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RESEARCH NOTE

Enhanced vincamine production in selected tryptophan-overproducing shoots of *Vinca minor*

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Abstract *Vinca minor* is the sole source of vincamine, an alkaloid known to be used in a variety of cerebral disorders. Three stable variant shoot lines (V10, V20 and V30) with tolerance thresholds of 10, 20 and 30 mg/l 5-methyltryptophan (5-MT; analogue of tryptophan), respectively, were selected. These lines showed twofold to threefold increase in tryptophan content and 1.5- to 2-fold increment in the total alkaloids in comparison to the wild line shoots. A maximum of 16-fold enhancement in vincamine production was recorded in V30 line followed by eightfold in V20 line. Inter simple sequence repeat (ISSR)-PCR amplification of all the three lines showed total of 65 bands; out of which 60 were monomorphic (92.3 %) and 5 were polymorphic (7.7 %). Tryptophan being a limiting factor in the indole alkaloid pathway plays a crucial role in modulating the flux towards vincamine production and its over-production positively resulted into enhanced vincamine production.

Keywords *Vinca minor* · Tryptophan · Vincamine · 5-methyl tryptophan · ISSR

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Introduction

Vinca minor, commonly known as myrtle, creeping myrtle, periwinkle or vinca, is an evergreen perennial herbaceous plant belonging to the family Apocynaceae. Known since ancient times, this plant has been heralded for its properties as astringent, wound-healing, antidermatosic, antigalactic etc. (Bruneton 1995). More than 40 terpenoid indole alkaloids (TIAs) have been identified in the aerial part of *V. minor* such as vincamine, isovincamine and vincine. The most important of these is vincamine, an alkaloid claimed to increase cerebral circulation and utilization of oxygen. It has been used in a variety of cerebral disorders (Belal et al. 2009; Molchan et al. 2012) and also as an active ingredient in dietary supplements promoted as smart drugs, cognitive enhancers or nootropics (Sabry et al. 2010). The growth of *V. minor* is comparatively slow (Tanaka et al. 1995). Moreover, this plant produces only a small amount of seeds which germinate rarely and so the plant is usually propagated from shoot cuttings rather than seeds (Bernath 2000; Omidbaigi 2005). However, the quality of herb material and alkaloid concentration is also greatly influenced by environmental conditions (Fard et al. 2008).

Tryptophan being precursor of TIAs, is the least abundant amino acid in a plant cell and its concentration is strongly regulated by feedback inhibition of the enzyme anthranilate synthase (Radwanski and Last 1995). To enhance the level of free tryptophan in the cellular metabolic pool of the cells the strategy to select 5-methyltryptophan (5-MT) resistant mutants has been employed in many crops (Kim et al. 2009). Five-methyltryptophan (5-MT) is an analogue of tryptophan and variant shoots develop resistance by over-producing tryptophan and through coding for a new form of anthranilate synthase that is less sensitive to feedback inhibition. In this regard, there have been several reports on isolation and

expression of feedback-insensitive forms of anthranilate synthase genes in an attempt to increase free tryptophan and secondary metabolite levels in several plants (Li and Last 1996; Cho et al. 2000; Tozawa et al. 2001; Hughes et al. 2004; Seth and Mathur 2005). In spite of rigorous biotechnological interventions applied to over-express respective genes, very little attention was paid to select variant shoots with ideal physiological background and with assured adequate availability of terpenoid indole alkaloid precursors drawn from shikimate and terpenoid routes of the primary metabolic pools (Verma et al. 2012). Failure of these efforts to get desired outcomes with exogenous feeding of these precursors in wild type cells might also have been a consequence of their inability to accommodate these molecules in cells due to their strong feedback inhibitory actions on other metabolic pathways. Based on these results, Canal et al. (1998) have, therefore, rightly inferred that the ultimate benefit of elevating the activity of a single key pathway enzyme can only be realized in the background of overall favourable physiological/biochemical environment of the engineered cell. Isolation of variant shoots of *V. minor* with better inherent capacity to over-synthesize tryptophan has been attempted in the present study to address this crucial issue of tryptophan availability for alkaloid synthesis. ISSR primers consist of a di or trinucleotide simple sequence repeat with a 5′ or 3′ anchoring sequence of 1–3 nucleotides. Compared with RAPD primers, The ISSRs have high reproducibility possibly due to the use of longer primers (16–25 mers) as compared to RAPD primers (10- mers) which permits the subsequent use of high annealing temperature (45–60 °C) leading to higher stringency (Culley and Wolfe 2000). ISSR being PCR based method that involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites, as primers in a single PCR reaction targeting multiple genomic loci to amplify mainly the inter- SSR sequences of different sizes (Reddy et al. 2002). ISSR markers has been used in population genetic studies of plant species as they effectively detect very low levels of genetic variation (Zietkiewicz et al. 1994; Bhatia et al. 2011; Mohanty et al. 2011).

