Gloriosa superba and Colchicum autumnale multi-tissue transcriptome analysis for colchicine pathway and rhizome development candidate gene identification

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Abstract

Background The continued emergence of side-effects caused by synthetic drugs underscores the need for plant-based drugs in human medicine. Medicinal rhizomatous crops are a “goldmine for modern drugs”, and include such species as *Gloriosa superba* L. and *Colchicum autumnale* L., the producers of colchicine, a plant-based medicine. The natural isomer of bioactive colchicine is used to effectively treat major diseases such as cancer, cardiovascular disease, and gout. The medicinal properties of colchicine are well characterized, however, almost nothing is known about its biosynthesis. The paucity of information on the colchicine biosynthetic pathway is a significant barrier to biomanufacturing of this biomedicine. A comparative transcriptome study of *G. superba* and *C. autumnale* serves as a sequence resource to aid with identification of this biomedicine pathway and rhizome development genes for synthetic biotechnology toolbox, which will enable improved colchicine biomanufacturing. **Result** Transcriptomes of two colchicine synthesizing monocots *G. superba* and *C. autumnale* were interrogated to identify putative cDNAs encoding enzymes and transcription factors involved in the colchicine biosynthetic pathway and rhizome development. Mining of the transcriptomes using Blast2GO led to the identification from *G. superba* and *C. autumnale*, respectively, of 20 and 29 candidate colchicine biosynthetic genes N-methyltransferases, 3-O-methyltransferases, cytochrome P450s, a class that could catalyze several steps in the pathway, and N-acetyltransferases. Similarly, 19 and 15 candidate rhizome developmental genes, which belongs to several classes including GIGANTEA, CONSTANS, Phytochrome B, Sucrose Synthase), Flowering Locus T, and REVOLUTA. Likewise, about 16 and 12 transcription factors involved in regulating rhizome development and secondary metabolic pathways in rhizomes such as MADS-box, AP2-EREBP, bHLH, MYB, NAC, and WRKY were also found in *G. superba* and *C. autumnale*, respectively. **Conclusion** The predicted genes in *G. superba* and *C. autumnale* encode colchicine pathway enzymes that provide fundamental information for plant-based biomedicine engineering in biorhizomes and microorganisms, a potentially important area of synthetic biotechnology. Additionally, increasing our understanding of rhizome functional genomics will lead to improved colchicine biomanufacturing, and generate important knowledge that can be applied to many other medicinal plant species, allowing for the engineered production of additional biomedicines in medicinal rhizomes.

Background

The growing need for ultrapure plant-based medicines to treat human disease often cannot be met due to a lack of feasible upstream biomanufacturing processes. For example, therapeutic colchicine alkaloid, a drug used to treat cancer, cardiovascular disease, and gout [1-25], is uniquely biosynthesized by the Colchicaceae family and extracted commercially from *Gloriosa superba* and *Colchicum autumnale*, important rhizome crops and the prime pharmaceutical source of colchicine. Further, colchicine is an FDA approved drug [26]. The colchicine analog CH-35 was more effective at inhibiting the β III isotype of breast cancer than taxol [27-30]. The results of clinical trials suggest that colchicine could prevent the recurrence of atrial fibrillation after cardiac surgery and renal diseases [31-34]. Moreover, colchicine is undergoing clinical trials to treat non-diabetic metabolic syndrome and diabetic nephropathy [35-37]. The best

chemical synthesis method available generates only a 9.2% yield of >99% (-)-colchicine [38-39]. On the other hand, plant-based colchicine from *G. superba* yields approximately 1% DW[22, 40]. However, the annual production of pharmaceutical colchicine is limited by its source [41-45].

Despite the availability of sequence data for the *G. superba* and *C. autumnale* transcriptomes (from the Medicinal Plant Transcriptome <https://medplantnaseq.org/>) and their chloroplast genomes, little is known about the colchicine biosynthetic pathway and its regulation in the plant [46]. The efficiency of colchicine biosynthesis likely depends on the interaction of gene circuit elements with other components within the biosynthetic network and how those gene circuits are regulated. This situation with colchicine production is not unusual—as more plant-based medicinally important compounds are discovered, especially where high purity and large amounts are required. To overcome the plant-based therapeutic colchicine production limitation, an advanced non-dormant *in vitro* biorhizome technology from *G. superba* has been established [47]. Biorhizomes are non-transgenic cultures that produce important biomedicine, which also serves as asexual reproductive organs, and are an advanced biotechnological platform compared to root and cell cultures due to their continuous and rapid colchicine production [48]. Nevertheless, the biochemical pathways and regulatory networks in the biorhizomes that control colchicine biosynthesis are yet to be characterized, leaving a significant barrier to improving colchicine biomanufacturing. Therefore, the first steps in building a synthetic biology toolbox for colchicine production include analysis of genes from the different Colchicaceae species in order to identify regulatory steps and factors that can then be adjusted to enhance colchicine biomanufacturing in the biorhizomes.

The current understanding of colchicine biosynthesis *in planta* is based on radiolabeling studies [49-51] and the transformation of *O*-methylandrocymbine to demecolcine by microsomes prepared from immature *C. autumnale* seeds [52]. The phenylalanine precursor in the phenylpropanoid pathway and trihydroxylated phenethylisoquinoline in the colchicine pathway have been studied [53-56]. However, research has not been performed at the molecular level to uncover enzymes and regulatory proteins in the colchicine biosynthetic pathway in *G. superba* or *C. autumnale*. To fully understand how colchicine is biosynthesized in *Gloriosa* biorhizomes, the key genes and enzymes that control the colchicine pathway from the alkaloidal precursor trihydroxylated phenethylisoquinoline to colchicine must be identified. To this end, we constructed a full-length cDNA library using mRNA isolated from *G. superba* leaves that consists of 2,790 processed sequences, of which 1,379 were assembled into 292 contigs and 1,411 singletons [47]. The cDNA library contains gene families expected to be involved in the colchicine pathway, including NMT, 3-OMT, cytochrome P450s: CYP96T1, CYP82E10, and NAT. Furthermore, we manually curated these gene families and identified specific genes whose corresponding enzymes are excellent candidates for involvement in the colchicine pathway, from the alkaloid formation steps to later steps in the pathway (Figure 1), including: 1) an NMT enzyme that catalyzes the conversion of the trihydroxylated phenethylisoquinoline intermediate to (*S*)-autumnaline; 2) a P450: CYP96T1 that catalyzes (*S*)-autumnaline to isoandrocymbine; 3) an OMT enzyme that catalyzes the transformation of isoandrocymbine to 3-*O*-methylandrocymbine; 4) a P450 that catalyzes *O*-methylandrocymbine to demecolcine; 5) an additional P450 CYP82E10 that catalyzes the conversion of demecolcine to

deacetylcolchicine; and 6) a NAT enzyme that catalyzes the transfer of an acetyl group from acetyl-CoA to a deacetylcolchicine nitrogen group to yield colchicine [47].

Although significant progress has been made in understanding rhizome-specific functions in plants, the mechanisms underlying the regulation of *Gloriosa* biorhizome growth, and the dormancy of field-grown *G. superba* and *C. autumnale* are not yet known [57-59]. Similar to many other rhizomatous species, natural *G. superba* and *C. autumnale* rhizomes undergo a dormancy period in their normal growth cycle, but the biorhizomes do not go dormant. Extensive studies examined the phenotypic variation between plant species, but why dormancy-free biorhizomes from different *Gloriosa* species produce different levels of colchicine in the controlled bioreactor environment remains unclear. The function of dormancy-associated genes (such as specific transcription factors) in biorhizomes and the core regulatory machinery that controls differential colchicine biosynthesis between species are not known. We hypothesize that *G. superba* and *C. autumnale* genes and gene networks are comparable, but that subtle differences in their regulation lead to changes in colchicine accumulation between the species. Comparison of the transcriptomes of these species will aid in filling the identified knowledge gaps.

