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# IDENTIFICATION OF ORGAN-SPECIFIC REGULATORY FRAMEWORKS OF *CATHARANTHUS ROSEUS* WITH EMPHASIS TO THE TIA BIOSYNTHETIC PATHWAY

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**Abstract**

*Catharanthus roseus* is a medicinal plant species having more than 100 alkaloids, including two anticancer alkaloids vinblastine and vincristine. RNA-seq data of a number of organs and treatments of *C. roseus* was utilized in order to identify organ-specific transcription factors (TFs) and those probably linked to the monoterpenoid indole alkaloids (MIA) pathway. Organ-specific TF transcripts as well as those probably regulate genes in the MIA pathway were identified. Expression of several TF transcripts was exclusive in organs like flower, mature leaf, root/hairy root, stem and seedling. Transcripts encoding peroxidases 1 and 12 were up-regulated in mature leaf, while down-regulated in the hairy roots. TF transcripts in hairy roots indicated no differential response when knocked down for the *tdc* gene (TDCi) compared to wild type. A number of eight transcripts of the MIA biosynthetic pathway concordantly expressed with TFs in the steps between tryptophan and vindoline biosyntheses. These transcripts are *tdc*, *str1*, *sgd*, *t16h*, *omt*, *nmt*, *d4h* and *dat*. The most common transcription factor families involved members of bHLH, MYB and WRKY whose genes are either induced by ABA or JA (or MeJA) or regulated during adverse condition. Results of virus induced gene silencing (VIGS) of two versions of *bHLH25* gene confirmed its role in driving expression of *str1* gene. This study highlights the regulatory frameworks in *C. roseus* with emphasis to the TIA pathway to be used in improving alkaloid biosynthesis via metabolic engineering.

**Key words:** RNA-seq, Concordant expression, MIA pathway.

## Introduction

Pathway databases (PDBs) constructed from annotated genomes of a number of biological systems are now available (ex., May *et al.*, 2009). Among these, PlantCyc (http://www.plantcyc.org) comprises 879 pathways and 3,455 compounds, which are far less than those available for plants (Ziegler & Facchini, 2008). More recently, Van Moerkercke *et al*. (2013) have conducted an RNA-Seq analysis to construct the metabolic map (CathaCyc) of different organs of the *C. roseus* transcriptomes under normal growth conditions. RNA-Seq analysis of few organs treated with methyl jasmonate (MeJA) or fungal elicitor, e.g., yeast extract (YE) was also done. The hormone MeJA is known for its positive effects on the accumulation of vindoline, one of the two prerequisite compounds of the production of vinblastine and vincristine in *Catharanthus roseus* L. (Rischer *et al.*, 2006). Expression of several TIA biosynthetic genes was reported to be induced by fungal elicitors such as YE to activate the production of ROS (Pauw *et al.*, 2004).

*Catharanthus roseus* has several applications in pharmaceuticals, as it is the main source of the two commercially used anticancer compounds namely vinblastine and vincristine (Rischer *et al.*, 2006). TIA biosynthetic pathway is induced by the central compound strictosidine due to the expression of three genes, e.g., *tdc* and *sls* (or *CYP72A1*) and *str* gene (Facchini & De Luca, 2008). In addition, vindoline is synthesized as one of the two building blocks, with catharanthine, for the formation of the two bisindole alkaloids (Rischer *et al.*, 2006) as a result of a six-step conversion of tabersonine to vindoline (Loyola-Vargas, 2007; Facchini & De Luca, 2008). A subsequent key step in the production of these two bisindoles is the condensation of catharanthine with vindoline (Costa *et al.*, 2008). This step is catalyzed by the enzyme peroxidase due to the action of *per1* gene.

TDCi hairy roots are transformed *C. roseus* (knocked down for *TDC* gene) cells with tryptamine biosynthesis blocked due to the knock down of *tdc* gene encoding tryptophan decarboxylase functioning in the conversion of tryptophan to tryptamine (Runguphan *et al*., 2009). Silencing was conducted in order to force the plant to use exogenously supplied precursors or substrate analogs for non-natural alkaloids biosynthesis. This strategy, termed mutasynthesis, is non-common in plant, while previously used to yield novel antibiotics in the soil bacterium *Streptomyces fradiae* (Shier *et al*., 1969).

Transcription factors (TFs) are key proteins required in the regulation of all biological aspects (Latchman, 1997). A TF binds to DNA sequences at a specific recognition site of target gene(s) in order to regulate cellular transcription. This action can result in promoting or blocking the recruitment of RNA polymerase to the target genes. TF is also a key determinant of gene-in-time and gene-in-site regulations during development of organisms and their responses to biotic and abiotic stresses (Gupta *et al*., 2005). Previous reports indicate plant-specific, MeJA-inducible transcription factors of the AP2-ERF family in *C. roseus*, namely ORCA2 and ORCA3 (van der Fits & Memelink, 2000; Rischer *et al*., 2006; Miettinen *et al*., 2014). These TFs regulate the expression of the three genes of the TIA pathway, e.g., *LAMT, SLS and STR*, acting consecutively during loganin, secologanin and strictosidine biosyntheses, respectively. These genes are induced in cell suspension culture and whole plant level. Several other TFs have been identified in *C. roseus* to regulate *STR* gene expression (Chatel *et al*., 2003), indicating the complexity in regulating TIA biosynthetic genes.

In the present study, we utilized the recovered *C. roseus* database in order to determine TF organ-specificity with emphasis to the TIA biosynthetic pathway towards the production of the two bisindoles vinblastine and vincristine. The results generally indicated that expression of members of basic helix-loop-helix (bHLH), WRKY and MYB TF families was organ-specific in addition to the concordant expressions of other members of these TF families and genes in the TIA pathway in *C. roseus*. A possible role of bHLH25 in driving *str1* gene in the TIA pathway was investigated via the use of the RNAi-based approach of virus induced gene silencing (VIGS).

## Materials and Methods

A number of 19 RNA-seq raw data of *C. roseus* was retrieved from SRA database of the NCBI (http://www.ncbi.nlm.nih.gov/sra, experiment SRP005953). To obtain high quality *de novo* transcriptome sequence data, the raw data was re-filtered to confirm that adaptor sequence and low-quality sequences were removed. Reads were then quantified and assembled via trinity software (r2013_08_14) to recover transcripts with a range of expression levels. The overall setup utilized during assembly was de Bruijn graph algorithm by *k*-mer with other parameters set at default levels for Trinity (Grabherr *et al*., 2011). Differentially expressed genes were detected by EdgeR (Robinson *et al*., 2010).