In the present study, we report for the first time, the selection and characterization of 5-methyl tryptophan resistant shoot lines of *V. minor* with enhanced production of vincamine and their ISSR marker based genetic profiling.

Materials and methods

Selection of 5-MT tolerant shoots

Shoots of *V. minor* were maintained on a shoot multiplication medium consisting of MS basal (Murashige and Skoog 1962) with 1.0 mg/l 6-benzyladenine (BA) and 0.1 mg/l naphthalene acetic acid (NAA). The nodal explants were placed on multiplication medium fortified with 10–50 mg/l 5-MT to determine the lethal and LD_{50} (lethal dose where 50 % population was allowed to survive) dose of the analogue. The survival of axillary buds, elongation and shoot growth were taken as the criteria for screening the 5-MT tolerant lines. The variants obtained were regularly sub-cultured on medium supplemented or devoid of 5-MT for 3–4 passages. Every sub cycle consisted of an 8-week passage. The best growing shoots were then transferred to two levels higher and one level lower than the original 5-MT concentration for 5–6 subsequent sub cycles. Shoot showing stable survival and shoot proliferation rate at the varying level of 5-MT were characterized on the basis of stability of acquired trait in presence and absence of the 5-MT, free tryptophan levels and alkaloid profiling. The temperature and relative humidity (RH) were maintained at 26 ± 2 °C and 70–80 %, respectively.

Glasshouse hardening

Before transplantation, shoots were shifted to rooting medium consisting of MS basal medium supplemented with 3 mg/l IBA (indole, 3- butyric acid). The 15 days-old rooted 5-MT-tolerant shoots were transferred to soil. Plantlets were taken out from the culture vessel and thoroughly washed with running tap water to remove the adhered phytagel. Finally they were planted in a mixture of sterilized soil and vermicompost (1:1) in earthen pots.

ISSR Profiling

Ten ISSR markers were utilized to amplify products from the isolated DNA of all the three selected lines (Table 1). Plant genomic DNA was isolated according to the procedure of Khanuja et al. (1999). The PCR mixture consisted of 2.5 µl of 10× taq buffer, 1.0 µl of 10 mM dNTPs mix, 1.25 unit of taq DNA polymerase, 6 pmol of primer i.e.1 µl of the primer. ISSR reactions were performed with 25 ng of DNA. PCR was carried out by denaturation at 94 °C for 5 min followed by 45 cycles of 1 min denaturation at 94 °C, annealing at 45 °C for 1 min and extension at 72 °C for 2 min with the final extension of 5 min at 72 °C. PCR reactions were performed in 90 well thermocycler (Eppendorf, Germany). Isolated DNA and the amplified products were separated by electrophoresis on 0.8 % (DNA yield) and 1.2 % (PCR product) agarose gel, respectively, stained with 0.5 mg/l ethidium bromide using 50 mA current for 3 h in 1x tris EDTA buffer and visualized by fluorescence under UV light. To calculate the band sizes, double digested λ DNA with EcoRI and HindIII was loaded

