Scientia Horticulturae 234 (2018) 300–311

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

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

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ARTICLE INFO

Keywords:
Mecardonia
Agrobacterium rhizogenes
Ri plasmid
Compact growth
Ornamental traits
Oncogene expression
Non-GMO

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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https://doi.org/10.1016/j.scienta.2018.02.047
Received 31 October 2017; Received in revised form 16 February 2018; Accepted 19 February 2018

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