The functional annotation and classification of differentially expressed contigs clusters were carried out by BLAST2GO software (Conesa *et al.*, 2005). *De novo* assembled transcripts with considerable homology to the genes in the TIA biosynthetic pathway were detected using cd-hit-2d and cd-hit-2d-est software. Values of FPKM for the recovered transcription factor-like (TF-like) and TIA related transcripts were calculated. Transcripts were clustered based on their expression patterns across different organs, treatments and genotypes. Clustering was based on log ratio RPKM data for transcripts of *C. roseus* SRA database in the different organs, treatments and genotypes. Clusters were studied in order to detect TF-like transcripts that are organ-specific, MeJA-induced or related to TIA biosynthetic pathway. Detection of DE transcripts required the occurrence of least two-fold of differential expression with a maximum rate of $10^{-3}$ false discovery rate (FDR). Accordingly, differential expression data were introduced in fold change (FC) of transcript levels. DE transcripts were compared with the NCBI non-redundant nucleotide database using BLASTX with an E-value cut off of $1^{e-06}$, and GO terms were mapped against b2g_sep15 database to the obtained hits. The mapped GO terms were annotated using default annotation threshold. GO annotations were refined using GO-Slims method (Min *et al*., 2005). Candidate coding regions within transcript sequences were identified using TransDecoder v 2.0.1 (Haas *et al*., 2013) and the longest open reading frames were compared with non-redundant protein database (E value cut-off of $1^{e-5}$) using BLASTP. Then, protein domains common in TFs were identified using HMMER3 software (Eddy, 2009).

VIGS lines of selected TFs were generated in 4-week-old *C. roseus* following the procedure outlined for tobacco (*N. benthamiana*) seedlings (Velásquez *et al.*, 2009). Primers used in constructing the gateway compatible pTRV2 vectors (Liu *et al*., 2002) were designed using Netprimer software (http://www.premierbiosoft.com/netprimer/index.html). Efficiency of VIGS was monitored by the use of TRV2-*PDS* vector as recently described (Bahieldin *et al*., 2016).

Semi-quantitative RT-PCR (or sqRT-PCR) was conducted twice, one to validate the RNA-Seq data of *C. roseus* for selected transcripts whose expression was organ-specific and the other to score knockdown of TF genes and the gene(s) driven by them in TF-VIGS lines. Eight randomly selected genes were used to validate the RNA-Seq data for transcription factors specifically overexpressed in mature leaf (cluster 2), flower (cluster 1), root (cluster 11) or stem (cluster 24). VIGS lines of selected TFs as well as transformed plants with empty pRTV2 were used in the second sqRT-PCR. Expression levels of the selected TF genes along with their driven gene(s) in flower and root were detected in TF-pTRV2 and empty pTRV2 transformed plants. In the validation experiment, plants were grown to maturity, while 4-wk-old plants were used in the VIGS experiment. Plants were grown in the greenhouses of King Abdulaziz University, Jeddah complying with the institutional, national and international guidelines. However, no ethics approval was required for any aspect of this study. Criteria of primer designing utilizing Netprimer software was the following: GC content of ~50%, nucleotide length of 20-27 bases, avoidance of secondary structure, annealing temperatures of the primer pairs within the range of 48-55ºC, and PCR products of 144-318 bp for the validation experiment, while 246-484 bp for the VIGS experiment. Total RNA was extracted from different genotype organs (e.g., mature leaf, flower, root and stem) using Trizol (Invitrogen) and treated with RNase-free DNase (Promega Inc.). sqRT-PCR was performed as recently described (Bahieldin *et al*., 2016). The *actin* gene (250 bp) was used in the two experiments as the house-keeping control. Primer sequences and PCR conditions are shown in Table 1.

**Table 1. Primer sequences along with the annealing temperature and expected amplicon sizes (bp) to be utilized in validating RNA-Seq dataset and VIGS lines of C. roseus via semi-quantitative RT-PCR. Single astrisks indicate primers used for generating VIGS lines. Double astrisks indicate transcripts used in scoring gene knockdown via sqRT-PCR. The "actin" gene was used as the stably expressed house-keeping gene (250 bp).**

| TF/gene | Primer sequence | | Anneal temp. (°C) | Amplicon size (bp) |
|---|---|---|---|---|
| | Forward | Reverse | | |
| | **Validation experiment** | | | |
| bHLH | AAGATCTCCAGGATGAACTCGAAGAG | AAGCCTCCAAAAGTCTCACAAATTCCC | 53 | 300 |
| WRKY35 | CCTCTTACTGAGTTTTCCTGTC | GTAAAAAGGACTACTGCCTGC | 48 | 150 |
| ERF114 | CCGCCGATGAGGAAAAAGAG | TGCTGCTGCTGCTACCTATG | 50 | 144 |
| bHLH49 | CAAGCATAACTGCTTTGCCAGTGACC | GATTTTCACAGCCAACATCGGATGCC | 55 | 300 |
| MYB | AGACTTTTTCCGGGTCGGACTG | TTCCAGCAGCAGCATCATCTCC | 53.5 | 308 |
| MYB44 | CATCACTTGCAGCCCTCTTTTG | CTCCTTCCTTTGGTTTTCGACG | 51 | 308 |
| bHLH30 | CCAGAAGCGGAAAGCAATAAACAC | CGAACCAAAAAGTTCATCGAAATGCTC | 52 | 305 |
| WRKY6 | GTTCATTAGGCCGTCAGCACTC | AAAGCTCGTGTCTCGGTTCG | 52 | 318 |
| | **VIGS experiment** | | | |
| CabHLH25 | ACCGAGCTCACGCGTCTCGAGTATGAA ACCCCACGAGAGG*,** | ACCGAATTCTCTAGAACGTTGCACCAT TTGGAAG* | 48 | 215 |
| | | TGAAAGACTCAATCTTGGTGTT** | 45 | 246 |
| GmbHLH25 | ACCGAGCTCACGCGTCTCGAGTGAGAA TTTGACCTCCCAAGG*,** | ACCGAATTCTCTAGATTCAATTTCAGG GAGCTGTG* | 48 | 400 |
| | | TCTTTTCGCAGTGAACTCTG** | 48 | 449 |
| str1 | TGAAGACACCCAAAAATGGC** | ACATGATGACAGTCCCGAAG** | 48 | 301 |
| | **House-keeping control** | | | |
| Actin | GTATTGTTGGTCGTCCAAGACACACTG | CTGTTGGCCTTGGGATTAAGAGGTGC | 55 | 250 |

