Over-Expression of Catharanthus roseus Apoplastic Peroxidase (CrPrx) Gene Associated with Late Downstream Steps of TIAs Pathway at whole plant level.

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Research Article

Keywords: Agrobacterium rhizogene, Catharanthus roseus, Apoplastic peroxidase gene (CrPrx), transgenic plants, Vincristine (VCR), Vinbastine (VLB)

Posted Date: January 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1089930/v1

Over-Expression of *Catharanthus roseus* Apoplastic Peroxidase (*CrPrx*) Gene Associated with Late Downstream Steps of TIAs Pathway at whole plant level.

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Abstract

Agrobacterium rhizogenes mediated genetic transformation for hairy root production was carried out to explore the possibility of recovering transgenic plants from hairy roots with over expressed limiting pathway Apoplastic peroxidase gene (*CrPrx*). For generating (*CrPrx*) over-expressing hairy roots, the leaves of *C. roseus* cv. Dhawal culture were infected with *A. rhizogenes* strain A_4 harboring Ri plasmid and a pBI 121-*CrPrx* construct. 15 independent roots clones finally established were christened as NV-C1 to NV-C15 clones. Maximum expressions of genes (RQ=8.23 ± 0.23) was observed in root lines NV-C7 followed by NV-C6(RQ=6.44 ± 0.21) and NV-C11(RQ=5.59 ± 0.30). The direct regeneration of shoots from hairy root explants co-cultured with *A.rhizogenes* resulted in integration of *CrPrx* gene in the regenerated progeny. Nearly 78.3 % root explants formed adventitious shoot buds on to an optimized regeneration medium (1.0 m/l NAA+5.0 mg/l BAP). *CrPrx* overexpressing tisssues registered 2.00-2.54 higher level of mRNA transcripted strictosidine synthase (STR), tryptophan decarboxylase (TDC) , 0.8 and 0.75 fold lesser level of *CrPrx*1 in NV-C7 root tissues as compare to their non-transformed controls. HPLC analysis revealed that *CrPrx* overexpressing NV-C7 roots had 3 folds more ajmalicine and serpentine content over parent NVST roots comparatively for Catharanthine, this enhancement was 2 folds, while shoots regenerated from NV-C7 and NVST showed ajmalicine accumulation elevated by a factor of 2.5 as well as also favored the near doubling of vindoline content which in turn resulted in better vincristine and vinblastine content. NV-C7 root line and its respective shoot progeny is promising tissue in terms of TIA's productivity at the whole plant level.

Keywords *Agrobacterium rhizogene* · *Catharanthus roseus* · Apoplastic peroxidase gene (*CrPrx*) · transgenic plants· Vincristine (VCR) · Vinbastine (VLB).

Introduction

Madagascar periwinkle, *Catharanthus roseus* is widely exploited for studies as a model of medicinal plant for many kinds of terpenoid indole alkaloids (TIAs). *C. roseus* is the best characterized sole source of two of the most valuable anticancer terpenoid indole alkaloids (TIAs), vinblastine and vincristine and their precursors, vindoline and catharanthine. *C. roseus* also produces monomeric alkaloids such as ajmalicine and serpentine in its roots that are also widely prescribed drugs for antihypertensive and sedative action, respectively (Mc Cormack, 1990; Jordan et al 1991; Verpoorte et al 1997, 2002; Duarte and Cooper-De Hoff 2010; Verma et al 2012, 2015). Several lines of evidence indicate that vacuolar class III plant peroxidases may be responsible for oxidation of ajmalicine to serpentine and for coupling of vindoline and catharanthine to a-3', 4'-anhydrovinblastine. In addition to the epidermis, idioblasts and laticifers may deposit catharanthine, as well as ajmalicine and its oxidation product serpentine, according to a recent study (Yamamoto et al., 2019). These bioactive TIAs have extremely low in planta yields (0.0001-0.0005 percent leaf dry weight), which makes extraction laborious and expensive.The current market price of these dimmers is 30-35 million dollars per Kg (Rs. 520 thousands/gram, as per Sigma-Aldrich catalog 2016). The adamant developmental, environmental, temporal and spatial regulatory mechanisms in plants of family Apocynaceae owes low TIAs accumulation (Verma et al., 2012b, 2017; Tatsis and O'Connor, 2016) and increasing their in *planta* is not always feasible because the accumulation of these compounds beyond certain limits might become detrimental to the plant (Benedito and Modolo, 2013). This emphasises the critical importance of having trustworthy alternatives to ensure the consistent manufacturing of key pharmaceuticals at reasonable rates around the world. The TIAs pathway is one of the most studied plant metabolic domains in the field of pathway modification research due to the commercial interest and increased stake. As a result, it's not surprising that the majority of TIAs are biosynthesized in response to a plant's needs, in levels just enough to complete an ecological purpose (Verma et al., 2015b, 2017).

With advanced knowledge of transcriptomics, molecular biology, metabolic engineering, and bioinformatics, 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, Verma et al 2017). The construction of a robust and efficient alternative production platform in *C. roseus* hairy roots by manipulating the TIA pathway has become a promising strategy in recent years (Sun and Peebles 2016, Pacurar et al., 2011). The root system is an unique plant organ, which presents several remarkable attributes such as easy in- vitro establishment, inherent genetic stability, fast metabolic activity , elevated growth rates, and amenable to genetically transform with Agrobacterium rhizogenes.

In this study, the aim was firstly, to push the lower pathway gene of metabolic route of TIAs pathway in C. roseus transformed hairy root tissues for higher accumulation of root specific alkaloids such as ajmalicine and /or serpentine. Secondly, to develop stable in-vitro whole plant level regeneration system with altered alkaloids spectra. The result demonstrates that overexpressing *CrPrx* gene in hairy roots of *C. roseus* is able to enhance flavonoids accumulation at whole plant level. Infact an outstanding observation was an increased accumulation of vincristine and vinblastine in backdrop of lower expression level of *CrPrx1* gene in *Crprx* over-expressing tissues. The following outcome might have been a consequence of positive global influence of *CrPrx* gene on TIAs pathway genes other than *CrPrx1* which is directly linked with dimerization step of their synthesis.