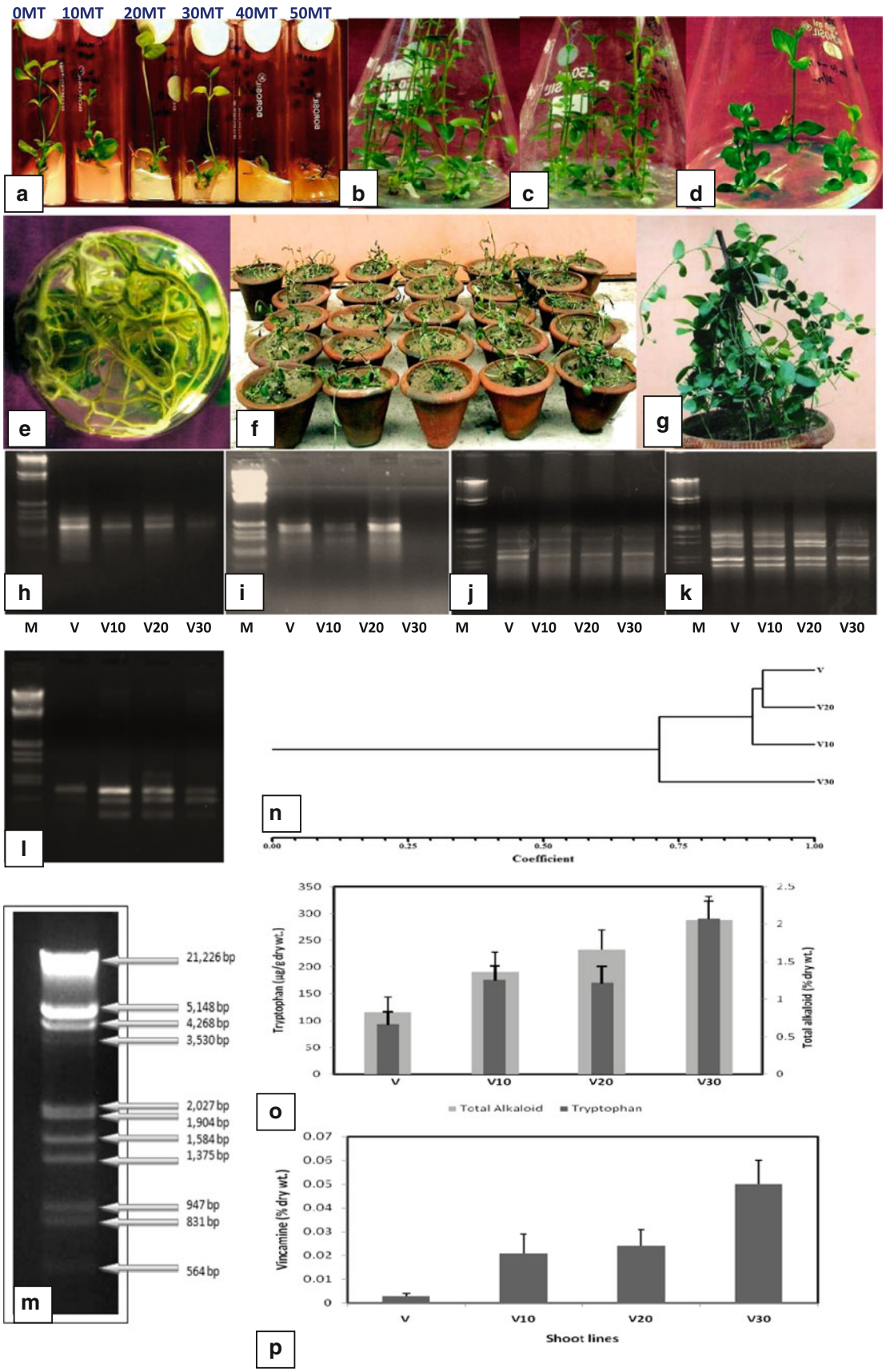
as standard molecular weight marker. The bands were visualized and photographed using Gel Documentation System (XR-Quantity One, Bio-Rad Laboratories). A similarity matrix was obtained after multivariate analysis using Nei and Li's coefficient (Nei and Li 1979). These similarity coefficients were used to generate a tree for cluster analysis using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method and the software NTSYS 2.1 (Numerical Taxonomy and Multivariate Analysis System).

