Expression analysis of *Cell wall invertase* under abiotic stress conditions influencing specialized metabolism in *Catharanthus roseus*

M. J. Nishanth¹, S. A. Sheshadri¹, Sudarshan Singh Rathore ², S. Srinidhi¹ & Bindu Simon¹

*Catharanthus roseus* is a commercial source for anti-cancer terpenoid indole alkaloids (TIAs: vincristine and vinblastine). Inherent levels of these TIAs are very low, hence research studies need to focus on enhancing their levels in planta. Since primary metabolism provides precursors for specialized-metabolism, elevating the former can achieve higher amounts of the latter. Cell Wall Invertase (CWIN), a key enzyme in sucrose-metabolism catalyses the breakdown of sucrose into glucose and fructose, which serve as carbon-skeleton for specialized-metabolites. Understanding CWIN regulation could unravel metabolic-engineering approaches towards enhancing the levels of TIAs in planta. Our study is the first to characterize CWIN at gene-expression level in the medicinal plant, *C. roseus*. The CWINs and their inter-relationship with sucrose and TIA metabolism was studied at gene and metabolite levels. It was found that sucrose-supplementation to *C. roseus* leaves significantly elevated the monomeric TIAs (vindoline, catharanthine) and their corresponding genes. This was further confirmed in cross-species, wherein *Nicotiana benthamiana* leaves transiently-overexpressing CrCWIN2 showed significant upregulation of specialized-metabolism genes: NbPAL2, Nb4CL, NbCHS, NbF3H, NbANS, NbHCT and NbG10H. The specialized metabolites- cinnamic acid, coumarin, and fisetin were significantly upregulated. Thus, the present study provides a valuable insight into metabolic-engineering approaches towards augmenting the levels of therapeutic TIAs.

Cell Wall Invertase (CWIN, EC: 3.2.1.26), a key enzyme in sucrose-metabolism catalyses the irreversible breakdown of sucrose into glucose and fructose. In addition, it also has several pleiotropic roles such as stress-response, sugar-signalling, flower, fruit and seed development. Besides, CWIN was also found to modulate specialized-metabolites levels in planta. Introduction of yeast CWIN into *Nicotiana tabacum* upregulated the levels of phenylpropanoids. Plant specialized-metabolites had been thought to be of little significance, but advancements in research have unravelled their physiological and therapeutic importance. Nearly 50,000 therapeutic specialized-metabolites have been identified in plants and characterized to date.

*Catharanthus roseus* (*C. roseus*) is a widely-known medicinal plant, used as the predominant source of the pharmaceutically-important specialized-metabolites, especially Terpenoid Indole Alkaloids (TIAs; vincristine and vinblastine are used in anticancer treatment). Dimerization of vindoline and catharanthine produces vinblastine in planta, which is further converted to vincristine. The monomeric precursors, vindoline and catharanthine are spatially separated in the plants (vindoline is localized in laticifers and idioblasts whereas catharanthine is secreted to the leaf surface and accumulates in the wax-exudates). Hence the production of vincristine and vinblastine is limited to trace amounts in planta. Owing to the inherent low-yields of the anti-cancerous TIAs, various biochemical and molecular studies have been conducted to unravel the specialized-metabolism and enhance TIA concentrations in *C. roseus*. Industrial production of vincristine and vinblastine is achieved by chemical coupling of more abundant monomeric precursors-vindoline and catharanthine. Therefore, increasing the yields of these precursors in planta, can be a plausible approach to obtain higher yields of the drugs via coupling process.

¹Phytoengineering Lab, School of Chemical and Biotechnology, SASTRA Deemed to be University, Thanjavur, Tamil Nadu, India. ²Actinomycetes Bioprospecting Lab, School of Chemical and Biotechnology, SASTRA Deemed to be University, Thanjavur, Tamil Nadu, India. M. J. Nishanth and S. A. Sheshadri contributed equally. Correspondence and requests for materials should be addressed to B.S. (email: bindusimon@scbt.sastra.edu)
Most studies have shown that primary and specialized-metabolisms are intimately interconnected, the former providing the precursors to the latter\[17\]-\[19\], but to date only a few attempts have been made towards understanding this interconnection, especially at the molecular level\[20\]. Sucrose and its hexose products (glucose and fructose) play important roles in both primary and specialized-metabolism. Besides acting as signalling molecules, they also provide carbon skeletons towards the production of specialized-metabolites\[21\]. The cross-talk between carbon and specialized-metabolisms has also been reported in glandular trichomes of tomato, wherein the energy and reducing power from photosynthesis are diverted towards specialized-metabolism, achieving high metabolic productivity\[22\]. Understanding the interplay between primary and specialized-metabolisms at molecular level involving the important genes and enzymes could unravel novel ways to enable manipulation of specialized-metabolites biosynthesis in planta. Despite the significant role of CWIN in primary and specialized-metabolism, as to our knowledge, so far no work has been done to understand CWIN regulation in any medicinal plants, including C. roseus.

As a part of our study, CWIN genes were identified in C. roseus genome and subjected to in-silico characterization, followed by tissue-specific expression analysis in the leaf, stem and roots. To understand the interrelationship between CWIN and major specialized-metabolism genes in C. roseus, a comparative expression analysis was performed for CWIN and other sucrose-metabolism genes (Sucrose Synthase, SUSY; Sucrose Phosphate Synthase, SPS), TIA pathway genes (Geraniol-10-Hydroxylase, G10H; Decacetylvinodoline-4-O-acetyltransferase, DAT; Secologanin synthase, SLS; Peroxidase, PRX1; 1-deoxoxyxylulose 5-phosphate synthase, DXS; Tryptophan Decarboxylase, TDC; STRICTOSIDINE synthase, STR), antioxidants and senescence-associated genes (Catalase, CAT; Superoxide dismutase, SOD and Senescence-associated gene, SAG) under different abiotic stress conditions. The gene-expression results were further supported by metabolite analysis (includes monomeric TIAs: vindoline, catharanthine; bis-indole SOD genes were identified in C. roseus CWIN isoforms; CRO_T000083 (CrCWIN1), CRO_T031716 (CrCWIN2), and CRO_T020329 (CrCWIN3). CrCWIN2 (CDS length: 1725bp; Genomic scaffold: cro_scaffold_3060381) had 7 exons and 6 introns whereas CrCWIN1 (CDS length: 1797bp; Genomic scaffold: cro_scaffold_3070386) and CrCWIN3 (CDS length: 1713bp; Genomic scaffold: cro_scaffold_3065222) were found to have 6 exons and 5 introns each. Previously characterized invertases from Agave tequilana\[20\], Populus trichocarpa\[21\], Sugarcane\[22\] and Cassava\[23\] have been shown to contain 6–8 exons. The genomic architecture of CrCWIN isoforms has been depicted in Fig. 1a.

The deduced amino acid sequences of CrCWIN1, CrCWIN2 and CrCWIN3 were predicted to contain 598 (67.8 kDa), 574 (65.0 kDa), 570 (64.9 kDa) amino acid residues. All the isoforms were predicted to localize in the cell wall. These findings have been summarized in Table 1. It is known that CWIN from Sugarcane, SoCIN1 encodes a protein 577 amino acids in length\[24\] and Arabidopsis thaliana CWIN with seven exons and six introns encodes 584 amino acids with mass 66.280kDa\[25\], thus highlighting the molecular similarities among CWINs from C. roseus and other plants.