Material and methods

Plasmid construction

These experiments were carried out only in cv. Dhawal genotype of *C. roseus*. For *CrPrx* over-expression studies, hairy roots were induced by agro-infection of leaf explants with A_4 strain of *A. rhizogenes* carrying the *CrPrx* gene construct. This gene construct was kindly gifted to us by Dr. Alok Krishna Sinha, National Institute of Plant Genome Research (NIPGR), New Delhi. The *CrPrx* (Accession No. AY924306; Kumar et al 2007) belongs to class III family of basic peroxidases whose transcript was found to be induced by MeJ in *C. roseus* with concurrent up- regulation of several TIAs pathway genes. According to Dr. Sinha's group *CrPrx* is apoplastic in nature and has a ubiquitous expression pattern in all tissues except in younger leaves of *C. roseus* plant. The *CrPrx* over-expression construct was produced by transferring *C. roseus CrPrx* cDNA from

pGEM-T easy (Promega, Madison, WI) cloning vector to a pBI121 binary vector by PCR amplification using gene-specific primers with targeted restriction sites (Jaggi et al 2011). The resulting vector pBI121-*CrPrx* has a *CaMV35S* promoter-driven expression of *CrPrx* cDNA. The construct was then mobilized first into E. coli and then to *A4* strain of *A. rhizogenes* for using in the present study. The freeze thaw method was used for the mobilization of the construct .The map of *CrPrx* construct is shown in (Supplementary material Appendix 1, Fig. A1)

Induction and selection of CrPrx over-expressing hairy root lines

Leaf explants obtained from 6 weeks-old multiple shoots cultures of *C. roseus* cv. Dhawal were transformed with *CrPrx*-harboring *A.rhizogenes* A4 strain. This bacterial strain was raised in liquid YEB (Yeast Extract Peptone) medium consisted of 10.0 mg/l bactopeptone, 5.0 mg/l Nacl, 10.0 mg/l yeast extract in 15 ml Falcon tube. The medium was further supplemented with 50 mg/l kanamycin and the tube was placed on a gyratory shaker (180 rpm) at 28^{o}C for incubation. Twenty four to 120 hrs old bacterial cultures in their exponential phase of growth with OD values greater than 1.0 at 660 nm were used for infecting the explants. Infection was made by wounding the explants with sterile needle dipped in the bacterial suspension. Untreated explants served as controls. The treated explants were blot dried and placed onto a semi solid hormone-free MS medium for incubation under dark. The co-cultivation duration varied from 24 to 144 hrs. After co-cultivation and subsequent elimination of bacteria on a 500 mg/l ampicillin and 500 mg/l cephalexin containing medium, the explants were incubated on a hormone-free MS medium for root induction. A total of 100 individual root clones were recovered. Out of these, 15 clones could be further multiplied in 1/4th B5 liquid medium on a shaker (95 rpm) at 24 ± 2ºC under ,diffused light through a 6 week culture cycle.These clones were regularly sub-cultured after every 5-6 weeks for biomass build up and further characterization.

Characterization of CrPrx over-expressing hairy root lines

The 15 root clones (designated as NV-C1 to NV-C15) were characterized in terms of relative expression of *CrPrx* by PCR and RT-PCR analysis. One of the root clone (NV-C7) that showed highest expression of *CrPrx* gene, was also checked for its plant regeneration potential. This root clone and the plants regenerated from it along with their non-transformed counterparts were then subjected to RT-PCR analysis to compare the relative expression of three other genes of TIAs pathway namely tryptophan decarboxylase (*tdc*), strictosidine synthase (*str*) and vacuolar peroxidase (*CrPrx1*). The transformed and non-transformed roots and plant regenerants were also compared for their alkaloid profiles through HPLC quantification of ajmalicine, serpentine, vindoline, catharanthine, vincristine and vinblastine. These tissues were also compared with respect to their peroxidase activity by biochemical assay.

DNA extraction and PCR analysis

For confirming the presence or absence of *CrPrx* gene in 15 putative transformed root clones, total genomic DNA was extracted from each of them. Non-transformed roots of multiple shoot culture-derived plantlets were used as control. For DNA extraction, method described by Dellaporta et al (1983) was used with minor modifications. Briefly, 1.0 g of root tissue was powdered in liquid nitrogen. The homogenized tissue was then transferred to centrifuge tube containing 15 ml extraction buffer (100mM Tris–HCl; 50mM EDTA; 500mM NaCl; 10mM β-mercaptoethanol; pH 8.0). After thorough mixing, 20% SDS was added to this followed by incubation at 65ºC for 10 min.5.0 ml of 5M potassium acetate was then added to each tube, mixed well and incubated on ice for 20 min. The tubes were then centrifuged at 13,000 rpm at 4ºC for 20 min. The supernatant was collected in fresh SS-34 tubes and DNA was precipitated using 10 ml of isopropanol, followed by incubation at -20ºC for 30 min. DNA was pelleted by spinning the tube at 12,000 rpm for 15 min at 4ºC. The pellet was washed with 70% ethanol, air dried and suspended in 700µl of re-suspension buffer (50mM Tris-HCL and 10mM EDTA; pH 8.0).After dissolution, the solution was again centrifuged at 10,000 rpm at 4ºC for 10 min. The pellet was washed twice with 70% ethanol, air dried and resuspended in appropriate volume of 1X TAE buffer prepared by mixing 10mM Tris-HCL and 1.0mM