Results

Benchmarking universal single-copy orthologue (BUSCO) analysis: Three *in vivo* tissues (leaf, fruit, and rhizome) were previously used to generate a combined RNA-seq dataset of *G. superba* and *C. autumnale* (<https://medplantnaseq.org/>). This investigation of the dataset indicated that the N50 for the assembly was fairly long at 2,134, given that contigs of ≥ 100 (instead of ≥ 200) were included in the assembly and that the data were generated from 50 bp single-end reads. The average contig length was somewhat short, however, as the statistic was skewed due to the inclusion of several contigs that were shorter than 200. BUSCO analysis, which in this case examined the core eukaryotic genes in plants, indicated that the dataset was ~89% complete (64% of the genes detected were found as a single sequence in the assembly, and 25% had duplicated sequences). In comparison, a total transcriptome analysis that we conducted in a different species and that focused on only a single sample type (containing combined RNA samples from the 1st and 2nd Asian Citrus Psyllid instars, including six biological replicates and utilizing 150 bp reads), led to BUSCO analysis results suggesting that the dataset was 96% complete (13% single sequence and 83% duplicate sequences), with only 1.4% fragments of core genes and 2.6% missing core genes. Thus, although the sequence data used in this analysis were of high quality, it is likely that some important genes may nevertheless still be missing from the dataset.

Annotation and comparative transcriptomes analysis: The *C. autumnale* and *G. superba* transcriptomes consist of 60,927 and 32,312 assembled multiple-tissue transcripts with 21,948 and 15,089 unigenes, respectively, identified as having functions belonging to known plant-specific gene ontology (GO) terms. Among these, 23,247 and 45,292 sequences were assigned and annotated as biological processes, 27,199 and 52,366 sequences as cellular components, and 22,760 and 44,942 sequences as molecular functions in *G. superba* and *C. autumnale*, respectively (Figure 2). The *G. superba* leaf tissue cDNA library was also annotated using the Blast2GO suite and contained a total of 1,703 unigenes with 588

sequences were assigned to biological process, 700 sequences to cellular component, and 568 sequences to molecular function categories (Figure 2). Figure 3 shows the percentages of GO terms assigned to the transcriptomes of *G. superba* and *C. autumnale*, and the cDNA library. Additional GO terms were used to identify Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway members, putative colchicine pathway enzymes, and rhizome developmental genes, along with transcription factors. KEGG annotated 15184 of 57580 (26.4%) and 33505 of 100528 (33.3%) sequences in *G. superba* and *C. autumnale* transcriptomes, respectively (Figure 4), suggesting that many of the transcripts belong to non-canonical pathways (e.g., are not involved in known signaling pathways or biosynthetic pathways). Many of these reads were characterized as “unknown”, as is still typical in transcriptomic analysis of non-model plant species [60-61]. *G. superba* cDNA library was also annotated by BlastKOALA, which annotated 66 of 848 (7.8%) sequences sets of proteins based on their role in specific pathways (Figure 4).

The mining of *G. superba* and *C. autumnale* transcriptomes revealed a total of 1299 genes that were possible initial candidates for involvement in the colchicine pathway, which included 647 sequences from *G. superba* and 652 from *C. autumnale*. From 647 candidate sequences in *G. superba*, 186 coded for NMTs, 105 for OMTs, 19 for NATs, and 337 for P450s; while from 652 candidate sequences in *C. autumnale*, 16 were putative NMTs, 106 OMTs, 20 NATs, and 510 P450s (Figure 5). Further, these transcripts were narrowed down to candidates for the genes that catalyze the reactions in the colchicine biosynthetic pathway. Moreover, a total of 339 putative rhizome developmental genes identified based on comparison to known rhizome developmental genes (119 sequences) from *Nelumbo nucifera* and tuber developmental genes (142 sequences) from *Solanum tuberosum* such as *Gl*, *CO*, *PHYB*, *SuSy*, *FT*, and *REV* were also identified in *G. superba* and *C. autumnale* transcriptomes (Figure 6). Notably, 113 sequences from *G. superba* and 226 from *C. autumnale*. In addition, a total of 1146 transcription factor sequences associated with rhizome development namely MADS-box, AP2-EREBP, bHLH, MYB, NAC, and WRKY were analyzed (Figure 7). This included 481 sequences from *G. superba* and 665 from *C. autumnale*. Phylogenetic trees of colchicine pathway, rhizome development, and transcription factors (with a total of 65, 58, and 59, respectively) of full-length amino-acid candidate and reference genes were constructed using the PHYLIP-3.697 software with the objective to examine the genetic divergence between two colchicine producing plants, *G. superba* and *C. autumnale*, and their reference sequences.

Discussion

What genes are expressed in rhizomes? Several recent investigations have identified rhizome-specific genes in different rhizomatous plants by directly comparing leaves, other tissues, and rhizomes, leading to the detection of genetic mechanisms responsible for controlling rhizome development, growth, and metabolism. Many genes exhibit significantly altered expression during rhizome development but genes associated with auxin hormone signaling appear to trigger rhizome induction [57-59]. The rhizome developmental gene *REV* is highly expressed in bamboo rhizome buds and plays an important role in meristem initiation [62]. In potato plants, calmodulin-binding protein plays a regulatory role in signal transduction for tuber formation [63]. In addition, the *FT*, *CO*, and *Gl* genes are involved in the

transduction of photoperiodic signals, which may promote rhizome budding in potatoes [64-65]. Tubers are not rhizomes, but there are some similarities to their growth that may include involvement of analogous genes. There are 14 other important rhizome formation-related genes, including a *MADS-box* that could be involved in rhizome enlargement [66]. Genes encoding PHYB, CO, GI, FT and SuSy were identified in Lotus rhizomes, but their expression and regulation differed in the shoot and rhizome [60]. Transcription factor families such as AP2-EREBP, bHLH, MYB, NAC, and WRKY were reported to be important in regulating specialized metabolic pathways in rhizomes [61]. Bioactive small molecules such as curcuminoid and candidate genes for gingerol synthesis are also highly expressed in rhizomes [67-71]. Notably, biosynthetic genes involved in benzyloquinoline alkaloid formation were highly upregulated during bulb development in *Corydalis yanhusuo* [72]. In wild rice, microRNAs were differentially expressed in aerial shoots and rhizomes [73]. However, the exact roles that the corresponding genes might play in biorhizomes are not known. This information provides a framework for our analysis of biorhizome-expressed genes and the identification of specific genes involved in biorhizome growth, resistance to dormancy and production of colchicine in this interesting biotechnology platform.

Why are colchicine pathway candidate NMT, P450s (CYP96T1 and CYP82E10), 3-OMT, and NAT genes from the transcriptomes the best targets? First, a cDNA contig consisting of three sequences showed significant sequence similarity to *Ricinus communis* NAT and partial similarity to an analogous gene from *Oryza sativa*. This contig, which contains a full-length transcript (cDNA *Gloriosa* 148), is a potential candidate for encoding the enzyme that catalyzes the acetyl transfer from acetyl-CoA to deacetylcolchicine to form colchicine, the final step in the proposed colchicine pathway [52]. The putative NAT gene also showed 100% homology to a full-length transcript in the *G. superba* transcriptome (*Gloriosa*-20120814|26859). Similarly, two NAT catalyzed reactions were identified in melatonin biosynthesis in *O. sativa*[74-75], carried out by SNAT1 and SNAT2 enzymes. The *SNAT1* transcript Q5KQI6.1 is a possible alternative reference sequence, with an identity of 69%, and 77%, respectively, to transcripts in the *G. superba* and *C. autumnale* transcriptomes (*Gloriosa*-20120814|70209_1 and *Colchicum*_20101112|6813; see Table 1 and Figure 8).