CrCWIN1 and CrCWIN3 lack the ‘mini-exon’, generally present as the 9 bp long second exon in all the functional CWIN\[23\]-\[28\]. This exon encodes ‘DPN’, the tripeptide core of the beta-fructofuranosidase motif, ‘NDPNG’ (sucrose-binding box, directly involved in the catalysis of sucrose-cleavage\[23\]). Such “defective invertases” lacking the NDPNG motif are thought to be ubiquitous in plant kingdom and are commonly found in tobacco, rice, maize, potato, poplar and chicory. They are known to possess regulatory functions during pollen development\[29\]. The other two important catalytic sites, ‘WECP’ and ‘RDP’\[28\] were present in all the three isoforms (Fig. 1b). The Cys-residue of ‘WECP’, is a conserved feature of CWINs\[28\].

The evolutionary relationship among CWINs of C. roseus and other plant species was analysed via phylogenetic analysis (MEGA7). CrCWIN1 grouped with CWINs of L. esculentum and N. tabacum; CrCWIN2 was found to be closely related to C. canephora CWIN whereas CrCWIN3 was found to group with CWIN of H. annuus, with well supported bootstrap values (Fig. 1c).

**Tissue specific expression profiling of C. roseus CWIN isoforms.** The expression pattern of CrCWINs was analysed in leaf, stem and roots via qRT-PCR followed by LinReg PCR analysis. SAND was used as the internal reference gene\[28\]. The result, as shown in Fig. 2 depicts the mean relative expression levels of each isoform in these tissues. Overall, CrCWIN2 (the isoform containing all the catalytic sites) showed the highest expression, followed by CrCWIN3 and CrCWIN1. Highest transcript levels of CrCWIN2 were seen in roots (mean relative expression ratio: 11.18), followed by leaves (0.73) and stem (0.24). CrCWIN3 was found to have a similar trend wherein its highest expression was seen in roots (2.54), followed by leaves (0.51) and stem (0.166). High demand for hexoses in roots (sink tissues)\[23\]-\[31\] is a plausible reason for the high transcript levels. A similar trend was seen in carrot, wherein the acid invertase activity correlated with the utilization and storage of sugars in sink organs\[29\]. In comparison to other two isoforms, the expression of CrCWIN1 was found to be very minimal. Similar tissue-specific differential expression was also observed among maize CWINs wherein Incw3 showed varied expression while Incw4 was constitutively expressed\[22\].

**Stress mediated gene expression profiling in C. roseus.** TIA metabolism; specifically vindoline and catharanthine biosynthesis is known to be influenced by abiotic stresses\[31\]. Extensive research has been done towards understanding transcriptional responses of TIA biosynthesis genes under conditions influencing alkaloid metabolism\[32\]-\[34\]. Multiple RNA-Seq experiments have been conducted to understand transcriptomic-modulations...
under various conditions. Gongora-Castillo et al. generated C. roseus transcriptome sequence and expression profiles, wherein it was found that vinblastine biosynthesis genes were up-regulated in response to methyl jasmonate treatment. Sun et al. investigated the transcriptional responses to Anthranilate Synthase (AS) overexpression in transgenic C. roseus hairy roots. It was found that aromatic amino acid, fatty acid, glutathione and alpha-linolenic acid metabolism-genes were significantly up-regulated, whereas glycolysis/gluconeogenesis, amino and nucleotide sugar, starch-sucrose, cysteine-methionine and pyruvate-metabolism genes were downregulated, indicating the possible modulations in primary and specialized metabolic pathways due to AS overexpression. Liu et al. studied the transcriptomic responses of C. roseus to Peanut-Witches’-broom Phytoplasma-infection via transcriptome sequencing. It was found that many of the abiotic and biotic stimulus-related genes as well as photosynthesis, chloroplast development and energy metabolism genes were up-regulated, indicating at the dynamic changes in primary metabolism and stress related gene-expression. Van Moerkercke et al. constructed CathaCyc, a metabolic pathway database of C. roseus, based on RNA-Seq data. Though gene-expression studies have been conducted in C. roseus, the correlation between the expression patterns of CWINs and TIA biosynthesis genes has not been investigated.

In the present study, C. roseus leaves were subjected to cold, drought, salinity, UV radiation, wounding and also exogenous sucrose treatment. The expression pattern of major genes involved in TIAs biosynthesis was monitored. Also, to study the simultaneous effect on other metabolic pathways, carbohydrate, phenylpropanoid metabolism and antioxidants/growth-associated genes were analysed. All the expression ratios have been depicted in Fig. 3 and the statistically significant (P < 0.05) results are detailed below.

**Cold stress.** As shown in Fig. 3a, cold stress resulted in the upregulation of sucrose-metabolizing genes (CrCWIN1, CrCWIN3 and SUSY), whereas SPS was downregulated. Sugars such as glucose, fructose, sucrose, raffinose and stachyose are well-known cryoprotectants, mainly involved in protecting cell-membrane integrity by reducing freeze-induced dehydration. Cold-responsive upregulation of SUSY and CWIN isoforms has been previously documented. While previous studies have shown an upregulation of SPS under cold stress, our results showed a slight decrease in its expression, probably owing to species-specific differences. In response to low temperature stress, plants modulate the expression of genes involved in soluble sugar metabolism and
transport, and also starch breakdown\(^\text{45,46}\), thereby accumulating sugars including sucrose and hexoses that act as cryoprotectants. Thus, a cascade of sugar metabolism genes, transporters and signalling components (such as kinases) is involved in cold stress-response in planta. The differential expression patterns of these genes vary in a species-specific manner\(^\text{47}\). As for the TIA metabolism genes, SLS, TDC, STR and PRX1 were induced significantly, indicating a possible role of TIAs in cold stress response. Peroxidases are known to impart cold-tolerance\(^\text{48}\).

A differential expression pattern was observed for the phenylpropanoid-biosynthesis genes. Phenylpropanoids, specifically flavonoids impart freeze tolerance by preventing protein aggregation\(^\text{49}\). A similar report in A. thaliana presented a slightly differing pattern, wherein PAL was found to be upregulated along with most of the carbohydrate metabolism genes\(^\text{50}\). Interestingly, the antioxidant gene CAT and SOD were downregulated while SAG was upregulated, indicating that PRX1 may have a more dominant anti-oxidant role compared to CAT and SOD.

### Drought stress

Drought stress was found to have adverse effects wherein most of the tested genes were downregulated (Fig. 3b). The carbohydrate metabolism genes were mostly downregulated. Drought inhibits plant growth, disturbs mineral-nutrient relations and impairs metabolism due to changes in photosynthetic carbon metabolism\(^\text{51,52}\). While two of the TIA-biosynthesis genes (DAT and TDC) were repressed, significantly high upregulation was observed for STR and PRX1, probably attributed to the increased demand of turgor pressure\(^\text{53}\). The phenylpropanoid biosynthesis genes again showed a differential expression pattern. Elevated levels of phenolics and their biosynthesis genes is a characteristic of drought-stressed tissues\(^\text{53}\). Cell wall toughening during drought was associated with enhanced lignin (a derivative of phenylpropanoid pathway) biosynthesis\(^\text{53,55}\). The antioxidant genes showed a differing trend, while SAG was upregulated, indicating that PRX1 may have a more dominant anti-oxidant role compared to CAT and SOD.