Tryptophan estimation and alkaloid analysis

Tryptophan estimation in the free amino acid pool was done by a modified procedure of Bieleski and Turner (1966) for sample preparation and Dalby and Tsai (1975) for colorimetric estimation. Two gram of glasshouse grown, oven dried tissue was grounded in mortar pestle and extracted twice with 10 ml MCW mixture (methanol: chloroform: water 12:5:3) and thrice with 10 ml 80 % ethanol during each centrifugation at 12,000–15,000 rpm for 15 min. To the combined extracts of MCW mixture, 7 ml distilled water and 5 ml chloroform was added and centrifuged to separate phases. Top layer was taken and added to the pooled ethanolic extracts. The volume was reduced to 10 ml under vacuum in a flash evaporator. The concentrated sample was then loaded onto a DOWEX 50X-2 column (15 cm) and allowed to stand for 30 min before fractionating out at 1.0 ml/min for complete adsorption on the resin. To remove soluble carbohydrates, the column was then washed with 25 ml distilled water. Tryptophan was eluted from the column by 4 × 25 ml washing with 0.3 % ammonia solution. The pooled ammonia fractions were evaporated to dryness on a water bath at a temperature 80 °C. The residue was then re-dissolved in 2.0 ml distilled water. For colorimetric estimation, 0.5 ml of that sample was mixed with the 2.0 ml of a reagent made freshly by mixing equal volume of reagent A and B [Reagent A- 270 mg of $FeCl_3.6H_2O$ dissolved in 0.5 ml water mixed with 1 litre of 2 % acetic anhydride in glacial acetic acid; Reagent B- 30 N Sulphuric acid]. The reaction mixture was kept on vortex and incubated at 60 °C on a water bath for 15 min. The reaction was stopped using an ice bath and the absorption was read at 575 nm. The free tryptophan was calculated using a standard curve of L-tryptophan (SIGMA-USA) prepared in the range of 0–50 µg/0.5 ml. For determining the total alkaloids content of glasshouse established shoots, 1.0 gm oven-dried (50–60 °C) tissue was extracted thrice with HPLC grade methanol (3 × 30 ml; 12 h each) at room temperature. The methanolic extracts were pooled and dried in vacuum to 10 ml, mixed with 10 ml dH_2O, acidified with10 ml of 3 % HCl and washed thrice with hexane (3 × 30 ml). The aqueous portion was basified with ammonia (till pH 8.0), extracted with chloroform (3 × 30 ml), dried over anhydrous sodium sulphate, concentrated in vacuum and weighed. For HPLC analysis of the crude alkaloid extracts, a modular HPLC apparatus (Waters Corp., Milford, MA, USA) equipped with a 600E multi-solvent delivery system and a 2,996 photodiode array (PDA) detector was used. Data was processed using Empower Pro (Waters Corp.) chromatographic software. On-line degassing of solvents was done with helium. 10 µl of the extract was loaded onto a RP-18e reversed-phase chromolith performance HPLC column (100 × 4.6 mm i.d.). A constant flow rate of 1.2 ml/min was used for all the analysis. The mobile phase used was consisted of 21:79 (v/v) acetonitrile-0.1 M phosphate buffer containing 0.5 % glacial acetic acid (pH 3.5). The detection was done at 254 nm. Standard of vincamine purchased from CHROMADEX-USA (0.25 mg/ml in methanol) was used for peak resolution based on retention time. Entire HPLC run took 40 min.

Results and discussion

The response of nodal explants under gradually increased 5-MT level supplemented in the medium was observed and LD_{50} value was found to be 15 mg/l. The direct selection scheme developed by our group earlier (Seth and Mathur 2005) allowed the recovery of sub to supra lethal variants. The selected lines grew well in the presence of 5-MT whereas the survival of corresponding wild type shoots was completely checked at 20 mg/l level of 5-MT supplementation. The acquired trait was stably maintained through sustained selection pressure for 8 sub-cycles and was retained even when the variants were grown for three continuous passages in the absence of the analogue. The repeated screening under 5-MT presence finally led to the isolation of 3 stable variants having the tolerance threshold of 10, 20 and 30 mg/l 5-MT (Fig. 1a–d) and has been designated as V10, V20 and V30, respectively. Lines V10 and V20 were found to be highly proliferating in terms of shoot numbers per culture while in V30 line, number of shoots was lesser, but leaves were found to be highly chlorophyllus. The selected shoots were showing full grown roots within 15 days of transfer to rooting medium (Fig. 1e). New buds had started emerging in the second week of transfer from the in vitro conditions and shoots were fully adapted to glasshouse within 30 days, with success rate of 80–85 % (Fig. 1f–g). ISSR profiling of above discussed three lines was also performed. Out of 10 primers only 5 primers, namely T-2, C-5, C-6, C-8 and A-9 were successfully responded (Table 1). Total of 65 bands were detected, out of which 60 were monomorphic