**Table 2. Description of RNA-Seq samples of *C. roseus* retrieved from SRA database (http://www.ncbi.nlm.nih.gov/sra) in terms of organ used, SRA accession number and treatment.**

| Sample no. | Sample code | Organ | SRA accession no. | Treatment |
|---|---|---|---|---|
| 1. | iML | Immature leaf | SRX047017 | Untreated |
| 2. | ML | Mature leaf | SRX047016 | Untreated |
| 3. | FL | Flower | SRX047002 | Untreated |
| 4. | R | Root | SRX047019 | Untreated |
| 5. | ST | Stem | SRX047018 | Untreated |
| 6. | S1 | Sterile seedling | SRX047007 | Untreated |
| 7. | S2 | Sterile seedling | SRX047009 | MeJA 6uM 12 d |
| 8. | SC1 | Suspension culture | SRX047006 | YE 0 mg/mL for 24 h |
| 9. | SC2 | Suspension culture | SRX047011 | YE 0.3 mg/mL for 6 h |
| 10. | SC3 | Suspension culture | SRX047004 | YE 0.3 mg/mL for 12 h |
| 11. | SC4 | Suspension culture | SRX047005 | YE 0.3 mg/mL for 24 h |
| 12. | SC5 | Suspension culture | SRX047015 | MeJA 100 uM 0 h |
| 13. | SC6 | Suspension culture | SRX047012 | MeJA 100 uM 6 h |
| 14. | SC7 | Suspension culture | SRX047013 | MeJA 100 uM 12 h |
| 15. | SC8 | Suspension culture | SRX047014 | MeJA 100 uM 24 h |
| 16. | HR1 | Hairy root (wild type) | SRX047022 | Untreated |
| 17 | HR2 | Hairy root (wild type) | SRX047024 | MeJA 250 uM for 24 h |
| 18 | HR3 | Hairy root (TDCi) | SRX047020 | Untreated |
| 19 | HR4 | Hairy root (TDCi) | SRX047026 | MeJA 250 uM for 24 h |

iML = Immature leaf, ML = Mature leaf, FL = Flower, R = Root, S = Sterile seedling, SC = Suspension culture, HR = Hairy root, MeJA = Methyl jasmonate, YE = Yeast extract, TDCi = *tdc* gene knocked-down

## Results and Discussion

We have assembled transcriptome retrieved from SRA database of different organs, treatments and genotypes (Table 2) of *C. roseus*. A number of 50,723 transcripts were recovered and calculated for abundance and differential expression using Rsem package and edgR method. The wild plant species was known for the production of monoterpenoid indole alkaloids (MIAs) with economic value. Accordingly, we have given special emphasis to the TF transcripts that might be organ-specific, MeJA- regulated or those controlling genes in the TIA biosynthetic pathway. A number of 290 TF-like transcripts along with 12 transcripts related to the TIA pathway, divided into 89 clusters, were differentially expressed across different organs, treatments and genotypes. Of 16 transcripts encoding TFs, eight transcripts encoding enzymes in the TIA pathway were co-expressed. The eight transcripts of the TIA pathway are shown in Figure 1. These transcripts co-expressed with TFs involved three transcripts functioning downstream tryptophan biosynthesis and the five transcripts downstream tabersonine biosynthesis towards the biosynthesis of vindoline. sqRT-PCR was conducted for eight transcripts overexpressed in mature leaf (cluster 2), flower (cluster 1), root (cluster 11) or stem (cluster 24). The results were in harmony with those of the RNA-Seq dataset (Fig. 2).

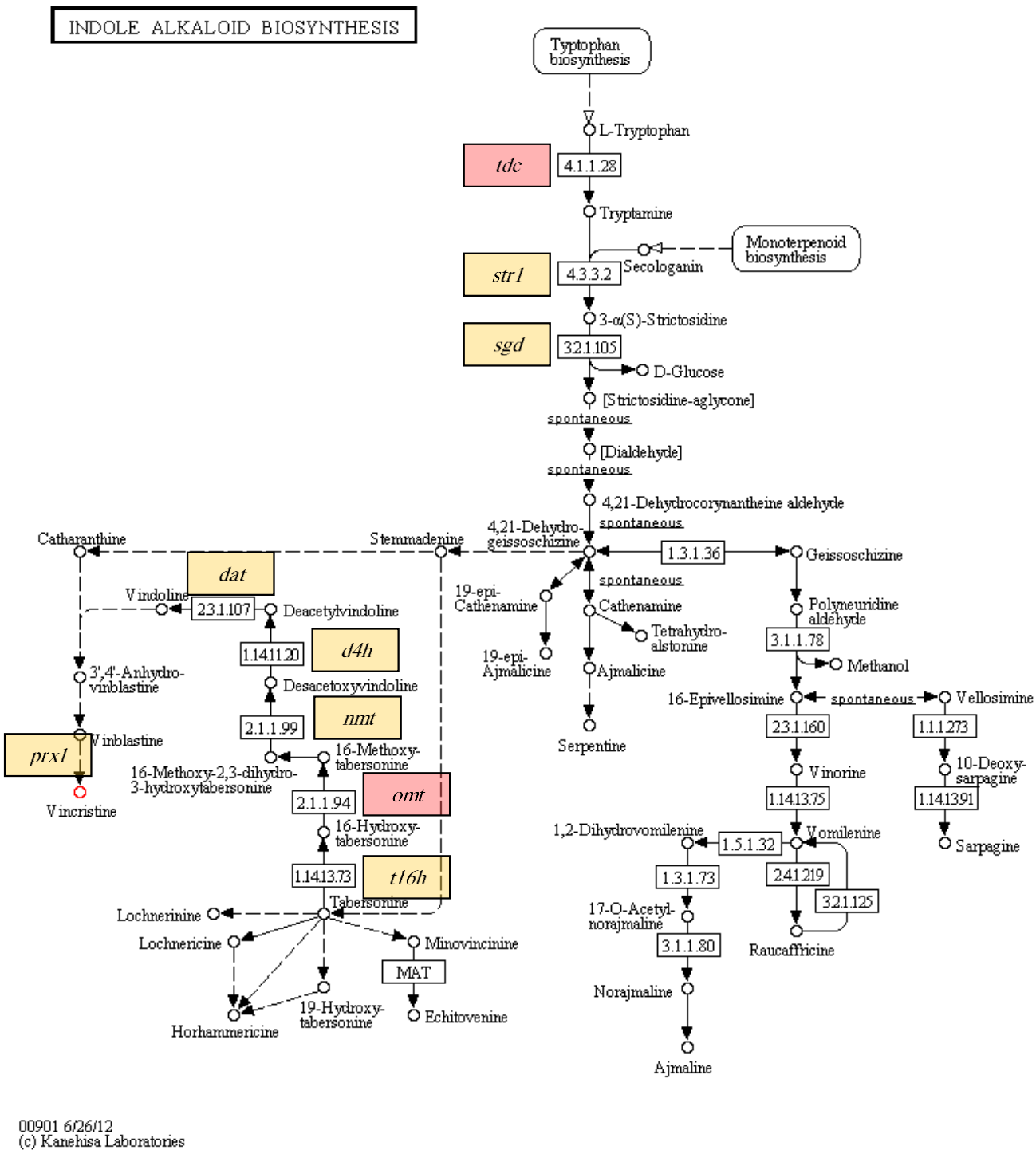



Fig. 1. TIA pathway for indole alkaloid biosynthesis with the regulated transcripts (in orange or red box) in *C. roseus* under different conditions (See Table 2). Transcripts in the red boxes are concordantly expressed.