### Salinity

As shown in Fig. 3c, salinity had varying effects on the expression of sucrose-metabolism genes, wherein CrCWIN1 and CrCWIN2 were highly induced, while CrCWIN3 and SPS were repressed. Osmoregulation

---

**Table 1. Cell Wall Invertases in Catharanthus roseus.**

| Gene Name (Sequence ID) | Scaffold         | Matching sequence details (Genbank ID)                           | % Identity | Query coverage in tBLASTx | Length of coding sequence (in bp) (Position in scaffold) | Length of genomic gene (in bp) | Predicted amino acid length (Molecular weight, kDa) | Predicted sub-cellular localization |
|------------------------|------------------|----------------------------------------------------------------|------------|--------------------------|----------------------------------------------------------|-------------------------------|-----------------------------------------------------|-----------------------------------|
| CrCWIN1 (CRO_T000083)  | cro_scaffold_3070386 | *Nicotiana tabacum* beta-fructofuranosidase, (XM_016633086) | 77%        | 95%                      | 1797 (3502 to 6638)                                      | 3137                          | 598 (67.8)                                         | Cell wall                         |
| CrCWIN2 (CRO_T031716)  | cro_scaffold_3060381 | *Coffea canephora* cell-wall invertase (DQ834314)                | 78%        | 90%                      | 1725 (12947 to 17217)                                     | 4271                          | 574 (65.0)                                         | Cell wall                         |
| CrCWIN3 (CRO_T020329)  | cro_scaffold_3065222 | *Chicorium intybus* mRNA for putative invertase (Y11124)         | 61%        | 90%                      | 1713 (27037 to 24482)                                     | 2924                          | 570 (64.9)                                         | Cell wall                         |

**Figure 2.** Tissue-specific expression pattern of the three CWIN isoforms in leaf, root and stem tissues of three month old C. roseus plants. The relative expression levels of CWIN isoforms in leaf, root and stem tissues were normalized against transcript levels of SAND. Results are represented as mean relative transcript levels and the error bars indicate standard deviation of triplicate samples.
is a key aspect in salinity tolerance in plants and some of the major osmolytes like proline, sugars and polyols play pivotal role in alleviating salt stress, thus explaining the elevated levels of sugar metabolism genes\(^5^7\). Among the TIA metabolism genes, TDC, STR and PRX1 were upregulated, while DAT was downregulated. Alkaloids are known to impart salinity tolerance to plants further corroborating their role in alleviating salinity stress\(^5^8,5^9\). Our results indicating the elevated levels of CWINs, STR, TDC and PRX1 under salinity therefore provide a prospective molecular-crosstalk between carbohydrate and TIA-biosynthesis pathways. The phenylpropanoid gene PAL was induced, while CHS was repressed. Similar reports have indicated that salt treatment could upregulate phenylpropanoid biosynthesis genes in safflower\(^6^0\), Salvia species\(^6^1\) and Caragana korshinskii\(^6^2\).

**Sucrose-supplementation.** Exogenous sucrose treatment had a marked effect on the soluble sugar, phenylpropanoid and TIA metabolism genes, wherein the expression of most of the genes examined was found to be upregulated (Fig. 3d). Sucrose treatment resulted in a highly pronounced upregulation of CrCWIN2, but not of CrCWIN1 or CrCWIN3, possibly due to the lack of the sucrose-binding box in these two isoforms. Further, SUSY was also found to be upregulated. The TIA-biosynthesis genes, G10H, SLS, TDC, STR, DXS and PRX1 were found to be significantly induced. Among the phenylpropanoid genes, PAL and C4H were considerably upregulated. It has been suggested that sucrose-supplementation induces CWIN in potato, along with principal phenylpropanoid genes, caused by a network of transcription factors (WD40, AN1 and bHLH\(^6^3\)). Sucrose-supplementation has been known to improve the therapeutic TIAs and phenylpropanoids\(^6^4\). Further, CAT and SAG were repressed, indicating a possible reduction in oxidative stress and senescence.

**UV stress.** As shown in Fig. 3e, UV treatment downregulated CrCWIN1, while TIA metabolism genes showed a varying trend. G10H, DAT and SLS were downregulated, while TDC, STR and PRX1 were upregulated. Previous reports also found an upregulation in the expression of STR and TDC\(^6^5\). UV-mediated alkaloid enhancement is attributed to their UV-absorbing properties, which prevents the damage to photosystems caused by UV-B radiation\(^4^5,4^6\). Further, CHS was considerably downregulated, which is in agreement with the previous observation in A. thaliana wherein several phenylpropanoid metabolism genes were downregulated in plants exposed to UV radiation\(^4^7\). However, the sensitivity of plants to UV radiation has been shown to vary with different plant species.

**Figure 3.** Gene expression profiles of soluble sugar, TIA metabolism genes, phenylpropanoid metabolism and antioxidant genes. Expression profile in (a) cold stress, (b) drought, (c) salinity, (d) sucrose, (e) UV radiation and (f) wound- treated tissues. The Results depict statistically significant (p < 0.05) Up/downregulation of the considered genes determined via three independent replicates in qRT-PCR. Data were analysed using LinREG PCR and REST software. Mean factors of gene expression compared to control group are represented as boxplots. Corresponding expression ratios of the genes significantly affected (p < 0.05) are shown next to the whisker-boxes. The median expression ratio values above/ below 1.0 indicate up/ downregulation of the target gene under stress treatment compared to the control leaves, indicated using upward and downward arrows.
species. The antioxidant and senescence marker genes SOD and SAG were upregulated, possibly indicating elevated demand for ROS scavenging mechanisms due to UV stress.

**Wounding stress.** As depicted in Fig. 3f, wounding stress resulted in a slight upregulation of CrCWIN2, while repressing SPS, indicating a possible enhancement in sucrose breakdown and reduction in its synthesis. Remarkably, sugars are known to regulate the expression of wound-inducible genes, such as pathogenesis-related genes, thereby corroborating the influence of wounding on soluble sugar metabolism genes. TIA-biosynthesis genes (SLS, STR, DXS and PRX1) were largely upregulated. In C. roseus, wounding is known to activate MAP-K mediated signalling cascade and subsequently, the genes and regulators of TIA-biosynthesis pathway. Our results further indicated that except CHI, all the phenylpropanoid biosynthesis genes were repressed. This observation showed that resource allocation might be directed towards lignin biosynthesis in response to wounding.

TIA metabolism in C. roseus is under tight regulation at transcriptional level by several transcription factors such as ORCAs, CrBPF1, CrWRKY1, CrMYC1, CrMYC2, BIS1, GBF2 and ZCT. CrWRKY1 binds to the promoter of TDC and its overexpression resulted in the upregulation of several genes, especially regulating the serpentine pathway. CrBPF1 (a MYB transcription factor) is known to repress TIA levels. CrGBF1, CrGBF2 and the Zinc Finger Transcription factors - ZCT-1, 2, 3 are known transcriptional repressors of TIA biosynthesis. ORCA3, an AP2/ERF factor is a master regulator of primary and specialized metabolism in C. roseus and is known to play critical role in TIA biosynthesis. ORCA3 transactivates the expression of Strychostisidine synthase (STR), a key TIA biosynthesis gene by binding to its 5′ upstream cis-element, jasmonate and elicitor-responsive element (JERE). Further, it upregulates the expression of several structural genes such as TDC, D4H, SLS, CPR, DXS and AS. Considering the importance of ORCA3 in TIA biosynthesis, its expression profile was monitored in our study. Remarkably, ORCA3 was found to be downregulated in sucrose, UV, salt and drought treated C. roseus leaves, while in cold and wounding, there was no significant change observed. However, the TIA-biosynthesis genes were found to be upregulated under these conditions, indicating the possibility of additional regulatory components besides ORCA3, operating under these conditions.