EDTA, pH 8.0). To remove RNA contamination, 5µl of RNaseA was added and incubated at 37ºC for 30 min. The mixture was then extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) by centrifugation at 10,000 rpm for 5 min. The aqueous layer was transferred to a fresh microcentrifuge tube and to this 1 vol of 3M sodium acetate (pH 5.2) and 2.0 vol of ethanol were added. The contents were centrifuged at 13,000 rpm and 4ºC for 10 min to obtain DNA pellet. The DNA was washed twice with 70% ethanol, air dried and finally resuspended in appropriate volume of 10mM Tris-HCL (pH 8.0). Plasmid DNA from *A. rhizogenes*(A4) strain was isolated by the alkaline lysis method. To verify the presence of the transgene in the hairy root clones, PCR of genomic DNA was carried out using pBI121 vector backbone-specific forward primer (PBIF) and gene-specific reverse primer (PBIPR). Details of these primer sequences are given in (Supplementary material Appendix 1, Table A1). The reaction was carried out in total volume of 25 µl containing 25-100 ng of template DNA, 2.5 µl of 10X PCR buffer, 1.0µl of $MgCl_2$ solution (50mM), 0.5 µl of Ampli Taq polymerase, 1.0 µl of 10mM dNTP mix, 10µM each of forward and reverse primers. Volume of the reaction mixture was made to 25µl with milliQ water. A thermal cycler (Gene Amp PCR system 1000^{TM}, BIO- RAD) was programmed for PCR run for 30 cycles of 94ºC for 4 min, 94ºC for 30 sec, 55ºC for 30 sec, 72ºC for 3 min and after 30 cycle, a final extension of 72ºC for 7 min and hold at 4ºC was given. The PCR products were electrophoresed in 1% (w/v) agarose gels and visualized using a Gel Documentation system (Protein Simple, Santa Clara, CA) after ethidium bromide staining.

Quantitative reverse transcription PCR (qrt-PCR) for CrPrx gene expression in hairy root lines

To investigate the transcriptional regulation of *CrPrx* in overexpressed hairy root lines, its RT-expression level was measured using qRT-PCR. The transgenic root lines and control root were harvested after 4 weeks of subculturing for extraction of total RNA using RNAiso Plus kit (Takara, Japan). High–capacity cDNA reverse transcription kit (Applied Biosystems, USA) was used to synthesize cDNA from the total RNA. The qRT-PCR was performed on Step One™ Real-Time PCR system (Applied Biosystems, Foster

City, California, USA).In each reaction, 2 µl of diluted cDNA was used as a template for amplification. The sample was added to 18 µl of reaction mixture containing 6.2 µl of sterile MQwater, 10 µl of Power SYBR green PCR master mix (Applied Biosystems, USA), and 0.9 µl of each forward and reverse 20 pmol of primers.The PCR conditions were kept as 95°C for 10 min, 95°C for 15 sec, 60°C for 1 min for 40 cycles, 95°C for 15 sec, and 60°C for 1 min. All reactions were performed with 3 biological replicates and 3 technical replicates of each biological replicate. The relative gene expression was determined based on the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). All real time PCR quantifications were performed with *Actin* as endogenous control and a non-template control as used in several previous studies. Real time transcript analysis was also carried out in hairy root line NV-C7 and plants regenerated from them (along with their non-transformed counterparts) to measure the relative expression of 3 other TIAs pathway enzymes namely tryptophan decarboxylase (*TDC*), strictosidine synthase (*STR*) and peroxidase (*CrPrx 1*). Details of primers pertaining to the study are provided in (Supplementary material Appendix 1, Table B1).

Determination of peroxidase activity

Hairy roots, transgenic shoots, non-transformed roots and shoots (200 mg each) were homogenized in 2 ml of 0.2M potassium phosphate buffer (pH 7.8 with 0.1mM EDTA). The homogenates were centrifuged at 15,000×g for 20 min at 4°C to extract total peroxidases. Enzyme activity was measured colorimetrically according to the method of Lin and Kao (1999). For this, 10µl of enzyme extract supplemented in 290 µl assay medium constituted 0.05M potassium phosphate buffer (pH 5.8), 7.2mM guaiacol and 11.8 mM H_2O_2. The change in absorbance was monitored at 470 nm for calculating peroxidase activity in a sample.

Change in $A_{470} = A_f - A_i$

A_f = final absorbance

A_i = initial absorbance

Activity was calculated by using the extinction coefficient (26.6 m/M/cm at 470 nm) for tetraguiaicol. One unit of peroxidase was defined as the amount of enzyme that resulted in the formation of 1µmol tetraguaiacol per min.

205 **Alkaloid extraction, HPLC detection**

206 The effects of *CrPrx* overexpression on TIAs productivity in the NV-C7 hairy roots and
207 plants regenerated from them were evaluated by measuring the concentration of total
208 alkaloids, two root-specific alkaloids: ajmalicine and serpentine and from leaf-specific
209 alkaloids: catharanthine, vindoline, vincristine and vinblastine. All tissues were harvested
210 after 4 weeks of subculturing and dried at 50℃ in an oven. 1.0g oven-dried (50–60°C)
211 samples were then processed for alkaloid extraction thrice with HPLC grade methanol
212 (3×30 ml; 12 h each) at room temperature. The MeOH extracts were pooled and dried in
213 vacuum to 10 ml, mixed with 10 ml dH_2O, acidified with10 ml of 3 % HCl, and washed
214 thrice with hexane (3×30 ml). The aqueous portion was basified with ammonia (till pH
215 8.0), extracted with chloroform (3×30 ml), dried over anhydrous sodium sulfate,
216 concentrated in vacuum, and weighed. For HPLC resolution and quantification, a
217 modular HPLC apparatus (Waters Corp., Milford, MA, USA) equipped with a 600E
218 multi-solvent delivery system and a 2,996 photodiode array (PDA) detector was used.
219 Data obtained were processed using Empower Pro (Waters Corp.) chromatographic
220 software. The injector, solvent delivery controller and Empower Pro chromatographic
221 manager were integrated to provide precise and reproducible results. On- line degassing
222 of solvents was done with helium. The Reverse-phase(RP-18e) chromolith performance
223 HPLC column was used for the analysis. An optimum mobile phase composition was
224 achieved by using 21: 79 (v/v) acetonitrile: 0.1 M phosphate buffer acidified with 0.5%
225 glacial acetic acid (pH 3.5). The other conditions employed were pH 3.5, flow rate of 1.0
226 ml/min, and detector wavelength at 254 nm. The injection volume was 10µl. Standard
227 mixture of vindoline, catharanthine, vincristine, vinblastine, ajmalicine and serpentine
228 (1mg/ml in methanol) was made. Each run took 35 min.