Albeit no incidence occurred of co-expression with TFs, three transcripts encoding peroxidases 1 and 12 were also regulated across different organs. The eight transcripts co-expressed with TFs involved three transcripts functioning downstream tryptophan biosynthesis and the five transcripts downstream tabersonine biosynthesis towards the biosynthesis of vindoline (Fig. 1). Comparison of the sequences of the assembled transcripts of the TIA biosynthetic pathway to those previously recovered in *C. roseus* indicated high percentages of sequence homology (Table 3). As shown in Figure 3, 13 clusters of TF-like transcripts that showed organ-specificity were selected for further analysis. They are clusters 1, 2, 7, 11, 15, 20, 24, 30, 31, 35, 43, 78 and 82. Expression levels of TFs in these clusters were stacked in order to detect the organ(s) where genes were either up- or down-regulated. The overall results indicated that expression levels of TF transcripts in the hairy roots of plants knocked down for *tdc* gene (namely TDCi) compared to the wild type or due to the treatment with MeJA as compared to their untreated controls except for in cluster 64 (Fig. 2).

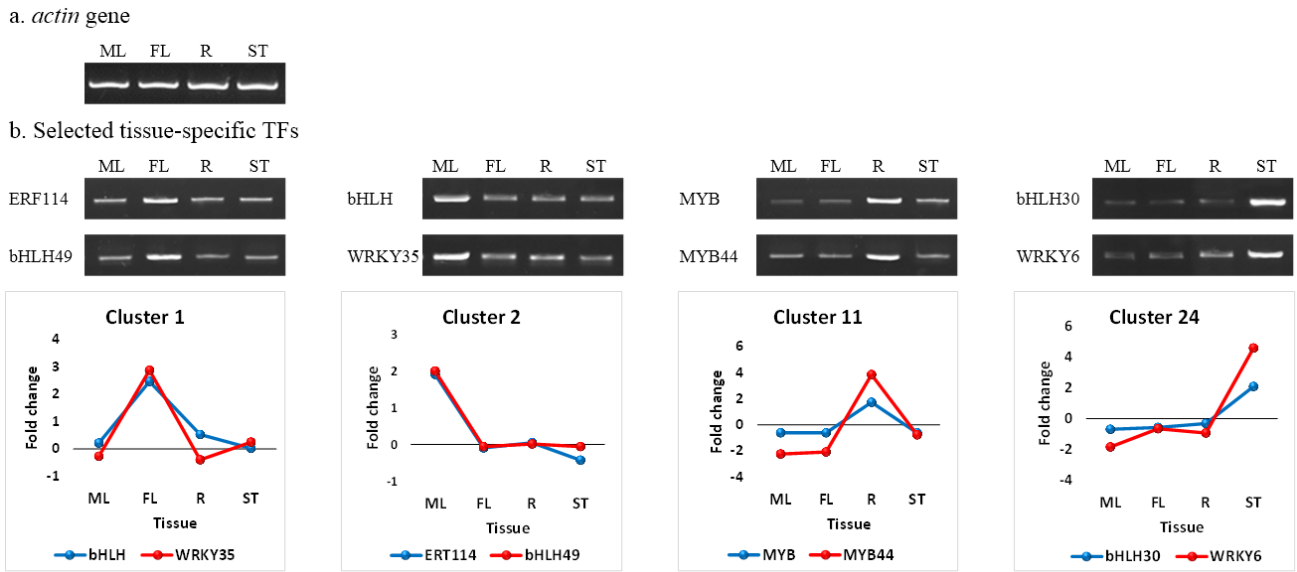



Fig. 2. Semi-quantitative RT-PCR and profiles of fold change values resulting from *C. roseus* RNA-Seq analysis for selected tissue-specific TFs of clusters 1, 2, 11 and 24. The "*actin*" gene was used as the stably expressed house-keeping gene. Primer sequences and PCR conditions are shown in Table 1.

**Table 3. Identity percentages of the regulated *C. roseus* transcripts of the TIA pathway.**

| Transcript | Gene | Accession | Identity (%) |
|---|---|---|---|
| comp659_c1 | tryptophan decarboxylase (or *tdc*) | M25151.1 | 94.83 |
| comp1806_c0 | strictosidine_synthase 1 (or *str1*) | X61932.1 | 100.00 |
| comp33625_c0 | strictosidine_beta-glucosidase (or *sgd*) | AF112888.1 | 61.81 |
| comp10495_c0 | tabersonine_16-hydroxylase (or *t16h*) | FJ647194.1 | 85.58 |
| comp223_c0 | 16-hydroxytabersonine O-methyltransferase (or *omt*) | EF444544.1 | 83.97 |
| comp15985_c0 | 16-methoxy-2,3-dihydro-3-hydroxytabersonine N-methyltransferase (or *nmt*) | KF896244.1 | 100.00 |
| comp46149_c0 | desacetoxyvindoline-4-hydroxylase(or *d4h*) | AF112888.1 | 96.39 |
| comp46568_c0 | | | 97.56 |
| comp14888_c0 | deacetylvindoline_4-O-acetyltransferase (or *dat*) | AF053307.1 | 100.00 |
| comp394_c0 | peroxidase_1 (or *per1*) | AM236087.1 | 100.00 |
| comp522_c0 | | | 94.88 |
| comp7302_c0 | | | 98.51 |

The results in Figure 3 also indicated several incidences of organ-specific expression of TF transcripts in several organs, e.g., flower (cluster 1), mature leaf (cluster 2), root/hairy root (cluster 11), stem (cluster 30), seedling (cluster 31), hairy root (cluster 35) and immature/mature leaves (cluster 82). Two of the three highly expressed TF transcripts in the flower (cluster 1) involved two analogs of bHLH aborted microspores transcription factor (encoded by *AMS* gene). This transcription factor plays an important role in tabidum development, male fertility and pollen differentiation (Thorstensen *et al.*, 2008). The third flower-specific transcript encodes WRKY35. This transcription factor is known for its action in embryo and pollen development and in iron transport (Eulgem *et al.*, 2000). The results also indicated occurrence of flower-specific TFs in many other clusters. Mature leaf-specific TFs involved ERF114 and bHLH49. The first TF functions as an activator of transcription targeting the pathogenesis-related promoter element, e.g., GCC-box and regulate genes in the stress signal transduction pathway (Nakano *et al.*, 2006). The bHLH49 is also a transcriptional activator but involved in cell elongation and regulates the expression of a subset of genes expressed in cell expansion by binding to the G-box motif (Ikeda *et al.*, 2012).