Second, the cDNA clone (*Gloriosa* 14D06) identified as encoding a putative NMT is a partial clone consisting mainly of the 3' end of the cDNA (Table 1 and Figure 8). The putative NMT clone had high sequence homology to a *Coptis japonica* NMT, which catalyzes a similar *N*-methylation reaction involved in (*S*)-*N*-methylcoclaurine formation in the benzyloquinoline pathway and has a near identical full-length transcript in the *G. superba* transcriptome (*Gloriosa*-20120814_33123). The putative NMT is a one candidate for the *N*-methylation step that converts the trihydroxylated phenethylisoquinoline intermediate to (*S*) autumnaline, the first alkaloidal precursor formation step in the proposed colchicine pathway [52, 76]. Other candidates include, the *G. superba* putative NMT transcript *Gloriosa*-20120814|64082_1, which was found to be 58% identical to *C. japonica* coclaurine NMT (BAB74802.1), and *Colchicum*_20101112|85, a transcript with 48.7% identity to pavine NMT (PNMT, sp|C3SBW0.1) from *Thalictrum flavum* (PNMT) converts (*S*)-tetrahydropapaverine to (*S*)-laudanoline in the benzyloquinoline alkaloid pathway that is common in many plants [77]. PNMT is also considered a possible alternative reference sequence due to its capability of adding a methyl group to (*S*)-

tetrahydropapaverine where the nitrogen is present, which is similar to the colchicine pathway mechanism. These putative transcripts also share homology with reticuline NMT involved in biosynthesis of the aporphine alkaloid magnoflorine in opium poppy roots [78].

Third, the cDNA clone (*Gloriosa* 8E03) identified as a putative P450 is a partial clone with 87% sequence homology to *Narcissus pseudonarcissus* CYP96T1, which catalyzes a C-C phenol coupling reaction in noroxomaritidine biosynthesis in the haemanthamine pathway and has an identical full-length transcript in the *C. autumnale* transcriptome (*Colchicum*-20101112_3005). This putative enzyme might be a candidate for the *para-para* phenol-tropolone oxidative coupling bridge-forming P450 CYP96T1, that is NADPH and O₂-dependent and converts (*S*)-autumnaline to isoandrocymbine [79-80]. In *Papaver somniferum*, salutaridine synthase enzyme (CYP719B1) is responsible for the C-C phenol coupling converting (*R*)-reticuline to salutaridine by connecting the 12 and 13 carbon [81-82]. *P. somniferum* CYP719B1 enzyme could also be considered as an alternative reference gene because of its C-C phenol-coupling mechanisms in the morphine pathway. Furthermore, in *C. japonica*, CYP80G2 has been shown to convert (*S*)-reticuline to (*S*)-corytuberine through C-C phenol-coupling in an isoquinoline alkaloid pathway [83]. The *G. superba* and *C. autumnale* transcriptomes contained transcripts (*Gloriosa*-20120814|30999_1 and *Colchicum*_20101112|76071) that shared of 52.7% and 51.7% identity, respectively, with CYP80G2 (sp|A8CDR5.1; see Table 1 and Figure 8).

Fourth, the cDNA clone (*Gloriosa* 7D05) identified as a putative OMT is a partial clone with high sequence homology to *Narcissus* sp. aff. *pseudonarcissus* norbelladine-4-OMT, which participates in a similar *O*-methylation reaction to form *O*-methylnorbelladine in the galanthamine pathway and has an identical full-length transcript in the *G. superba* transcriptome (*Gloriosa*-20120814_6585 and *Gloriosa*-20120814|82787_1). This putative OMT is a likely candidate for the proposed 3-*O*-methylation step that would catalyze the conversion of isoandrocymbine to *O*-methylandrocymbine [84]. The 3-OMT is expected to be a class I methyltransferase, similar to other members of this gene family that are involved in plant alkaloid metabolism. Additional reference 3-OMT sequences, such as AFB74613.1 from *P. somniferum* and sp|A0A077EWA5.1| from *N. pseudonarcissus*, were considered when filtering *G. superba* and *C. autumnale* transcriptomes for possible alternative 3-OMTs. *Gloriosa*-20120814|16875_1 and *Gloriosa*-20120814|82787_1 were found to possess 50.9% and 72.8% identity, respectively, to the sequences. The transcript *Colchicum*_20101112|23611 showed an identity of 58.1% to the *N. pseudonarcissus* gene (Table 1 and Figure 9). In *P. somniferum* 3-OMT catalyzes the conversion of 4'-*O*-desmethyl-3-*O*-acetylpapaveroxine to 3-*O*-acetylpapaveroxine by forming a heterodimer with 2-OMT in the noscapine pathway [85-86].

Fifth, the cDNA clone (*Gloriosa* 1F02) identified as another putative P450 is a partial clone with 79% sequence homology to CYP82E10 from *Fragaria x ananassa* and *Nicotiana tabacum*, which catalyzes an *N*-demethylation reaction from nicotine to nornicotine and has a full-length homolog in the *C. autumnale* transcriptome (*Colchicum*-20101112_5364). This putative P450 enzyme might be a candidate for the *N*-demethylation that converts demecolcine to deacetylcolchicine [87]. Nevertheless, in *N. tabacum* three cytochrome P450 superfamily of monooxygenases that catalyze the *N*-demethylation of nicotine have

been identified, CYP82E4, CYP82E5v2, and CYP82E10[88]. CYP82E4 is the major nicotine demethylase enzyme responsible for converting nicotine to nornicotine [89]. The demethylase P450 enzymes CYP82E5v2 and CYP82E10 were expressed in nonsenescent green leaves and/or root tissue in *N. tabacum* [87, 89-93]. The transcripts, *Gloriosa*-20120814|5091 and *Colchicum*_20101112|74349 were found to possess 53.4%, and 54% identity, respectively, to NP_001312976.1 (CYP82E4; see Table 1 and Figure 9). Thus, enzymes belonging to the predicated NMT, 3-OMT, CYP96T1, CYP82E10, and NAT families are likely to be early targets for cloning and characterization in the colchicine pathway. However, the possibility exists that one or more of the candidate genes will not function as predicted. In this case, other genes from the *G. superba* and *C. autumnale* transcriptomes that show homology to colchicine pathway enzymes would also be considered as candidate genes.

What genes are candidate rhizome developmental genes from G. superba and C. autumnale?

Underground rhizome (storage organ) development and the molecular mechanisms controlling rhizome enlargement and dynamics remain unclear, even in well-studied medicinal and invasive plants [60, 68-69]. Biorhizomes can be a suitable system to study rhizome growth and biomedicine biomanufacturing because they are much more accessible than plant tissues growing in the soil. In addition, it is useful to identify genes related to rhizome development as they likely control key resources involved in biomass yield. Several genes have been identified from other species that affect rhizome formation in different rhizomatous crops, but comparable genes have not been characterized in the Colchicaceae. Therefore, characterizing rhizome developmental genes from *G. superba* and *C. autumnale* could allow clarification of molecular intricacies involved in biorhizome biomass production and lead to enhanced biomanufacturing. To screen for rhizome developmental genes in colchicine producing species, the transcriptomes from *G. superba* and *C. autumnale* were analyzed as described above for metabolism related genes. Specific results related to major development-related genes are outlined below.

G1 is a sensor gene that involved in seasonal growth, which includes tuber development in potato [61, 94-96]. The reference *G1* sequence from *N. nucifera* transcriptome (gi|720040388|ref|XP_010268589.1) and *S. tuberosum* transcriptome (PGSC0003DMT4000048370) were compared with *G. superba* and *C. autumnale* transcriptomes. Two potential full-length candidate genes *Gloriosa*-20120814|8521_1 and *Gloriosa*-20120814|11998_1 shared an identity of 70%, and 76%, respectively, to these genes (Table 2 and Figure 9).

CO genes play an important role in the circadian clock and rhizome development in radish and potato [97-98]. Five full-length sequences revealed *CO* functionality in *Gloriosa*-20120814|18001_1, *Gloriosa*-20120814|51159_1, *Gloriosa*-20120814|17037_1, *Gloriosa*-20120814|20079_1, and *Gloriosa*-20120814|72100_1. The reference sequence from *S. tuberosum* (NP_001274795.1) shared an identity with candidate sequences of 64%-73%. While *Colchicum*_20101112|3159, *Colchicum*_20101112|5391, *Colchicum*_20101112|26221, and *Colchicum*_20101112|27602 shared an identity of 72%-82%. The alternate reference sequence from *S. tuberosum* (PGSC0003DMT400067656) shared an identity with candidate sequences *Gloriosa*-20120814|51159_1 of 76% and *Colchicum*_20101112|3159 of 82%. The reference sequence from *N. nucifera* (gi|719967386|ref|XP_010261698.1) shared an identity with

candidate sequences *Gloriosa*-20120814|51159_1 of 84% and *Colchicum*_20101112|3159 of 82% (Table 2 and Figure 9).

