their genomes. We selected four root inducing (Ri)-lines, designated GME, GCA, GVI and GIN, based on their different degree of compact growth habit. Under greenhouse conditions representative Ri-lines displayed reduced internode and shoot length, shoot and root biomass, aerial plant coverage, individual leaf area, flower width, pedicel length and the number of flowers per plant; and increased the number of nodes, axillary shoots, flower density and flower width relative to aerial plant coverage and mostly normal flowering when compared to non-transformed plants. To correlate phenotypical traits with gene expression, quantitative PCR analysis was performed. Ri-line GIN showed the highest *rolA-D* and *ORF8*, *ORF13-14* gene expression which correlated with its super-dwarf phenotype, whereas the most weak Ri-phenotype observed in Ri-line GME showed no presence of *rolA* and ORF8 genes in plant genome. Expression of *rolD* and *ORF13* correlated with reduced aerial plant coverage, shoot weight, shoot: root ratio and increased flower density and flower width relative to plant coverage, thus being considered of particular interest in *Mecardonia* breeding. Expression of *ORF8* and *rolA* correlated with reduced aerial plant coverage, pedicel length, the total number of flowers per plant and increased flower width relative to the aerial plant coverage. Moreover, ORF8 and ORF13 may have a more prominent role in plant development than previously assumed and assigned to *rol*-genes. Overall, a better-organized compact growth without affecting other traits could be generated.

1. Introduction

Mecardonia Ruiz & Pav. is a Plantaginaceae genus mainly distributed in South America that includes nine species, five of them native to Argentina (Greppi et al., 2017; Souza, 1997). It is characterized by annual or perennial herbs with erect or creeping habit, densely branched with attractive small yellow flowers, sometimes white or pink. Its distribution extends from east of USA to north of Argentine Patagonia and central Chile, being the region of south of Brazil, northeast of Argentina and Uruguay, the diversification centre of the genus (Rossow, 1987; Souza, 1997). *Mecardonia* species, characterized by their fast growth and blooming from early spring to the end of the summer, make them suitable in the industry of ornamental plants, which constantly demands new floricultural crops (Shibata, 2008). In fact, commercial

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cultivars of *Mecardonia* have been recently introduced to the global market for their use as pot or border plants (e.g. Suntory® Flowers Limited, Proven Winners™ and Sakata Seed America, Inc.). In floriculture production, compact growth is an essential criterion of plant quality. Mainly when the production is for potting, compact and homogeneous plants are aesthetically preferred by consumers and are easier to handle and transport. To achieve this goal, growers often prune the plants and/or use chemical growth retardants (Milošević et al., 2015). However, pruning is laborious and the use of chemicals is expensive and undesirable because their negative effects on the environment and human health (Lütken et al., 2012a; Milošević et al., 2015; Silva et al., 2003). *Mecardonia* species from Argentina have a natural elongated growth habit that had been reduced in hybrids through interspecific crosses (Soto et al., 2011a). However, incompatibility barriers and sterility related problems often attempt to the generation of compact genotypes. An alternative approach is to produce stable compact plants by the introduction of *rol* genes from wild-type strains of *Agrobacterium rhizogenes*, a molecular breeding strategy that counteracts the limitations of classical breeding. This natural genetic transformation involves the infection, transfer, stable integration and expression of oncogenes present in the transferred DNA (T-DNA) of a root-inducing (Ri) plasmid. After transformation, typical hairy roots develop from the sites of infection which are able to grow in hormone-free media, creating the so-called “hairy root syndrome” or “hairy root disease” (Cardarelli et al., 1987; Christensen and Müller, 2009; Milošević et al., 2015; Spano et al., 1981; Tepfer, 1984). Whole plant regeneration from hairy roots, with or without intermediary calli, normally leads to the development of dwarf plants (Tepfer, 1990, 1984). Induction of hairy roots and regenerated dwarf plants (Ri-plants), are produced by modification of plant cell growth and development caused by the expression of bacterial oncogenes (Alpizar et al., 2008; Casanova et al., 2005; Christensen and Müller, 2009; Veena and Taylor, 2007; Milošević et al., 2015; Prinsen et al., 1994; Schmülling et al., 1993). In herbaceous and woody species transformed with wild-type Ri-plasmids or specific *rol*-genes, phenotypic alterations normally involve increased rooting ability, reduced internode length and apical dominance, altered flowering and atypical morphology and size of leaves and flowers (Christensen et al., 2008; Gentile et al., 2004; Giovannini et al., 1997; Godo et al., 1997; Holefors et al., 1998; Lütken et al., 2012a; Milošević et al., 2015; Mishiba et al., 2006; Rugini et al., 2015, 1991; Schmülling et al., 1988; Welander et al., 2004). The use of wild-type strains of *A. rhizogenes* offer the advantage to select primary transformants by their “hairy root” phenotypes, establishing a marker-free selection (Christey, 2001; Lütken et al., 2012a; Roychowdhury et al., 2013). Interestingly, plants regenerated through this method are not considered GMOs in USA (USDA APHIS, 7 CFR part 340), Japan (Mishiba et al., 2006) and Argentina (SAGYP Nº701/11) because they are produced by a natural transformation process that do not involve in vitro recombinant DNA techniques. In the European Union (EU), according to the current legislation plants obtained through this method are classified as non-GMO (European Union, 2001; Lütken et al., 2012b; Lütken et al., 2012c). However, the adverse public opinion about GMOs in Europe may have contributed to the lack of clear laws, since the method using non-engineered *A. rhizogenes* is not clearly recognized as a non-transgenic technique (Rugini et al., 2015). In this context, *Agrobacterium* T-DNAs with expressed oncogenes were recently detected in cultivated sweet potato (Kyndt et al., 2015). Interestingly, since natural transformation events occurred during domestication of this food crop, evidences would contribute to change the current perspective of transgenic crops (Kyndt et al., 2015).

In *A. rhizogenes* agropine type strains like A4 or ATCC15834 (Porter and Flores, 1991), two T-DNAs present in the Ri plasmid are transferred independently to the plant genome (Slightom et al., 1986). The T_L-DNA contains at least 18 open reading frames (ORFs) that include the oncogenes *rolA* (ORF10), *rolB* (ORF11), *rolC* (ORF12) and *rolD* (ORF15) that are the main determinants of hairy root initiation and generation of dwarf Ri-plants (Casanova et al., 2005; Christensen et al., 2008; Hegelund et al., 2017; Slightom et al., 1986; White et al., 1985). However, other genes highly conserved between strains and present in the T_L-DNA, such as ORF8, ORF13 and ORF14 have roles in root induction (Aoki and Syono, 1999; Capone et al., 1989; Ouartsi et al. 2004; Stieger et al. 2004). Moreover, overexpression of *ORF8* and *ORF13* alter morphogenesis producing plants with shortened internodes and alterations in other traits. Observations suggest that these regulators may have a substantial contribution in the generation of dwarf phenotypes when they are synthesized from genes of a wild-type Ri plasmid (Kodahl et al., 2016; Lemcke and Schmülling, 1998; Stieger et al., 2004; Umber et al., 2005). Six ORFs are present in the T_R-DNA and two auxin biosynthetic genes designated *aux1* and *aux2* are involved in hairy root induction although their contribution in the manifestation of dwarf phenotypes is not clearly understood (Camilleri and Jouanin, 1991; Gaudin and Jouanin, 1995; Slightom et al., 1986, 1985; Vilaine and Casse-Delbart 1987; White et al., 1985).

Here, we examined the ability of a wild-type strain of *A. rhizogenes* to produce compact growth and modify other traits that can be useful in *Mecardonia* breeding. Twenty independent Ri-lines of *Mecardonia* cv. ‘Guaraní amarilla INTA’ were produced by a highly efficient transformation method and four Ri-lines were selected and tested in vitro and under greenhouse conditions. The *rol*-gene products do not function equally in all host plant species and it has been proposed that their functions may be replaced or modified by other oncogenes (Christensen et al., 2008; Kodahl et al., 2016; Lemcke and Schmülling, 1998; Porter and Flores, 1991). Hence, to gain insight into the developmental regulation modulated by transferred T-DNA genes in *Mecardonia*, correlations between phenotypical traits and expression of *rolA-D*, *ORF8*, *ORF13-14* and *aux1-2* genes was analyzed.