Root-specific TFs (cluster 11) involved several MYBs, ex., MYB12, MYB32 and MYB44, as well as WRKY13. MYB12 is part of subgroup 7 belonging to members of the R2R3-MYB family. This TF regulate expression of genes encoding several enzymes, e.g., chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), flavonol synthase (FLS) (Mehrtens *et al.*, 2005). Previous reports indicated that this TF had a main role in flavonol biosynthesis in roots of Arabidopsis (http://www.ncbi.nlm.nih.gov/gene/819359). A transcript of other undefined member of R2R3-MYB was also highly expressed in seedlings of *C. roseus* (cluster 31). Transcripts of MYB32 (Kranz *et al.* 1998) and MYB44 (Jung *et al.*, 2008) were also reported to be expressed in the roots of Arabidopsis. MYB44 was reported to be induced in roots, stems, leaves, inflorescence and flowers due to abscisic acid (ABA) accumulation (Jung *et al.*, 2008). Its expression represses the production of protein phosphatases 2C and confers resistance to different abiotic stresses. Concordantly, MYB32 was reported to be induced in root by ABA, ethylene, light, cold and high salinity (Kranz *et al.*, 1998).

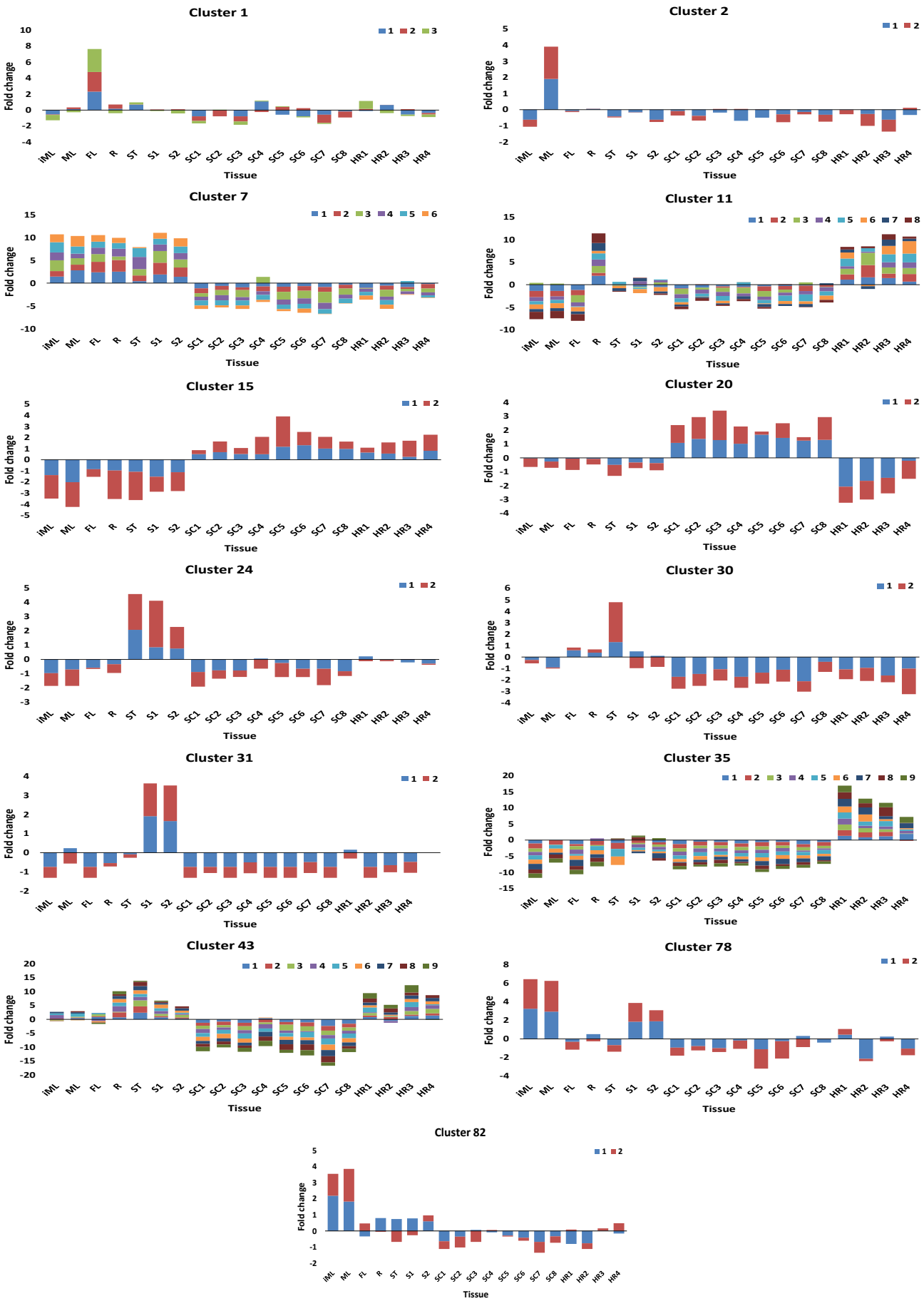



Fig. 3. Stacked expression levels of TF-like transcripts of selected clusters generated from RNA-Seq database of *C. roseus* organs under different conditions (See Table 2). iML = immature leaf, ML = mature leaf, FL = flower, R = root, ST = stem, S1-2 = sterile seedlings 1-2, SC1-8 = suspension cultures 1-8, HR1-4 = hairy roots 1-4. S2, SC5-8, HR2, HR4 = MeJA-treated, HR3, HR4 = *tdc*-knocked down.

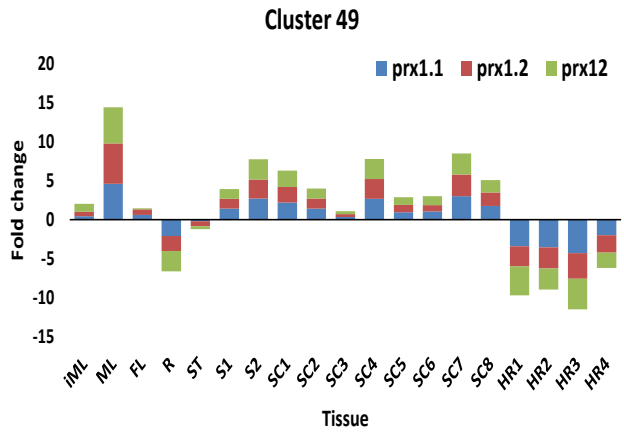



Fig. 4. Stacked expression levels of transcripts encoding peroxidase in *C. roseus* organs under different conditions (See Table 2). iML = immature leaf, ML = mature leaf, FL = flower, R = root, ST = stem, S1-2 = sterile seedlings 1-2, SC1-8 = suspension cultures 1-8, HR1-4 = hairy roots 1-4. S2, SC5-8, HR2, HR4 = MeJA-treated, HR3, HR4 = *tdc*-knocked down.