0MT 10MT 20MT 30MT 40MT 50MT
a
b
c
d
e
f
g
h
i
j
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M V V10 V20 V30 M V V10 V20 V30 M V V10 V20 V30 M V V10 V20 V30
l
V
V20
V10
V30
n
0.00 0.25 0.50 0.75 1.00
Coefficient
21,226 bp
5,148 bp
4,268 bp
3,530 bp
2,027 bp
1,904 bp
1,584 bp
1,375 bp
947 bp
831 bp
564 bp
m
Tryptophan (µg/g dry wt.)
Total alkaloid (% dry wt.)
V V10 V20 V30
Total Alkaloid
Tryptophan
o
Vincamine (% dry wt.)
V V10 V20 V30
Shoot lines
p

◀ **Fig. 1** Selection and establishment of 5-MT tolerant shoots of *V. minor* and their ISSR based molecular and chemical analysis. **a** Wild line shoots of *V. minor* on graded series of 5-MT supplemented MS + 1 mg/l BA + 0.1 mg/l NAA medium; **b–d** selected V10, V20 and V30 lines, respectively; **e** rooted V10 shoot line on MS + 3 mg/l IBA; **f**, **g** glasshouse acclimatized V30 shoot line; **h**, **i** ISSR profile generated by T-2, C-5, C-6, C-8, and A-9 primers; (**m**) band size of loaded Double digested λ DNA with EcoRI and HindIII molecular weight marker (**n**) Nei and Li's similarity matrix based UPGMA dendrogram; (**o**) tryptophan, total alkaloid content and vincamine production (**p**) in selected shoot lines

(92.3 %) and 5 were polymorphic (7.7 %). The primer C-8 scored maximum 5 bands. The banding pattern of all the responsive primers has been shown in Fig. 1h–l. The similarity matrix obtained after multivariate analysis using Nei and Li's coefficient was used to generate a tree for cluster analysis using UPGMA method. The cluster analysis revealed that the wild line is closer to line V20 followed by V10 line and the maximum variability was recorded by V30 line (Fig. 1n). This distant nature of V30 in the dendrogram and very less number of polymorphic bands stand greater chances of having a linkage with the 5-MT resistance genes and could be target of further studies. Microsatellite markers revealed high degree of allelic variation that results from variation in number of repeat-motifs at a locus caused by replication slippage and/or unequal crossing-over during meiosis (Kalia et al. 2011). In spite of limited understanding of the functions of the ISSR motifs within the plant genes, ISSRs are being widely utilized in plant genome analysis. ISSR markers have gained considerable importance in plant genetics and breeding owing to many desirable attributes including hypervariability, multiallelic nature, codominant inheritance, reproducibility, relative abundance, extensive genome coverage (including organellar genomes), chromosome specific location, amenability to automation and high throughput genotyping (Kalia et al. 2011). ISSRs segregate mostly as dominant markers following simple Mendelian inheritance (Reddy et al. 2002). As 5-MT resistance is associated with single dominant nuclear gene mutation (Wasaka and Widholm 1987), variability among selected lines determined by ISSR markers was found to be highly significant. In comparison to wild line shoots, all selected lines had shown twofold to threefold increase in tryptophan concentration in amino acid pool when grown in presence of 5-MT. The maximum tryptophan production was observed in V30 (290.34 ± 57.1 µg/g dry wt.) followed by V10 (175.87 ± 39.4 µg/g dry wt.) and V20 (170.24 ± 30.9 µg/g dry wt.). The elevated levels of free tryptophan in these shoots lines were also reflected by their higher total alkaloid contents in presence of analogue which was found to be 1.36 ± 0.32, 1.42 ± 0.56 and 2.05 ± 0.42 in V10, V20 and V30 lines, respectively in comparison to the wild line shoots (0.83 ± 0.29). The HPLC profile of crude alkaloid extracts revealed increased production of vincamine. The maximum of 16-fold increment in vincamine production was detected in V30 line (0.05 ± 0.01) followed by eightfold in V20 (0.024 ± 0.007) and sevenfold in V10 line (0.021 ± 0.008) compared with the wild line shoots (0.003 ± 0.001) (Fig. 1o–p). Tryptophan represents not only an essential amino acid but also the precursor of various primary and secondary metabolites. Production efficiency of tryptophan is relatively low and its level in plants is limited (Ikeda Ikeda 2006; Ishihara et al. 2007). Analogues of amino acids may function as feedback inhibitors if they are stereo-chemically close enough to the natural amino acids. This is apparently the case with 5-MT because tryptophan effectively reverses the 5-MT inhibition (Kim et al. 2004). The basis for adopting such an approach was its successful implementation in several earlier studies aimed to enhance the nutritional quality of many agricultural crops in terms of the improved content of essential dietary amino acids like tryptophan, arginine, lysine, phenylalanine, tyrosine, methionine, threonine etc. in them (Negrutiu et al. 1984; Lee and Kameya 1991; Brotherton et al. 1996; Kim et al. 2004; Galili et al. 2005). It was also well documented that transgenic *Catharanthus roseus*