2. Materials and methods

2.1. Plant material

Aseptic cultures of commercial *Mecardonia* cv. ‘Guaraní amarilla INTA’ obtained at Instituto de Floricultura (CIRN-INTA, Buenos Aires, Argentina) were used for transformation. Vegetative propagation was made in vitro, every approximately 30 days, by the use of nodal cuttings placed in glass test tubes (150 mm × 25 mm) containing 10 mL of hormone-free WPM medium (Lloyd and McCown, 1981) supplemented with 30 g/L sucrose (Merck, Darmstadt, Germany) and 6 g/L agar (Britania, Buenos Aires, Argentina). Explants were cultured at 24 °C and long-day photoperiods (16 h light/8 h darkness, 45 μmol $m^{-2} s^{-1}$) provided by cool-white fluorescent lamps (Interelec, China).

2.2. Bacterial strain and generation of transformed roots

Wild-type *Agrobacterium rhizogenes* ATCC15834 was used for the induction of adventitious roots. This strain was cultured in solid YMB medium supplemented with 50 μg/L rifampicin (Richet, Argentina) for 48 h at 28 °C in darkness. Leaf explants were initially used for adventitious root induction. Fully expanded leaves were cut from in vitro grown plantlets and injured with *A. rhizogenes* using a scalpel, while non-inoculated but injured explants were used as controls. Explants were placed in Petri dishes containing hormone-free WPM medium supplemented with 30 g/L sucrose for 3 days at 24 °C and 16 h-photoperiod. Explants were then transferred to fresh WPM medium supplemented with 30 g/L sucrose and antibiotics to eliminate *A. rhizogenes* (400 mg/L cefotaxime sodium [Cefacolin Northia, Buenos Aires, Argentina] and 100 mg/L Ampicillin-Sulbactam 2:1 [Ampi-Bis Plus Northia, Buenos Aires, Argentina]), and cultured at 24 °C and 16 h-photoperiod. Since explants did not induce adventitious roots and died after a week of culture, an alternative method was attempted to obtain transformed roots. Using this approach, the internodes of established in vitro plantlets of about 20 days were injured with *A. rhizogenes* using a

scalpel, while control treatment consisted of non-inoculated injured plants. Plantlets were maintained at 24 °C and 16 h-photoperiod for 15–20 days until putative transformed root primordia developed from injured internodes. Internode portions (about 5–10 mm length) carrying adventitious roots, were excised and placed in Petri dishes containing solid WPM medium supplemented with 30 g/L sucrose and the same antibiotics and concentrations used previously to eliminate *A. rhizogenes*, and cultured at 24 °C and 16 h-photoperiod. Explants were weekly subcultured to the same fresh medium.

2.3. Plant regeneration

Root explants developing spontaneous shoot primordia were transferred to round jar glasses (65 mm × 85 mm) containing 40 mL of solid WPM medium supplemented with 30 g/L sucrose and antibiotics as described previously and cultured at 24 °C and 16 h-photoperiod for additional 30–45 days until shoots of 2–3 cm length regenerated from the roots. Nodal cuttings of these plantlets were excised and cultured in glass test tubes containing antibiotic-free WPM medium supplemented with sucrose (30 g/L). After PCR analysis, confirmed and independent transformed Ri-lines were propagated by cuttings. Transformed and control plants were transplanted to potting soil and used for the experiments.

2.4. PCR analysis

To determine the presence of T-DNA genes in the putative transformed lines, total DNA was extracted from leaves of control plants and 20 independent Ri-lines using the CTAB method described by Pérez de la Torre et al. (2010). We selected leaves because these organs are widely used to extract total DNA in different plant species and usually deliver quantity and quality DNA. To amplify fragments of *rolA-D* genes we used specific primers previously utilized by Gennarelli et al. (2009) (Supplementary Table 1). To amplify fragments of ORF8,13–14 and *aux1-2* genes, primers for PCR and quantitative real-time RT-PCR analyses (qPCR) were designed from DNA sequences of ATCC15834 strain (Kajala et al., 2014; Supplementary Table 1) using Beacon designer 6.0 software (Premier Biosoft International, Palo Alto, CA). As positive controls, gene amplifications were obtained from ATCC15834 by colony PCR. In addition, primers for *virC* gene (Soto et al., 2011b) present in Ri-plasmid but not in T-DNAs transferred to the plants, were used to discard the possibility of *A. rhizogenes* residues in the transformed plants. PCR reaction mixture (25 µL) consisted of PCR buffer 10X, 2 mM $MgCl_2$, 0.2 mM dNTPs (Inbio Highway, Tandil, Argentina), 0. µM of each primer (Eurofins MWG Operon, KY, USA), 0.5U Taq DNA polymerase (Inbio Highway, Tandil, Argentina) and 30 ng template. DNA amplifications were performed in a gradient cycler (Eppendorf Mastercycler nexus, Hamburg, Germany) using an initial denaturation at 94 °C for 3 min followed by 35 cycles of 1 min at 94 °C, 1 min at 47 °C and 1 min at 72 °C, and a final extension for 5 min at 72 °C. PCR products were analyzed on 2.5% TAE agarose gel stained with 0.01 mg/mL ethidium bromide (Promega, WI, USA) and visualized by fluorescence under UV-light.

2.5. Gene expression analysis

Expression of transferred genes was analyzed by quantitative real-time RT-PCR (qPCR). Total RNA isolation was performed on day 44 from plants grown in the greenhouse. Shoots containing leaves with approximately ten nodes were harvested on a sunny day three hours after sunrise. One shoot per plant from 18 different plants of each genotype were divided in three biological samples with six shoots each. Samples were rapidly frozen in liquid nitrogen and stored at −80 °C until they were grinded. High quality total RNA was isolated from 100 mg of frozen tissue using TRIzol (Invitrogen, Argentina). Genomic DNA was eliminated after treatment with DNase I for 20 min at room temperature (Invitrogen, USA). RNA concentration was measured using Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Purity and integrity of RNA was determined by 260/280 nm ratio and the integrity was further checked by electrophoresis in 1.5% agarose gel. For each sample, 500 ng DNase-treated RNA was reverse-transcribed using Superscript III first strand synthesis system (Invitrogen, USA) and random hexamer primers according to the manufacturer's instructions. Specific primer pairs for qPCR were designed as described in the previous section. qPCR was carried out in a 13-µL reaction mix containing 200 nM of each primer, 1 µL of cDNA sample and FastStart Universal SYBR Green Master (Roche Diagnostics, Mannheim, Germany). Negative controls (no RT added and non-template control) were incorporated in the assays. qPCRs were performed using a 96-well plate StepOne Plus cycler and software (Applied Biosystems, USA). The thermal profile was set to 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, and hybridization temperature set to 60 °C for 1 min. Amplicon specificity was verified by melting curve analysis (60 to 95 °C) after 40 PCR cycles. The qPCR assay was carried out using three biological and two technical replicates. Amplification efficiencies and raw Ct values were determined for each gene with the slope of a linear regression model using LinRegPCR software (Ruijter et al., 2009). These profiles were estimated in relation to the Ri-line with the lowest expression and the reference gene using fgStatistics software (Di Rienzo, 2009) based on previously published algorithms (Pfaffl, 2001). Two highly conserved reference genes (*EF1a* and *18SrRNA*) were tested. Since genomic or transcriptomic sequences of *Mecardonia* were not publicly available, primers were designed from highly conserved regions by aligning different known plant cDNA sequences, including those of the Plantaginaceae family, by using BioEdit software (Hall, 1999). Each reference gene showed similar Cq values through different samples, although the *18SrRNA* gene showed very high levels of expression reflected by their very low Cqs (Bustin et al., 2009), thus we selected *EF1a* gene for further relativization.

