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Evaluation of tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) hairy roots for the production of geraniol, the first committed step in terpenoid indole alkaloid pathway

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ABSTRACT

The terpenoid indole alkaloids are one of the major classes of plant-derived natural products and are well known for their many applications in the pharmaceutical, fragrance and cosmetics industries. Hairy root cultures are useful for the production of plant secondary metabolites because of their genetic and biochemical stability and their rapid growth in hormone-free media. Tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) hairy roots, which do not produce geraniol naturally, were engineered to express a plastid-targeted geraniol synthase gene originally isolated from *Valeriana officinalis* L. (*VoGES*). A SPME-GC–MS screening tool was developed for the rapid evaluation of production clones. The GC–MS analysis revealed that the free geraniol content in 20 hairy root clones expressing *VoGES* was an average of 13.7 μg/g dry weight (DW) and a maximum of 31.3 μg/g DW. More detailed metabolic analysis revealed that geraniol derivatives were present in six major glycoside forms, namely the hexose and/or pentose conjugates of geraniol and hydroxygeraniol, resulting in total geraniol levels of up to 204.3 μg/g DW following deglycosylation. A benchtop-scale process was developed in a 20-L wave-mixed bioreactor eventually yielding hundreds of grams of biomass and milligram quantities of geraniol per cultivation bag.

1. Introduction

Plants synthesize a vast range of low-molecular-weight compounds also described as natural products or secondary metabolites. Tropical rainforests are characterized by the richest species diversity on earth (up to 942 species/ha) including up to 298,000 species of higher terrestrial plants (Wilson et al., 2012; Mora et al., 2011). Many of the resulting secondary metabolites can be used as food, feed, flavors, fragrances, cosmetics, agrochemicals and pharmaceuticals. Secondary metabolites therefore contribute substantially to human wellbeing and can provide backbone structures or leads for new semi-synthetic or synthetic pharmaceutical compounds.

The complex stereochemical structure of many secondary metabolites means that total chemical synthesis is not feasible. Extraction from a natural source or biotechnology-based production may be considered, but the latter is required when the source species is rare or endangered. Key secondary metabolites often accumulate naturally in minute quantities, and the source species may also be difficult to cultivate and recalcitrant to genetic transformation. Therefore alternative approaches are required in which the corresponding metabolic pathways are engineered in more amenable hosts for the production of valuable secondary metabolites.

Hairy root cultures provide a useful platform for the production of plant secondary metabolites, particularly alkaloids and their derivatives, which are produced at high levels in hairy roots (Giri and Narasu, 2000). For example in *Hyoscyamus niger* hairy roots scopolamine contents of 4.1% of the DW have been recorded (Sevón and Oksman-Caldentey, 2002; Rischer et al., 2013). The advantages of hairy roots include relatively fast growth (0.1–2 g DW

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per liter and day), genetic and biochemical stability and growth in hormone-free medium (Shanks and Morgan, 1999; Sevón and Oksman-Caldentey, 2002). Hairy roots possess the full spectrum of biochemical activity required to produce the same secondary metabolites as the complete plants from which they are derived, but their capacity for the biosynthesis of natural products is often greater (Kim et al., 2002). Hairy roots therefore play an important role as a model system for the investigation of plant metabolism and genetic engineering (Shanks and Morgan, 1999).

Despite these advantages, it is more difficult to scale-up hairy root cultures than plant cell suspension cultures, which are therefore used more often in commercial production processes. The main challenge is the heterogenic morphology of hairy roots, as well as their irregular growth and high shear stress sensitivity. These issues have been addressed by the development of single-use (disposable) bioreactors as an alternative to glass or stainless steel cultivation systems such as spray reactors and bubble columns. Single-use wave-mixed systems such as BIOSTAT RM have been established that simplify the mass propagation of hairy roots by inducing waves within a cultivation bag, ideally operating in ebb-and-flow mode (Eibl and Eibl, 2006, 2009). Recent investigations have also shown that orbitally shaken single-use bags are also useful for the mass propagation of hairy roots (Lampart, 2013).

Terpenoid indole alkaloids (TIAs) are one of the major classes of natural products derived from plants. All terpenoids originate from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) and the corresponding metabolic pathway has been characterized both at the genetic and biochemical levels (Phillips et al., 2008). The terpenoid indole alkaloids are then derived from tryptophan and the iridoid terpenoid secologanin, the latter originating from the triose phosphate/pyruvate or non-mevalonate pathway (Verpoorte et al., 1997; O'Connor and Maresh, 2006). Strictosidine synthase catalyzes the Pictet–Spengler reaction between tryptamine and secologanin to form strictosidine, the common precursor of TIAs (Stöckigt et al., 2008). The first committed step in the pathway is the formation of geraniol, an acyclic monoterpene alcohol that is synthesized in one step from geranyl diphosphate (GPP). Geraniol not only represents the first step of the TIA pathway, but also has important properties as a pharmaceutical (Carnesecchi et al., 2001) and fragrance (Chen et al., 2006). The geraniol is jointly transferred with a cytochrome P450 mono-oxygenase geraniol-10-hydroxylase (G10H) and NADPH:cytochrome P450 reductase (CPR) to 10-hydroxygeraniol (Meijer et al., 1993). The 10-hydroxygeraniol in turn undergoes dehydrogenation to 10-oxogeraniol which is further oxidized to 10-oxogeranial (Ikeda et al., 1991). It is postulated that 10-oxogeranial is first cyclized and reduced to form iridodial, which in turn undergoes hydroxylation, dehydrogenation and again a cyclisation. Loganic acid is then synthesized from the resulting iridotrial by hydroxylation, glucosylation and hydroxylation reactions (Verpoorte et al., 1997). Then two further steps lead to the formation of secologanin: a methylation and an acyclisation catalyzed by S-adenosyl-L-methionine:loganic acid methyltransferase (LAMT) and secologanin synthase (SLS), respectively (Murata et al., 2008; Irmler et al., 2000).