FT and *FT*-like protein StSP6A are key components of tuberigen and a systemic floral inducer in potato [99]. By employing computational analysis using RNA-Seq data, we identified five full-length *FT* candidate genes such as *Gloriosa*-20120814|55554_1, *Gloriosa*-20120814|55004_1, *Gloriosa*-20120814|26010_1, *Gloriosa*-20120814|45011_1, and *Gloriosa*-20120814|52745_1), while two candidate genes from *Colchicum*_20101112|8829 and *Colchicum*_20101112|22914. The *FT* reference sequence from *S. tuberosum* (StSP6A NP_001274897.1) shared an identity range with candidate genes of 60%-74%. The alternative *FT* reference sequence from *S. tuberosum* (PGSC0003DMT400060057) shared an identity with candidate sequence *Gloriosa*-20120814|55554_1 of 76% and two sequences with *Colchicum*_20101112|8829 and *Colchicum*_20101112|22914 of 75% and 79%, respectively. The *FT* reference sequence from *N. nucifera* (gi|720039388|ref|XP_010268289.1) shared an identity with *Gloriosa*-20120814|55554_1 of 84% and two candidate genes with *Colchicum*_20101112|8829 and *Colchicum*_20101112|22914 of 81% and 86%, respectively (Table 2 and Figure 9).

The photoreceptor *PHYB* is involved tuber induction and microRNA, miR172 highly expressed in potato tuber [100]. It has been shown in *N. nucifera* that *PHYB* and/or other phytochromes might measure the length of the light period to affect rhizome girth enlargement [61]. The *PHYB* candidate gene *Gloriosa*-20120814|36050_1 and *Colchicum*_20101112|1221 shared an identity of 81% with the reference sequence from *N. nucifera* (gi|720038316|ref|XP_010267948.1). The *PHYB* reference sequence from *S. tuberosum* (PGSC0003DMT400061712) shared an identity of 81% with candidate sequence *Gloriosa*-20120814|85746_1) and 80% with *Colchicum*_20101112|1221 (Table 2 and Figure 9).

SuSy plays a key role in tubers biomass allocation, starch biosynthesis and storage, and cleaves sucrose into fructose and UDP-glucose [101-102]. Two full-length *SuSy* sequences were identified in *G. superba* such as *Gloriosa*-20120814|9073_1 and *Gloriosa*-20120814|69933_1); whereas five candidate genes in *C. autumnale* *Colchicum*_20101112|583, *Colchicum*_20101112|593, *Colchicum*_20101112|2693, *Colchicum*_20101112|13826, and *Colchicum*_20101112|33099, which shared an identity of 81-82% with *N. nucifera* (gi|720050864|ref|XP_010271909.1). The reference sequence from *S. tuberosum* (PGSC0003DMT400007506) shared an identity of 78% with candidate sequence *Colchicum*_20101112|2693 (Table 2 and Figure 9).

REV plays an important role during morphogenesis and controlling the apical meristem formation during rhizome development in bamboo [61, 103]. Since, biorhizomes are compressed scale leaves that replicate rhizomes possessing vegetative buds [47], at the molecular level an intricate regulatory network determines initial biorhizome development and *REV* could antagonistically regulate lamina structures that trigger formation of specialized organs. The *REV* reference sequence from *N. nucifera* (gi|720097995|ref|XP_010247499.1) shared an identity with candidate *Gloriosa*-20120814|11056_1 sequence of 86% and *Colchicum*_20101112|467 of 85%. The reference sequence from *S. tuberosum* (PGSC0003DMT400030829) shared an identity with *Gloriosa*-20120814|17486_1 of 81% and

Colchicum_20101112|467 of 81%. Additionally, the *REV*-like homolog (*PpHB1*) was found in bamboo, which highly expressed in the tips of lateral buds at several developmental stages [104]. BLAST analysis of *Gloriosa-20120814|15982_1* and *Colchicum_20101112|6503* showed a 79% and 78% identity when compared to *PpHB1*, respectively (Table 2 and Figure 9).

What are the transcription factors in G. superba and C. autumnale? Currently, no information is known about transcription factors in *G. superba* and *C. autumnale*. Besides rhizome developmental genes, an important subset of rhizome transcription factors genes was also identified. It was suggested that MADS-box (containing the MADS domain), AP2-EREBP (APETALA2/ethylene-responsive element binding protein), bHLH (basic helix-loop-helix), MYB (myeloblastosis related), NAC (no apical meristem), and WRKY (contain the highly conserved amino acid sequence WRKYGQK and the zinc-finger-like motifs Cys(2)-His(2) or Cys(2)-HisCys, and bind to the TTGAC(C/T) W-box *cis*-element in the promoter of their target genes) proteins can act as rhizome developmental transcription factors [105-108]. Several genome-wide analyses have been conducted on these transcription factors in *S. tuberosum*, *N. nucifera* and *Sinopodophyllum hexandrum* [61, 105, 109]. The screened candidate transcription factors listed below could contribute to our understanding of the molecular mechanism of biorhizome development. Moreover, a better understanding of the regulation of colchicine production will be gained by identifying how candidate colchicine biosynthetic gene family members interact with and are controlled by rhizome developmental genes and transcription factors in *G. superba* and *C. autumnale*.

MADS-box genes were present on all 12 potato chromosomes, and StMADS1 and StMADS13 were proposed to be likely downstream targets of StSP6A and involved in tuber development [105]. Therefore, the known *S. tuberosum* and *N. nucifera* *MADS-box* sequences were used as query to perform BLAST against the *G. superba* and *C. autumnale* protein databases. One full-length candidate sequence *Gloriosa-20120814|19828_1* shared an identity of 77% with an *N. nucifera* gene (*gi|720053055|ref|XP_010272608.1*). The reference sequence from *S. tuberosum* (PGSC0003DMT400000026) shared an identity of 81 and 82% with candidate sequences *Colchicum_20101112|22985* and *Gloriosa-20120814|35037_1*, respectively (Table 3 and Figure 9).

AP2 (APETALA2) and EREBPs are some of the largest and the prototypic families of transcription factors unique to plants and are upregulated in rhizomes [110-111]. Four *AP2-EREBP* candidate genes were *Gloriosa-20120814|77954_1*, *Gloriosa-20120814|7267_1*, *Gloriosa-20120814|54352_1*, and *Gloriosa-20120814|45526_1* based on homology to the reference sequences in *N. nucifera* (*gi|720011136|ref|XP_010259468.1*) with identities of 66%, 69%, 82%, and 86%, respectively. In addition, full-length alternative sequences were identified using a reference sequence from *S. tuberosum* (PGSC0003DMT400016585) such as *Colchicum_20101112|4936* and *Colchicum_20101112|10921* from *C. autumnale* with identities of 76% and 82%, respectively. Of the six *AP2-EREBP* candidate genes, two (*Gloriosa-20120814|7267_1* and *Gloriosa-20120814|77954_1*) were found to be 100% identical to *G. superba* leaf tissue cDNA *Gloriosa* 8E03 (Table 3 and Figure 9).

The bHLH superfamily of proteins is the second largest transcription factor family in plants and *StbHLH76* and *StbHLH86* had relatively high expression levels in the potato tuber compared to other tissues [112]. The full-length sequence of *Gloriosa*-20120814|6279_1 demonstrated possible *bHLH* functionality with a reference sequence from *N. nucifera* (gi|720006121|ref|XP_010257880.1) with an identity of 90%. The reference sequence from *S. tuberosum* (PGSC0003DMT400022702) shared an identity of 89% with the *bHLH* candidate sequence *Gloriosa*-20120814|53746_1). Additionally, the reference sequence from *N. nucifera* (gi|719971259|ref|XP_010273628.1) shared an identity of 82% with the *bHLH* candidate sequence from *C. autumnale Colchicum*_20101112|7042. Likewise, *S. tuberosum* (PGSC0003DMT400022701) shared an identity of 86% with the candidate sequence *Colchicum*_20101112|21881 (Table 3 and Figure 9)

The MYB transcription factors are considered potentially important regulators of secondary metabolism, where the R2R3-MYB proteins are specific to plants [113-115]. The reference *R2R3-MYB* sequence from *N. nucifera* (gi|719972251|ref|XP_010277005.1) shared an identity of 91% with the *MYB* candidate sequence (*Gloriosa*-20120814|15461_1). The reference sequence from *S. tuberosum* (PGSC0003DMT400012203) shared an identity of 91% with the *MYB* candidate sequence *Gloriosa*-20120814|15461_1. In *C. autumnale*, candidate sequence *Colchicum*_20101112|11314 shared an identity of 87% with *N. nucifera* (gi|719993192|ref|XP_010253806.1). Likewise, the *S. tuberosum* sequence (PGSC0003DMT400017709) shared an identity of 86% with candidate sequence *Colchicum*_20101112|20668 (Table 3 and Figure 9).