Table 1 Band analysis of 5-MT tolerant shoots of *V. minor* generated by 10 ISSR primers

S. no.	Primer code	Sequence	Total bands	Band size (range in base-pairs)	Monomorphic bands	Polymorphic bands
1	A-1*	5′CCACCACCACCACCA3′	–	–	–	–
2	A-4*	5′CTACTACTACTACTA3′	–	–	–	–
3	A-9	5′GGAGGAGGAGGAGGA3′	13	947–400	12	1
4	C-5	5′AGCAGCAGCAGCAGC3′	12	2,100–1,000	12	0
5	C-6	5′GTCGTCGTCGTCGTC3′	14	2,100–947	12	2
6	C-8	5′TACTACTACTACTAC3′	20	1,800–700	20	0
7	G-3*	5′TCGTCGTCGTCGTCG3′	–	–	–	–
8	G-7*	5′GAGGAGGAGGAGAG3′	–	–	–	–
9	T-2	5′GGTGGTGGTGGTGGT3′	6	1,375–947	4	2
10	T-11*	5′CAGCAGCAGCAGCAG3′	–	–	–	–
		Total	65		60	5

* Not responded

cultures over-expressing Tryptophan decarboxylase (TDC) and/or Strictosidine synthase (STR) did not produce higher levels of TIAs as compared to the non-transformed control cells (Canel et al. 1998; Whitmer et al. 1998). Further experimentation in these studies suggested that limited availability of indole and terpenoid precursors (i.e., tryptophan and secologanin, respectively) for TIAs synthesis was one of the primary reasons for this failure because when loganin and/or tryptamine were exogenously fed to such cultures, the pathway effectively advanced to higher TIAs accumulation even in the wild type cells in some of these studies (Whitmer et al. 2002a, b). These studies also pointed out that cultured cells responded better to loganin feeding than tryptophan or tryptamine supplementations because these indole precursors are not allowed to accumulate in larger amounts in the cells because of their strong feed-back inhibitory effects on cellular metabolism and overall growth. It was also observed that tryptamine utilization through its coupling with secologanin during strictosidine synthesis was a more limiting step than its formation per se (Moreno et al. 1993; Whitmer et al. 1998, 2002a, b; Morgan and Shanks 2000). These findings explained why transgenic callus and cell suspension cultures of *C. roseus* with over-expressed TDC gene generally show signs of metabolic stress resulting in poor growth and proliferation due to tryptophan scarcity for other life sustaining processes like auxin synthesis. 5-MT resistance has also been reported to have a variety of functions, and yield remarkably diverse expression patterns upon 5-MT treatment (Kim et al. 2009). Many genes that were identified tend to be related to defense and stress responses, suggesting "cross-talking" between biotic/abiotic stresses including the 5-MT treatment (Kim et al. 2009). Therefore, 5MT resistant mutants might be of value for identifying genes related to plant defenses and stresses. The work presented here lead to generation of novel tissue with an ideal physiological state utilized ultimately by plant based pharmaceutical industry.