To summarize, sucrose treatment was found to simultaneously upregulate CrCWIN2 and major specialized-metabolism genes, along with downregulation of antioxidant systems (CAT) and senescence marker (SAG), indicating a reduction in oxidative stress and senescence. This observation points at a possible pattern of co-regulation in primary and specialized-metabolism gene networks in response to sucrose feeding in C. roseus leaves.

**Metabolite analysis of stress-treated leaf tissues.** Plant metabolome undergoes profound changes in response to abiotic stress. In order to study the effect of stress treatments on specialized-metabolites in C. roseus, we assessed the levels of the monomeric precursors of anticancer TIA's-vindoline and catharanthine, along with the bis-indole alkaloid-vinblastine, cinnamic acid (product of PAL catalysed reaction), coumarin, fisetin (phenylpropanoid) and geraniol (an aldehyde compound) in plants subjected to PEG-induced drought stress. Geraniol content was found to decrease upon drought treatment. The levels of geraniol in plants was shown to depend on the intensity of drought stress, duration and the species. Accumulation of cinnamic acid was found to be decreased. These results further correlate with the expression profile of phenylpropanoid genes, wherein PAL was found to be downregulated. However, existing reports present contrasting findings, indicating the species-specific nature of phenylpropanoid regulation under low temperature stress.

It was observed that cold treatment led to a significant decrease in vindoline, while no change was observed in the levels of catharanthine. In a previous report, catharanthine and vindoline accumulation was shown to be downregulated in response to cold. A precursor of TIAS, geraniol was found to be enhanced in cold-treated leaves. However, a previous report on geranium indicated that low temperature decreased geraniol levels, although the precise mechanisms are unclear. Phenylpropanoids form the first line of defence against abiotic stress, owing to their inherent antioxidant potential. Accumulation of cinnamic acid was found to be decreased. These results further correlate with the expression profile of phenylpropanoid genes, wherein PAL was found to be downregulated. However, existing reports present contrasting findings, indicating the species-specific nature of phenylpropanoid regulation under low temperature stress. There was no change observed in the levels of coumarin. In Arabidopsis leaves, the levels of scopoletin, a coumarin-derivative was found to increase in response to cold treatment. The differences in observations can be due to the differences in metabolic reorganization of individual plants in response to stress. Cold stress resulted in a remarkable increase in the levels of fisetin. Flavonoids are known to accumulate in response to abiotic stresses, thereby conforming to low temperatures.

Drought stress led to a decrease in levels of all the metabolites analysed, except for fisetin. Drought has been shown to cause dynamic variations in the levels of vindoline and catharanthine, wherein vindoline displayed a decline-rise trend while catharanthine showed a gradual decline in its levels in C. roseus tissues subjected to PEG-induced drought stress. Geraniol content was found to decrease upon drought treatment. The levels of geraniol in plants was shown to depend on the intensity of drought stress, duration and the species. Cinnamic acid levels were found to be decreased, which correlate with our gene-expression data, wherein PAL was significantly downregulated. However, previous reports have shown contrasting results. Accumulation of fisetin showed a marked increase in drought-mediated upregulation of flavonoid biosynthesis. A differential effect of drought on flavonoid biosynthesis was reported in wheat. Flavonoids act as ROS quenchers, thereby forming first-line of defense against oxidative stress. Coumarin levels were found to be decreased. A similar result was observed in case of Vitis vinifera leaves, wherein most of the abundant phenolic compounds underwent a significant decline, despite other reports indicating at the converse, owing to the species-specificity of drought-induced changes in metabolite levels.

Salinity stress resulted in a significant increase in the levels of vindoline, while catharanthine was severely reduced. Previous reports suggest conflicting effects of salinity on TIA levels. Geraniol concentration increased in salt-treated tissues, thereby pointing at an upregulation of monoterpenoid biosynthesis. A similar observation was made in Coriandrum sativum, attributed to the increased density of oil glands. Flavonoid concentrations showed a significant decrease in salt-treated tissues. However, a differential effect of salt stress on...
PAL isoforms was observed in diverse plant species. It could therefore be inferred that the effect of salinity on cinnamic acid is species-dependent. The levels of fisetin showed a drastic decline under salinity. Research reports have indicated that pattern of flavonoid accumulation under salinity stress is species-specific, governed mainly via the predominant flavonoid present.

UV stress resulted in a downregulation of TIAs and phenylpropanoids. On the contrary, previous studies have reported UV based induction of TIAs under Nitrogen-supplementation to leaves. An increase in vindoline and catharanthine levels was also reported in suspension cell cultures subjected to UV radiation. UV radiation has been proposed to induce the biosynthesis of UV-absorbing and ROS-scavenging phenols however, we report that the levels of phenylpropanoids either show a decrease (cinnamic acid and fisetin) or no change (coumarin). This could be attributed to plant-specific differences in response mechanisms to UV-exposure.

Wounding stress downregulated the production of catharanthine, vindoline, cinnamic acid and coumarin, whereas geraniol and fisetin were not significantly affected. Alkaloid formation was shown to be reduced in detached plant parts subjected to wound stress in leaves. Wounding stress downregulated the production of cinnamic acid and ajmalicine levels of wounded leaves, while catharanthine levels remained unaffected. Cinnamic acid esters are known to have wound-protectant effects and phenylpropanoid-derived metabolites such as acetosyringone play roles in wound stress-response. The observed decline in cinnamic acid levels can be due to the channelling of this compound towards the synthesis of other downstream wound-protectant metabolites.

Exogenous sucrose-supplementation resulted in highly pronounced upregulation in the biosynthesis of all the metabolites analysed. Sucrose can act as signalling molecule inducing the biosynthesis of various plant secondary metabolites.

Figure 4. Quantification of TIAs (vindoline, catharanthine and vinblastine), specialized metabolites (geraniol, cinnamic acid, coumarin and fisetin) and tryptophan (TIA precursor amino acid) in the (a) leaf, stem, root and calli of as well as (b) leaves subjected to different conditions influencing TIA metabolism: sucrose supplementation, drought, salinity, UV, wounding and cold stress. The error bars represent standard deviation of duplicate measurements. Statistical significance between sucrose-treated and control samples was tested using student’s t-test (\( * < 0.05; ** < 0.01; *** < 0.001 \)).
specialized-metabolites such as flavonoids and anthocyanins. Though the levels of vindoline and catharanthine were significantly increased upon sucrose treatment, the dimeric alkaloid, vinblastine was not upregulated upon any of the stress treatments. This could be attributed to the spatial separation of its precursors, vindoline and catharanthine in the leaf tissues. Previous attempts towards enhancing TIAs in *C. roseus* have also shown upregulation of the monomeric precursors, rather than the dimeric TIAs. Overexpression of ORCA3 and G10H had a more pronounced effect on the accumulation of the precursors (strictosidine, vindoline, catharanthine and ajmalicine) than the dimeric TIAs (anhydrovinblastine and vinblastine). Transient overexpression of CrMPK3 also resulted in a higher upregulation of vindoline, catharanthine and serpentine compared to vincristine (dimeric TIA). Moreover, the levels of vindoline and catharanthine in planta are inherently higher than those of the dimers, thereby enabling researchers to commercialize their in-vitro coupling to obtain the dimeric TIAs. Thus, strategies for increasing the levels of vindoline and catharanthine via sucrose-metabolism in *C. roseus*, followed by their isolation and chemical-coupling to obtain the dimeric TIAs would be promising towards enhanced production of anti-cancer TIAs. In summary, sucrose-supplementation could enhance the production of specialized-metabolites in *C. roseus* leaves without causing damage to growth-associated processes. Further studies to dissect the mechanistic aspects of this effect could open up novel avenues in metabolic engineering of medicinal plants. Figure 4b summarizes the effect of the stress treatments on metabolite accumulation in *C. roseus*. The chromatograms (recorded at 210 nm, 250 nm and 269 nm) are available in Supplementary Fig. 1a–c.