There is abundant evidence indicating that NAC proteins play crucial roles in hormone signaling, lateral root development and are upregulated during rhizome formation [61, 116-117]. *NAC* candidate sequence *Gloriosa*-20120814|28710_1 shows 84% identity with reference gene from *N. nucifera* (gi|720085419|ref|XP_010243512.1). An alternative gene *Gloriosa*-20120814|18396_1, was 81% identical to *S. tuberosum* PGSC0003DMT400045294. The reference sequence from *N. nucifera* (gi|720046186|ref|XP_010270427.1) shared an identity of 71% with candidate sequence *Colchicum*_20101112|44481 and *S. tuberosum* (PGSC0003DMT400079789) shared an identity 71% with *Colchicum*_20101112|794 (Table 3 and Figure 9).

WRKY proteins can regulate diverse responses in rhizome-related gene networks, including sprouting mechanisms in potato tuber and in specialized metabolism [118-119]. Two full-length candidate sequences, *Colchicum*_20101112|3957 and *Colchicum*_20101112|49701, shared significant homology with the reference sequence from *Sinopodophyllum hexandrum* (ALD83482.1), with 76% and 80% identity, respectively. A similar sequence from *Gloriosa*-20120814|20138_1 shared an identity of 72%. The reference sequence from *N. nucifera* (gi|720006863|ref|XP_010258120.1) shared an identity of 77% with *Gloriosa*-20120814|20164_1. The reference sequence from *S. tuberosum* (PGSC0003DMT400028529) shared an identity of 77% with candidate sequence *Gloriosa*-20120814|66904_1. The alternative reference sequence from *N. nucifera* (gi|720007162|ref|XP_010258216.1) shared an identity of 81% with candidate sequence *Colchicum*_20101112|22998 and *S. tuberosum* (PGSC0003DMT400072835) shared an identity percentage with *Colchicum*_20101112|22998 of 81% (Table 3 and Figure 9).

Phylogenetic analysis of colchicine pathway, rhizome developmental, and transcription factor protein in G. superba and C. autumnale: A total of 20 and 29 candidate colchicine pathway enzymes in *G. superba* and *C. autumnale* transcriptomes, respectively, were split into four primary clusters and mapped against 4 cDNA and 12 reference enzymes (Table 1 and Figure 10A). Among these, step 1 NMT contained 7 genes in the clade such as 3 from *G. superba* transcriptome, 1 from *G. superba* cDNA, and 1 from *C. autumnale* transcriptome. Both step 2 and step 4 P450s genes were closely related and aligned in the same clade but step 2 P450 genes was nested within step 4. Within the step 2 P450's nest, 2 genes in *G. superba* and 5 in *C. autumnale* transcriptomes and 1 cDNA. While in step 4, 1 in *G. superba* and 4 in *C. autumnale* transcriptomes and 1 in cDNA, which indicates that the P450 genes could be evolutionarily similar. The largest clade represented by OMT, which encompassed 15 *G. superba* and 10 *C. autumnale* genes. The last cluster NAT was comprised of 4 genes in *G. superba* and 3 in *C. autumnale* transcriptomes and 1 in cDNA.

Additionally, rhizome developmental gene identification of potential orthologous between *G. superba* and *C. autumnale* is one of the most important bottlenecks in transcriptomics [120]. Notably, rhizome developmental genes such as *Gl*, *CO*, *FT*, *PHYB*, *SuSy*, and *REV* have been mapped in *G. superba* and *C. autumnale* transcriptomes using reference genes from *S. tuberosum*, *N. nucifera*, *A. thaliana*, and *P. praecox*. A total of 19 *G. superba* and 15 *C. autumnale* candidate genes were mapped against 24 reference genes to identify possible rhizome developmental unigenes (Figure 10B). Furthermore, rhizome developmental phylogenetic analysis shows 6 distinct clusters, among them *CO* represents the largest clade. In addition, the transcription factor plays a crucial role in rhizome development [121]. A total of 16 and 12 transcription factors were mapped in the *G. superba* and *C. autumnale* transcriptomes, respectively (Figure 10C). To better reveal the genes associated with rhizome development, 6 transcription factor families were analyzed such as *MADS-box*, *AP2-EREBP*, *bHLH*, *MYB*, *NAC*, and *WRKY* that showed good correlation between the clusters of protein families. The mapped rhizome developmental gene families explains the conserved sequence homology between reference and candidate genes, which indicates a network of genetic mechanisms in *G. superba* and *C. autumnale* are crucial for rhizome development.

Interestingly, the phylogenetic trees of colchicine pathway, rhizome developmental, and transcription factors indicate that the candidate genes of *G. superba* and *C. autumnale* were common to Colchicaceae due to high homology and evolution similarity of the candidate genes. This suggests that the predicted candidate genes not only are involved in colchicine biosynthesis in rhizomes but are also involved in the rhizome metabolism. In comparison with tuber producing *S. tuberosum* and *N. nucifera*, rhizome developmental and transcription factor genes from *G. superba* and *C. autumnale* showed nearly the same transcriptional regulation map that are possible downstream targets of rhizome initiation or biomass production. The annotated genes from *G. superba* and *C. autumnale* can contribute to identifying candidate genes in the biosynthesis of different groups of secondary metabolites in bio-rhizome and rhizome developmental genes, which could serve as a comprehensive resource for molecular mechanism research of colchicine biosynthesis in *G. superba*. This provides a molecular platform and resource for future genetic and functional rhizomatous medicinal crops genomic research.

Conclusions

In this study, we have interrogated two evolutionarily diverse transcriptomes of *G. superba* and *C. autumnale* to predict genes that encode candidate proteins of colchicine biosynthesis and rhizome metabolism. *G. superba* and *C. autumnale* are rhizomatous medicinal plants that lack reference genomes. Collectively, transcriptomes and cDNA approaches were applied to select candidate genes for each predicted colchicine pathway step, which should help to elucidate the colchicine biosynthetic pathway. Additionally, our work will be useful to identifying rhizome developmental genes and transcription factors in *G. superba* and *C. autumnale*, with the ultimate goal of improving biorhizome biomass. The predicted genes in this work now need to be functionally validated, and are an important resource for metabolic engineering or synthetic biotechnology that could improve colchicine biomanufacturing.

Methods

cDNA library

A full-length cDNA library was constructed from a month-old *G. superba* biorhizome derived leaves. Total RNA isolation and cDNA library construction were performed according to previous protocols [122]. The assembled cDNA sequences were BLASTed against the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/>), and *G. superba* and *C. autumnale* transcriptomes.

Prediction of colchicine pathway and rhizome developmental proteins

The *G. superba* and *C. autumnale* transcriptomes were obtained from our Medicinal Plant Transcriptome database (<https://medplantrnaseq.org/>). BUSCO analysis of transcriptomes were processed according to Waterhouse et al. [123]. Colchicine pathway and rhizome developmental along with its transcription factors encoding proteins were predicted from the NCBI database.