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References

Belal TS, Barary MH, Ibrahim MEAL, Sabry SM (2009) Kinetic spectrophotometric analysis of naftidrofuryl oxalate and vincamine in pharmaceutical preparations using alkaline potassium permanganate. J Food Drug Anal 17:415–423

Bernath J (2000) Medicinal and Aromatic Plants. Mezo. Publ. Budapest. pp 667

Bhatia R, Singh KP, Sharma TR, Jhang T (2011) Evaluation of the genetic fidelity of in vitro-propagated gerbera (*Gerbera jamesonii* Bolus) using DNA-based markers. PCTOC 104:131–135

Bieleski RL, Turner NA (1966) Separation and estimation of amino acids in crude plant extracts by thin layer electrophoresis and chromatography. Annal Biochem 17:278–293

Brotherton JE, Schechter S, Ranch JP, Widholm JM (1996) Inheritance and stability of 5-methyltryptophan resistance in *Datura innoxia* selected in vitro. Plant Cell Physiol 37:389–399

Bruneton J (1995) Pharmacognosy, Phytochemistry, Medicinal Plants. Lavoisier Publ. Paris, pp 915

Canel C, Lopes-Cardoso MI, Whitmer S, Van der Fits L, Pasquali G, Van der Heijden R, Hoge JH, Verpoorte R (1998) Effects of over-expression of strictosidine synthase and tryptophan decarboxylase on alkaloid production by cell cultures of *Catharanthus roseus*. Planta 205:414–419

Cho HJ, Brotherton JE, Song HS, Widholm JM (2000) Increasing tryptophan synthesis in a forage legume *Astragalus sinicus* by over expressing the tobacco feedback insensitive anthranilate synthase(ASA2) gene. Plant Physiol 123:1069–1076

Culley TM, Wolfe AD (2000) Population genetic structure of the Cleistogamous plant species *Viola pubescens* Aiton (Violaceae), As indicated by allozyme and ISSR molecular markers. Heredity 86:545–556

Dalby A, Tsai CY (1975) Acetic anhydride requirement in the colorimetric determination of tryptophan. Annal Biochem 63:283–285

Fard FR, Moieni A, Omidbaigi R (2008) Effects of different concentrations of naphthaleneacetic acid and 6-benzylaminopurine on shoot regeneration of *Vinca minor* L. J Agric Sci Tech 10:337–344

Galili G, Amir R, Hoefgen R, Hesse H (2005) Improving the levels of essential amino acids and sulfur metabolites in plants. Biol Chem 386:817–831

Hughes EH, Hong SB, Gibson SI, Shanks JV, San KY (2004) Expression of a feedback-resistant anthranilate synthase in *Catharanthus roseus* hairy roots provides evidence for tight regulation of terpenoid indole alkaloid levels. Biotechnol Bioeng 86:718–727

Ikeda M (2006) Towards bacterial strains overproducing L-tryptophan and other aromatics by metabolic engineering. Appl Microb Biotechnol 69:615–626

Ishihara A, Matsuda F, Miyagawa H, Wakas K (2007) Metabolomics for metabolically manipulated plants: effects of tryptophan overproduction. Metabolomics 3:319–334

Kalia RK, Rai MK, Kalia S, Singh R, Dhawan AK (2011) Microsatellite markers: an overview of the recent progress in plants. Euphytica 177:309–334

Khanuja SPS, Shasany AK, Darokar MP, Kumar S (1999) Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. Plant Mol Biol Rep 17:1–7

Kim DS, Lee IS, Jang CS, Hyun DY, Seo YW, Lee YI (2004) Selection of 5-methyltryptophan resistant rice mutants from irradiated calli derived from embryos. Euphytica 135:9–19

Kim DS, jang CS, Kim JB, Lee GJ, Kang SY, Kim W, Seo YW (2009) Identification of differentially expressed genes associated with 5-methyltryptophan resistance in rice mutants. Biol Plant 53:444–450