229 **Statistical analysis**

230 The statistical differences between the treatments were evaluated by one-way analysis of
231 variance accompanied with Duncan test, two-way analysis of variance (ANOVA) using
232 the Assistat Software Version 7.7 . Values at $P < 0.01$ and $P < 0.05$ were considered
233 statistically significant.

Results

Over-Expression of *CrPrx* Gene Construct in Transformed Hairy Roots

For generating *CrPrx* over expressing hairy roots, the leaves of cv Dhawal cultivar of *C. roseus* were infected with *A. rhizogenes* strain A_4 harboring the Ri plasmid and a pBI121-*CrPrx* construct (courtesy: Professor Alok K Sinha, NIPGR, New Delhi). A total of 100 independent transformation events were induced. Out of these, 15 independent root clones could finally be established in $1/4^{th}$ strength B_5 liquid medium. They were christened as NVC1 to NVC15 clones. Presence of *CrPrx* gene was successfully detected in 12 hairy root clones by PCR analysis of genomic DNA using vector backbone specific reverse primer (Fig.1A). Root lines designated as NVC4, NVC9 and NVC10 did not show its presence.

Expression level of *CrPrx* in the root clones was compared using qRT- PCR (Fig. 1 B). data suggested that 10 of the 12 transgenic lines showed increased *CrPrx* transcript level when compared with non-transformed roots. Transcript level in root clones NVC1 and NVC13 were at par with that of non-transformed roots. Maximum expression of the gene (RQ=8.23 ± 0.23) was observed in root line NV-C7 followed by NV-C6 (RQ=6.44±0.21) and NV-C11 (RQ=5.59± 0.30). All other transgenic lines showed 1.43 to 4.0 folds increase in transcript levels of *CrPrx* in comparison to that of control. Based on these results, line NV-C7 was further advance to biochemical characterization and plantlet regeneration studies.

Regeneration of transgenic plants from NV-C7 hairy root clone

Approximately 2–3 cm root segments excised from the *CrPrx* over-expressing hairy root line NV-C7 when transferred onto a regeneration medium (1.0 mg/l NAA + 5.0 mg/l BAP) that was optimized in my earlier experiments, showed appearance of green protuberances near the root tips within two weeks of culturing . These protuberances assumed the shape of organized shoot buds in another two weeks' time (Fig. 2). On the contrary, hairy roots in growth regulator-free MS medium for 4 weeks continued to elongate but formed no lateral roots. Nearly 78.3% root explants formed adventitious shoot buds on this NAA/BAP combination medium. The regenerated buds upon shifting

to MS basal elongated into micro-shoots. Rooting in these micro-shoots occurred when they were transferred to a ½ MS+ 5.0 mg/l IBA medium. Plantlets thus, derived from hairy roots exhibited prolific rooting and had shorter internodes when transplanted to potted soil.

Detection of peroxidase activity in *CrPrx* expressing NV-C7 roots and shoots

It is necessary to highlight here again that Class III peroxidases like *CrPrx* are multifunctional enzymes that catalyze the oxidation of small molecules at the expense of H2O2. In general, peroxidases are known to regulate H2O2 metabolism and hence, play an important role in the regulation of plant responses to a range of endogenous signals/stimuli, environmental stresses and secondary metabolic needs of a cell. H2O2 also serve as a signal for the induction of defense mechanism or can be used in peroxidase catalyzed cross-linking reactions to reinforce the cell wall.

In case of *C. roseus*, earlier studies have indicated that while expression of a peroxidase gene *CrPrx1* is directly associated with the dimerization steps of vindoline and catharanthine in the cell vacuole in the leaves, the expression of ubiquitously present *CrPrx* gene is indirectly linked with hyperexpression of several other TIAs pathway genes that are also elicited by H2O2. Keeping these considerations in mind, an attempt was made in this study to determine whether 35S-*CrPrx* over-expressing hairy root line NV-C7 and the transgenic shoots regenerated from it had increased peroxidase activity or not (Fig. 3).

It was observed that *CrPrx* over-expressing root line and shoots recovered from it showed higher peroxidase activity when compared with non-transformed tissues. The root tissue of NV-C7 line and shoots regenerated from it recorded 1.60 and 1.22 fold higher peroxidase enzyme activity that was calculated as GPOX scavenging action in terms of increase in the absorbance of tetra-guaiacol at 470 nm.

Transcript analysis of other TIAs pathway genes in the back-drop of CrPrx over expression

The influence of *CrPrx* over-expression in NV-C7 hairy root line and the shoots recovered from it was also analyzed in terms of transcript expression of three other TIAs pathway genes namely Strictisidine synthase (*STR*), tryptophan decarboxylase (*TDC*) and *CrPrx1* by qRT-PCR (Fig. 4). *CrPrx* over-expressing tissues registered 2.0-2.5 folds

higher levels of mRNA transcripts of *STR* and *TDC* genes as compared to their non-transformed controls. Interestingly, the transcript expression of *CrPrx1* in NV-C7 tissues was 0.8 and 0.75 times less than in the corresponding controls.