2.6. Phenotypic analysis

Characterization of the plants was carried out in vitro and under greenhouse conditions. For in vitro experiments, at least 20 apical cuttings (two nodes each) of control (non-transformed) plants and the four selected Ri-lines were cultured in a growth chamber at 24 °C and 16h-photoperiod ($45\ \mu mol\ m^{-2}\ s^{-1}$) on glass test tubes (150 mm × 25 mm) containing 10 mL of solid WPM medium supplemented with 30 g/L sucrose. The number of primary adventitious roots, developing from the cut surface of the shoot explant, and second order lateral roots were recorded every two days until day 28. On day 36 plantlets were removed from test tubes and several traits were analyzed, including shoot and internode length, shoot diameter, number of nodes and axillary shoots on the main stem and shoot and root dry weight.

Greenhouse experiments were conducted at Instituto de Floricultura, CIRN-INTA, Buenos Aires, Argentina during early June to late October 2016. At least 20 in vitro grown plants of each genotype were acclimated in a temperate propagation room for 11 days in plugs containing a moistened Grow Mix (Terrafertil, Argentina). After acclimation, plants were transplanted into pots (Ø 14 cm) containing the same substrate and transferred to a temperate greenhouse with natural photoperiod (11 to 14 h), average irradiation and temperature of $700\ \mu mol\ m^{-2}\ s^{-1}$ and 24 °C, respectively. Plants of the different genotypes were randomly distributed and regularly cycled to avoid any potential effect of position. Plants were watered as needed and fertilized twice a week (Hakaphos® Rojo, COMPO). Images were taken from the top to estimate the evolution of vegetation coverage per plant and individual leaf area using Adobe® Photoshop® CS6. Plants transplanted to soil showed a more horizontal growth habit represented by several main shoots growing radially. For morphological measurements, on day 44 (vegetative stage) the longest main shoot was selected to determine

shoot length, internode length, shoot diameter and the number of nodes and axillary shoots (≥0.5 cm). In parallel experiments, roots and shoots were exposed for five days at 70 °C to determine aerial and hypogeum dry biomass. Days to flowering were recorded and reproductive traits were evaluated including flower width, pedicel length, the number of opened flowers per plant, flower density, the number of flowers per shoot, flower width relative to aerial plant coverage and flower longevity. The latter trait was determined using at least 40 flowers of each genotype and no differences between control and transformed plants were observed (about 7 days). For all experiments, measurements were made with a ruler and a caliber was used for shoot diameter measurements.

2.7. Statistical analysis

Expression and phenotypic data were subjected to ANOVA followed by Tukey's posttest using InfoStat software (InfoStat v.2009) and expressed as the mean value ± standard error means (SEM).

3. Results

3.1. Transformation of mecardonia plants

To generate Ri-plants of *Mecardonia*, detached leaves of in vitro grown plantlets were inoculated with a wild-type strain of *A. rhizogenes*. After 10 days of culture on solid WPM medium leaf explants showed necrosis and were not able to induce adventitious roots. We tested an alternative transformation method in which bacteria was inoculated in the internodes of active growing plantlets. Most inoculated injuries (about 70%) developed adventitious roots with a distinctive hairy root phenotype close to the shoot stem (Fig. 1a and b), while root development was not observed in any of the non-inoculated injured plantlets. Adventitious roots of 1–3 cm length were cut with a neighboring portion of the internode and placed on hormone-free WPM medium supplemented with antibiotics to eliminate bacteria (Fig. 1c). Roots quickly turned green and direct shoot regeneration was observed (Fig. 1d and e). Regenerated shoots were excised after about 45–60 d of aseptic culture and propagated in WPM medium (Fig. 1f and g). PCR amplification confirmed the presence of T_L-DNA genes (*rolA-D*) in 20 independent Ri-lines (see oligonucleotides used in Supplementary Table 1), demonstrating that all root events were transformed. In order to select an affordable number of Ri-lines to conduct the experiments, transformed lines were preliminary tested in a long-day growth chamber in comparison with non-transformed plants. Four Ri-lines designated GME, GCA, GVI and GIN were selected based on their different degree of compact growth habit. The presence of ORF8, ORF13-14 oncogenes was also detected in selected Ri-lines with the exception of ORF8 and *rolA* in Ri-line GME (Supplementary Table 2). *Aux1* and *aux2* genes were not detected in any of the four Ri-lines (Supplementary Table 2). In addition, amplification of *virC* gene was not detected in established Ri-plantlets, demonstrating that *A. rhizogenes* was eliminated.

3.2. Phenotypic analysis

3.2.1. In vitro experiments

We assessed several vegetative traits in selected Ri-lines GME, GCA, GVI and GIN cultured in vitro. The number of primary adventitious roots and second order roots were significantly increased in all Ri-lines respect to control plantlets, which correlated with their higher root biomass (Table 1). The number of nodes, axillary shoots and shoot diameter were increased in all Ri-lines respect to control plantlets and correlated with their higher shoot biomass (Table 1). All Ri-lines showed a strong reduction in the shoot: root ratio when compared to control plantlets (Table 1). Internode length was reduced in Ri-lines GVI and GIN (Table 1). In these Ri-lines shoot length was similar to control plantlets because they increased the number of nodes (Table 1). Shoot length was slightly higher in Ri-lines GME and GCA as a consequence of an increased number of nodes but similar internode length. Under our experimental conditions, control and Ri-plantlets were not induced to flower. Overall, transformed lines of *Mecardonia* showed usual Ri-phenotypes when cultured in vitro.

3.2.2. Greenhouse experiments

Transformed Ri-lines were tested in a temperate greenhouse. Control and transformed plants were characterized by the presence of several shoots growing radially (hereafter called main shoots). The evolution of vegetative coverage was measured from day 11 to day 44 after transplant. Ri-lines GCA, GVI and GIN showed different reductions in coverage rates (Fig. 2k). At day 44, when differences between genotypes were evident, these lines showed a reduction in coverage of about 25–50% (Fig. 2l). In this day, the longest main shoot of each plant was selected to measure different vegetative traits, which are described as follows. Improved compact growth in Ri-lines was caused by the reduction of internode length and as a result shoot length was reduced (Fig. 2a and b, k–l); however, all Ri-lines showed main shoots with a higher number of nodes, a characteristic that attenuated shoot length reductions in the plants (Fig. 2a and b, d). Each node has one pair of opposite leaves and two competent buds are found in the axilla of each leaf. Axillary shoots were significantly increased in all Ri-lines (Fig. 2e). This trait when relativized to the number of nodes revealed no changes in three out four Ri-lines respect to control plants (Fig. 2f), suggesting that increased branching could be explained by their higher number of nodes. However, Ri-line GCA showed an increased number of axillary shoots per node, suggesting improved bud outgrowth in this Ri-line. Individual leaf area was reduced in all Ri-lines (Fig. 2j) although leaf morphology was not evidently affected and wrinkled leaves, often associated to Ri-phenotypes, were not observed. Aerial and hypogeum biomass were determined earlier, on day 19 from a parallel experiment, to avoid restrictions in root growth. Aerial biomass was reduced in Ri-lines GME, GVI and GIN (Fig. 2g) and root biomass in Ri-lines GVI and GIN (Fig. 2h), showing reductions in total biomass in Ri-lines GME, GVI and GIN. The shoot: root ratio was fairly affected in Ri-line GVI (Fig. 2i). Reduced aerial biomass in Ri-lines GVI and GIN was correlated with their reduced aerial vegetative coverage (Fig. 2g, k–l). Despite reductions in individual leaf area and internode length in Ri-line GCA, aerial biomass was not affected when compared to control plants, suggesting that increased number of nodes, leaves and axillary shoots would compensate biomass reduction in this line. In general, shoot diameter was not affected in Ri-lines, although a slight decrease was detected in Ri-line GVI (Fig. 2c). Overall, a better-organized compact growth habit was obtained in transformed plants.

