Journal of Plant Physiology 219 (2017) 12–21

Contents lists available at ScienceDirect

Journal of Plant Physiology

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

Differential rubisco content and photosynthetic efficiency of *rol* gene integrated *Vinca minor* transgenic plant: Correlating factors associated with morpho-anatomical changes, gene expression and alkaloid productivity

Priyanka Verma[a,d,*], Shamshad Ahmad Khan[a,d], Nusrat Masood[c], N. Manika[b], Abhishek Sharma[a], Neha Verma[a], Suaib Luqman[b], Ajay K. Mathur[a]

[a] Department of Plant Biotechnology, Central Institute of Medicinal and Aromatic Plants (CIMAP), Council of Scientific and Industrial Research, PO-CIMAP, Lucknow 226015, India
[b] Botany and Pharmacognosy Department, Central Institute of Medicinal and Aromatic Plants (CIMAP), Council of Scientific and Industrial Research, PO-CIMAP, Lucknow 226015, India
[c] Molecular Bio-prospection Department, Central Institute of Medicinal and Aromatic Plants (CIMAP), Council of Scientific and Industrial Research, PO-CIMAP, Lucknow 226015, India
[d] Division of Biochemical Sciences, CSIR-National Chemical Laboratory(NCL), Dr. Homi Bhabha Road, Pashan, Pune 411008, India

ARTICLE INFO

Keywords:
Photosynthetic efficiency
Rubisco
rol gene
Transgenic plant
Vincamine
Vinca minor

ABSTRACT

Transgenic plants obtained from a hairy root line (PVG) of *Vinca minor* were characterized in relation to terpenoid indole alkaloids (TIAs) pathway gene expression and vincamine production. The hairy roots formed callus with green nodular protuberances when transferred onto agar-gelled MS medium containing 3.0 mg/l zeatin. These meristematic zones developed into shoot buds on medium with 1.0 mg/l 2, 4-dichlorophenoxyacetic acid and 40 mg/l ascorbic acid. These shoot buds subsequently formed rooted plants when shifted onto a hormone-free MS medium with 6% sucrose. Transgenic nature of the plants was confirmed by the presence of *rol* genes of the *Ri* plasmid in them. The transgenic plants (TP) had elongated internodes and a highly proliferating root system. During glass house cultivation TP consistently exhibited slower growth rate, low chlorophyll content (1.02 ± 0.08 mg/gm fr. wt.), reduced carbon exchange rate (2.67 ± 0.16 μmol m^{-2} s^{-1}), less transpiration rate (2.30 ± 0.20 mmol m^{-2} s^{-1}) and poor stomatal conductance (2.21 ± 0.04 mmol m^{-2} s^{-1}) when compared with non-transgenic population. The activity of rubisco enzyme in the leaves of TP was nearly two folds less in comparison to non-transgenic controls (1.80 milliunits ml^{-1} mg $protein^{-1}$ against 3.61 milliunits ml^{-1} mg $protein^{-1}$, respectively). Anatomically, the TP had a distinct tetarch arrangement of vascular bundles in their stem and roots against a typical ploy arched pattern in the non-transgenic plants. Significantly, the transgenic plants accumulated 35% higher amount of total TIAs (3.10 ± 0.21% dry wt.) along with a 0.03% dry wt. content of its vasodilatory and nootropic alkaloid vincamine in their leaves. Higher productivity of alkaloids in TP was corroborated with more than four (RQ = 4.60 ± 0.30) and five (RQ = 5.20 ± 0.70) times over-expression of TIAs pathway genes tryptophan decarboxylase (*TDC*) and strictosidine synthase (*STR*) that are responsible for pushing the metabolic flux towards TIAs synthesis in this medicinal herb.