The two highly expressed transcripts encoding stem-specific TFs (cluster 30) involved transcripts encoding GRAS and ethylene-responsive abr1. Gene family of the first TF was reported to have a diverse role in root and shoot development (Hirsch & Oldroyd, 2009), gibberellic acid (GA) signaling and phytochrome A signal transduction (Bolle, 2004). The second TF is a negative regulator of the abscisic acid (ABA) signaling pathway and involved in the responses to stress conditions (Pandey *et al.*, 2005). Several highly expressed transcripts were detected in hairy roots (cluster 35). Expression of many of them cannot be justified as no enough information is available to support this incidence. There is no explanation for the high expression of bHLH123 in hairy roots, which was reported to have a role in guard cells (https://www.arabidopsis.org/ servlets/ TairObject?type=locus&name=At3g20640). Transcript of the AP2-like ethylene-responsive transcription factor BBM2 was mostly expressed in developing seeds (http://www.uniprot.org/uniprot/ Q8LSN2). Transcripts encoding two HBP-1b TFs were highly expressed in the hairy root. This TF was reported in wheat to bind to the hexamer motif, ACGTCA, of histone gene promoters (Tabata *et al.*, 1991). We speculate that this action might help hairy roots in its continuous cell division. The highly expressed transcripts encoding hairy root-specific TF that can also be justified is the fer-like iron deficiency-induced TF. This TF is mainly involved in roots especially in the differentiation zone (Heim *et al.* 2003) and to lower extent in leaves and stem. Interestingly, three transcripts encoding peroxidase that might function in the TIA pathway in the oxidation of bisindole vincristine (Ahn *et al.*, 1997) were highly down regulated in root and hairy root, while highly upregulated in mature leaf (cluster 49, Fig. 4). However, previous reports indicated the existence of low amounts of vinblastine and vincristine in plant leaves (Gupta *et al.*, 2005). This phenomenon might be explained by the low expression levels of genes upstream that encoding peroxidase in the TIA pathway.

Leaf-specific expression of the heat stress transcription factor a-9 (cluster 82) is not justified. However, the ethylene-responsive transcription factor win1, highly expressed in the leaf (cluster 82), was reported to function in inducing the expression of enzymes involved in wax biosynthesis. This TF acts only in aerial organs (Broun *et al.*, 2004). There are clusters with organ-specific TFs involving stem and seedling organs (cluster 24), in one hand, and leaf and seedling organs (cluster 78), on the other hand. The transcription factors in cluster 24 involved bLHL30 and WRKY6. Overexpression of transcript encoding the first TF resulted in the upwardly curly leaves (An *et al.*, 2014), while that encoding the second is expressed in leaves and shoots to control processes related to senescence and pathogen defense (Robatzek & Somssich, 2002). The transcription factors in cluster 78 involved MYB28 and GATA22. The first prevents insect performance (e.g. lepidopteran insect *Mamestra brassicae* and *Spodoptera exigua*) by promoting glucosinolates (Gigolashvili *et al.*, 2007), while the second is involved in the regulation of chlorophyll biosynthesis and the response to light stimulus (Reyes *et al.*, 2004). Other regulations involved TFs that are up regulated (cluster 15) or down regulated (cluster 7) in both suspension cultures and hairy roots. Up regulation (cluster 20) and down regulation (cluster 43) of TFs solely in suspension cultures also took place (Fig. 3).

Transcripts of TFs that were concordantly co-expressed with those of the TIA pathway are shown in Figure 5. These co-expressed transcripts are displayed in seven clusters, e.g., 3, 21, 25, 28, 53, 64 and 74. The eight co-expressed transcripts of the TIA pathway involve three genes downstream tryptophan biosynthesis, e.g., *tdc, str1* and *sgd*, while five genes in the six-step conversion of tabersonine to vindoline, e.g., *t16h*, *omt*, *nmt*, *d4h* (two analogs) and *dat* (Fig. 1). The *tdc* and *omt* transcripts co-existed in one cluster (cluster 64) with a TF-like transcript harboring a bHLH domain. No information is available to predict the type of this TF, hence, its possible function. However, fold change data of this TF and its two co-expressed genes indicated that the three transcripts were highly expressed in hairy roots and may be MeJA-induced (cluster 64, Fig. 5). The other two transcripts downstream tryptophan biosynthesis, e.g., *str1* and *sgd*, co-expressed with three (cluster 25) and one (cluster 28) TFs, respectively. Two of the first three TFs belong to bHLH25, while the third is MYB12 whereas the TF co-expressed with *sgd* is WRKY2. Previous reports on the action of bHLH25 in Arabidopsis (namely AtbHLH025) indicated that it is mainly expressed in flower and induced by ethylene (ACC) and jasmonic acid (JA) (Heim *et al.*, 2003 see Figure 3; http://www.uniprot.org/uniprot/ Q9T072). MYB12 was indicated earlier to have a main role in flavonol biosynthesis in roots of Arabidopsis (http://www.ncbi.nlm.nih.gov/gene/819359). WRKY2 was reported as an ABA-inducible during seed germination and post germination growth arrest (Jiang & Yu, 2009). This data complements the results of the present study where the transcript encoding this TF along with the co-expressed *sgd* transcript were highly downregulated in all organs, treatments and genotypes, except in immature leaf (cluster 28).