**Isolation, cloning and transient overexpression of CrCWIN2 CDS in *N. benthamiana***. The overexpression of CrCWIN2 in *N. benthamiana* resulted in 138-fold higher accumulation of CrCWIN2 (Fig. 5a,b). The CWIN activity in infiltrated tissues was found to be ~2.2 times more than the control (*N. benthamiana* leaves infiltrated with recombinant agrobacterium carrying the vector pcAMBIA2301), thereby validating the functionality of CrCWIN2 (Fig. 5c). The results further showed changes in the expression of endogenous genes belonging to diverse metabolic pathways. Most notably, among the sucrose-metabolism genes, NbCWIN3 was significantly enhanced, while NbSPS1 and NbSUSY were downregulated. This pattern suggested that heterologous expression of CrCWIN2 could simultaneously alter the sucrose-synthesis as well as breakdown processes, which are respectively governed mainly via SPS and invertases. Among the phenylpropanoid biosynthesis genes, it was observed that NbPAL2 was upregulated significantly, pointing at the possible role of metabolic-restructuring caused by CWIN overexpression. Further, the genes downstream to *PAL* revealed an interesting pattern, wherein the lignin-pathway genes (*Nb4CL* and *NbHCT*) and anthocyanin-biosynthesis genes (*F3H* and *ANS*) were also found to be significantly induced (Fig. 5b). In the isoprenoid-biosynthesis pathway, the mevalonate-biosynthesis genes (*NbHMGR, NbHMGS*) showed no pronounced changes in their expression, while the non-mevalonate pathway genes (*NbDXR, NbDXS*) were repressed. However, the downstream genes of isoprenoid biosynthesis pathway: *NbHDS*, and *NbG10H* were found to be upregulated (Fig. 5b). This observation further points at the possible interconnection between sucrose-metabolism and other specialized-metabolic processes.

The metabolite analysis of CrCWIN2 overexpressing *N. benthamiana* leaves against the agroinfiltrated control showed a significant increase in the levels of specialized metabolites - cinnamic acid, coumarin, and fisetin (Fig. 5d). This indicated that CWIN overexpression could possibly enable partitioning of intermediates towards biosynthesis of specialized-metabolites. A previous study also reported that overexpression of yeast CWIN could enhance the levels of phenylpropanoids as an inherent mechanism towards protecting plant systems from pathogen-induced stress. Moreover, research evidence pointed that anthocyanins and flavonoids were recruited mostly under stressed conditions in planta, wherein tissue ROS content is usually higher. It is also known that the metabolite influx for lignin biosynthesis occurs through sucrose via the shikimate acid and phenylpropanoid biosynthesis pathway. The chromatograms (recorded at 210 nm, 250 nm and 280 nm) are available in Supplementary Fig. 2.

**Conclusion**

Primary and specialized-metabolisms in plants are interconnected as primary metabolites can serve as precursors and signals for the synthesis of specialized-metabolites. A clear molecular understanding of this interconnection can lead to novel metabolic engineering approaches for enhancing the biosynthesis of therapeutically important plant specialized-metabolites. *C. roseus*, the source of anti-cancer TIAs is an important medicinal plant in which the specialized-metabolism, especially TIA-biosynthesis has been extensively studied. But, the genetic understanding of TIA-biosynthesis in relation to central carbon metabolism is lacking. A sucrose-cleaving enzyme, CWIN plays pivotal roles in modulating diverse specialized-metabolic pathways in planta. In spite of research reports indicating at the profound effects of CWIN on specialized-metabolites biosynthesis, as to our knowledge, there have been no studies done towards understanding CWIN expression, regulation and its influence on specialized-metabolism in medicinal plants. The present work is the first to understand the possible interrelation between CWIN expression and TIA biosynthesis in the anti-cancer medicinal plant *C. roseus*.

This study identified three CWIN isoforms in *C. roseus*, which exhibited tissue-specific differential expression patterns. Among the three isoforms, only one (CrCWIN2) was found to possess the catalytic sites required for invertase functionality. Gene-expression analysis was performed to decipher the possible correlation between the expression patterns of CWIN isoforms, TIAs, phenylpropanoid biosynthesis genes, sucrose metabolism genes and also growth/antioxidant genes under abiotic stress conditions known to influence vindoline and catharanthine production in *C. roseus*. Sucrose-supplementation was found to enhance the expression of CWIN and specialized-metabolism genes, and also improved the levels of vindoline, catharanthine, geraniol, fisetin, coumarin and cinnamic acid. The interconnection between primary and specialized-metabolism was further confirmed via transient overexpression of full-length CrCWIN2 in *N. benthamiana*.
These results can give us cues for further metabolic engineering approaches to enhance the production of medicinally/economically important phytochemicals without compromising the overall plant health and vegetative growth. In this regard, future studies to identify the regulatory factors that can co-regulate CWIN and specialized-metabolism genes can be of interest.

Materials and Methods

Plant materials and stress treatments. Seeds of C. roseus (var. Pacifica Cherry red) were germinated on coco peat and maintained at 25 °C and 65% relative humidity in green house. Two months old C. roseus plants were subjected to different abiotic stress treatments. UV treatment was done by exposing the plants to UV radiation (48 μW cm⁻²) in LAF for two minutes. Wounding was performed by damaging ~50% of the leaf lamina with a surgical blade. Cold stress was induced by incubating the plants at 4 °C. The detached leaves were subjected to salt stress by dipping them in 200 mM NaCl solution. Drought treatment was performed by placing the
detached leaves on dry blotting paper in petri dishes. Exogenous sucrose treatment was performed by placing the detached leaves in 90 mM sucrose solution. The tissues were harvested after 24 hours of stress treatments, snap-frozen in liquid nitrogen and stored at −80 °C.

**RNA isolation and Quantitative RT-PCR.** To study the tissue-specific expression patterns of CWIN, total RNA was isolated from leaf, stem, root and callus tissues of C. roseus. To examine the stress mediated expression of CWIN along with other genes, RNA was extracted from pooled tissues of C. roseus leaves subjected to stress treatments using Plant RNA isolation kit following the manufacturer’s instructions (MN, Germany). 6 μg of total RNA was subjected to DNase treatment using RNase free DNase (Thermo Scientific, Lithuania) followed by first strand cDNA synthesis using PrimeScript RT reagent kit (TaKaRa, Japan). qRT-PCR was performed on Mastercycler Realplex qRT-PCR instrument (Eppendorf). Reaction mix contained 1 μl of diluted (3.5 times) cDNA, 5 pmol each of forward and reverse primer, 1X SYBR green (Roche, Germany) in 7.5 μl reaction. Cycling parameters were: Initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 40 s, extension at 72 °C for 30 s followed by final extension at 72 °C for 5 min.

The leaf tissues subjected to stress treatments were used for analysis of the expression levels of primary and specialized-metabolism genes using specific primers listed in Supplementary Table S1. SAND served as the reference gene. The expression patterns of sucrose-metabolism genes: CWIN, SUSY and SPS along with predominant TIA metabolism genes: G10H, DAT, SLS, PRX1, DXS; Phenylpropanoid metabolism genes: PAL, C4H, CHS, TDC, STR, CAT, SOD, SAG and APETALA2-domain transcription factor ORCA3, (a known transcriptional regulator of TIA-biosynthesis genes) were monitored.