The influence of *CrPrx* over-expression in NV-C7 roots and shoots was also studied in terms of their TIAs productivity profiles. For this, the *CrPrx* over-expressing roots and three shoot lines regenerated from it were analyzed for their alkaloid spectra after 5 weeks of growth. Ajmalicine, serpentine, vindoline, catharanthine, vincristine and vinblastine content in them was measured and compared with control hairy root line NVST and its shoot progeny obtained in earlier experiments done with wild type A4 strain without peroxidase gene construct (Table 1).

The *CrPrx* over-expressing NV-C7 roots recorded an impressive 3-folds increase in ajmalicine and serpentine content over NVST roots whereas for catharanthine the enhancement was two folds. While vindoline and vinblastine levels in the two types of transgenic roots did not differ much, vincristine content was nearly half in the NV-C7 roots. When shoots regenerated from NV-C7 and NVST lines were compared for TIAs spectrum, ajmalicine was again accumulated by a factor of 2.5 or more in the *CrPrx* over-expressing shoots but serpentine and catharanthine level in all the three *CrPrx* expressing shoot lines was nearly same (0.20-0.25 % dry wt.) and that did not differ much from what was present in the NVST root derived shoot (0.017 % dry wt.). *CrPrx* over-expression in shoots also favored the near doubling of vindoline content which in turn, also resulted in better vincristine accumulation in them. Two of the shoot cultures derived from NV-C7 root line also had 2-3 fold improvement in their vinblastine content. The increased accumulation of vincristine and vinblastine in the back-drop of lower expression level of *CrPrx1* gene in *CrPrx* over-expressing tissues as observed in my study might have been a consequence of positive global influence of the later on TIAs pathway genes other than the one (*CrPrx1*) that is directly linked with dimerization step of their synthesis.

The plant regeneration protocol developed in *A. rhizogenes* mediated study, was used for the production of transgenic over- expressing TIAs downstream pathway gene peroxidase(*CrPrx*). Maximum expression of the gene (RQ=8.23 ± 0.23) was observed in root line NV-C7. The root tissue of NV-C7 line and shoots regenerated from it recorded

1.60 and 1.22 fold higher peroxidase enzyme activity. *CrPrx* over-expressing tissues registered 2.0-2.5 folds higher levels of mRNA transcripts of *STR* and *TDC* genes as compared to their non-transformed controls. Interestingly, the transcript expression of *CrPrx1* in NV-C7 tissues was 0.8 and 0.75 times less than in the corresponding controls. The *CrPrx* over-expressing NV-C7 roots recorded an impressive 3-folds increase in ajmalicine and serpentine content over NVST roots whereas for catharanthine the enhancement was two folds. While vindoline and vinblastine levels in the two types of transgenic roots did not differ much, vincristine content was nearly half in the NV-C7 roots. When shoots regenerated from NV-C7 and NVST lines were compared for TIAs spectrum, ajmalicine was again accumulated by a factor of 2.5 or more in the *CrPrx* over-expressing shoots but serpentine and catharanthine level in all the three *CrPrx* expressing shoot lines was nearly same (0.20-0.25 % dry wt.).

Discussion

The most common and effective strategy for increasing the metabolic flux of a pathway toward the synthesis of a target metabolite(s), as repeatedly emphasised in the literature, is to overcome rate limiting steps at the gene/enzyme level. This method might be used to over-express a limiting step, reduce branch point diversion of a common intermediate, or activate pathway-specific transcription factors/transporter proteins (Oksman- Caldentey and Saito 2005; Verpoorte et al 2007; Guirimand et al 2009; Yoon et al 2013; O'Connor 2015; Bahieldin et al 2016). Nonetheless, our limited understanding of the regulatory architectures of plant secondary metabolite biosynthesis pathways and their biochemical integration with other metabolic networks makes it difficult to predict the outcomes of gene manipulation attempts centred on a specific biogenetic stage. One intentional genetic change in a pathway can result in many other limits in the same or other over-lapping paths (Qu et al 2015; Liu et al 2017). Since pathway gene expressions in medicinal crops are often closely linked with tissue differentiation, it is always desirable to assess the overall influence of a genetic alteration at the whole plant level. In this perspective, the direct plant regeneration methodology developed in the study for *A. rhizogenes*-mediated hairy root clone gains significance. The protocol was successfully utilized to generate and evaluate transgenic plants of *C. roseus* wherein downstream

pathway gene peroxidase (*CrPrx*) was over-expressed. The Over-expression of this apoplastic peroxidase was carried out for the first time in *C. roseus* research and sought answers for two important questions: (a) can over-expression of a *CrPrx* gene (which is associated with late oxidative steps of TIAs pathway in transformed hairy roots improve the production of root-specific alkaloids such as ajmalicine and/or serpentine?; (b) can transgenic plants regenerated from *CrPrx* over-expressing hairy roots show altered alkaloids spectrum at whole plant level? The obtained results clearly indicated that modulating the *CrPrx* levels had a significant impact on expression profiles of many other TIAs pathway genes like TDC (tryptophan decarboxylase), STR (strictosidine synthase) and vacuolar peroxidase (CrPrx1). Ten of the twelve transgenic root lines that showed CrPrx PCR expression registered increased but variable *CrPrx* transcript level when compared with non-transformed roots. Maximum expression of the gene (RQ=8.23 ± 0.23) was observed in root line NV-C7. All other transgenic lines showed 1.43 to 4.0 folds increase in transcript levels of *CrPrx* in comparison to that of control. Such variability is common in *Agrobacterium*-transformed cultures and is attributed to gene copy number and position effects (Rothstein et al 1987; van der Krol et al 1988). Looking into the potential of root line NV-C7, it was subjected to transgenic plant regeneration efforts. The roots upon shifting to 1.0 mg/l NAA + 5.0 mg/l BAP medium , showed appearance of green protuberances near the root tips and assumed the shape of organized shoot buds in another two weeks' time. Nearly 78.3% root explants formed adventitious shoot buds on this NAA/BAP combination medium. The regenerated buds upon shifting to MS basal elongated into micro-shoots. Rooting in these micro-shoots occurred when they were transferred to a ½ MS+ 5.0 mg/l IBA medium. *CrPrx* over-expressing shoots registered 2.0-2.5 folds higher levels of mRNA transcripts of *STR* and *TDC* genes as compared to their non-transformed controls. Interestingly, the transcript expression of *CrPrx1* in NV-C7 tissues was 0.8 and 0.75 times less than in the corresponding controls. Transcripts of *CrPrx1* are known to be absent or present with low levels in C. roseus hairy roots (Costa et al 2008) and might account for this observation. Peroxidase (POD) are associated with TIAs pathway in C. roseus either at the coupling steps of monomeric indole alkaloids, vindoline and catharanthine into vinblastine in the leaf tissue (*CrPrx1*) or during ajmalicine to serpentine conversion in roots (Blom et al