1. Introduction

Vinca minor is an evergreen perennial plant of the family Apocynaceae with a strong folklore history as an astringent, wound-healing, antidermatosic and antigalactic medicinal herb (Grujic et al., 2015). More than 50 monomeric eburnamine-types of terpenoid indole alkaloids (TIAs) have so far been isolated from this plant (Bruneton, 1995; Verma et al., 2012a). In modern clinical medicine, *V. minor* is valued for its leaf alkaloid vincamine that has a strong modulatory influence on brain circulation and neuronal homeostasis. The molecule has also been shown to possess antihypoxic and other neuroprotective potencies in the animal model (Belal et al., 2009; Sabry et al., 2010; Molchan et al., 2012; Verma et al., 2014a, 2014b, 2015a). Like several other high-value phytomolecules that are in clinical use, the *in-planta* availability of vincamine is also very low (> 0.001% dry wt.) and this poses a serious problem in meeting its industrial demand. The low

* Corresponding author at: Division of Biochemical Sciences, CSIR-National Chemical Laboratory (NCL), Dr. Homi Bhabha Road, Pashan, Pune 411008, India.
E-mail address: p.verma@ncl.res.in (P. Verma).

http://dx.doi.org/10.1016/j.jplph.2017.09.004
Received 15 March 2017; Received in revised form 9 September 2017; Accepted 15 September 2017
Available online 20 September 2017
0176-1617/

accumulation of TIAs in plants of family Apocynaceae in general, has been traced back to very rigid developmental, environmental, temporal and spatial regulatory mechanisms (Verma et al., 2012b; Tatsis and O'Connor, 2016). This may be required for three possible reasons: auto protection of the plant from the cellular toxicity of biosynthesized alkaloids; maintenance of cellular carbon economy and energy budgeting because for TIAs synthesis precursors are drawn from growth-sustaining primary metabolic pool and: limited space for storage of a finished product (Facchini and De Luca, 2008; Pan et al., 2016). It is therefore not surprising that majority of TIAs are biosynthesized in a need-based manner and in quantities that are just sufficient to carry out an ecological task for the plant (Verma et al., 2015b). Metabolome designing by modern -omic tools of pathway engineering is being viewed as a possible mechanism to overcome some of these barriers (Gandhi et al., 2015; O'Connor 2015). Among others, over-expression or down-regulation of the genes and transcription factors associated with a given pathway by genetic engineering approach is the most frequently followed strategy today (Tran et al., 2010; Yoon et al., 2013). Development of robust *Agrobacterium*-mediated genetic transformations protocols are integral pre-requisites of these pathway engineering efforts (Pacurar et al., 2011). Prospecting *A. rhizogenes*-mediated transformed hairy roots as *in vitro* production platforms for TIAs or regenerating transgenic plants from them for cultivation, therefore, constitute exciting research target in *Vinca* biotechnology (Verma et al., 2014a, 2014b). It may be recalled here that the bulk of bioengineering efforts to manipulate TIAs metabolism in plants such as *Catharanthus roseus*, *Rauvolfia serpentina* and *V. minor* have mainly relied on the use of undifferentiated cell suspension that lack requisite level of cell, tissue and organ differentiation for executing the entire pathway (Gandhi et al., 2015; O'Connor 2015; Verma et al., 2015, 2017). Recalcitrant nature of such cultures for regenerating complete plants has been another serious impediment in these plant systems (Tanaka et al., 1994; Choi et al., 2004; Verma et al., 2012c). The present study was, therefore, initiated to break this technological impasse and provide detail of an efficient protocol for recovery of higher vincamine yielding transgenic plants from *A. rhizogenes*-mediated hairy root cultures of *V. minor*. The transgenic and non-transgenic plants were compared in terms of their morphological, anatomical and physiological attributes in relation to their TIAs metabolism under glass house environment.