Reaction efficiencies and Cq values of triplicate qRT-PCR assays were obtained through LinReg PCR software. Using these values, the relative gene-expression ratios were computed via the Relative Expression Software Tool (REST®). REST® performs randomization tests to determine the expression ratio of a sample, using the efficiency-corrected comparative Cq values. The up/down-regulation of a gene is determined by taking into account the individual amplification efficiencies of target and reference genes.

**High-Performance Liquid Chromatography (HPLC) analysis.** The HPLC analysis was done as described in Singh et al. and Lin et al., with modifications. The C. roseus and N. benthamiana tissues were harvested and ground frozen in liquid nitrogen. The samples were sequentially extracted with 1:10 w/v ratio of tissue solvent in a sequence of chloroform, followed by ethyl acetate and finally methanol each for 30 min with vigorous shaking. The supernatants were collected by centrifugation and freeze dried. The extracts were filtered through a 0.22 μm syringe-driven filter and analysed via a reverse-phase HPLC system (Agilent 1260-Infinity, C-18 column 4.7 × 250 mm; 5 μm particle size) at 25 °C. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B). The elution profile was as follows: 0 min: 100% A; 0–5 min: 100–70% A; 5–25 min: 70–50% A; 25–28 min: 50–30% A; 28–30 min: 30–100% A; 30–35 min: 100% A. The flow rate was maintained at 1 ml.min⁻¹. The injection volume was 20 μl and the eluent was monitored using a PDA-DAD detector between 190 nm and 400 nm. The concentrations of the selected compounds were calculated by comparing the peak area, retention time (RT) and wavelength of the designated compound and expressed in μg of compound per mg of fresh tissue (C. roseus) and ng of compound per mg of fresh tissue (N. benthamiana).

**Identification and bioinformatic analysis of C. roseus CWIN coding sequences (CDS).** The amino acid sequences of the well characterized CWINs of Arabidopsis thaliana (AT3G13790), Nicotiana tabacum (X81834), Coffea canephora (DQ834314), Lycopersicon esculentum (AF506006) and Orzya sativa (AT432199) were used as query sequences to find the CWIN coding sequence isoforms in C. roseus via tBLASTn analysis of medicinal Plant Genomics Resources (MPGR) Consortium (http://medicinalplantgenomics.msu.edu/). Subsequently, amino acid translate tool of ExpASY, (http://web.expasy.org/translate/) and their molecular features were analysed using EBI-Tools (http://www.ebi.ac.uk/Tools/emboss/).

The homology with known sequences was analysed using BLASTrn and BLASTx tools of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Subcellular localization was predicted using Plant-mPloc prediction tool (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/). Evolutionary relationships of the sequences were compared using Maximum likelihood method with thousand bootstrap values employing MEGA7 program. Genomic architecture of introns and exons was obtained using Gene Structure Display Server 2.0 (http://gsds.cbi.pku.edu.cn/) (111).

**Isolation and cloning of full-length C. roseus CWIN CDS.** Full-length C. roseus CWIN CDS was isolated via RT-PCR. Total RNA was isolated from leaf tissues of three month old C. roseus plants using RNAeasy Plant Mini kit (Qiagen, Germany) following the manufacturer’s instructions. Subsequently, 3 μg of total RNA was subjected to DNase treatment and cDNA was synthesized using Transcriptor first strand cDNA synthesis kit (Roche, Germany). Full-length C. roseus CWIN CDS was amplified using gene specific primers (LP: 5′-GGATCCCATGGCCAATTCTTACATTTGGTTCTTCT-3′; RP: 5′-GCTAGCCCTTAATCTTCACAGGATGAAAATTT-3′). Underlined bases contained BamHI and SstI sites respectively. The PCR-amplified cDNA was cloned into pGEMT-Easy vector and validated by sequencing, restriction analysis and PCR. Next, the C. roseus CWIN CDS was cloned into the modified expression vector pCAMBIA2300 as BamHI-SstI insert, downstream to CaMV 35S Promoter.

**Transient overexpression of C. roseus CWIN CDS in Nicotiana benthamiana.** Recombinant pCAMBIA2300 was transformed into Agrobacterium tumefaciens strain EHA105 via freeze thaw method. Transformed clones were verified by PCR followed by agroinfiltration into N. benthamiana as previously reported. Leaves infiltrated with recombinant agrobacterium carrying the vector pCAMBIA2300 were used as
control. After four days, leaves were harvested, snap frozen in liquid nitrogen and stored at -80°C until further analysis.

To study the effect of CWIN overexpression on other genes in N. benthamiana, total RNA was extracted from agroinfiltrated tissues. 6µg of total RNA was used to synthesize cDNA and the gene-expression analysis was carried out via qRT-PCR. PP2A114 was used as an internal reference gene. The N. benthamiana genes analysed in this study were 4-coumarate:coenzyme a ligase (4-CL; Nb6.1trP58793), Anthocyanin Synthase (ANS; Nb6.1trP1132), Cinnamoyl-CoA Reductase (CCR; Nb6.1trP67697), Chalcone Synthase (CHS; Nb6.1trP67289), Dihydroflavonol 4-Reductase (DFR; Nb6.1trP53078), Flavannone 3-Hydroxylase (F3H; Nb6.1trP67389), Shimikate o-Hydroxycinnamoyltransferase (HCT; Nb6.1trP21540), Peroxidase 9 (PRX; Nb6.1trP50659), Catalase Isozyme 1 (CAT; Nb6.1trP54093), Superoxide Dismutate (SOD; Nb6.1trP67255), Sucrose Synthase (SUSY; Nb6.1trP69162), Sucrose Phosphate Synthase isozymes (SPS; Nb6.1trP64694, SPS2, Nb6.1trP56089), Cell Wall Invertase isozymes (CWIN2; Nb5.1trv202472, CWIN3; Nb5.1trv228617), Phenylalanine Ammonia Lyase isozymes (PAL2, Nb6.1trP20094; PAL3, Nb6.1trP49210 and PAL4, Nb6.1trP56366), Flavannone 3-hydroxylase (F3H; Nb6.1trP67389), 3-hydroxy-3-methylglutarlyl-coenzyme-a-reductase 1 (HMGR; Nb6.1trP54761), Hydroxymethylflavanyl- synthase-like (HMGS; Nb6.1trP33093), Probable 1-deoxy-d-xylulose- 5-phosphate chloroplastic (DXS; Nb6.1trP16938), 1-deoxy-d-xylulose-5-phosphate reductoisomerase (DXR, Nb6.1trP48271), Geraniol 8-hydroxylase-like (G8H; Nb6.1trP70636), Cytochrome p450 cyp72a19-like (SLS, Nb6.1trP5153), 4-hydroxy-3-methylbut-2-en-1-yl diphosphate chloroplastic (HDS; Nb6.1trP30454), Phytone Synthase (PS; Nb6.1trP21364). The gene IDs have been obtained from Benth Genome (http://benthgenome.qut.edu.au). Primers used have been enlisted in Supplementary Table S1.