Time to anthesis was similar in most Ri-lines when compared to control plants, although it was delayed about 20 days in Ri-line GIN (Fig. 3a). Plants accomplished full bloom and commercial size after four months. On day 120, flowering phase related traits were assessed. Flower width was significantly reduced (15–32%) in all Ri-lines and pedicel length was reduced in three Ri-lines (about 20%; Fig. 3b and c) when compared to control plants. Flower morphology was rather different in Ri-line GVI, which showed less air space between petal lobes. Pale corollas and barely visible nectar guides were observed in Ri-line GIN (Fig. 3i). The total number of opened flowers per plant was significantly reduced in Ri-lines GCA, GVI and GIN (about 30%) when compared to control plants (Fig. 3d). All Ri-lines showed different reductions in aerial plant coverage (about 15–58%; Fig. 3e), as previously observed on early stages of plant development for three out four Ri-lines (Fig. 2l). In this reproductive developmental stage Ri-line GME also showed a reduction in aerial plant coverage respect to control plants (Fig. 3e). Flower density was calculated from aerial photos (Fig. 3f). This parameter was similar to control plants in Ri-lines GME and GCA while consistent higher values were observed in Ri-lines GVI (about 70%) and GIN (about 85%) (Fig. 3f), indicating that ornamental

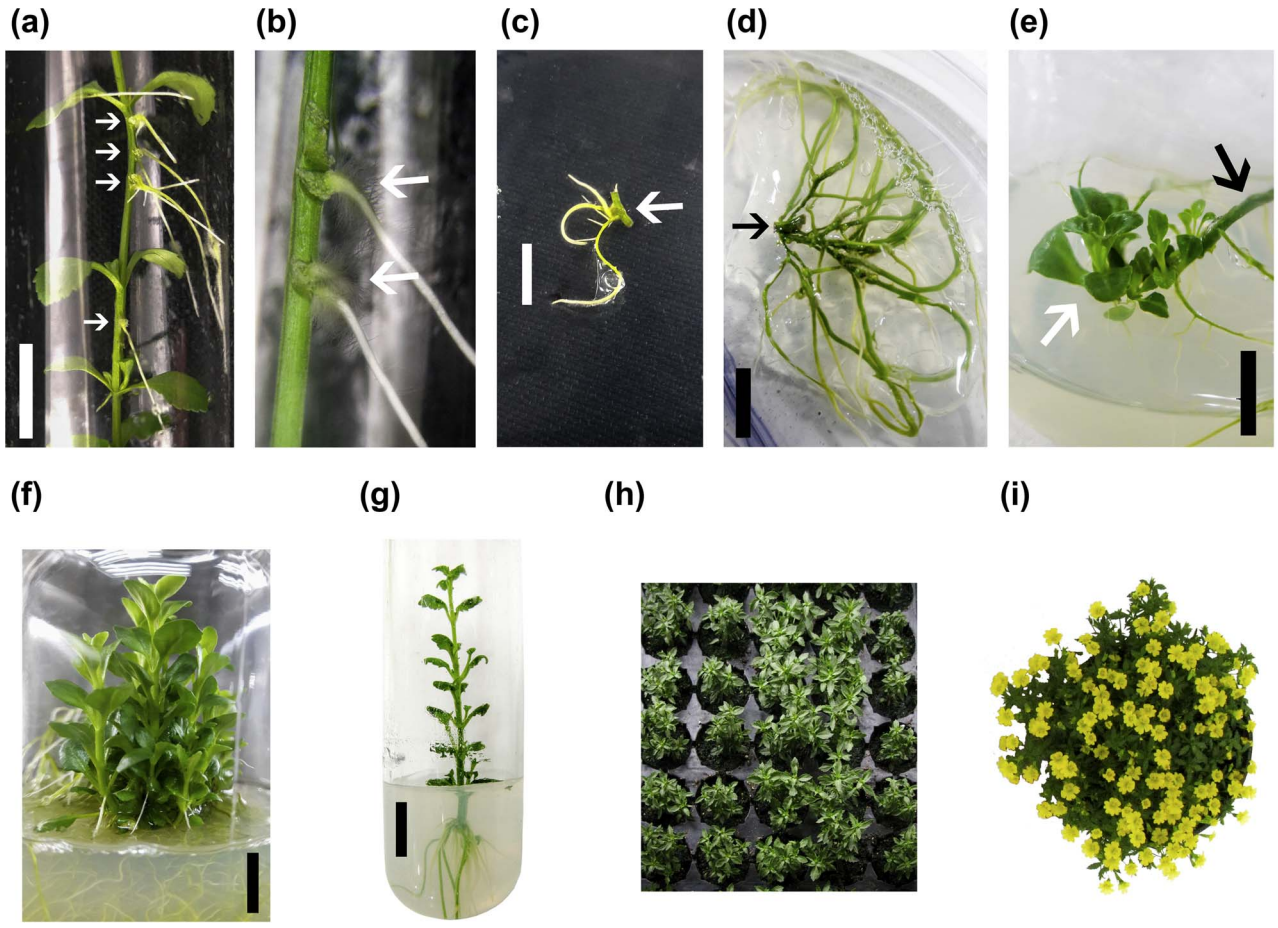

Fig. 1. Generation of *Mecardonia* Ri-lines. (a, b) Adventitious roots produced from injured internodes after 18 d of inoculation with *A. rhizogenes* ATCC15834 strain. Arrows indicate root development from injury sites. (c, d) Root cultures of 5 and 14 d respectively, characterized by their vigourous growth and increased lateral branching on hormone-free WPM medium supplemented with antibiotics. Roots turned green after about 7 d and spontaneous shoot primordia were visible after about 12 d. Arrows indicate the internode portion from which adventitious roots developed. (e) White arrow indicates shoot development from root cultures of 34 d on WPM medium. Black arrow indicates shoot primordia initiations. (f) Shoots of 54 d. (g) Regenerated plantlet obtained from an excised shoot of (f) with roots growing from the cut surface on hormone- and antibiotic-free WPM. (h) Regenerated Ri-plants growing in plugs during the acclimation stage. (i) Full bloom Ri-plant in the greenhouse. Bars = 1 cm.

value was improved in Ri-lines GVI and GIN. Inspection of flowers produced by main shoots (without considering flowers produced by axillary shoots) indicated that improved flower density in Ri-lines GVI and GIN was associated to an increased proportion of opened flowers per shoot respect to the rest Ri-lines and control plants (Fig. 3g). A concomitant reduction in the number of flower primordia was also observed in GVI and GIN lines (Fig. 3g). The number of total flowers per shoot (opened plus primordia) was similar to controls in Ri-lines GME, GCA and GVI, although increased in Ri-line GIN (Fig. 3g). Since flower longevity was similar between controls and Ri-lines, results suggest accelerated flower development in Ri-lines GVI and GIN. In addition, the relationship between flower width and aerial plant coverage was calculated. This parameter was similar in control plants and Ri-line GME, while it was increased by about 42, 100 and 63% in Ri-lines GCA, GVI and GIN, respectively (Fig. 3h). Results indicate that in three Ri-lines reduction in flower width occurs concomitantly with a larger

Table 1
Vegetative traits in *Mecardonia* Ri-plantlets[a].