2. Material and methods

2.1. Raising and establishment of transgenic plant

Leaf explants from multiple shoot cultures of *V. minor* were used for hairy root induction. The mother stock shoot cultures were maintained on a MS medium supplemented with 1.0 mg/l 6-benzylaminopurine (BAP), 0.1 mg/l α-naphthaleneacetic acid (NAA), 0.4 mg/l thiamine hydrochloride and 4.0 g/l phytagel. For co-cultivation, the bacterial suspension of *Agrobacterium rhizogenes* strain (Agropine type) A4 was raised in liquid YMB medium (Yeast Mannitol Broth) supplemented with 50 mg/l kanamycin (engineered MTCC strain). Twenty-four-hour-old bacterial culture (OD 0.5 at 600 nm) was used for infecting the explants by wounding them with sterile needles dipped in the bacterial suspension. Untreated explants were plated to serve as controls. The treated explants were placed on a hormone-free MS medium for five days. After co-cultivation, the explants were shifted to a MS basal medium supplemented with cephalexin and ampicillin (500 mg/l each) for 15–20 days for bacterial elimination and root induction. The resultant PVG (hairy root clone) roots was carefully excised and transferred to one-fourth strength of Gamborg B5 liquid medium for growth and multiplication *via* regular sub-culturing through a five-week culture cycle. For shoot bud regeneration from hairy roots, 15 days old shake flask cultures of hairy root clone PVG were transferred to a semi-solid MS (Murashige and Skoog, 1962) basal medium supplemented with different combination of zeatin (1.0–5.0 mg/l) and naphthaleneacetic acid [NAA (0.5–2.5 mg/l)]. A minimum of 10 replicated cultures per hormonal combination were raised. A fast growing green nodular calli obtained with 3.0 mg/l zeatin upon transfer to MS + 1 mg/l 2, 4-D containing medium with 40 mg/l ascorbic acid formed organized shoot buds. The regenerated shoot buds were excised from the callus tissue and multiplied using a hormone-free MS basal medium fortified with 60 g/l sucrose. The buds elongated and formed both shoot and root in this medium simultaneously. The rooted plantlets were carefully taken out from the culture vessels, washed with running tap water to remove the adhered phytagel and, planted in a mixture of sterilized soil and vermicompost (1:1) and kept in glass house for acclimatization. For maintaining high humidity around these plantlets the pots were covered with glass jars during initial phase of the hardening. Watering was kept to a minimum to keep the soil moist.

The glasshouse hardened plants were subsequently checked for the presence of *rol* a, *rol* b and *rol* c genes by PCR analysis. For this DNA isolation and PCR amplification was carried out according to previous report (Verma et al., 2012c).

2.2. Histological studies

To trace the anatomical differences between control and transgenic plants, leaf, stem and root samples were fixed in a 70% ethanol: acetic acid: formaline (18:1:1 v/v) solution. The fixed tissues were dehydrated through a graded ethanol-butyl alcohol series and embedded in paraffin wax (Johanson, 1940). Serial transverse sections (10–15 μm) were cut on a rotary microtome, deparaffinized in a xylene series, stained with safranine (1% w/v) and mounted in Canada balsam for microscopic examination. For stomatal counting, the adaxial surface of the leaf was cleaned with wet cotton and a thin epidermal film was peeled off, mounted in glycerin on a glass slide with a cover slip, and then gently pressed with fine-point tweezers before observing under the microscope. All microscopic observation and photography was done using an Olympus stereo-microscope (BH-2) fitted with a C35AD-2 camera.

2.3. Measurement of photosynthetic parameters

The total chlorophyll and chlorophyll *a/b* contents were estimated in leaves present on the third node from the top of the primary stem of glass house acclimatized transgenic and control plants. For this, 200 mg leaf tissue was extracted with 80% acetone and absorbance was taken at 645 and 663 nm using a Milton Roy spectrophotometer- Spectronic 21 D. Following Arnon (1949) equations, the total chlorophyll, chlorophyll *a* and chlorophyll *b* content was calculated using the formula [(20.2 X A_{645}) + (8.02 X A_{663})] X V/1000 X W; [(12.7 X A_{663}) − (2.64 X A_{645})] X V/1000 X W and; [(22.9 X A_{645}) − (4.68 X A_{663})] X V/1000 X W, respectively. In all cases V was taken as 25 ml and W as 200 mg. A computerized photosynthesis system model Li-6000 (LiCOR, Lincoln, USA) was used to measure photosynthetic rate, transpiration rate and stomatal conductance.

2.4. Rubisco assay

Rubisco activity was measured by spectrophotometric method (Racker, 1957, 1962) with minor modifications. Stock solutions of individual components of reaction mixture was made which contained 4.4 ml Tris HCl [1 M], 1.5 ml $MgCl_2$ [100 mM], 1 ml $KHCO_3$ [2 M], β-NADH [2.5 mM], 1.5 ml ATP [100 mM], 1.5 ml GSH [1.5 ml] and 0.6 ml RuDP [25 mM] and three enzyme systems, namely α-GDH/TPI, GAPDH/3PGK and Rubisco/*Vinca* plant tissue lysate were made. Reactions were mixed properly and the pH was adjusted to 7.8 at 25 °C with 0.1 M NaOH and 0.1 M HCl. Before addition of rubisco standard enzyme or *Vinca*