1991). The higher POD activity in *CrPrx* over-expressing root line and shoots indicated that it had a higher capacity for the decomposition of H_2O_2 which possibly triggers the signaling pathways to modulate the expression of TIA pathway genes which in turn resulted in the elevated levels of TIAs. The *CrPrx* over-expressing NV-C7 roots recorded an impressive 3-folds increase in ajmalicine and serpentine content over NVST roots whereas for catharanthine the enhancement was two folds. As expected, vindoline and vinblastine levels in the two types of transgenic roots did not differ much and vincristine content was nearly half in the NV-C7 roots. When shoots regenerated from NV-C7 and NVST lines were compared for TIAs spectrum, ajmalicine was again accumulated by a factor of 2.5 or more in the *CrPrx* over-expressing shoots but serpentine and catharanthine not differ much from what was present in the NVST root derived shoot (0.017 % dry wt.). *CrPrx* over-expression in shoots also favored the near doubling of vindoline content which in turn, also resulted in better vincristine accumulation in them. Two of the shoot cultures derived from NV-C7 root line also had 2-3 fold improvement in their vinblastine content. The increase accumulation of vincristine and vinblastine in the back-drop of lower expression level of *CrPrx1* gene in *CrPrx* over-expressing tissues as observed in my study might have been a consequence of positive global influence of the later on TIAs pathway genes other than the one (*CrPrx1*) that is directly linked with terminal dimerization step of the pathway. Another reason for increase in specific alkaloids might also be due to the induction of the native vacuolar peroxidase gene which catalyzes conversion of ajmalicine to serpentine as a result of stress signal modulation by *CrPrx* as proposed by Jaggi et al (2011).

Acknowledgements

The authors gratefully acknowledge the Council of Scientific and Industrial Research (CSIR), New Delhi, and Director, CSIR-CIMAP, Lucknow, for providing the facility and financial support to carry out this work. NV thanks the University Grants Commission, New Delhi, for awarding her the UGC-Rajiv Gandhi National Fellowship to conduct part of this investigation. We also thank Dr. Alok Krishna Sinha, National Institute of Plant Genome Research (NIPGR), New Delhi for providing the *35s-CrPrx-GUS-GFP* gene construct (Accession No. AY924306).

Ethical Statement

The presented work is the part of doctoral thesis of Dr. Neha Verma and have not been published or under consideration in any publication house.

Author Contributions

NV, AKM and PV designed the research.VG and LUR assisted in molecular analysis.RKV analyzed metabolic data and performed compound identification. SL assisted in biochemical analysis. NV wrote the manuscript with consent from all other authors.

Conflict of interest

All authors declare no conflict of interest.

Data availability statement

The present work have Supplementary data associated as Supplementary Appendix 1.

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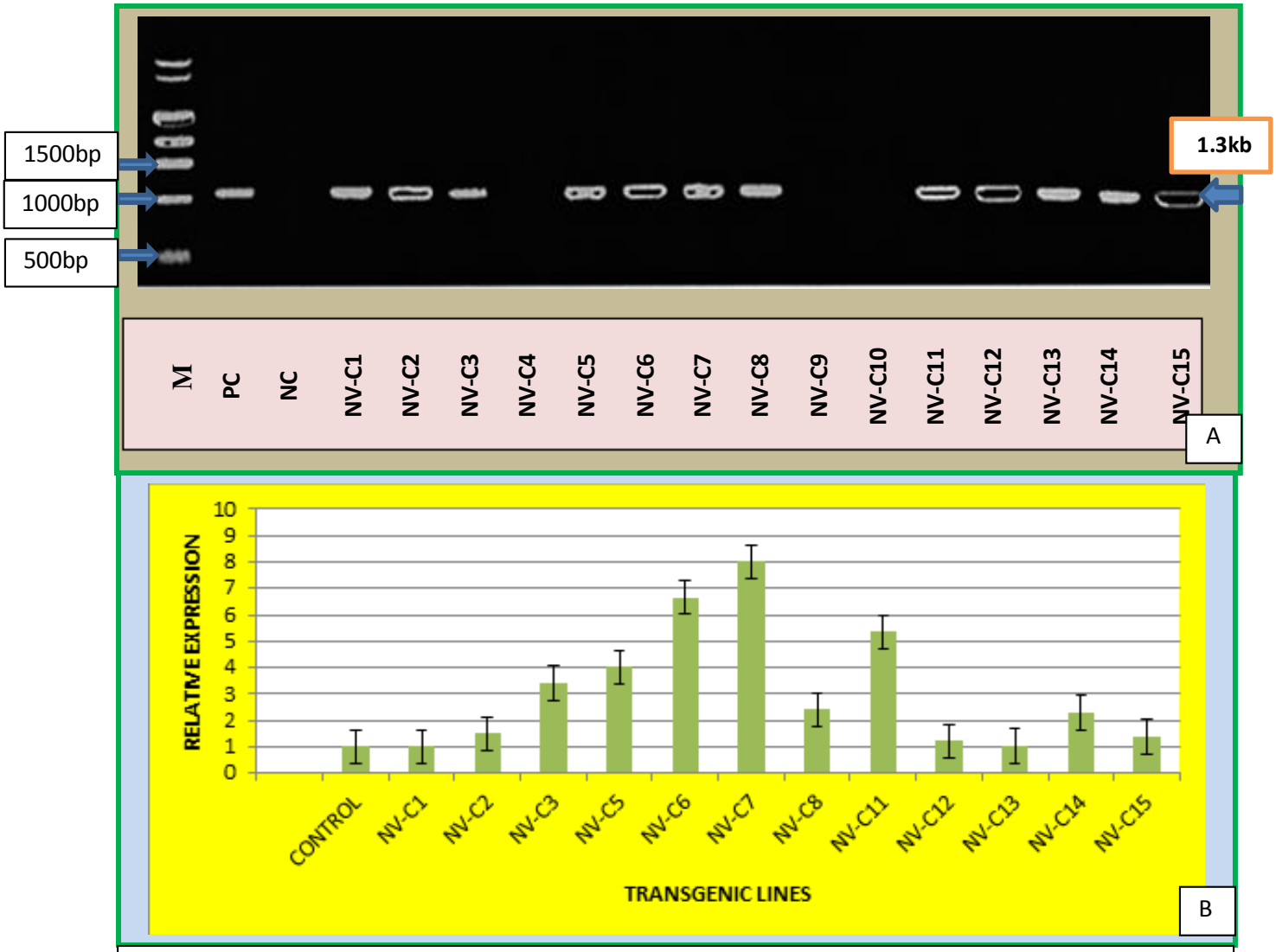