Line	Control	GME	GCA	GVI	GIN
Shooth length (mm)	75.08 ± 4.35 ab	96.66 ± 1.15 d	94.04 ± 2.35 cd	74.02 ± 1.40 a	84.44 ± 1.41 bc
Internode length (mm)	10.41 ± 0.40 bc	11.57 ± 0.32 c	9.13 ± 0.37 ab	7.79 ± 0.41 a	8.67 ± 0.20 a
Shoot diameter (mm)	0.92 ± 0.04 a	1.12 ± 0.02 b	1.11 ± 0.03 b	1.04 ± 0.03 b	1.13 ± 0.02 b
Node number	8.78 ± 0.22 a	10.67 ± 0.17 b	10.25 ± 0.16 b	10.22 ± 0.15 b	10.67 ± 0.29 b
Axillary shoot number	1.40 ± 0.23 a	8.78 ± 0.22 bc	7.88 ± 0.40 b	7.67 ± 0.29 b	9.78 ± 0.36 c
Axillary shoots per node	0.17 ± 0.03 a	0.82 ± 0.02 bc	0.77 ± 0.04 b	0.75 ± 0.03 b	0.92 ± 0.04 c
Shoot dry weight (mg)	16.29 ± 1.25 a	34.23 ± 1.36 c	38.53 ± 1.18 c	23.99 ± 0.57 b	44.37 ± 0.97 d
Root dry weight (mg)	1.92 ± 0.12 a	26.13 ± 1.65 c	28.24 ± 1.63 c	14.12 ± 0.84 b	64.16 ± 2.62 d
Shoot : root ratio	8.21 ± 0.68 b	1.34 ± 0.09 a	1.39 ± 0.07 a	1.74 ± 0.09 a	0.70 ± 0.04 a
Adventitious root number	3.40 ± 0.22 a	19.70 ± 0.77 d	13.00 ± 0.97 c	7.60 ± 0.67 b	21.40 ± 1.03 d
Second order root number	0.90 ± 0.29 a	3.80 ± 0.78 b	4.67 ± 0.64 b	5.00 ± 1.05 b	4.80 ± 0.41 b

[a] Shoot and internode length, shoot diameter and node number were measured from the main shoot of in vitro cultured plantlets. Internode length is an average of three full expanded apical internodes. Adventitious root number indicates primary roots generated from the cut surface of shoot explants. Second order root number indicates the roots derived from primary roots. Different letters denote significant differences among means determined using Tukey posttest ($P < 0.05$).

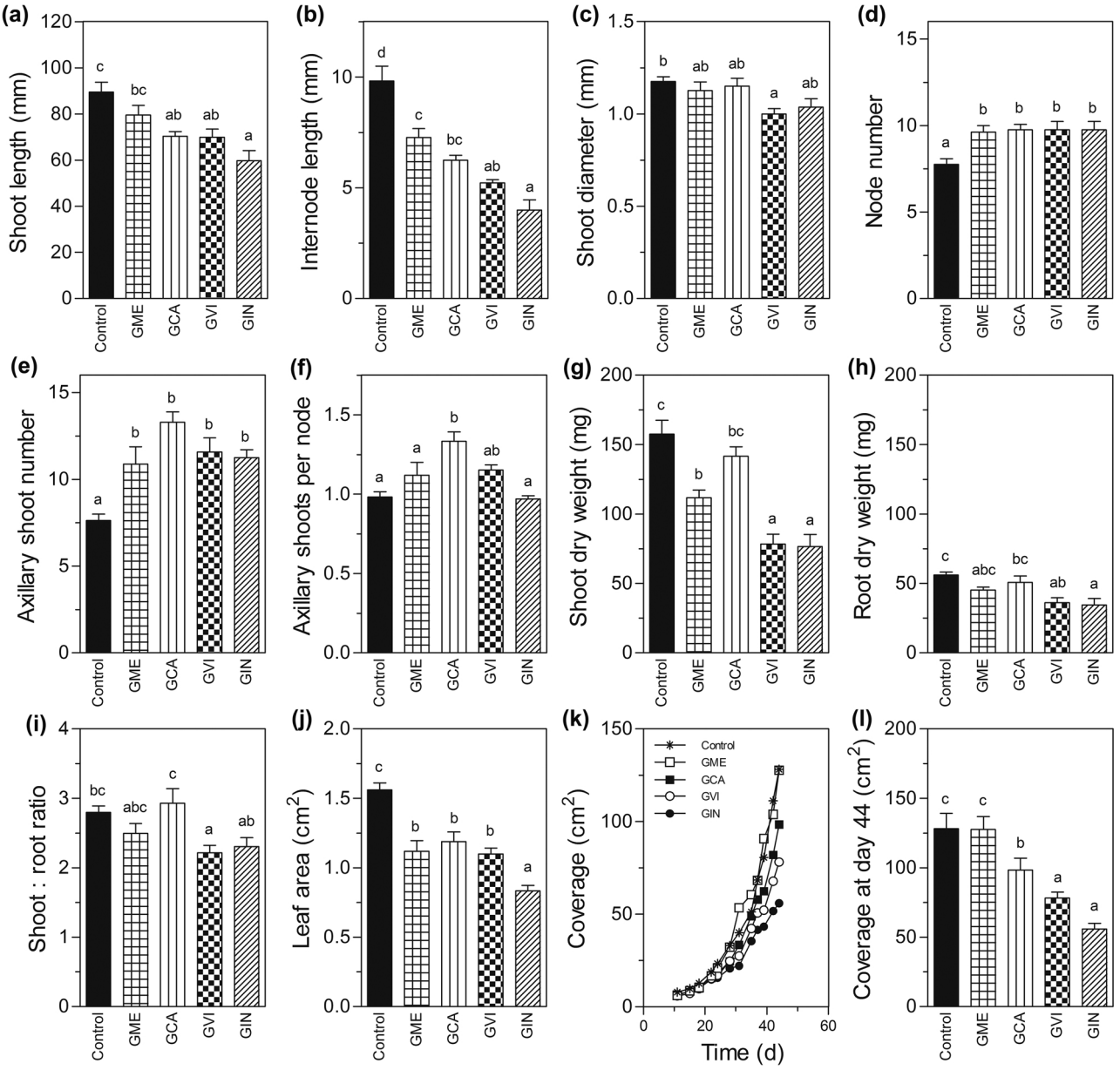

Fig. 2. Vegetative phase traits in *Mecardonia* Ri-plants. (a) Main shoot length. (b) Internode length (average of three full expanded apical internodes per plant). (c) Main shoot diameter. (d) Number of nodes. (e) Number of axillary shoots. (f) Number of axillary shoots per node. (g) Aerial biomass. (h) Hypogeum biomass. (i) Shoot: root ratio. (j) Individual leaf area. (k) Aerial plant coverage. (l) Aerial plant coverage at day 44. Different letters denote significant differences among means determined using Tukey posttest ($P < 0.05$).

reduction in aerial plant coverage, therefore ornamental value related to this parameter was improved in these lines.

3.3. Analysis of gene expression and correlation with phenotypical traits

PCR analysis showed the presence of T_L-DNA oncogenes in Ri-lines including *rolA-D*, ORF8, ORF13-14 genes with the exception of *rolA* and ORF8 in Ri-line GME. Genes of the T_R-DNA like *aux1-2* were not detected in any of the Ri-lines (Supplementary Table 2). To correlate phenotypical traits of greenhouse grown plants with gene expression, real-time qPCR analysis for T-DNA genes was performed in established plants. Ri-lines showed detectable expression of *rolA-D* and *ORF8*, *ORF13-14* genes, and as expected, they were not detected in non-transformed plants (Fig. 4). Expression of *aux1-2* was not detected in any Ri-line, which confirmed previous results and suggest that T_R-DNA genes were not transferred to the plants. Relative expression in Ri-line GIN was clearly increased respect to the rest three lines for five of the seven measured genes, including *ORF8* (Fig. 4a), *rolA* (Fig. 4b), *rolB* (Fig. 4c), *rolC* (Fig. 4d) and *ORF14* (Fig. 4f). Expression of these genes in the other three Ri-lines was lower and similar and, as expected for Ri-line GME, expression of *ORF8* and *rolA* was not detected (Fig. 4 a and b; Supplementary Table 2), suggesting that latter genes are not present in this line.

