Metabolic Engineering 12 (2010) 561–572

Contents lists available at ScienceDirect

Metabolic Engineering

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

Genetic alterations for increased coumarin production lead to metabolic changes in the medicinally important *Pelargonium sidoides* DC (Geraniaceae)

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ARTICLE INFO

Article history:
Received 17 April 2010
Received in revised form
12 August 2010
Accepted 17 August 2010
Available online 24 August 2010

Keywords:
Agrobacterium rhizogenes
Genetic transformation
Hairy root
Metabolic engineering
rol Genes
Secondary metabolism

ABSTRACT

The medicinal plant *Pelargonium sidoides* is fast becoming threatened due to the overharvest of its tubers from the wild to produce a phytopharmaceutical for treating respiratory infections. The action of the coumarins is implicated in the efficacy of the commercial herbal extract with the highly oxygenated coumarins exhibiting the best anti-bacterial and anti-viral activity. Through this work we aimed at exploring the metabolic effects of *Agrobacterium rhizogenes* transformation. After confirmation of transgenesis using PCR amplification of the *rol* A (320 bp), *rol* B (400 bp) and *rol* C (600 bp) genes, metabolite profiles indicated a high level of variability between the different transgenic clones but these had more compounds compared to non-transgenic control cultures. This was represented by a two- to four-fold increase in detected metabolites in transgenic clones. We quantified several commercially important coumarins, flavonoids and phenolic acids. One of the clones had six out of nine of these metabolites. Overall, the concentration of these metabolites of interest were significantly changed in transgenic root cultures, for instance shikimic acid was recorded at the highest level in clone A4T-A. Production of key metabolites at significantly higher concentrations due to transgenesis and positive anti-bacterial activity exhibited by transgenic roots lends support to the idea of developing these clones as an alternative source that will allow for sustainable access to economically valuable secondary compounds of *P. sidoides*.

1. Introduction

Pelargonium sidoides DC (Family Geraniaceae) is an herbaceous, perennial plant predominantly found in the Eastern Cape Province of South Africa but it is also distributed in areas of the Free State, southern and south-western Gauteng and Lesotho (Van der Walt and Vorster, 1988; Brendler and Van Wyk, 2008). This plant is characterised by thickened underground tubers or rhizomes which are red on the inside. Its flowers are arranged in a rosette of dark maroon red to black petals (Lewu et al., 2007a, 2007b; Brendler and Van Wyk, 2008). A large part of the South African population is still reliant on traditional herbal remedies for healthcare and these tubers are used for multiple purposes as a traditional herbal medicine by several South African ethnic groups (Kolodziej and Kiderlen, 2007). For instance, root and leaf water extracts are used to treat stomach ailments such as diarrhoea and gastritis (Lewu et al., 2007a, 2007b; Brendler and Van Wyk, 2008) plus ear, nose and throat disorders, respiratory tract infections, fatigue and weakness of the body, hepatic disorders and menstrual complaints (Kolodziej, 2002, 2007; Van Wyk and Wink, 2004; Lewu et al., 2006; Mativandlela et al., 2006; Kolodziej and Kiderlen, 2007). Extracts are also used for ethnoveterinary purposes (Lewu et al., 2007a, 2007b; Brendler and Van Wyk, 2008), for example cleansing wounds in livestock (Watt and Breyer-Brandwyk, 1962; Hutchings, 1996; Lewu et al., 2007b). For an excellent comprehensive review on *P. sidoides* summarising historical and scientific aspects including biological and pharmacological activity refer to Brendler and Van Wyk (2008). A commercial product (EPs® 7630), more commonly known as Umckaloabo® (Spitzner Arzneimittel, Germany) (Kolodziej, 2007; Kolodziej and Kiderlen, 2007) has become a popular remedy and is widely used to treat bronchitis (Van Wyk and Gericke, 2003) and one kilogram of tubers is generally required to manufacture 975 mL commercial extract (Mayet, 2010).

