Comprehensive RNA-Seq-based study and metabolite profiling to identify genes involved in podophyllotoxin biosynthesis in Linum album Kotschy ex Boiss. (Linaceae)

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Article

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Abstract

Linum album is a well-known rich source of anticancer compounds, *i.e.*, podophyllotoxin (PTOX) and other lignans, which play an important role in the plant defensive system. In the present study, the RNA-Seq data of flax (*L. usitatissimum*) under aluminum toxicity, zinc and K^+ deficiency, drought stress, ABA, and *Fusarium* treatments were analyzed to identify common DEGs and then examined in *L. album*, using HPLC and qRT-PCR. Commonly DEGs analysis identified *EP3* with a significant increase in all stresses. The highest expression increase was found for laccase (*LAC11*), lactoperoxidase (*POD*), 4-coumarate-CoA ligase (*4CL*), and secoisolariciresinol (SECO) dehydrogenase (*SDH*). A quantitative expression showed that the *SDH* gene had an increasing trend in *L. album* root and its expression trend was different in the plant shoot. The gene expressions of deoxyPTOX synthase (*2-ODD*), excluding drought stress, and 5'-desmethyl-yatein O-methyltransferase (*OMT1*) revealed a rising trend. HPLC analysis confirmed the results of gene expression. SECO content increased under drought stress, and 6-metoxyPTOX content was more than PTOX in both tissues. Identified modifications of critical genes related to PTOX biosynthesis in response to multiple stresses can provide a baseline for improving PTOX content.

Introduction

The genus *Linum*, belonging to the family Linaceae, comprises about 230 species, including *L. album* Kotschy ex Boiss., *L. flavum* L., and *L. mucronatum* Bertol that are known as valuable sources of lignans, especially podophyllotoxin (PTOX) [1,2,3]. Although, the accumulation pattern of lignans may vary from one species to another [4]. Lignans have broad applications in traditional and modern medicine and food industries. Due to the cytotoxicity of PTOX, its semi-synthetic derivatives, *i.e.*, Etoposide and Teniposide with anticancer activities, have been currently produced in the pharmaceutical industries [5,6].

PTOX and its derivative compounds, particularly 6-methoxypodophyllotoxin (6-MPTOX), are the major aryltetralin lignans resulting from the shikimic acid/phenylpropanoid pathway [7] (Fig. 1). In this biosynthetic pathway, the matairesinol is generated by secoisolariciresinol (SECO) oxidation through SECO dehydrogenase (SDH). The role of lignans in the plant defense systems against abiotic and biotic stresses has been reported [5].

Expression of some genes encoding enzymes in the biosynthetic pathway of PTOX was recognized in *L. flavum*, *Forsythia koreana* Rehder Nakai and *Podophyllum hexandrum* Royle [8]. However, downstream genes in *L. album* have not yet been completely elucidated [9]. Hence, transcriptome and metabolome profiling is necessary to explore gene expression patterns of PTOX biosynthesis in *L. album* to improve the production of this valuable anticancer compound.

Gene expression studies increasingly rely on high-throughput sequencing (RNA-Seq), whereas they are usually performed on a limited number of biological replicates and experimental conditions due to the high cost of RNA-Seq. Therefore, the desired results can be explored by integrating and analyzing the datasets. Several RNA-Seq studies have been reported on genes associated with an individual's response to stress and textile properties in flax so far. For example, changes in the gene expression have been previously evaluated using RNA-Seq sequencing in flax genotypes with different tolerance under aluminum toxicity, drought stress, *Fusarium* treatment, and zinc deficiency [10–13,]. Gene expression changes were unique and specialized for each stimulus. Under aluminum toxicity, NAC (NAM, ATAF, and CUC) transcription factors (TFs), along with MADS-box (for MCM1, AG, DEFA, and SRF), regulate plant growth and lead to aluminum tolerance by interfering with cell wall alterations [13]. However, under drought stress, gene expression patterns were changed to cope with water deficit toward proline biosynthesis and DNA repair, indicating the important role of proline in drought tolerance of flax [10].

Drought stress is one of the major limiting factors which can be affected the growth and geographical distribution of plants worldwide, followed by a plenty reduction in crop yields. Severe climate change increases the frequency of harsh drought conditions leading to multiple stresses such as drought and heat or drought and K^+ deficiency stress [14,15]. During evolution, plants have adapted themselves to elude the lethal effects of stresses via the favorable physiological, biochemical, and molecular properties as well as transcriptional and epigenetic regulations [16].