**CWIN activity assay.** CWIN activity assay was performed as described previously115 with minor modifications. Briefly, the rapidly harvested leaf tissue was weighed, ground in liquid nitrogen followed by homogenization with 1 ml extraction buffer [all as mol m⁻³: Heps-KOH (pH 8.0), 50; MgCl₂; 5 Ethylenediaminotetraacetic acid (EDTA), 2; MnCl₂; 1; CaCl₂; 1; Benzamidine, 1; Dithiotreitol, 1; Phenyl-methylsulphonyl sulphonyl fluoride, 0-1] on ice. The homogenate was centrifuged at 13000 × g for 15 min at 4°C and the pellet was resuspended in 500 µl extraction buffer. Total protein concentration in the extracts was determined using Bradford method116. The reaction mixture containing 10µg of total protein, 200 mM sucrose and 50 mM sodium acetate buffer at pH 4.7 was incubated at 37°C for 30 min. After incubation, reaction was alkalinized by adding 100 µl 1 M Tris-HCL, pH 8.5 and heated at 85°C for 3 min. Two blanks were set up to measure acid hydrolysis of sucrose (contained no extract) and endogenous glucose levels (contained no sucrose). The amount of hexoses released was measured enzymatically using Sucrose, D-Fructose, D-Glucose assay kit (Megazyme, Ireland). Activity was expressed as micromoles of hexoses released per minute per milligram of total protein.

**Statistics.** qRT-PCR data were analysed using REST™ and represented as box-and-whiskers plot, with central line indicating median of expression ratio with respect to control; box borders represent 95% confidence intervals and whiskers depict standard error margins. All other data are expressed as mean values and standard deviation of three independent experiments. Statistical significance was evaluated using t-test via GraphPad Prism 5 (GraphPad Software, La Jolla California USA, www.graphpad.com). All the graphs were plotted using GraphPad Prism 5 (GraphPad Software, La Jolla California USA, www.graphpad.com).

**References**

1. Li, Z. et al. High invertase activity in tomato reproductive organs correlates with enhanced sucrose import into, and heat tolerance of young fruit. J. Exp. Bot. 63(3), 1153–66, https://doi.org/10.1093/jxb/err329 (2012).
2. Liu, Y. H., Offler, C. E. & Ruan, Y. L. Cell Wall Invertase promotes fruit set under heat stress by suppressing ROS-independent cell death. Plant Physiol. 1, 163–80, https://doi.org/10.1104/pp.116.028599 (2016).
3. Ru, L. et al. Transcriptomic and metabolomics responses to elevated Cell Wall Invertase activity during tomato fruit set. J. Exp. Bot. 68(15), 4263–4279, https://doi.org/10.1093/jxb/erx129 (2017).
4. Prools, R. K. & Hückelhoven, R. Cell-wall invertases, key enzymes in the modulation of plant metabolism during defence responses. Mol Plant Pathol. 15(8), 858–64, https://doi.org/10.1111/mpp.12139 (2014).
5. Baumert, A. et al. Patterns of phenylpropanoids in non-inoculated and potato virus Y-inoculated leaves of transgenic tobacco plants expressing yeast-derived invertase. Phytochemistry. 56(6), 535–41, https://doi.org/10.1016/S0031-9422(03)00242-2 (2001).
6. Jimenez-Garcia, S. et al. Current Approaches for Enhanced Expression of Secondary Metabolites as Bioactive Compounds in Plants for Agronomic and Human Health Purposes – a Review. Pol. J. Food Nutr. Sci. 63(2), 67–78, https://doi.org/10.2478/v10222-012-0076-2 (2013).
7. Ibrahim, M. H. et al. Primary, Secondary Metabolites, Photosynthetic Capacity and Antioxidant Activity of the Malaysian Herb Kacip Fatimah (Labisia Pumila Bentham) Exposed to Potassium Fertilization under Greenhouse Conditions. Int. J. Mol. Sci. 13(11), 15231–15342, https://doi.org/10.3390/ijms131115321 (2012).
8. Moudi, M., Go, R., Yien, C. Y. S. & Nazre, M. Vinca Alkaloids. Int. J. Prev. Med. 4(11), 1231–1235 (2017).
9. Ahmed B. et al. Structural and functional characterization of the Vindoline biosynthesis pathway enzymes of Catharanthus roseus. J. Mol. Model. 24(3), https://doi.org/10.1007/s00894-018-3593-7 (2018).
10. Roepke, J. et al. Vinca drug components accumulate exclusively in leaf exudates of Madagascar periwinkle. PNAS. 107(34), 15257–15292, https://doi.org/10.1073/pnas.0911451107 (2009).
11. de Bernonville, D. T. et al. Phytochemical genomics of the Madagascar periwinkle: Unravelling the last twists of the alkaloid engine. Phytochemistry. 113, 9–23, https://doi.org/10.1016/j.phytochem.2014.07.023 (2014).
12. Tan, Q. et al. Overexpression of ORCA3 and G10H in Catharanthus roseus Plants Regulated Alkaloid Biosynthesis and Metabolism Revealed by NMR-Metabolomics. PLoS ONE. 7(8), e43038, https://doi.org/10.1371/journal.pone.0043038 (2012).
13. Mujib, A. et al. Catharanthus roseus alkaloids: application of biotechnology for improving yield. Plant Growth Regul. 62(2), 111–127, https://doi.org/10.1007/s10725-015-0109-x (2012).
14. Alam, M. M., Naeem, M., Khan, M. M. A. & Uddin, M. Vincristine and vinblastine anticancer catharanthus alkaloids: pharmaceutical applications and strategies for yield improvement in Catharanthus roseus: Current research and future prospects, (eds Naim, M., Afzal, T. & Khan, M.), https://doi.org/10.1007/978-3-319-51620-2_11 (Springer, Cham, 2017).
15. Ishikawa, H. et al. Total synthesis of vinblastine, vincristine, related natural products, and key structural analogues. J. Am. Chem. Soc. 31(13), 4904–4916, https://doi.org/10.1021/ja608942b (2009).
16. Chung et al. Screening 64 cultivars Catharanthus roseus for the production of vindoline, catharanthine, and serpentine. Biotechnol. Prog. 27(4), 937–943, https://doi.org/10.1002/btp.557 (2011).

17. Singh, N., Luthra, R. & Sangwan, R. S. Mobilization of starch and essential oil biogenesis during leaf ontogeny of lemongrass (Cymbopogon flexuosus Staff.). Plant and Cell Physiol. 32(6), 803–811, https://doi.org/10.1093/oxfordjournals.pcp.0078147 (1991).

18. Dubey-Shankar, V., Bhalla, R. & Luthra, R. Sucrose mobilization in relation to essential oil biogenesis during Palmerosa (Cymbopogon martini Roxb. Wats. var. motia) indolence development. J. Bioenerg. Biomembr. 28(4), 479–487 (2003).

19. Wang, L. et al. System level analysis of transcriptome revealing a sequential integration of primary and secondary metabolism leading to polyphenol accumulation and preparation of stress resistance. Plant J. 87(3), 318–322, https://doi.org/10.1111/tpj.13201 (2016).

20. Becerra-Moreno, A. et al. Combined effect of water loss and predation stress on gene activation of metabolic pathways associated with phenolic biosynthesis in carrot. Front Plant Sci. 6, 837, https://doi.org/10.3389/fpls.2015.00837 (2015).

21. Caretto, S., Linsalata, V., Colella, G., Mita, G. & Lattanzio, V. Carbon fluxes in a turbulent and non-turbulent plant canopy and net primary productivity. Net Primary Prod. 24(2), 1–52 (2010).

22. Balle, G. K. et al. Multiple Omics of Tomato glandular trichomes reveal distinct features of central carbon metabolism supporting high productivity of specialized metabolites. Plant Cell 29(5), 960–983, https://doi.org/10.1093/pcp/pcv080 (2017).