Fig. 1: A. PCR analysis of 15 hairy root clones (NVC1 to NVC15) generated through transformation with *A. rhizogenes* A_4 strain harboring the pBI121-*CrPrx* construct. PC-Positive control of plasmid DNA; NC-Negative control of genomic DNA of normal hairy root; M-Molecular marker
B. qRT- detection of *CrPrx* transcript level in 35s-*CrPrx* expressing hairy root lines of *C.roseus*

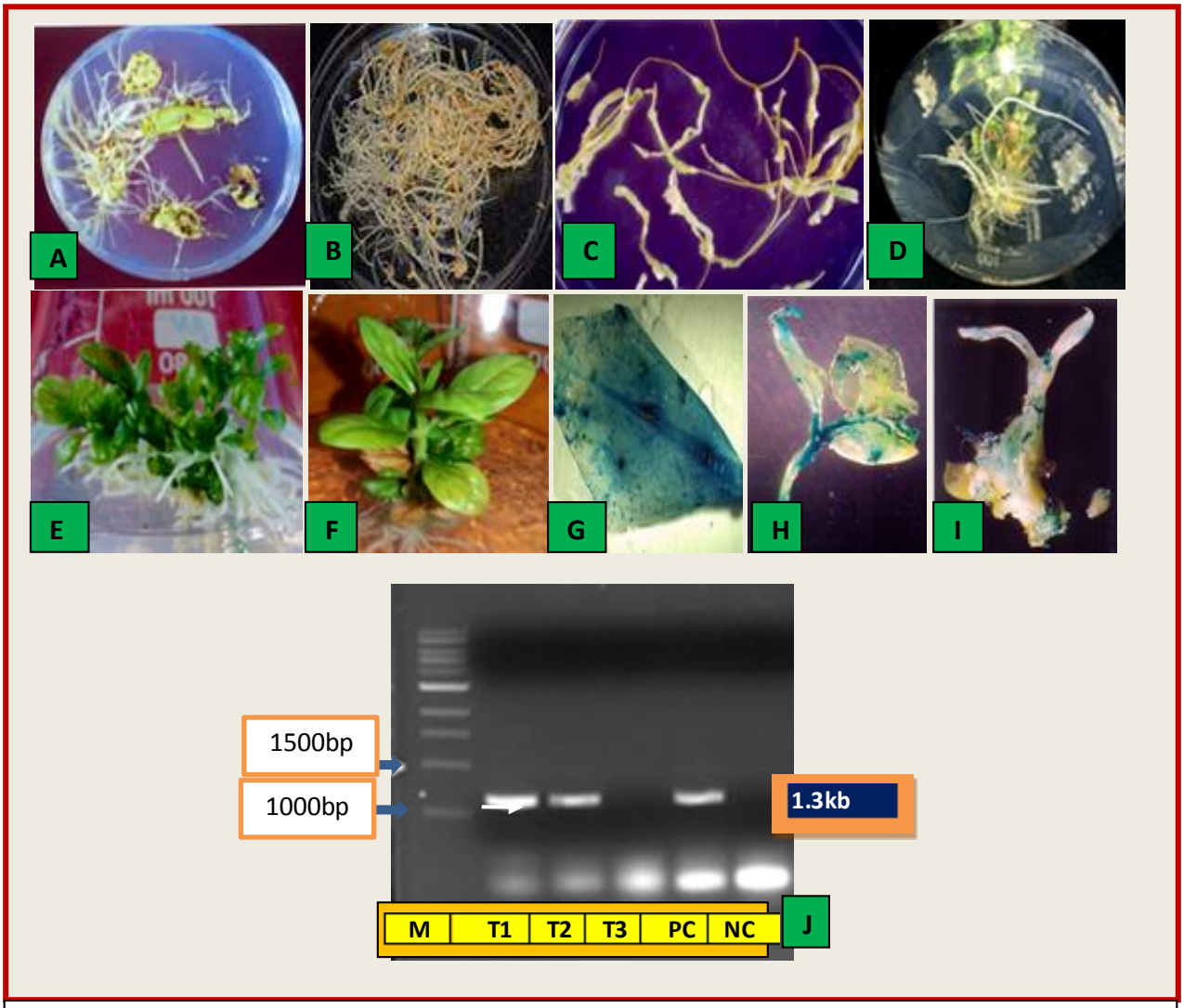

Fig. 2 Transgenic plant regeneration from *CrPrx* over-expressing NV-C7 hairy root line of *C. roseus*. A-Origin and B-*in vitro* maintenance of NV-C7 line in ¼ strength liquid B5 medium; (C-F) progressive stages of shoot bud, micro-shoot and plantlet formation; (G-I) *GUS* staining in regenerated shoots; J-*CrPrx* gene expression in transgenic progeny (M-Marker, PC- positive control, NC- negative control; T1, T2, T3– Randomly analyzed transformed plants .