Lee HY, Kameya T (1991) Selection and characterization of a rice mutant resistant to 5-methyltryptophan. Theor Appl Gene 82:405–408

Li J, Last RL (1996) The *Arabidopsis* trp5 mutant has a feedback resistant anthranilate synthase and elevated soluble tryptophan. Plant Physiol 110:51–59

Mohanty S, Parida R, Singh S, Joshi RK, Subudhi E, Nayak S (2011) Biochemical and molecular profiling of micropropagated and conventionally grown *Kaempferia galangal*. PCTOC 106-39-46

Molchan O, Romashko S, Yurin V (2012) L-tryptophan decarboxylase activity and tryptamine accumulation in callus cultures of *Vinca minor* L. PCTOC 108:535–539

Moreno PRH, Van der Heijden R, Verpoorte R (1993) Effect of terpenoid precursor feeding and elicitation on formation of indole alkaloids in cell suspension cultures of *Catharanthus roseus*. Plant Cell Rep 12:702–705

Morgan JA, Shanks JV (2000) Determination of metabolic rate-limitations by precursor feeding in *Catharanthus roseus* hairy root cultures. J Biotechnol 79:137–145

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plant 15:473–497

Negrutiu IC-RA, Verbruggen I, Jacobs M (1984) Lysine overproducer mutants with an altered dihydro-dipicolinate synthase from protoplast culture of *Nicotiana sylvestris* (Spegazzini and Comes). Theor Appl Gene 68:11–20

Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. PNAS 76:5269–5273

Omidbaigi R (2005) Production and Processing of Medicinal Plants. Vol 2, Behnashr Publ. Iran, pp 438

Radwanski ER, Last RL (1995) Tryptophan biosynthesis and metabolism: biochemical and molecular genetics. Plant Cell 7:921–934

Reddy MP, Sarla N, Siddiq EA (2002) Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica 128:9–17

Sabry SM, Belal TS, Barary MH, Ibrahim MEAL (2010) A validated HPLC method for the simultaneous determination of vincamine and its potential degradant (metabolite), vincaminic acid: applications to pharmaceutical formulations and pharmacokinetic studies. J Food Drug Anal 18:447–457

Seth R, Mathur AK (2005) Selection of 5-methyltryptophan-resistant callus lines with improved metabolic flux towards terpenoid indole alkaloid synthesis in *Catharanthus roseus*. Curr Sci 89:554–557

Tanaka N, Takao M, Matsumoto T (1995) Vincamine production in multiple shoot culture derived from hairy roots of *Vinca minor*. PCTOC 41:61–64

Tozawa Y, Hasegawa H, Terakawa T, Wasaka K (2001) Characterization of rice anthranilate synthase α- subunit genes OASA1 and OASA2, Tryptophan accumulation in transgenic rice expressing feedback-insensitive mutant of OASA1. Plant Physiol 126:1493–1506

Verma P, Mathur AK, Masood N, Luqman S, Shankar K (2012) Tryptophan over producing cell suspensions of *Catharanthus roseus* (L) G. Don and their up-scaling in stirred tank bioreactor: detection of phenolic compound and antioxidant potential. Protoplasma. doi:10.1007/s00709-012-0423-5

Wasaka K, Widholm JM (1987) A 5-methyltryptophan resistant rice mutant MTR1, selected in tissue culture. Theor Appl Genet 74:49–54

Whitmer S, Canel C, Hallard D, Goncalves C, Verpoorte R (1998) Influence of precursor availability on alkaloid accumulation by transgenic cell line of *Catharanthus roseus*. Plant Physiol 116:853–857

Whitmer S, Van der Heijden R, Verpoorte R (2002a) Effect of precursor feeding on alkaloid accumulation by a strictosidine synthase over-expressing transgenic cell line S1 of *Catharanthus roseus*. PCTOC 69:85–93

Whitmer S, Van der Heijden R, Verpoorte R (2002b) Effect of precursor feeding on alkaloid accumulation by a tryptophan decarboxylase overexpressing transgenic cell line T22 of *Catharanthus roseus*. J Biotechnol 96:193–203

Zietkiewicz E, Rafalski JA, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20:176–183