23. Cortés-Romero, C., Martínez-Hernández, A., Mellado-Mojica, E., Lópeze, M. G. & Simpson, J. Molecular and Functional Characterization of Novel Fructosyltransferases and Invertases from Agave tequilana. PLoS ONE 7(4), e55878, https://doi.org/10.1371/journal.pone.0055878 (2012).

24. Chen, Z., Gao, K., Su, X., Rao, P. & An, X. Genome-wide identification of the invertase gene family in Populus. PLoS ONE 10(9), e0138540, https://doi.org/10.1371/journal.pone.0138540 (2015).

25. Niu, J. Q., Wang, A. Q., Huang, J. L., Yang, L.-T. & Li, Y.-R. Isolation, characterization and promoter analysis of Cell Wall Invertase gene SoCIN1 from Sugarcane (Saccharum spp.). Sugar Techn. 17(1), 5–76, https://doi.org/10.1007/s12355-014-0348-8 (2015).

26. Yao, Y. et al. Genome-wide identification of 3D modeling, expression and enzymatic activity analysis of Cell Wall Invertase gene family from cassava (Manihot esculenta Crantz). Int J Mol Sci. 15(5), 7313–7331, https://doi.org/10.3390/ijms15057313 (2014).

27. Schwebel-Dugué, N. Characterization of 10-hydroxygeraniol dehydrogenase from Catharanthus roseus. PLoS One 12, 124–131, https://doi.org/10.1371/journal.pone.0121104 (2017).

28. Sun, J., Mannnahan, E., Sun, C. & Peebles, C. A. Examining the transcriptional response of overexpressing anthranilate synthase in the hairy roots of an important medicinal plant. Plant cell Physiol. 54(5), 673–685, https://doi.org/10.1093/pcp/pcp0359 (2013).

29. Gongora-Castillo, E. et al. Development of transcriptomic resources for investigating the biosynthesis of monoterpene indole alkaloids in medicinal plant species. PLoS one 7, e52506, https://doi.org/10.1371/journal.pone.0052506 (2012).

30. Kritthka, R. et al. Characterization of 10-hydroxycorynanthe dehydrogenase from Catharanthus roseus reveals cascaded enzymatic activity in iridoid biosynthesis. Sci Rep. 5, 8258, https:// doi.org/10.1038/srep08258 (2015).

31. Kumar, S., Shah, N., Garg, V. & Bhata, S. Large scale in-silico identification and characterization of simple sequence repeats (SSRs) from de novo assembled transcriptome of Catharanthus roseus (L.) G. Don. Plant cell rep. 33, 905–918, https://doi.org/10.1007/s00207-014-1569-8 (2014).

32. Liu, L.-Y. et al. High-throughput transcriptome analysis of the leafy flower transition of Catharanthus roseus induced by peanut witches'-broom phytoplasma infection. Plant Cell Physiol. 55, 942–957, https://doi.org/10.1039/c3ppct00289 (2014).

33. Shukla, A. K., Shasany, A. K., Gupta, M. M. & Khanuja, S. P. Transcriptome analysis in Catharanthus roseus leaves and roots for comparative terpenoid indole alkaloid profiles. J Exp Bot. 57, 3921–3932, https://doi.org/10.1093/jxb/erl146 (2006).

34. Sun, J., Mannnahan, E., Sun, C. & Pheeles, C. A. Examining the transcriptional response of overexpressing anthranilate synthase in the hairy roots of an important medicinal plant. Catharanthus roseus by RNA-seq. BMC plant. 6(1), 108, https:// doi.org/10.1186/s12870-016-0794-4 (2016).

35. Verma, M., Ghangal, R., Sharma, R., Sinha, A. K. & Jain, M. Transcriptome analysis of Catharanthus roseus for gene discovery and expression profiling. PLoS one 9(7), e103583, https://doi.org/10.1371/journal.pone.0103583 (2014).

36. Yuanyuan, M. Y., Ali, Z., Jiang, L., & Hongbo, S. Roles of plant soluble sugars and their responses to plant cold stress. AJ R. 8 (10) (2009).

37. Kaplan, F. et al. Transcript and metabolite profiling during cold acclimation of Arabidopsis reveals an intricate relationship of cold-regulated gene expression with modifications in metabolite content. Plant J. 5(6), 967–981, https://doi. org/10.1111/j.1365-311X.2007.03100.x (2007).

38. Redondo-Gomez S. Abiotic and biotic stress tolerance in plants in Molecular stress physiology of plants. (eds Rout, G. R, Das, A. B. Springer India, https://doi.org/10.1007/978-81-322-0807-5_1 (2013).

39. Yue, C. et al. Effects of cold acclimation on sugar metabolism and sugar-related gene expression in tea plant during the winter season. Plant mol. boil. 88, 591–608, https://doi.org/10.1113/pcp.ptc009 (2014).

40. Lee, J. H., Yu, D. J., Kim, S. J., Choi, D. & Lee, H. J. Intraspaces differences in cold hardness, carbohydrate content and β-amylase gene expression of Vaccinium corymbosum during cold acclimation and deacclimation. Tree Physiol. 22(12), 1533–1540, https://doi.org/10.1093/treephys/tps122 (2012).

41. Tang, K. & Pan, Q. Strategies for enhancing alkaloids yield in Catharanthus roseus via metabolic engineering approaches in Catharanthus roseus: Current research and future prospects (eds Naem, M., Aftab, T. & Khan, M.) Springer, Cham, https://doi.org/10.1007/978-3-319-51620-2_3 (2017).

42. Schulz, E., Tohge, T., Zuther, E., Fernie, A. R. & Hincha, D. K. Flavonoids are determinants of freezing tolerance and cold acclimation in Arabidopsis thaliana. Sci Rep. 6, 34027, https://doi.org/10.1038/srep34027 (2016).

43. Lee, B., Henderson, D. A. & Zhu, J.-K. The Arabidopsis cold-responsive transcriptome and its regulation by ICE1. Plant Cell. 17, 3153–3175, https://doi.org/10.1105/tpc.105.035568 (2005).

44. da Silva, E. C., de Albuquerque, M. B., de Azevedo, A. D. N. & da Silva, C. D. J. Drought and Its Consequences to Plants – From Individual to Ecosystem: Responses of Organisms to Water Stress, (ed. Akin, S.) https://doi.org/10.5772/25833 (Intech, 2013).

45. Banu, S., Ramegowda, V., Kumar, A. & Pereira, A. Plant adaptation to drought stress. F1000Research, 5, pii: F1000 Faculty Rev-1554, https://doi.org/10.12688/f1000research.7678.1 (2016).
Acknowledgements

The authors are thankful to Department of Science and Technology, Govt. of India (Grant sanction number: SB/YS/LS-188/2014) and Department of Biotechnology, Govt. of India (Grant sanction number: BT/Bio-CARE/02/10078/2013–14) for funding the research. We thankfully acknowledge the HPLC facility provided by Dr. Jayapradha Ramakrishnan, SASTRA Deemed to be University (EMR scheme, SERB: SR/S0/HS/0073/2012). The authors thank SASTRA Deemed to be University for providing the necessary infrastructural facilities.

Author Contributions

The research work was conceptualized and designed by B.S. (corresponding author). N.M.J. and S.A.S. performed the experiments; N.M.J., S.A.S. and S.S. interpreted the data. S.S.R. performed the HPLC analysis, S.S.R. and S.A.S. performed the chromatography data interpretation. N.M.J., S.A.S., S.S. and B.S. wrote the article.
Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-33415-w.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018