Functional annotation of transcriptomes, colchicine pathway and rhizome developmental proteins

The *G. superba* and *C. autumnale* transcriptomes GO classification of the identified know plant proteins was performed using the web-accessible Blast2GO v5 annotation system (<https://www.blast2go.com/>) [124]. Blast2GO is an all-in-one bioinformatics software for protein functional prediction and the genome-wide analysis of annotation data. KEGG pathway enrichment analysis was used to analyze the functional significance of biochemical pathways using BlastKOALA and GhostKOALA. The first step in Blast2GO is to align cDNA nucleotide sequences against the NCBI non-redundant database by Basic Local Alignment Search Tool protein (BLASTp/BLASTn) with an expectation value of $1e^{-5}$. Next, transcriptomes FASTA protein sequences were uploaded to Blast2GO for BLAST analysis to identify homologous sequences, then mapping and annotation were performed. The significantly enriched biological processes, molecular function, cellular component and KEGG pathway were identified by *p* value less than threshold value 0.05. The colchicine pathway candidate genes with their specific enzymatic function and high homology

were identified using alkaloid metabolic reference genes such as *NMT*; *3-OMT*; two cytochrome P450s: *CYP96T1* and *CYP82E10*; and *NAT* for comparison [74-89]. Rhizome developmental genes namely *GI*, *CO*, *PHYB*, *SuSy*, *FT* and *REV* as well as transcription factors such as *MADS-box*, *AP2-EREBP*, *bHLH*, *MYB*, *NAC*, and *WRKY*, were identified in like manner using reference genes from *S. tuberosum* and *N. nucifera* [https://plants.ensembl.org/Solanum_tuberosum/Info/Index and <ftp://ftp.ncbi.nih.gov/genomes/>].

Phylogenetic analysis of possible total colchicine pathway, rhizome developmental, and transcription factor proteins

Gloriosa superba and *Colchicum autumnale* possible candidate colchicine pathway, rhizome developmental, and transcription factor protein sequences were aligned using ClustalW version 2.1. The phylogenetic trees were constructed from the PHYLIP-3.697 package using the Seqboot, Protdist, Neighbor, and Consense programs [125]. For tree visualization, Interactive Tree of Life (iTOL) software was used [<https://itol.embl.de/>] [105].

Abbreviations

AP2-EREBPAPETALA2/ethylene-responsive element binding protein

bHLH Basic-Helix-Loop-Helix

BLASTBasic Local Alignment Search Tool

BUSCOBenchmarking universal single-copy orthologue

cDNA Complementary DNA

CO CONSTANS

DW Dry weight

FT Flowering Locus T

GI GIGANTEA

GO Gene Ontology

iTOL Interactive Tree of Life

KEGGKyoto Encyclopedia of Genes and Genomes

MADS Mini chromosome maintenance 1

MYB Myeloblastosis related

NAC No apical meristem

NAT *N*-acetyltransferase

NCBI National Center for Biotechnology Information

NMT *N*-methyltransferase

OMT *O*-methyltransferase

PHYB Phytochrome B

PHYLIPPHYLogeny Inference Package

qRT-PCR Quantitative Real Time Polymerase Chain Reaction

REV REVOLUTA

SuSy Sucrose Synthase

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The *Gloriosa superba* and *Colchicum autumnale* transcriptomes are available at Medicinal Plant Transcriptome database at <https://medplantrnaseq.org/>.

Competing interests

The authors declare that they have no competing interests.

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Author's contribution

JSM carried out the sequence annotations and bioinformatic analyses. GS provided cDNA and the design of the study as well as wrote the manuscript. TMK established transcriptomes and DG conceived of,

designed and coordinated the rhizome developmental gene verification. All authors read and approved the final manuscript.

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Tables

Table 1. Predicted candidate colchicine pathway genes in *Gloriosa superba* and *Colchicum autumnale* transcriptomes.

Colchicine Pathway Genes	Candidate Gene ID	Reference Gene ID	% Identity	e- Value
Step 1: NMT	Gloriosa-20120814 64082_1	BAB71802.1 [Coptis japonica]	57.792	1.88E-63
	Gloriosa-20120814 31585_1	BAB71802.1 [Coptis japonica]	52.866	2.28E-123
	Colchicum_20101112 85_	BAB71802.1 [Coptis japonica]	51.862	4.03E-127
	Colchicum_20101112 85_	C3SBW0.1 [Thalictrum flavum subsp. glaucum]	48.703	3.10E-116
	Gloriosa-20120814 64082_1	C3SBW0.1 [Thalictrum flavum subsp. glaucum]	51.299	9.25E-53
	Gloriosa-20120814 12877_1	C3SBW0.1 [Thalictrum flavum subsp. glaucum]	51.29	7.38E-111
Step 2: P450	Colchicum_20101112 29610_	CYP719B1 [Papaver somniferum]	50	8.81E-17
	Colchicum_20101112 67076_	CYP96T1 [Narcissus pseudonarcissus]	51.282	9.95E-24
	Gloriosa-20120814 30999_1	CYP80G2 [Coptis japonica]	52.778	1.79E-06
	Gloriosa-20120814 85639_1	CYP80G2 [Coptis japonica]	52.206	8.23E-35
	Colchicum_20101112 76071_	CYP80G2 [Coptis japonica]	51.724	1.24E-27
	Colchicum_20101112 88526_	CYP80G2 [Coptis japonica]	50	2.01E-07
	Colchicum_20101112 99151_	CYP80G2 [Coptis japonica]	50	7.77E-31
Step 3: OMT	Gloriosa-20120814 31595_1	AFB74613.1 [Papaver somniferum]	55.814	6.62E-10
	Gloriosa-20120814 18437_1	AFB74613.1 [Papaver somniferum]	54.795	2.60E-21
	Gloriosa-20120814 13320_1	AFB74613.1 [Papaver somniferum]	52	5.10E-20
	Gloriosa-20120814 46280_1	AFB74613.1 [Papaver somniferum]	51.429	3.68E-07
	Gloriosa-20120814 6585_1	AFB74613.1 [Papaver somniferum]	51.282	4.34E-08
	Gloriosa-20120814 2006_1	AFB74613.1 [Papaver	51.163	7.24E-

	somniferum]		08
Gloriosa-20120814 16875_1	AFB74613.1 [Papaver somniaferum]	50.909	1.37E- 33
Gloriosa-20120814 31794_1	AFB74613.1 [Papaver somniaferum]	50.847	1.32E- 15
Gloriosa-20120814 36043_1	AFB74613.1 [Papaver somniaferum]	50.588	4.80E- 22
Gloriosa-20120814 25318_1	Q9LEL5.1 [Coptis japonica]	62.162	5.4E- 11
Gloriosa-20120814 2864_1	Q9LEL5.1 [Coptis japonica]	50.725	4.88E- 16
Gloriosa-20120814 25750_1	Q9LEL5.1 [Coptis japonica]	50.685	1.15E- 22
Colchicum_20101112 51346_	Q9LEL5.1 [Coptis japonica]	53.333	1.16E- 28
Colchicum_20101112 72754_	Q9LEL5.1 [Coptis japonica]	52.809	1.86E- 20
Colchicum_20101112 59167_	Q9LEL5.1 [Coptis japonica]	50.485	9.05E- 29
Gloriosa-20120814 82787_1	A0A077EWA5.1 [Narcissus pseudonarcissus]	72.857	1.42E- 26
Gloriosa-20120814 42115_1	A0A077EWA5.1 [Narcissus pseudonarcissus]	68.636	2.31E- 109
Gloriosa-20120814 238_1	A0A077EWA5.1 [Narcissus pseudonarcissus]	64.017	3.14E- 104
Gloriosa-20120814 10195_1	A0A077EWA5.1 [Narcissus pseudonarcissus]	59.031	1.08E- 92
Colchicum_20101112 68404_	A0A077EWA5.1 [Narcissus pseudonarcissus]	71.25	2.12E- 35
Colchicum_20101112 14671_	A0A077EWA5.1 [Narcissus pseudonarcissus]	67.234	3.44E- 114
Colchicum_20101112 34468_	A0A077EWA5.1 [Narcissus pseudonarcissus]	65.236	3.19E- 108
Colchicum_20101112 9022_	A0A077EWA5.1 [Narcissus pseudonarcissus]	63.983	4.49E- 108
Colchicum_20101112 75393_	A0A077EWA5.1 [Narcissus pseudonarcissus]	63.194	2.07E- 62
Colchicum_20101112 29556_	A0A077EWA5.1 [Narcissus pseudonarcissus]	59.633	9.11E- 89