Linum usitatissimum and *L. album* are growing in arid and semi-arid regions of Iran. Thus, they are often exposed to drought and other coupled stresses in their natural habitat [17]. It can be concluded that both species have good resistance to the stresses mentioned above, but the commonly regulated responsive genes of the plants have not been investigated using transcriptome studies under multiple stresses.

The present work conducted a comprehensive RNA-Seq analysis of published datasets on *L. usitatissimum*, a model plant containing lignans, using the same pipeline. This approach would let us find common and unique transcriptional changes at the genome-wide level, particularly changes associated with lignans biosynthesis occurring during short-term abiotic and biotic stresses [10–13, 18]. The present study aimed to focus on the changes in gene expression patterns involved at the main core of the PTOX biosynthesis pathway, which can be directly affected by different stresses and limiting factors, *i.e.*, drought and K^+ deficiency. These findings can be interestingly considered to better understanding of the molecular basis of PTOX biosynthesis in the genetic engineering or genome-editing approaches for the improvement of PTOX production.

Results

RNA-Seq data and transcriptome responses

A total of 1358545065 pair- and single-end raw reads were obtained from six RNA-Seq datasets, ranging from 18.15 to 90.14 million per sample. Among these, 275217314, 130295728, 109809110, 402917502, 220197810 and 220107601 million reads were belonged to drought, ABA, K^+ deficiency, *Fusarium*, aluminum toxicity and zinc deficiency treatments, respectively. After trimming, 10% of reads were almost removed, giving the quality of the datasets. The clean reads with an average of 94.13% read mapping rate were aligned to the *L. usitatissimum* genome (Table 1).

Table 1
Overview transcriptome data used in this study

Stress	No. SAMPLE	Cultivar	Sample tissue	Time afer stress	Replicates per Sample	Accession	Number of reads	mapping rate (%)
drought stress	12	Z141	leaf	20 day	3	PRJNA598287	275217314	94.72
		NY17						
ABA	2	NIKE	plant	14 day	-_	PRJNA515812	130295728	92.71
K + deficiency	12	Sofie	plant	21 day	3	PRJNA414507	109809110	96.31
fusarium	8	Regina	plant	14 day	3	PRJNA504749	402917502	92.99
		Nike						
Aluminum toxicity	8	TMP1919-N	root	7day	2	PRJNA343117	220197810	92.36
		Hermes						
Zinc deficiency	6	Norlin	root	21 day	4	PRJNA497472	220107601	94.96

The results from RNA-Seq analysis showed that the total number of differentially expressed genes (DEGs) was in the range of 314 for *Fusarium* wilt to 15659 for drought stress, depending on stress treatment. In drought stress, the number of

upregulated DEGs (4328) was more than that of downregulated DEGs (2709). While in other stresses, the number of downregulated genes was higher (Fig. 2).

Investigation of the common genes among the datasets revealed 238 commonly regulated genes responsive to abiotic stresses and 20 commonly regulated genes responsive to both biotic and abiotic stresses in the shoot. Also, 38 commonly regulated gene-responsive abiotic stresses were observed in the root. Only one gene, AT3G54420, showed a significant change in gene expression between shoot and root among all the studied stresses (Fig. 3). The gene encodes an *EP3* chitinase, found in different organs such as hydathodes, stipules, root epidermis, and emerging root hairs is catalyzed the hydrolysis of chitin and is involved in response to fungal infections and some of the abiotic stresses. As well as, 787 commonly regulated genes were obtained from data on drought stress and K^+ deficiency, and this study experimentally examined both stresses.

A gene ontology (GO) analysis was performed on all commonly regulated genes in the shoot (20 genes) and root (38 genes) to predict their functions. The commonly regulated genes were grouped into the three main GO categories, including biological processes, cellular components, and molecular functions (Fig. 4). The present work focused on the GO terms category of biological processes. Functional classification of the 20 commonly regulated genes in the shoot showed two stress-responsive GO terms. In these GO terms, there are three genes encoding transcripts belonging to apetala2/ethylene responsive factor (AP2/ERF, *DRE2B*), homeobox (HB-7, *ATHB-7*), and NAC (*anac047*) TFs families that significantly upregulated in ABA treatment and drought stress, and downregulated *Fusarium* wilt and K^+ deficiency. The subtilisin-like serine protease (*ARA12* and *SDD1*) genes, encoding a serine-type endopeptidase, showed the highest upregulation (respectively, 7.88 and 7.21), while the lowest downregulation (-6.10) was observed in the myo-inositol oxygenase 2 (*MIOX2*) gene under the fusarium stress. Out of 12 functional categories of 38 specific commonly regulated genes in the root, 8 GO terms significantly exhibited enrichments of response to stress, including defense response to a bacterium, insect, oomycetes, water deprivation, wounding, oxidative stress, and plant-type hypersensitive response. The GO terms 'response to stress (GO:0006950)', 'response to stimulus (GO:0050896)', 'cellular process (GO:0009987)', and 'metabolic process (GO:0008152)' were all enriched within both shoot and root tissues (Fig. 4).