The maximum expression of *rolA-D*, *ORF8* and *ORF13-14* genes observed in Ri-line GIN was correlated with its marked phenotypical traits, like the lowest leaf area and flower width (Fig. 2j; Fig. 3b), delayed flowering (Fig. 3a) and increased number of open and total flowers per shoot (Fig. 3g). Expression of *ORF13* (Fig. 4e) was higher and similar in Ri-lines GVI and GIN and expression of *rolD* (Fig. 4g) was also higher in these lines respect to the other two Ri-lines (Fig. 4e, g). These observations are more correlated with similar reduction in their shoot weight (Fig. 2g) and aerial plant coverage (Fig. 2l; Fig. 3e) and increased flower density associated to the higher proportion of opened flowers in the shoots (Fig. 3f and g) and flower width respect to aerial

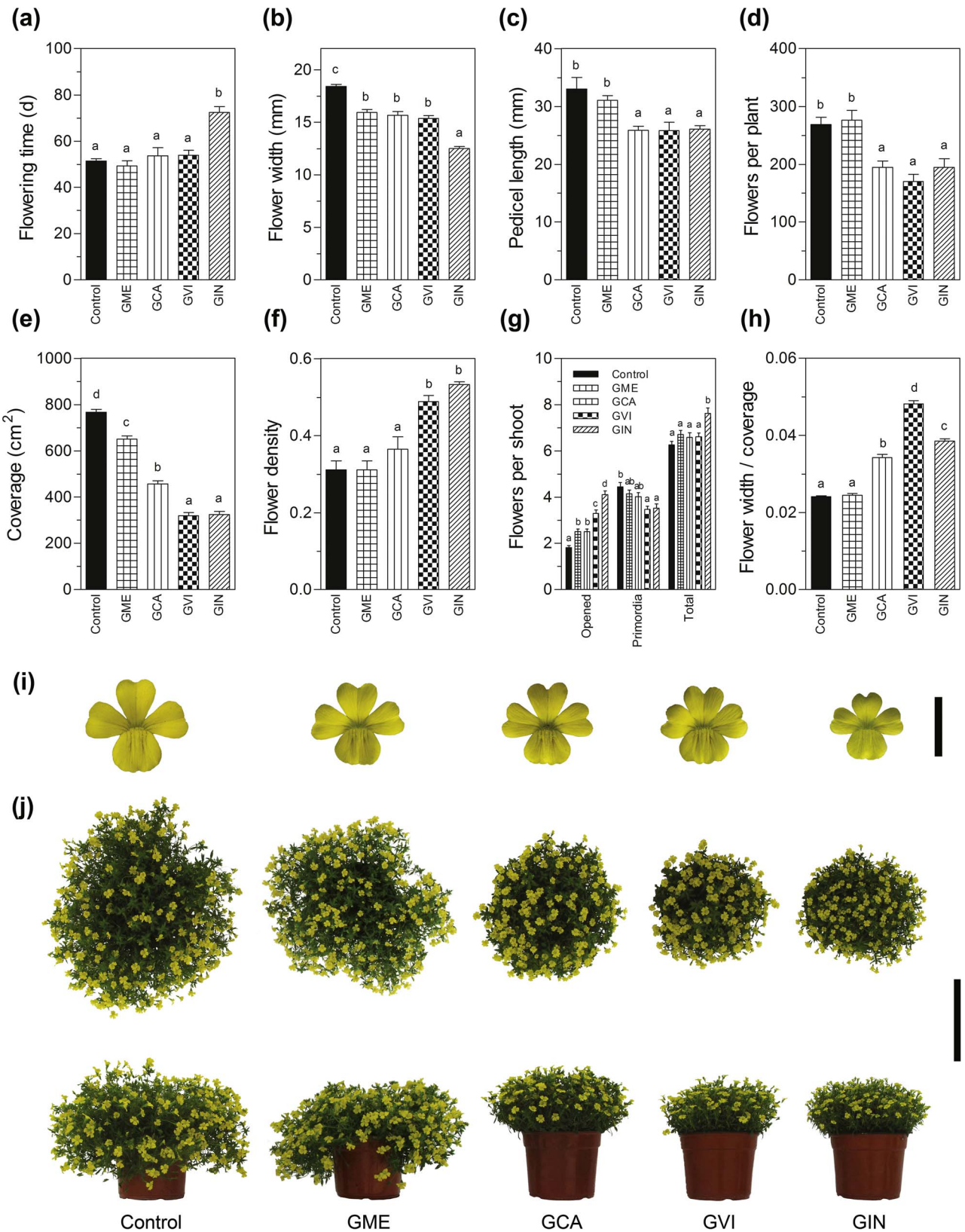

Fig. 3. Flowering phase traits in *Mecardonia* Ri-plants. (a) Flowering time (days to anthesis). (b) Flower width. (c) Pedicel length. (d) Total number of opened flowers per plant. (e) Aerial plant coverage at day 120. (f) Flower density. (g) Number of flowers produced by main shoots. (h) Flower width relative to plant coverage. (i) Flower size and morphology (bar 10 mm). (j) Aerial and side views of representative four-month-old plants (bar 14 cm). Different letters denote significant differences among means determined using Tukey posttest ($P < 0.05$).

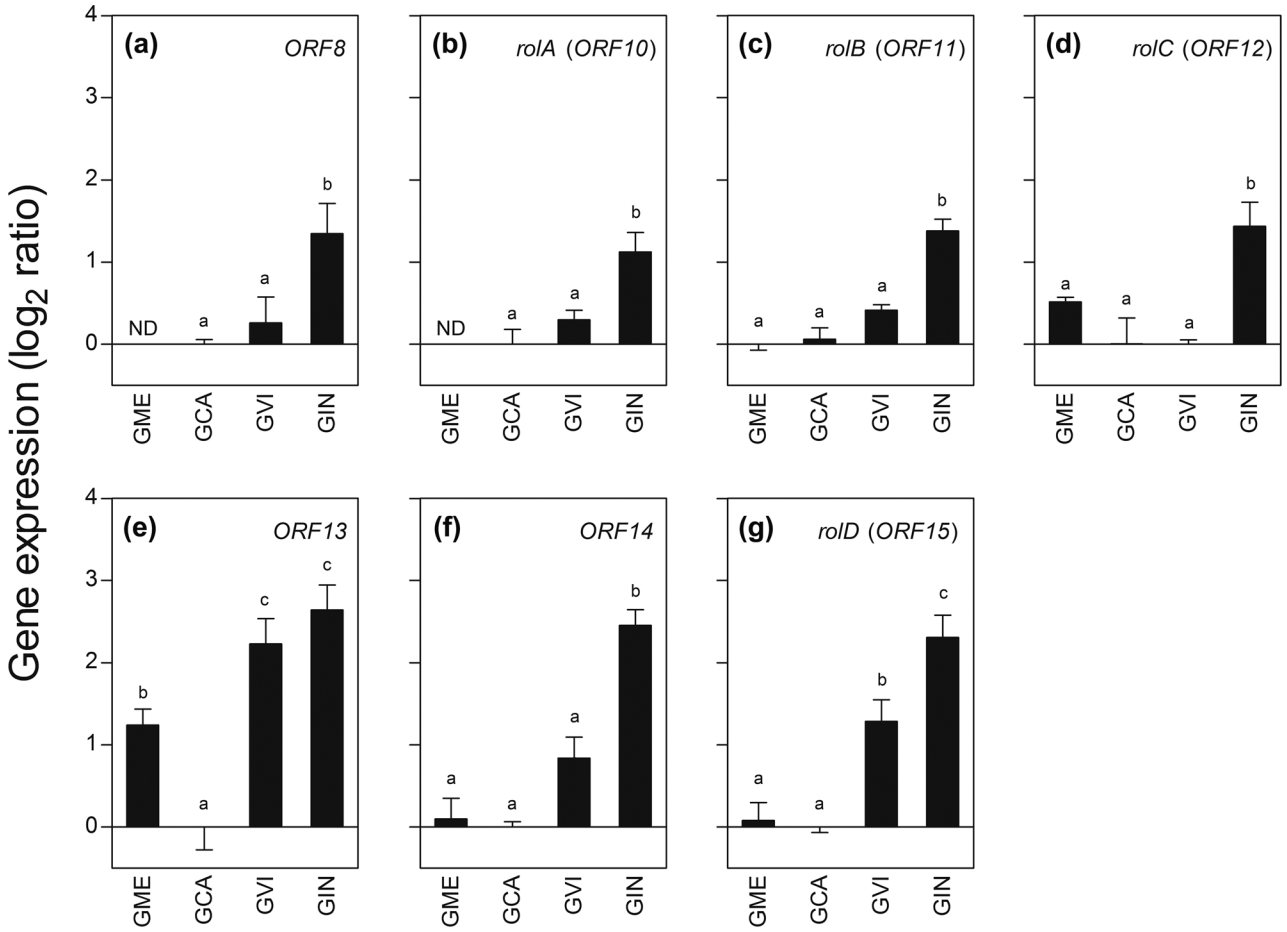

Fig. 4. Expression analysis of *rolA-D*, *ORF8*, *ORF13* and *ORF14* genes in shoots of *Mecardonia* Ri-lines. Relative transcript levels of (a) *ORF8*, (b) *rolA*, (c) *rolB*, (d) *rolC*, (e) *ORF13*, (f) *ORF14* and (g) *rolD* genes were determined by qPCR and expressed as the difference between each Ri-line in relation to the lowest Ri-line and reference gene. (ND) indicates non-detectable expression. Different letters denote significant differences among means determined using Tukey posttest ($P < 0.05$).