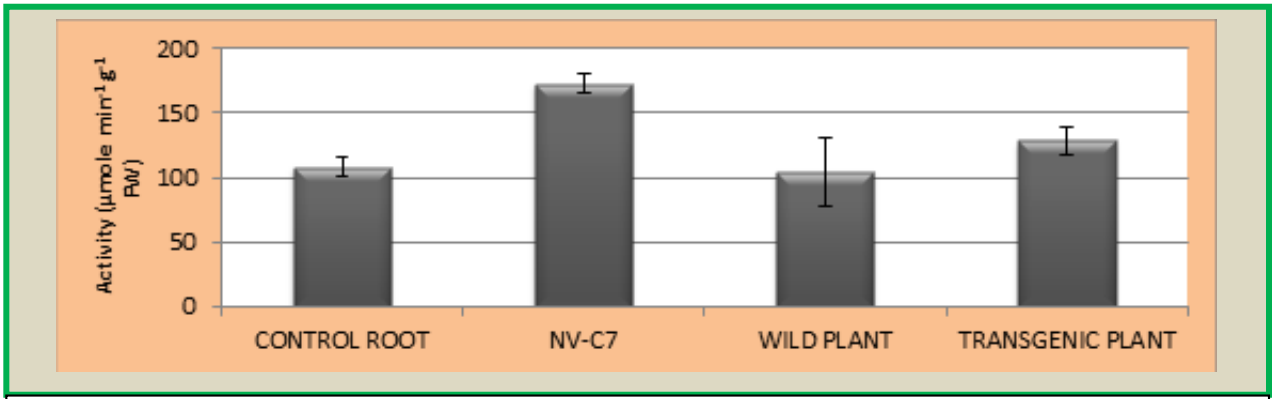

Fig. 3: Comparative peroxidase activity measurements in 35S-CrPrx over expressing NV-C7 hairy rots and plants against control tissues

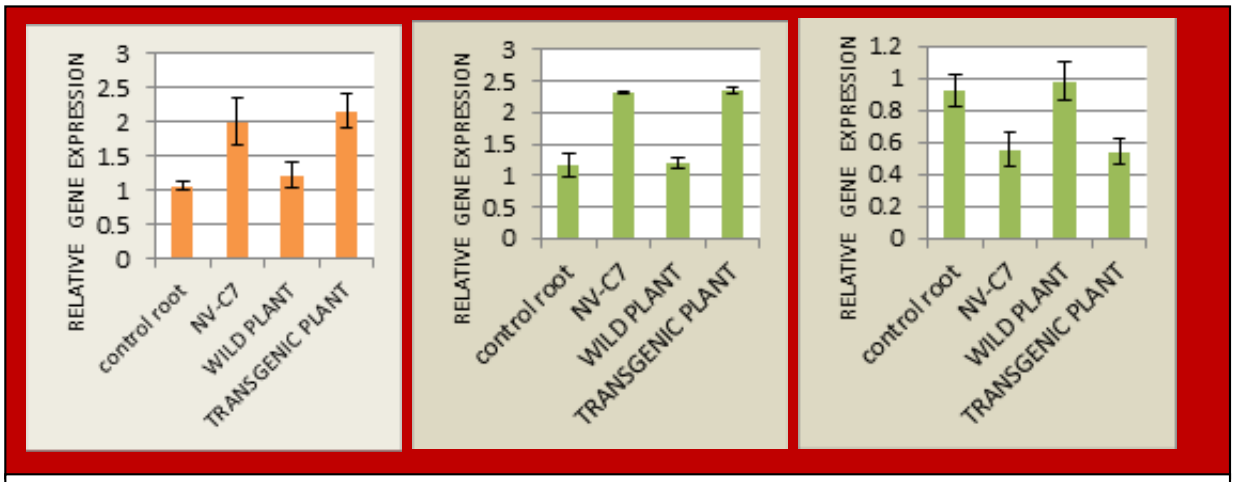

Fig. 4: qRT-PCR analyses of TIAs pathway genes *STR* (A), *TDC* (B) and *CrPrx1* (C) in *CrPrx* over-expressing NV-C7 root clone and the transgenic plants regenerated from it

Table 1: Comparative alkaloid profiles of *CrPrx* over-expressing hairy root line NV-C7and its regenerated shoots in comparison to *CrPrx* non-expressing NVST hairy root line and its regenerants

Tissue analyzed	Alkaloid content (% dry wt.)					
	AJM	SERP	CATH	VDL	VCR	VLB
NVST roots	0.142	0.083	0.090	0.030	0.015	0.024
***CrPrx* over-expressing NV-C7 roots**	0.482	0.232	0.207	0.031	0.006	0.026
NVST shoot	0.031	0.017	0.035	0.040	Trace	0.003
***CrPrx* over-expressing shoot (T1)**	0.089	0.024	0.042	0.071	0.003	0.006
***CrPrx* over-expressing shoot (T2)**	0.082	0.020	0.044	0.076	0.001	0.009
***CrPrx* over-expressing shoot (T3)**	0.090	0.025	0.046	0.084	0.003	0.005

AJM-Ajmalicine; SERP-Serpentine; CATH-Catharanthine; VDL-Vindoline; VCR-Vincristine; VLB-Vinblastine.

Supplementary Files

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