We previously showed that heterologous geraniol synthesis can be achieved by expressing the geraniol synthase from *Valeriana officinalis* L. (Valerianaceae) (*VoGES*) in tobacco (*Nicotiana tabacum* L. Samsun NN) leaves (Dong et al., 2013a). GC–MS and LC–MS analysis of leaf extracts revealed that the aerial parts of the plant produced free geraniol as anticipated. However, LC–MS analysis showed that endogenous tobacco enzymes oxidized the geraniol and conjugated these products into glycosides (Dong et al., 2013a). The monoterpenoid geraniol is produced in the plastids/chloroplasts so it is difficult to predict how hairy roots would perform, although they are known to contain plastids and do produce plastidial isoprenoids including monoterpenes (Lourenco et al., 1999) and diterpenes (Zhi and Alfermann, 1993). To determine the potential of hairy roots as a production platform for TIAs, we evaluated the capacity of hairy roots, generated by transformation with *Agrobacterium rhizogenes* containing the 35S::*VoGES* construct, to produce geraniol and its derivatives. We also investigated a range of scale-up systems to determine whether the tobacco hairy root production platform was in principle suitable for commercial applications.

2. Materials and methods

2.1. Generation of tobacco hairy roots

The hairy roots of *N. tabacum* (cv. Petit Havana SR1) were initiated by infecting leaves by *Agrobacterium rhizogenes* LBA9402carrying the pBIN2.4VoGES1 vector for the expression of VoGES. This vector contained a geraniol synthase gene isolated from *V. officinalis* (L.) augmented with an artificial plastid targeting signal, driven by the double-enhanced *Cauliflower mosaic virus* 35S promoter (www.pri.wur.nl/UK/products/ImpactVector/). The artificial plastid targeting was selected on the basis of data described in Dong et al. (2013a,b), where they compared different targeting signals with truncated *VoGES* and did not observe any difference between the native and the new artificial plastid transit signals. In addition, we generated wild-type (WT) hairy roots by transformation with wild-type *A. rhizogenes* LBA9402 and control hairy root clones by transformation with bacteria containing the empty vector (BIN). Leaves from tobacco plants grown *in vitro* were sliced into ~1 cm^2 pieces and placed mid-rib uppermost on hormone-free modified Gamborg's B5 medium as described by Häkkinen et al. (2007). The leaf mid-rib was inoculated with recombinant *A. rhizogenes* using a sterile needle and the leaves were incubated at 24 °C in the dark. After 48 h, the leaves were transferred to cultivation medium supplemented with 500 ppm cefotaxime to eliminate the bacteria. Hairy roots emerged approximately 2–3 weeks later and single root tips were placed on solid culture medium supplemented with 500 ppm cefotaxime. After one round of selection, hairy root clones originating from single root tips were transferred to solid culture medium without antibiotics. The hairy root clones were grown at 24 °C in the dark and subcultured at 4-week intervals. For metabolic analysis, 50 mg fresh weight (FW) of hairy root biomass was added to 50 ml of liquid modified Gamborg's B5 medium in 250-ml shake flasks and cultivated in a rotary shaker (70 rpm, 24 °C, in the dark) for 21 days. The biomass was then harvested by Büchner-filtration, frozen in liquid nitrogen and stored at −80 °C.

2.2. DNA analysis

Genomic DNA from tobacco hairy roots was isolated using the CTAB method of Murray and Thompson (1980). Putative transgenic hairy roots were screened by PCR, using forward primer 5′-CGA CAC TTA TGG CTC GTA TG-3′ and reverse primer 5′-ACC GAC TCG TTA CAA GAA GG-3′ to amplify a 850-bp fragment of *VoGES* and 100 ng of genomic DNA as the template. The presence of *rol*B was verified by PCR using forward primer 5′-ATG GAT CCC AAA TTG CTA TTC CTT CCA CGA-3′ and reverse primer 5′-TTA GGC TTC TTT CTT CAG GTT TAC TGC AGC-3′ to amplify a 780-bp fragment. To confirm the absence of *A. rhizogenes* in the hairy root clones, a 450-bp diagnostic fragment of the *vir*D gene was amplified by PCR using forward primer 5′-ATG TCG CAA GGA CGT AAG CCC A-3′ and reverse primer 5′-GGA GTC TTT CAG CAT GGA GCA A-3′. The pBIN2.4VoGES1 plasmid (1 ng) was used as a positive control, and for *rol*B and *vir*D a boiled preparation of WT *A. rhizogenes* LBA9402 was used as a control. PCR products were separated by 0.8% (w/v) agarose gel