	Colchicum_20101112 23611_	A0A077EWA5.1 [Narcissus pseudonarcissus]	58.15	1.12E-90
Step 4: P450	Gloriosa-20120814 5091_1	CYP82E5v2 [Nicotiana tabacum]	52.326	1.27E-24
	Colchicum_20101112 74349_	CYP82E5v2 [Nicotiana tabacum]	55.172	4.87E-29
	Colchicum_20101112 75506_	CYP82E5v2 [Nicotiana tabacum]	50.962	6.75E-34
	Gloriosa-20120814 5091_1	CYP82E10 [Nicotiana tabacum]	51.163	6.91E-25
	Colchicum_20101112 74349_	CYP82E10 [Nicotiana tabacum]	55.172	4.41E-29
	Colchicum_20101112 54622_	CYP82E10 [Nicotiana tabacum]	51.24	3.41E-34
	Colchicum_20101112 39301_	CYP82E10 [Nicotiana tabacum]	50	4.66E-22
	Colchicum_20101112 75506_	CYP82E10 [Nicotiana tabacum]	50	4.23E-34
	Gloriosa-20120814 5091_1	CYP82E4 [Nicotiana tabacum]	53.488	1.63E-24
	Colchicum_20101112 74349_	CYP82E4 [Nicotiana tabacum]	54.023	1.36E-27
	Colchicum_20101112 75506_	CYP82E4 [Nicotiana tabacum]	50	8.59E-32
Step 5: NAT	Gloriosa-20120814 32279_1	BAG90782.1 [Oryza sativa Japonica Group]	67.702	1.42E-74
	Colchicum_20101112 8688_	BAG90782.1 [Oryza sativa Japonica Group]	71.034	3.05E-67
	Gloriosa-20120814 70209_1	Q5KQI6.1 [Oryza sativa Japonica Group]	69.136	1.54E-108
	Gloriosa-20120814 51165_1	Q5KQI6.1 [Oryza sativa Japonica Group]	68.016	2.70E-106
	Gloriosa-20120814 30717_1	Q5KQI6.1 [Oryza sativa Japonica Group]	64.615	6.09E-104
	Colchicum_20101112 6813_	Q5KQI6.1 [Oryza sativa Japonica Group]	76.768	5.79E-109
	Colchicum_20101112 37095_	Q5KQI6.1 [Oryza sativa Japonica Group]	70.476	1.26E-102

Table 2. Predicted candidate rhizome developmental genes in *Gloriosa superba* and *Colchicum autumnale* transcriptomes.

Rhizome Developmental Genes	Candidate Gene ID	Reference Gene ID	% Identity	e-Value
GIGANTEA	Colchicum_20101112 2617_	XP_010261025.1 <i>Nelumbo nucifera</i>	82.004	0
	Gloriosa-20120814 11998_1	XP_010268589.1 <i>Nelumbo nucifera</i>	76.132	0
	Gloriosa-20120814 8521_1	PGSC0003DMT4000048370 <i>Solanum tuberosum</i> *	69.743	0
CONSTANS	Colchicum_20101112 2617_	PGSC0003DMT4000048370 <i>S. tuberosum</i> *	82.609	1.68E-173
	Gloriosa-20120814 51159_1	XP_010261698.1 <i>Nelumbo nucifera</i>	84.091	7.14E-12
	Colchicum_20101112 3159_	XP_010257847.1 <i>Nelumbo nucifera</i>	81.818	1.79E-11
	Gloriosa-20120814 51159_1	PGSC0003DMT400067656 <i>S. tuberosum</i> *	75.51	1.14E-13
	Colchicum_20101112 3159_	PGSC0003DMT400026065 <i>S. tuberosum</i> *	81.818	4.61E-12
	Gloriosa-20120814 17037_1	NP_001274795.1 <i>Solanum tuberosum</i>	72.5	4.85E-30
	Gloriosa-20120814 51159_1	NP_001274795.1 <i>Solanum tuberosum</i>	68.627	3.75E-10
	Gloriosa-20120814 18001_1	NP_001274795.1 <i>Solanum tuberosum</i>	68.627	4.24E-10
	Gloriosa-20120814 20079_1	NP_001274795.1 <i>Solanum tuberosum</i>	67.442	2.97E-11
	Gloriosa-20120814 72100_1	NP_001274795.1 <i>Solanum tuberosum</i>	64	4.59E-29
	Colchicum_20101112 3159_	NP_001274795.1 <i>Solanum tuberosum</i>	81.818	2.96E-10
	Colchicum_20101112 5391_	NP_001274795.1 <i>Solanum tuberosum</i>	73.75	1.17E-23
	Colchicum_20101112 27602_	NP_001274795.1 <i>Solanum tuberosum</i>	79.365	7.90E-29
	Colchicum_20101112 26221_	NP_001274795.1 <i>Solanum tuberosum</i>	71.795	1.62E-12
Phytochrome B	Gloriosa-20120814 36050_1	XP_010267948.1 <i>Nelumbo nucifera</i>	81.445	0

	Colchicum_20101112 1221_	XP_010267948.1 <i>Nelumbo nucifera</i>	80.142	0
	Gloriosa-20120814 85746_1	PGSC0003DMT400061712 <i>S. tuberosum</i> *	80.927	0
	Colchicum_20101112 1221_	PGSC0003DMT400061712 <i>S. tuberosum</i> *	79.539	0
Sucrose	Gloriosa-20120814 69933_1	XP_010271909.1 <i>Nelumbo nucifera</i>	81.886	0
Synthase	Colchicum_20101112 13826_	XP_010271909.1 <i>Nelumbo nucifera</i>	81.39	0
	Colchicum_20101112 593_	XP_010271909.1 <i>Nelumbo nucifera</i>	80.15	0
	Colchicum_20101112 2693_	XP_010271909.1 <i>Nelumbo nucifera</i>	81.404	0
	Colchicum_20101112 33099_	XP_010271909.1 <i>Nelumbo nucifera</i>	71.535	0
	Colchicum_20101112 583_	XP_010271909.1 <i>Nelumbo nucifera</i>	71.411	0
	Gloriosa-20120814 9073_1	PGSC0003DMT400007506 <i>S. tuberosum</i> *	76.923	0
	Colchicum_20101112 2693_	PGSC0003DMT400007506 <i>S. tuberosum</i> *	76.289	0
Flowering	Colchicum_20101112 8829_	XP_010268289.1 <i>Nelumbo nucifera</i>	80.925	1.75E-109
Locus T	Colchicum_20101112 22914_	XP_010268289.1 <i>Nelumbo nucifera</i>	86.232	1.56E-92
	Gloriosa-20120814 55554_1	XP_010268289.1 <i>Nelumbo nucifera</i>	83.815	2.85E-113
	Colchicum_20101112 22914_	PGSC0003DMT400060057 <i>S. tuberosum</i> *	78.788	9.86E-81
	Colchicum_20101112 8829_	PGSC0003DMT400060057 <i>S. tuberosum</i> *	75	2.51E-97
	Gloriosa-20120814 55554_1	PGSC0003DMT400060057 <i>S. tuberosum</i> *	75.595	4.17E-99
	Colchicum_20101112 8829_	NP_001274897.1 <i>Solanum tuberosum</i>	73.81	5.00E-93
	Colchicum_20101112 22914_	NP_001274897.1 <i>Solanum tuberosum</i>	77.273	1.98E-76
	Gloriosa-20120814 55554_1	NP_001274897.1 <i>Solanum tuberosum</i>	74.405	8.62E-95

	Gloriosa-20120814 55004_1	NP_001274897.1 <i>Solanum tuberosum</i>	67.066	3.03E-82
	Gloriosa-20120814 26010_1	NP_001274897.1 <i>Solanum tuberosum</i>	64.968	1.36E-71
	Gloriosa-20120814 45011_1	NP_001274897.1 <i>Solanum tuberosum</i>	72.034	4.46E-63
	Gloriosa-20120814 52745_1	NP_001274897.1 <i>Solanum tuberosum</i>	60	1.55E-66
REVOLUTA	Gloriosa-20120814 11056_1	XP_010247499.1 <i>Nelumbo nucifera</i>	86.244	0
	Colchicum_20101112 467_	XP_010247499.1 <i>Nelumbo nucifera</i>	85.221	0
	Colchicum_20101112 467_	PGSC0003DMT400030829 <i>S. tuberosum</i> *	81.02	0
	Gloriosa-20120814 17486_1	PGSC0003DMT400030829 <i>S. tuberosum</i> *	81.182	0
	Gloriosa-20120814 15982_1	AAY32332.1 <i>Phyllostachys praecox</i>	78.851	0
	Colchicum_20101112 6503_	AAY32332.1 <i>Phyllostachys praecox</i>	78.091	0

*Sequence from *Solanum tuberosum* transcriptome
[\[https://plants.ensembl.org/Solanum_tuberosum/Info/Index\]](https://plants.ensembl.org/Solanum_tuberosum/Info/Index)

Table 3. Predicted candidate transcription factors in *Gloriosa superba* and *Colchicum autumnale* transcriptomes.