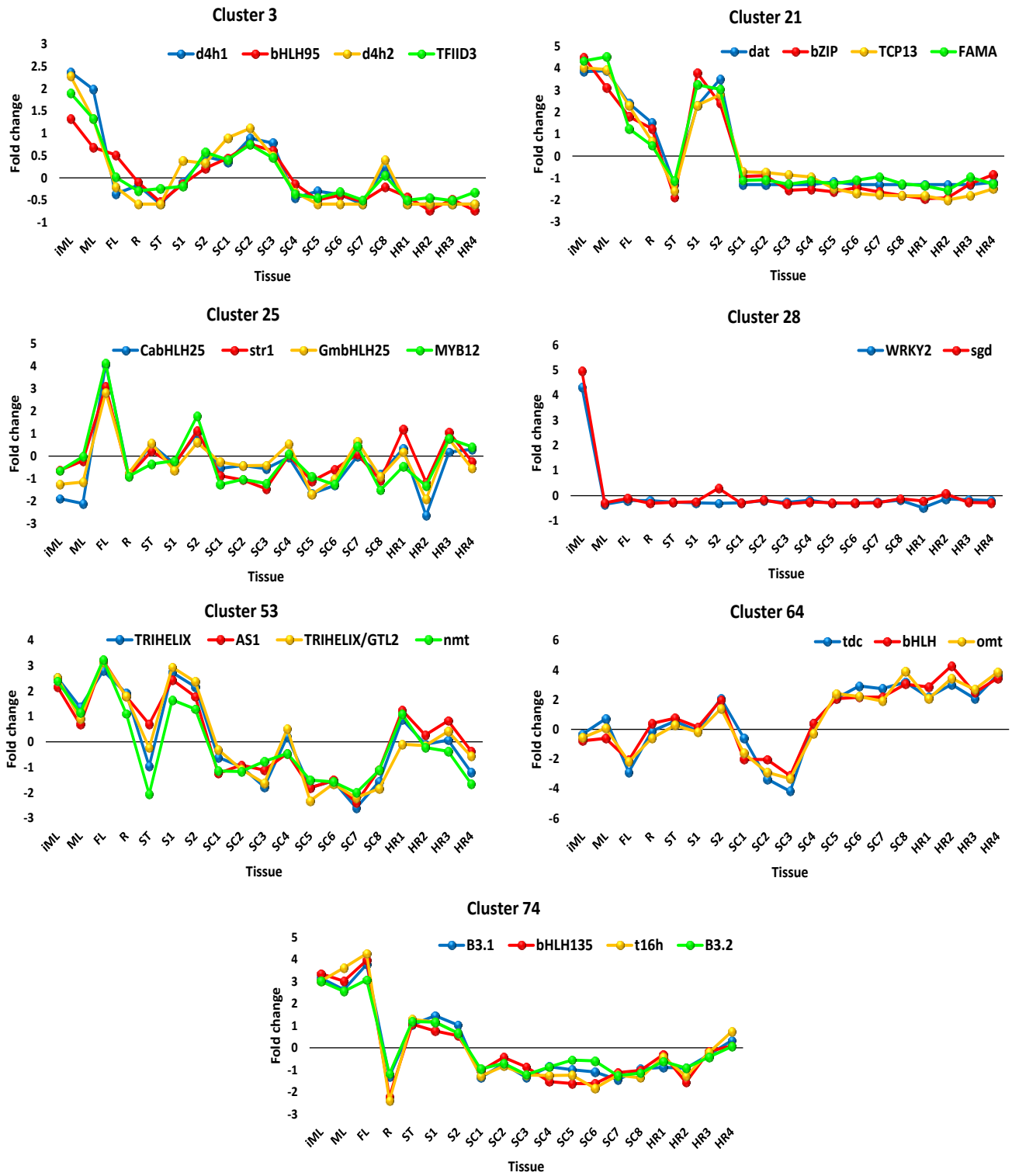
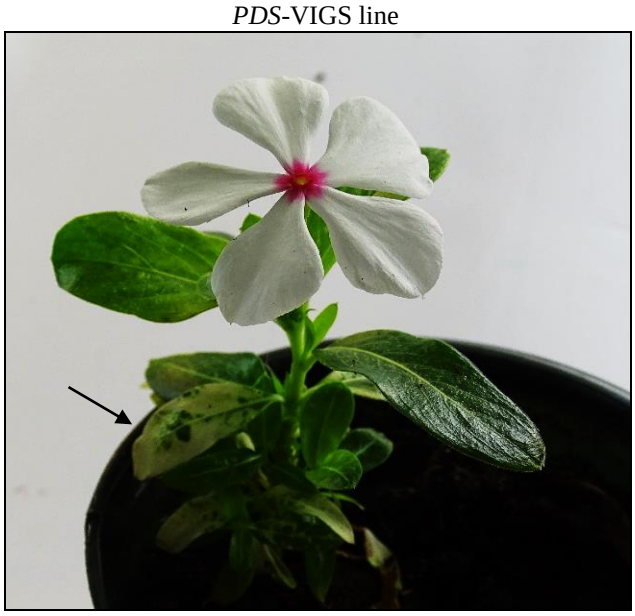



Fig. 5. Clusters indicating co- expression of transcripts related to the TIA biosynthetic pathway and TF-like transcripts of *C. roseus* organs under different conditions (See Table 2). iML = immature leaf, ML = mature leaf, FL = flower, R = root, ST = stem, S1-2 = sterile seedling 1-2, SC1-8 = suspension culture 1-8, HR1-4 = hairy root 1-4. S2, SC5-8, HR2, HR4 = MeJA-treated, HR3, HR4 = *tdc*-knocked down. *t16h* = tabersonine 16-hydroxylase, *d4h1* = desacetoxyvindoline-4-hydroxylase 1, *d4h2* = desacetoxyvindoline-4-hydroxylase 2, *dat* = deacetylvindoline 4-O-acetyltransferase, *str1* = strictosidine synthase 1-like, *sgd* = strictosidine beta-glucosidase, *nmt* = 16-methoxy-2,3-dihydro-3-hydroxytabersonine N-methyltransferase, *omt* = 16-hydroxytabersonine O-methyltransferase, *tdc* = L-tryptophan decarboxylase, bHLH95 = transcription factor bHLH95-like, TFIID3 = transcription initiation factor TFIID subunit 3-like isoform x1, leu zip = basic-leucine zipper transcription factor, tcp13 = transcription factor tcp13-like transcript variant, fama = transcription factor fama-like transcript variant, CabHLH25 = transcription factor bHLH25-like (*Cicer arietinum*), GmbHLH25 = *Glycine max* transcription factor bhlh25-like transcript variant, MYB12 = transcription factor myb12-like, WRKY2 = transcription factor WRKY 2, TRIHELIX = trihelix transcription factor, AS1 = transcription factor as1-like transcript variant, GTL2 = trihelix transcription factor GTL2-like, bHLH = basic helix-loop-helix, B3.1 = B3 domain-containing protein At5g60140-like, bHLH135 = transcription factor bhlh135-like, B3.2 = B3 domain.

*PDS*-VIGS line



empty pTRV2 line

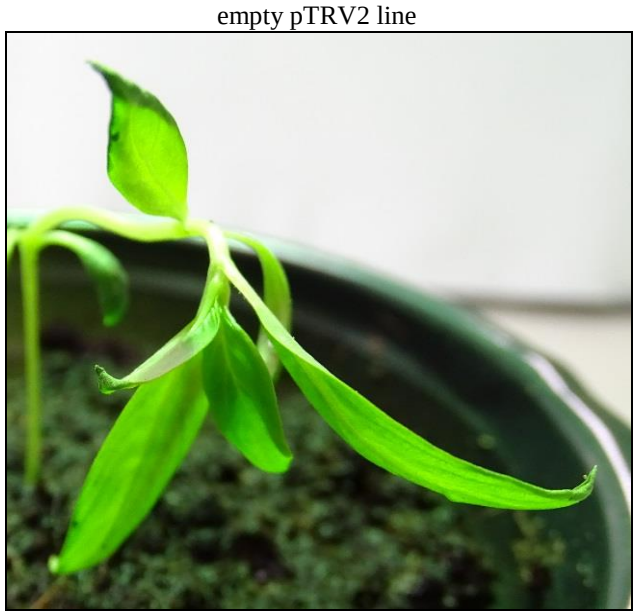


Fig. 6. *PDS*-VIGS line indicating photo-bleaching due to *PDS* gene silencing in leaves of *C. roseus* plants transformed with pTRV2 (Left) compared to the leaves of transformed plant with empty pTRV2 (Right).