For a better insight into the functions and the metabolic roles, DEGs were analyzed through MapMan. The commonly regulated genes related to the shoot and root of six stresses were common in the 8 MapMan bincode classifications. The commonly regulated genes related to the shoot were specifically altered within minor CHO metabolism and RNA regulation of transcription (Table 2), whereas commonly regulated genes related to the root were specifically altered within the process of TC/org transformation, redox, cell, transport, hormone metabolism, and signaling receptor kinases (Table 3). The MapMan results were shown that commonly regulated genes share the same trend of regulation in ABA treatment and drought stress. As well as, observed the same trend of regulation in K^+ deficiency and fusarium wilt while drought stress and K^+ deficiency were the almost opposite trend. The GO terms of commonly regulated genes under both drought stress and K^+ deficiency were highly grouped in metabolic process, cellular process, and response to stress (31 GO terms), indicating complex regulatory mechanisms controlling gene expression in the face of stress. In addition, secondary metabolic processes are also involved in responding to stress. This classification observed that 32 genes are involved in specialized metabolites (SMs) biosynthesis, including phenylpropanoids, phenolic compounds, terpenoids, etc.

Table 2
DEGs identified in the studies analyzed of shoot (universal DEGs)

BinCode	BinName	fusarium	ABA	k	drought
3	minor CHO metabolism	up	down	up	down
16	secondary metabolism	down	up	down	up
20	stress	up	down	up	up
26	misc	down/up	down/up	down/up	up
27	RNA.regulation of transcription	up	down	up	down
33	development	up	down	up	down
35	not assigned	up	down/up	up	down/up
10	cell wall	down	up	down	up
11	lipid metabolism	up	down	down	down
29.5	protein.degradation	down/up	up	down/up	down/up
Up: Gene sets belonging to the corresponding group were upregulation.					
Down: Gene sets belonging to the corresponding group were downregulation.					
Down/up: Gene sets belonging to the corresponding group were up/downregulation.					

Table 3
DEGs identified in the studies analyzed of root (universal DEGs)

BinCode	BinName	Zinc deficiency	Aluminum toxicity
8	TCA / org. transformation	down	down
16	secondary metabolism	down/up	down
20	stress	down	down
21	redox	up	up
26	misc	down/up	down/up
31	cell	down	down
33	development	down	down/up
34	transport	down	down
35	not assigned	down	down
10	cell wall	down	down
11	lipid metabolism	down	down/up
17	hormone metabolism	up	down
29.5	protein.degradation	down	down
30.2	signalling.receptor kinases	down	down
Up: Gene sets belonging to the corresponding group were upregulation.			
Down: Gene sets belonging to the corresponding group were downregulation.			
Down/up: Gene sets belonging to the corresponding group were up/downregulation.			

Overview Transcriptome Profiling Of Sms

This study investigated transcripts abundance in SMs biosynthetic pathways in flax as a model plant for lignan (using homologous genes analysis with MapMan). Among all the SMs biosynthetic pathways, phenylpropanoid and flavonoid pathways were highly affected by stresses that led to PTOX biosynthesis. The highest increase in transcript abundance was *AT4G12300* (7.6-fold) and *AT5G47950* (7.26-fold) for flavonoids and phenylpropanoids under drought stress, respectively (Fig. 5A). The K^+ deficiency increased 2.5-fold transcripts abundance for *AT4G16740* and *AT1G59960* genes involved in the biosynthetic pathways of terpenoids and flavonoids, respectively (Fig. 5B). The ABA treatment was also caused an increase of 6.7-fold (*AT5G49555*) and 4.5-fold (*AT4G35160*) in biosynthetic pathways of carotenoids and phenylpropanoids (Fig. 5C). Fusarium treatment was induced an increase of 3.8-fold (*AT5G05390*) biosynthetic pathways of simple phenols (Fig. 5D).