The roots of *P. sidoides* contains coumarins, flavonoids, phenolic and hydroxycinnamic acid-derivatives, phytosterols and tannins (Seidel and Taylor, 2003; Brendler and Van Wyk,

Abbreviations: APCI, atmospheric pressure chemical ionisation; GCMS, gas chromatography mass spectrometry; LCMS, liquid chromatography mass spectrometry; LCMSMS, liquid chromatography tandem mass spectrometry; LMS, liquid half-strength MS medium; LOD, limit of detection; MIC, minimum inhibitory concentration; MS, Murashige and Skoog (1962) medium; MRM, multiple reaction monitoring; MSTFA, *N*-methyl-*N*-methyl-*N*-(trimethylsilyl)-triflouro acetamide; PGRs, plant growth regulators; RSD, relative standard deviation; SMS, solid half-strength MS medium; S/N, signal-to-noise ratio; untransformed, Untr'

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doi:10.1016/j.ymben.2010.08.001

2008). Root extracts display anti-bacterial and anti-fungal activity (Kayser and Kolodziej, 1997) and has immunomodulating properties (Seidel and Taylor, 2003; Mativandlela et al., 2006); amongst others. This pharmacological action has been assigned to coumarins (Ding et al., 1988; Kiderlen and Kaye, 1990; Wagner and Jurcic, 1991; Marcucci et al., 1992; Kayser and Kolodziej, 1997); gallic acid-derivatives (Brendler and Van Wyk, 2008) and other phenolic compounds (Van Wyk and Wink, 2004). It is mainly the highly oxygenated coumarins, 7-hydroxy-5,6-dimethoxycoumarin (termed umckalin) and 6,8-dihydroxy-5,7-dimethoxycoumarin which display the best anti-bacterial and anti-viral potential (Kayser and Kolodziej, 1997; Lewu et al., 2006; Brendler and Van Wyk, 2008). Apart from coumarins, the commercial extract EPs® 7630 also contains purine derivatives, benzypyranones, peptides, monomeric and oligomeric carbohydrates, minerals and oligomeric prodelphinidins (Schötz and Nöldner, 2007; Schoetz et al., 2008).

In recent years, the commercial demand for *P. sidoides* tubers for local and international trade has caused an enormous increase in the rate of uncontrollable, illegal and indiscriminate wild harvesting, which is fast leading to irreparable reductions in the number of natural populations, causing a biodiversity threat. Even though these tubers are highly sought-after, they are currently only cultivated on a small, virtually negligible scale. Current estimates indicate that 26 tonnes are wild harvested on a monthly basis (Lewu et al., 2007a; Brendler and Van Wyk, 2008), this is often before plants reach maturity. These plants regenerate slowly in nature, with re-sprouting of harvested areas taking up to six months (De Wet, 2005). The viability of seeds collected from the wild is also low and this is coupled to low seed germination (Lewu et al., 2006). White et al. (2008a) estimated that it would take about 56 years for plants which were replanted in a harvested area to recover to their pre-harvested mass. Furthermore, this species is also often confused with its sister species, *P. reniforme*, and both species are then collected from wild populations. Overexploitation of *P. sidoides* has thus become increasingly alarming for conservationists (White et al., 2008a) with recent estimates indicating the extraction of 3328 million tubers from the wild since 2000 to 2008 for export to Germany (Mayet, 2010). Illegal harvesting in the rural Eastern Cape Province despite tight conservation regulations against this practice persists and in some cases, harvesters have been caught with over 392 bags of *P. sidoides* tubers containing approximately 61 000 tubers from one harvest (Mayet, 2010).

Alternative solutions such as harvesting the leaves and stems (Lewu et al., 2006) and large-scale cultivation (Fennell et al., 2006) are not put into practice as the culture of selling wild crafted tubers is strongly favoured. The idea to domesticate *P. sidoides* has generated growing scientific interest in terms of the cultivation requirements of these plants. Greenhouse cultivation investigated by White et al. (2008a) has no deleterious effects on the production of bioactive compounds of *P. sidoides* as the production of umckalin (6-hydroxy-5,7-dimethoxycoumarin) was not significantly repressed. To our knowledge, the use of biotechnological tools to increase umckalin and other coumarins has never been studied in this species. Such a strategy would be useful for metabolic engineering for increased production of the valuable secondary metabolites which are thought to be responsible for the biological activity.