The first transcript in the six-step conversion of tabersonine to vindoline, e.g., *t16h*, co-expressed with three TFs. Two of which harbor the B3 domain, while the third TF is a bHLH135. The B3 super family plays a central role in plant life from embryogenesis to seed maturation and dormancy (Wang *et al*., 2012). bHLH135 was recently reported to be involved in the regulation of light signaling pathway (Castelain *et al*., 2012). The latter data complements that of the present study as the four co-expressed transcripts were upregulated in all organs, except for the root, suspension cultures and hairy roots (cluster 74), where cells are not likely to be exposed to light. Transcripts of three TFs co-expressed with *nmt* transcript (cluster 53). Two out of these TFs belong to TRIHELIX family, while the third is an AS1. TRIHELIX was reported earlier to function in fruit and/or seed development (Smalle *et al.*, 1998). The TRIHELIX/GTL2 was recently reported to function in calmodulin/calcium binding in many plant organs and responds to cold and salt stresses (Xi *et al*. 2012). AS1 is regulated during leaf morphogenesis and is required for normal cell differentiation (Xu *et al.*, 2006). The results of the four co-expressed transcripts in this study (cluster 53) indicated highly down regulation only in stem and suspension cultures. Transcripts of two TFs co-expressed with two analogs of *d4h* transcripts (cluster 3). These two TFs are bHLH95 and TFIID3. The first TF was reported to act during seed development and in siliques only, while had no role in root, rosette leaves or flower development (Kondou *et al.*, 2008). TFIID3 is one of several factors that make up the RNA polymerase II preinitiation complex with no emphasis on certain organs or environmental conditions (Lee & Young, 2000). The results of the four co-expressed transcripts in cluster 3 indicated the high expression level in plant leaves. This data contradicted those available in the literature where expression of bHLH95 was scored only in siliques. The last gene in the six-step conversion of tabersonine to vindoline, e.g., *dat*, co-expressed with three TFs namely basic-leucine zipper (bZIP), TCP13 and FAMA (cluster 21). bZIP was reported to be upregulated by drought, NaCl and ABA treatments in vegetative organs (Uno *et al.*, 2000). TCP13 plays central roles during morphogenesis of shoot organs and defense response by negatively regulating the expression of boundary-specific genes, e.g., *CUC* genes (Koyama *et al*., 2007). FAMA is also required to promote differentiation and morphogenesis of stomatal guard cells and regulate stomata formation (Ohashi-Ito & Bergmann, 2006).

From the previous results, one solid evidence indicates that *str1* gene is likely driven by bHLH25 due to the following reasons. First, the results indicated that two, rather than one, versions of this TF (analogues of TF in *Cicer arietinum* and *Glycine max*) co-expressed with *str1* gene (Fig. 5). Second, previous reports indicate that *str1* gene is regulated by several TFs in *C. roseus* (Chatel *et al.*, 2003), which enlarges the chance of having other TFs, like bHLH25, driving the gene. Third, Figure 5 (cluster 25) indicated that *str1* and the two *bHLH25* genes were all highly expressed in the same tissue, e.g., flower. This data is in harmony with those of Heim *et al*. (2003). Forth, Heim *et al*. (2003) also diagrammatically indicated that this TF was particularly MeJA-inducible, which is an important criterion for regulating genes in the TIA pathway, especially *str1*gene (van der Fits & Memelink, 2000; Rischer *et al*., 2006; Miettinen *et al*., 2014). Accordingly, the two versions of this TF were analyzed further in order to confirm the current speculation. These two versions were knocked down separately utilizing the RNAi-based approach (VIGS) and expression levels of the two TF genes and the co-expressed *str1* gene were analyzed in flower and root via sqRT-PCR. The results of Figure 6 indicated the success of utilizing the tobacco approach (Velásquez *et al*., 2009) to induce VIGS line of the marker *PDS* (*phytoene desaturase*) gene (for photo-bleaching) in the new leaves 21 days after infiltration.

Figure 5 indicates that expression levels of *bHLH25-like* and *str1* genes in WT plants are high in flower, while low in root. The results of sqRT-PCR of the two *bHLH25*-VIGS lines indicated the occurrence of knockdown of either corresponding gene (*CabHLH25* or *GmbHLH25*) in the flower and confirmed the consequent knockdown of *str1* gene in the same organ compared to plant transformed with empty pTRV2 used as a control (Fig. 7). In other words, levels of expression of the three genes *CabHLH25*, *GmbHLH25* and *str1* in roots of the two *bHLH25*-VIGS lines and plant transformed with empty pTRV2 were low, while high only in the flower of plant transformed with empty pTRV2. Interestingly, either *bHLH25* genes was knocked down in the two *bHLH25*-VIGS lines confirming that *CabHLH25* and *GmbHLH25* are derivatives of one ancestral gene. This finding was further confirmed by the results of *str1* gene, where the latter gene was knocked down in both *bHLH25*-VIGS lines (Fig. 7). The results of house-keeping *actin* gene indicated no differential gene expression in VIGS lines and plants transformed with empty pVTR2 either in the flower or in the root (Fig. 7). Hence, we conclude that either version of bHLH25 likely regulates expression of *str1* gene. Further analysis is required on the effects of this TF on the other genes acting downstream *str1* gene in the TIA pathway.

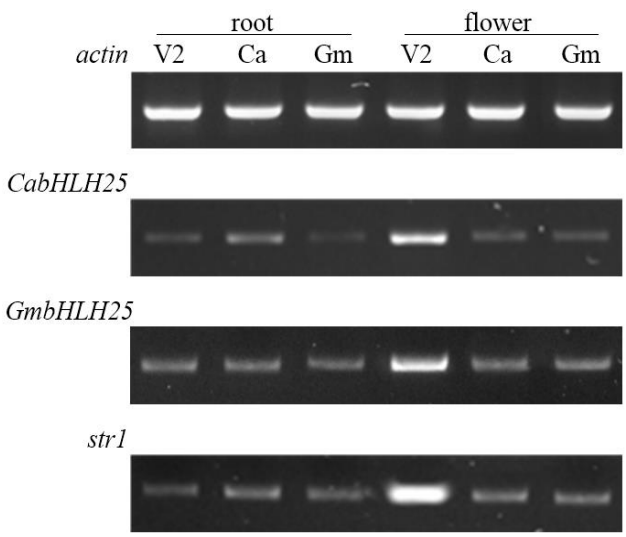



Fig. 7. sqRT-PCR of relative abundance of the two *C. roseus* TFs transcripts, e.g., *CabHLH25-like* (Ca) and *GmbHLH25-like* (Gm) and their driven gene (e.g., *str1*) in root and flower of its corresponding VIGS line compared to those in transformed line with empty pTRV2 plants (V2).Reactions of each *bHLH* gene (Ca or Gm) were done only for its corresponding VIGS line, while reactions of *actin* and *str1* genes were done for VIGS of the two *bHLH* (Ca and Gm) genes. The "*actin*" gene was used as the stably expressed house-keeping gene (250 bp). Primer sequences and PCR conditions are shown in Table 1.

In conclusion, the data of the present study highlights the possibility to manipulate genes in the TIA pathway via the over expression of hormone-regulated TFs. We anticipate that this information might be useful in future efforts towards the enhancement of metabolite biosynthesis in *C. roseus* via metabolic engineering of the TIA biosynthetic pathway.

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