plant coverage when compared to the other Ri-lines (Fig. 3h). Comparison of Ri-lines GCA and GVI only showed differences in the expression of *ORF13* and *rolD* genes (Fig. 4e, g). Therefore, higher levels of *ORF13* and/or *rolD* in Ri-line GVI respect to GCA, could explain its reduced shoot weight (Fig. 2g), shoot: root ratio (Fig. 2i) and aerial plant coverage (Fig. 2l; Fig. 3e) and increased flower density, produced by changing the ratio between opened flowers and primordia in the shoots, and increased flower width relative to aerial plant coverage (Fig. 3f and g, h). Comparison of Ri-lines GME and GCA showed differences in the expression of *ORF8*, *rolA* and *ORF13* genes (Fig. 4a and b, e). Reduced aerial plant coverage (Fig. 2l; Fig. 3e), pedicel length (Fig. 3c), number of flowers per plant (Fig. 3d) and increased flower width relative to aerial plant coverage (Fig. 3h) in Ri-line GCA respect to GME, would be explained by the expression of *ORF8* and/or *rolA* genes in this Ri-line. Axillary shoots per node (Fig. 2f) were increased in Ri-line GCA respect to GME suggesting also roles for ORF8 and/or *rolA* in the modulation of this trait. However, the number of axillary shoots per node was similar to control plants in GVI and GIN lines, supporting oncogene interactions. In addition, the possible action of ORF13, previosuly proposed, may depend of *rolA* and/or *ORF8* gene expression. Other possibility is that higher levels of ORF13 expression, similar to those observed in GVI and GIN, would be necessary in Ri-line GME. Reduction in individual leaf area (Fig. 2j) was more correlated with expression of *rolB*, *rolC* and *ORF14*. Finally, the number of nodes and axillary shoots were higher and similar in all Ri-lines respect to control plants (Fig. 2d and e) and correlation with specific gene expression was not observed.

4. Discussion

In this study we report efficient transformation of ornamental *Mecardonia* with a wild-type strain of *A. rhizogenes*. Plants regenerated from hairy roots showed varying degrees of compact growth habit and various traits related to ornamental value were improved. Shoots inoculated with *A. rhizogenes* produced distinctive fast growing and highly branched roots when grown in hormone-free medium, allowing selection of transformants without using antibiotic resistance genes (Breyer et al., 2014; Christensen et al., 2008; Ebinuma and Komamine, 2001). A great advantage of *Mecardonia* plants is that transformed roots regenerated spontaneously into shoots as it was reported in other ornamentals (Chaudhuri et al., 2006; Giovannini et al., 1997; Jaziri et al., 1994; Oksman-Caldentey et al., 1991), avoiding calli formation and the use of plant growth regulators. In vitro Ri-plantlets showed increased root growth and root branching when compared to controls, two common charactersitics observed in plants transformed with wild-type *A. rhizogenes* (Jung and Tepfer, 1987; Spena et al., 1987; Tepfer, 1984; Tzfira et al., 1999). In general, they showed a slight reduction of internode length and a clear increment in the number of nodes, axillary shoots, shoot diameter and shoot and root biomass, reducing severely the shoot: root ratio respect to control plantlets. Shoot length was similar or increased in *Mecardonia* Ri-lines because reductions of internode length were compensated by a higher number of nodes. Compensation effects have been reported in aspen and gentian (Suginuma and Akihama, 1995; Tzfira et al., 1999), indicating that shoot length in Ri-plantlets can vary among species. In addition, the number of shoots per node was clearly increased in all Ri-lines indicating enhanced

axillary bud break as it was also reported in aspen and gentian (Suginuma and Akihama, 1995; Tzfira et al., 1999).

Under greenhouse conditions *Mecardonia* Ri-plants showed different degrees of dwarfness, characterized by reductions of internode and shoot length, apical dominance, individual leaf area, aerial plant coverage, aerial and hypogeum biomass and similar shoot: root ratios when compared to control plants, evidencing striking differences when compared to Ri-plantlets. Decreased coverage in Ri-lines was produced by reduced internode lenght and leaf area despite an increased number of nodes, axillary branches and leaves per shoot. As a result, *Mecardonia* Ri-plants were more erect, compact and dense and did not expand radially as non-transformed plants, demonstrating the potential of *rol* and possibly other oncogenes to produce useful developmental alterations. Ri-line GME showed a subtle Ri-phenotype with small differences in shape and structure respect to control plants. Ri-line GCA showed a semi-dwarf Ri-phenotype, while Ri-lines GVI and GIN showed marked morphological alterations and could be included in the super-dwarf Ri-phenotype described by Casanova et al. (2005). Decreased shoot length was evidenced despite an increased number of nodes. Interestingly, similar results were observed in other ornamental plants like *Pelargonium* spp., *Angelonia salicariifolia* and *Kalanchoe blossfeldiana* (Christensen et al., 2008; Koike et al., 2003; Pellegrineschi and Davolio-Mariani, 1996), demonstrating that compact growth can be generated despite an increased number of nodes, although in *Nierembergia scoparia* and aspen compensation effects were observed (Godo et al., 1997). The increased number of axillary shoots in *Mecardonia* Ri-lines correlated with a higher number of nodes. Reduced apical dominance has been observed in different species transformed with wild-type *A. rhizogenes*, including ornamentals like *Nierembergia*, scented geranium, *Eustoma* and *Atropa* (Godo et al., 1997; Handa, 1992; Jaziri et al., 1994; Pellegrineschi and Davolio-Mariani, 1996; Tzfira et al., 1999). As mentioned previously, improved bud break was evident in all *Mecardonia* Ri-plantlets, although under greenhouse conditions increased bud break was only obseved in Ri-line GCA. Altogether, differences between *Mecardonia* Ri-plantlets and plants grown in the greenhouse reinforce the idea that mechanisms that control shoot length and other traits depend strongly on host species and growth conditions.

Alterations in flowering time have been observed in several species transformed with wild-type *A. rhizogenes*, including delayed flowering in tobacco, *Hyoscyamus muticus* and *Ipomoea trichocarpa*; and accelerated flowering in *Convolvulus arvensis*, carrot, Belgian endive, *Gentiana scabra* and *Limonium* spp. (Mercuri et al., 2001; Oksman-Caldentey et al., 1991; Otani et al., 1996; Suginuma and Akihama, 1995; Sun et al., 1991; Tepfer, 1984). In ornamental production, delayed flowering is not desirable since it increases production time and costs. In the present study three out four Ri-lines showed normal flowering, indicating that transformation with *A. rhizogenes* can be considered valuable for further breeding.