Hairy root cultures are the product of genetic transformation mediated by *Agrobacterium rhizogenes* through transfer of the *rol* genes of the root inducing (Ri) plasmid (Casanova et al., 2005). These adventitious, non-geotropic transgenic roots maintain a high growth rate, usually producing high levels of secondary metabolites (Bulgakov, 2008). This was investigated as a means of possibly producing coumarins and other secondary metabolites which are generally synthesised by the tubers of *P. sidoides*.

Genetic transformation using *A. tumefaciens* has been reported for a few horticulturally important *Pelargonium* species, for example: *Pelargonium* sp. 'Fresham' (KrishnaRaj et al., 1997; Bi et al., 1999); *P.* × *domesticum* 'Dubonnet' (Boase et al., 1998); *P.* × *hortorum* (Robichon et al., 1995; Hassanein et al., 2005); *P. capitatum* (Hassanein et al., 2005); *P. zonale* and *P. peltatum* (Winkelmann et al., 2005). A transformation system using *A. rhizogenes* which simultaneously altered several morphological characteristics (plant stature, leaf and branch production, root system architecture and flowering) of the regenerated *Pelargonium* plants has been reported (Pellegrineschi et al., 1994; Pellegrineschi and Davolio-Moriani 1996). Recently, *P. graveolens* cv. Hemanti was transformed using *A. rhizogenes* strains LBA 9402 and A4 and whole plants regenerated from hairy roots were used to study the effects on essential oil production (Saxena et al., 2007). No published reports on the genetic transformation of *P. sidoides* using *A. rhizogenes* could be found in the literature.

Development of an efficient transformation protocol to induce hairy roots was viewed as an important platform for metabolite engineering in *P. sidoides* in our laboratories. We therefore determined the ability of two *A. rhizogenes* strains, i.e. A4T and LBA 9402, and one *A. tumefaciens* (C58C1) strain, which carries the *rol* genes, to transform different explants, in order to induce the hairy root syndrome. As the aims of this study were two-fold, this paper not only reports on the transformation of *P. sidoides* with the *rol* genes but also on the effect of transgenesis on the biochemical properties of the transgenic clones with particular reference to coumarin synthesis and accumulation.

2. Materials and methods

2.1. Seed germination

P. sidoides seeds, donated by Parceval Pharmaceuticals (Wellington, South Africa) in March (2006), were stored at room temperature prior to germination experiments. All seeds were surface-decontaminated with 70% (v/v) ethanol for 1 min, soaked in a 3.5% (w/v) NaOCl commercial bleach solution for 22 min and rinsed three times for 5 min in sterile distilled water. The seeds were germinated on 1/4-strength Murashige and Skoog (1962) medium (Highveld Biological, South Africa) solidified with 10 g L^{-1} agar (Agar-agar Biolab, South Africa) adjusted to pH 5.8 using 0.1 M NaOH or HCl. All media were autoclaved at 122 kPa and 120 °C for 20 min and once cool, poured into Petri dishes (100×20 mm^2, Corning®, USA). Thereafter laminar flow conditions were used for experimental procedures. Two separate experiments were conducted. The first experiment involved testing the effect of light on germination by incubating seeds under a 24 h light regime (50 µmol m^{-1} s^{-2}; photosynthetic photon flux density (PPFD)) provided by cool white fluorescent tubes (L75W/20X Osram, USA; Code number F96T12), and henceforth referred to as Treatment A. As a control, seeds were placed in the dark. Secondly, the seeds were scarified by partially removing the seed coat with sand paper before decontamination. Thereafter, the scarified and untreated seeds were incubated under continuous light conditions (detailed above). This treatment is from here onwards referred to as Treatment B.

2.2. Ri transformation of explants

A. rhizogenes (A4T) and *A. tumefaciens* (C58C1) strains were cultured on YMA medium (5 g L^{-1} yeast extract; 0.5 g L^{-1} casein hydrolysate; 8 g L^{-1} mannitol, 2 g L^{-1} ammonium sulphate; 5 g L^{-1} NaCl solidified with 15 g L^{-1} agar, pH 6.6), whilst