Transcription Factors	Candidate Gene ID	Reference Gene ID	% Identity	e-Value
MADS-box	Gloriosa-20120814 19828_1	XP_010272608.1 <i>Nelumbo nucifera</i>	77.366	2.02E-138
	Gloriosa-20120814 35037_1	PGSC0003DMT400000026 <i>Solanum tuberosum</i> *	82.432	3.32E-42
	Gloriosa-20120814 39825_1	PGSC0003DMT400000026 <i>Solanum tuberosum</i> *	60.317	9.37E-23
	Colchicum_20101112 22985_	PGSC0003DMT400000026 <i>Solanum tuberosum</i> *	81.159	3.61E-37
AP2-EREBP	Colchicum_20101112 4936_	XP_010263474.1 <i>Nelumbo nucifera</i>	73.684	3.88E-88
	Gloriosa-20120814 45526_1	XP_010271250.1 <i>Nelumbo nucifera</i>	86.047	9.56E-22
	Gloriosa-20120814 54352_1	XP_010271250.1 <i>Nelumbo nucifera</i>	82.353	1.69E-16
	Gloriosa-20120814 7267_1	XP_010271250.1 <i>Nelumbo nucifera</i>	68.831	3.04E-32
	Gloriosa-20120814 58739_1	XP_010271250.1 <i>Nelumbo nucifera</i>	66.667	1.15E-18
	Gloriosa-20120814 77954_1	XP_010271250.1 <i>Nelumbo nucifera</i>	65.854	4.44E-33
	Gloriosa-20120814 23228_1	PGSC0003DMT400016585 <i>Solanum tuberosum</i> *	80.905	1.16E-97
	Colchicum_20101112 10921_	PGSC0003DMT400016585 <i>Solanum tuberosum</i> *	75.701	6.49E-99
	Colchicum_20101112 4936_	PGSC0003DMT400016584 <i>Solanum tuberosum</i> *	83.929	5.12E-64
	Gloriosa-20120814 6279_1	XP_010257880.1 <i>Nelumbo nucifera</i>	89.744	3.68E-18
bHLH	Colchicum_20101112 7042_	XP_010273628.1 <i>Nelumbo nucifera</i>	81.818	1.93E-48
	Gloriosa-20120814 53746_1	PGSC0003DMT400022702 <i>Solanum tuberosum</i> *	88.889	1.27E-11
	Colchicum_20101112 21881_	PGSC0003DMT400022701 <i>Solanum tuberosum</i> *	86.364	9.32E-08
	Gloriosa-20120814 15461_1	XP_010277005.1 <i>Nelumbo nucifera</i>	90.698	8.02E-86
MYB	Colchicum_20101112 11314_	XP_010253806.1 <i>Nelumbo</i>	87.097	1.43E-

		<i>nucifera</i>		14
	Gloriosa-20120814 15461_1	PGSC0003DMT400012203 <i>Solanum tuberosum</i> *	90.698	1.34E-87
	Colchicum_20101112 20668_	PGSC0003DMT400017709 <i>Solanum tuberosum</i> *	86.301	2.98E-41
NAC	Gloriosa-20120814 28710_1	XP_010243512.1 <i>Nelumbo nucifera</i>	84.397	1.10E-89
	Colchicum_20101112 44481_	XP_010270427.1 <i>Nelumbo nucifera</i>	71.429	1.09E-07
	Gloriosa-20120814 18396_1	PGSC0003DMT400045294 <i>Solanum tuberosum</i> *	81.212	2.64E-101
	Colchicum_20101112 794_	PGSC0003DMT400079789 <i>Solanum tuberosum</i> *	71.429	7.85E-91
WRKY	Colchicum_20101112 22998_	XP_010258216.1 <i>Nelumbo nucifera</i>	80.952	5.53E-07
	Gloriosa-20120814 20164_1	XP_010258120.1 <i>Nelumbo nucifera</i>	76.596	7.13E-50
	Gloriosa-20120814 66904_1	PGSC0003DMT400028529 <i>Solanum tuberosum</i> *	77.419	3.60E-28
	Colchicum_20101112 22998_	PGSC0003DMT400072835 <i>Solanum tuberosum</i> *	80.952	5.16E-07
	Colchicum_20101112 49701_	ALD83482.1 <i>Sinopodophyllum hexandrum</i>	80	2.46E-14
	Colchicum_20101112 3957_	ALD83482.1 <i>Sinopodophyllum hexandrum</i>	76	2.38E-19

*Sequence from *Solanum tuberosum* transcriptome
[\[https://plants.ensembl.org/Solanum_tuberosum/Info/Index\]](https://plants.ensembl.org/Solanum_tuberosum/Info/Index)

Figures

Figure 1

Proposed colchicine pathway in *Gloriosa superba*. TH: Tyrosine hydroxylase; L-DOPAD: L-Dihydroxyphenylalanine decarboxylase; PAL: Phenylalanine ammonia-lyase; NMT: N-methyltransferase; OMT: O-methyltransferase; NAT: N-acetyltransferase [47]

Figure 2

Gene ontology (GO) annotation of *G. superba* and *Colchicum autumnale* transcriptomes.

Figure 3

Percentage of gene ontology annotations for molecular function, biological process, and cellular components in *G. superba* and *C. autumnale* transcriptome and *G. superba* leaf cDNA library.

Figure 4

Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation of *G. superba* and *C. autumnale* transcriptomes and *G. superba* leaf cDNA library predicted proteins.

Figure 5

Total number of transcripts in colchicine pathway in *G. superba* and *C. autumnale* transcriptomes and *G. superba* leaf cDNA library.

Figure 6

Total number of transcripts of rhizome developmental genes in *G. superba* and *C. autumnale* transcriptomes and *G. superba* leaf cDNA library. *Solanum tuberosum* and *Nelumbo nucifera* were reference transcriptomes.

Figure 7

Total number of transcripts of transcription factors in *G. superba* and *C. autumnale* transcriptomes and *G. superba* leaf cDNA library. *S. tuberosum* and *N. nucifera* were reference transcriptomes.

Figure 8

Possible candidate colchicine pathway genes in *G. superba* and *C. autumnale* transcriptomes and *G. superba* leaf cDNA library.

Figure 9

Possible candidate rhizome developmental genes and transcription factors in *G. superba* and *C. autumnale* transcriptomes.

Figure 10

A. Predicted colchicine pathway phylogenetic tree of *G. superba* and *C. autumnale* transcriptomes with reference genes. Neighbor-joining tree of pathway enzymes shown with NMT, P450s, OMT, and NAT was generated from PHYLIP-3.697 software suite with 1000 replicates in bootstrap values. B. Neighbor-joining phylogenetic tree of rhizome developmental genes in *G. superba* and *C. autumnale* transcriptomes with reference genes from *S. tuberosum*, *N. nucifera*, *A. thaliana*, and *P. praecox*. The phylogenetic relationship of GI, CO, FT, PHYB, SuSy, and REV were generated from the PHYLIP-3.697 package with 1000 replicates in bootstrap values. C. Phylogenetic tree of rhizome transcription factors such as MADS-box, AP2-EREBP, bHLH, MYB, NAC, and WRKY in *G. superba* and *C. autumnale* transcriptomes with reference genes from *S. tuberosum*, *N. nucifera*, *A. thaliana*, and *S. hexandrum*. The phylogenetic relationship were conducted from the PHYLIP-3.697 package with 1000 replicates in bootstrap values.