Plants with increased branching are expected to produce more flowers per plant. In *Antirrhinum majus* and ornamental gentian transformed with wild-type *A. rhizogenes*, increased number of axillary shoots correlated with a higher number of flowers (Handa, 1992; Hosokawa et al., 1997; Mishiba et al., 2006). In the present study, the total number of flowers per plant was significantly reduced in three Ri-lines despite the increased number of axillary shoots that derived from main shoots. This is explained by the reduced plant coverage observed in Ri-lines and because axillary branches did not contribute substantially to the production of flowers as main shoots did. Despite this apparent negative effect, flower density was similar to control plants in Ri-lines GME and GCA and increased in GVI and GIN, demonstrating that ornamental value was improved in the latter Ri-lines. Reductions in flower width in Ri-lines GCA, GVI and GIN were compensated by a higher decrease in aerial plant coverage. Therefore, the ratio between flower width and aerial plant coverage was increased respect to control plants, leading to improved ornamental value. Christensen et al. (2008) reported similar results in the ornamental plant *Kalanchoe blossfeldiana* for the number of flowers and flower size when relativized to plant height. Our results together with previous reports show the potential of *A. rhizogenes* to succesfully improve flower related traits useful in ornamental production.

Reductions in aerial biomass in *Mecardonia* Ri-plants correlated with reductions in shoot length, leaf area, the number of flowers and flower width, being these results in line with those observed in *K. blossfeldiana* (Christensen et al., 2009). Reductions in hypogeum biomass were observed in *Mecardonia* Ri-lines as well as in tomato plants overexpressing *rolABC* genes (van Altvorst et al., 1992), while increased in others like *Aralia elata* and ornamental *Datura*, *Angelonia salicariifolia* and scented geranium (Giovannini et al., 1997; Kang et al., 2006; Koike et al., 2003; Pellegrineschi and Davolio-Mariani, 1996). In general, phenotypical variations among species would depend on how oncogene products interact with endogenous plant growth regulators, indicating the importance to advance in the knowledge of the roles of these genes in different plant species and in the development of derived applications (Khan et al., 2016; Mauro et al., 2017).

Aux1 and *aux2* genes were not detected in PCR and qPCR analysis, suggesting that T_R-DNA was not integrated in any of the transformed *Mecardonia* Ri-lines. Our results are in line with previous findings that showed lower frequencies of T_R-DNA integration in plant genomes and absence of integration was also reported in some species, reflecting the importance of T_L-DNA to develop dwarf phenotypes (Alpizar et al., 2008; Hegelund et al., 2017; Schmülling et al., 1988). In addition, ORF8 and *rolA* genes were not detected in Ri-line GME. Since both genes are physically contiguous, it is possible that a truncated T_L-DNA was integrated in this line.

Correlations between phenotypical traits and T-DNA oncogenes expressed under their natural gene promoters have been poorly explored in plants transformed with wild-type *A. rhizogenes*. Here, Ri-line GIN showed the highest *rolA-D* and *ORF8, ORF13-14* gene expression that correlated with the lowest leaf area and flower width, delayed flowering and increased number of open and total flowers per shoot. Tobacco plants expressing *rolABC* genes displayed a more severe phenotype than plants expressing *rolBC*, *rolAC* or single *rol*-gene combinations (Schmülling et al., 1988). Accordingly, the weakest Ri-phenotype observed in Ri-line GME, which do not express *rolA* and *ORF8* genes, suggest the importance of collective oncogene contribution to generate more accentuated Ri-phenotypes in *Mecardonia* plants. Ri-lines GVI and GIN showed higher expression of *rolD* and *ORF13* genes that correlated with reductions in their aerial plant coverage, shoot weight, shoot: root ratio and increased flower density caused by the higher proportion of opened flowers in the shoots and flower width relative to the aerial plant coverage. These genes displayed the highest changes in relative expression between Ri-lines. Tobacco plants expressing *rolD* under its native promoter caused reduced plant height, leaf and flower size (Mauro et al., 1996). Similarly, carrot plants expressing *rolD* by its native promoter showed extreme dwarfism (Limami et al., 1998). Interestingly, transgenic tobacco and tomato plants overexpressing ectopically ORF13 exhibited reduced height and smaller leaves and flowers (Hansen et al., 1993; Lemcke and Schmülling, 1998; Stieger et al., 2004). Moreover, ectopic overexpression of *ORF13* in Arabidopsis produced extremely dwarf plants with reduced leaf and flower size (Kodahl et al., 2016). Overall, evidences in *Mecardonia* and previous studies suggest roles for *rolD* and ORF13 as inhibitors of plant growth. Transgenic tobacco and tomato plants expressing *rolD* by its native promoter showed increased number of inflorescences and flowers (Bettini et al., 2003; Mauro et al., 1996). Axillary inflorescences of tobacco plants showed fast growth and development (Mauro et al., 1996). Tissues expressing *rolD* displayed enhanced and earlier flower organogenesis *in vitro*, indicating that transformed plants were highly committed to produce flowers (Mauro et al., 1996). In addition, expression of *rolD* in Arabidopsis caused enhanced and precocious formation of vegetative and reproductive meristems (Falasca et al., 2010). Furthemore, ORF13 was the only T-DNA gene that induced cell proliferation

when inoculated in carrot discs and tobacco leaf discs (Fründt et al., 1998; Hansen et al., 1993). Tomato plants expressing *ORF13* enhanced the number of cell divisions in the shoot apical meristems and accelerated the production of leaf primordia (Stieger et al., 2004). Altogether, these evidences show that *rolD* and ORF13 are involved in organogenesis. Hence, the increased flower density in Ri-lines GVI and GIN, caused by accelerated flower development in the shoots, maybe explained by the higher expression of *rolD* and/or *ORF13* in these lines.

Expression of *ORF8* and/or *rolA* in *Mecardonia* would contribute to reduce aerial plant coverage, pedicel length, the total number of flowers per plant and increase flower width relative to aerial plant coverage. Plants of tobacco overexpressing ectopically *ORF8* produced dwarf plants with reduced internode length, small leaves and coverage and slightly increased size of the flowers respect to control plants (Umber et al., 2005). Plants of tobacco overexpressing ectopically *rolA* displayed a dwarf phenotype with delayed flowering and reduced number of flowers, pedicel length and the size of the flowers (Dehio et al., 1993; Martin-Tanguy et al., 1996). Evidences suggest that *ORF8* and/or *rolA* expression would contribute to the modulation of these traits in *Mecardonia*. Moreover, the highest expression of *rolA* in Ri-line GIN may explain its delayed flowering phenotype. In the case of ORF8 and ORF13, they may have a more prominent role in the regulation of plant development than previously assumed and assigned to *rol*-genes when wild-type Ri-plasmids are used for transformation, as recently proposed by Kodahl et al. (2016) for ORF13.

Finally, *Mecardonia* Ri-lines showed decreased leaf area and flower width which correlated with expression of *rolB*, *rolC* and *ORF14* genes. Accordingly, *rolB* and mainly *rolC* reduce the size and morphology of leaves and flowers in various species (Arshad et al., 2014; Bettini et al., 2010; Boase et al., 2004; Kurioka et al., 1992; Schmülling et al., 1988; van Altvorst et al., 1992; Winefield et al., 1999; Zuker et al., 2001), although no morphological changes were reported in plants overexpressing *ORF14* (Lemcke and Schmülling, 1998). Therefore, expression of *rolB* and/or *rolC* in *Mecardonia* plants may modulate these traits. Since expression of oncogenes was determined in established vegetative stage plants of *Mecardonia*, correlations of gene expression and reproductive traits should be taken carefully. Despite this fact, the possible functions of different oncogenes in plant development that emerged from these correlations matched well with previous evidences. Nevertheless, future investigations would be necessary to determine oncogene expression in reproductive stages and if the identified candidates have actually roles in the modulation of *Mecardonia* traits.

5. Conclusions

This study is the first report of *Mecardonia* transformation with a wild-type strain of *A. rhizogenes*. Ri-plants showed reduced growth and enhanced plant architecture and flower related traits, providing a powerful alternative to the use of chemical growth retardants and pruning techniques. Since plants produced by this molecular approach are not considered GMO in several countries, modified plants can be quickly introduced in the ornamental market. Moreover, identified oncogenes would be considered candidates in *Mecardonia* breeding.

Conflict of interests

All authors approved the manuscript and its submission to the journal and declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2018.02.047.

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