The highest decreases were observed in the biosynthetic pathways of glucosinolates (-5.4 for *AT4G39950*), flavonoids (-4.15 for *AT5G54010*), and phenylpropanoids (-3.36 for *AT4G35160*), sulfur-containing. misc.alliinase (-7.3 for *AT1G70560*), phenylpropanoids (-3.4 for *AT3G50280*) under drought, K^+ deficiency, ABA, and *Fusarium* treatment in *L. usitatissimum*, respectively. Root tissue subjected to stressful conditions showed little changes in the gene expression of SMs (Fig. 5E, 5F); however, most alterations were related to the *AT2G19070* gene in phenylpropanoid biosynthetic pathways.

Modulating Lignans Biosynthetic Pathway Genes During Stress Exposure

The mining of transcriptome data of *L. usitatissimum* obtained from shoots and roots was used to identify various genes implicated in the biosynthesis of PTOX. In this regard, 12 transcripts encoding enzymes attributed to the PTOX biosynthetic pathway in *L. usitatissimum* were found, of which 11 transcripts revealed significant expression in at least one studied stress. Coniferyl alcohol has been known as a critical precursor in PTOX biosynthesis, so earlier and later its biosynthetic stages were considered as upstream and downstream, respectively. Transcripts encoding enzymes, namely 4-coumarate-CoA ligase (4CL), shikimate O-hydroxycinnamoyltransferase (HCT), *trans*-caffeoyl-CoA 3-O-methyltransferase (CCoAOMT), cinnamoyl-CoA reductase (CCR) and coniferyl-alcohol dehydrogenase (CAD) were upstream. The highest number of upstream transcripts was obtained for CAD. Transcripts encoding enzymes, namely LAC11, pinoresinol/lariciresinol reductase (PLR1-4), lactoperoxidase (POD), and SDH, were downstream. The highest increase of expression was 6.2, 5.92, 5.3, and 3.93-fold change for LAC11, POD, 4CL, and SDH under drought stress, respectively (Fig. 6). Interestingly, gene expressions of encoding these enzymes were only downregulated under aluminum toxicity treatment. Therefore the heatmap results illustrated that drought stress highly affected enzymes of the PTOX biosynthetic pathway. POD and SDH from downstream and 4CL from upstream were positively adjusted, leading to the process progressing toward PTOX biosynthesis in the shoot.

To further explore the protein interactions, Fisher's test was performed to find TFs which possess significantly over-represented targets in the transcripts encoding enzymes, subsequently constructing a protein-protein interactions (PPI) network of all transcripts encoding enzymes and TFs. Hub genes were defined by the maximum neighborhood component (MNC) ranking algorithm, which obtained higher utilization. PPI network analysis indicated that TFs with the strongest interaction, namely NAC, ARF (auxin response factors), HB, MADS, MYB (myeloblastosis), and AS2, were hub genes, then selected first neighbors including AP2/ERF, MYB, MADS, CPP (cystein-rich polycomb-like protein), WRKY, ZF_HD (zinc finger homeodomain), HSF (heat stress transcription factor), ARF, C2H2ZnF (C2H2 zinc-finger), AS2 (asymmetric leaves2), NAC, TCP (TB1, CYC and PCFs), B3, Jmj (JUMONJI), GARP (golden2, ARR-B, Psr1), BBR_BPC (barley b-recombinant/basic pentacysteine), SBP (SQUAMOSA promoter binding proteins), bZIP (basic leucine zipper domain), YABBY, DOF (DNA binding with one finger), and HD. In this network analysis, LAC11 has more interactions with hub TFs, whereas CCoAOMT has more interactions with other transcripts encoding enzymes (Fig. 7).

Gene expression changes in response to multiple stresses in L. album

The end of the PTOX biosynthetic pathway is not fully understood in *L. album*, while several genes were elucidated in the *Podophyllum* species [19]. On the other side, the genes encoding enzyme SDH, which is involved in the branching of downstream lignans of the PTOX biosynthesis pathway, and converts the SECO to matairesinol, were shown an increase in RNA-Seq analyses associated with the shoots of *L. usitatissimum* (Fig. 6). Therefore, the qRT-PCR analysis of *SDH*, 5'-desmethyl-yatein O-methyltransferase (*OMT1*), and deoxypodophyllotoxin synthase (*2-ODD*) genes from downstream was performed to better understand the expression patterns under drought stress, K^+ deficiency, and combination of both conditions in shoot and root of *L. album*. The relative expression of these genes ranged from 0.00001 to 6.96, with the lowest fold change being for the *SDH* in shoot under combined stress and the highest fold change for the *OMT1* in root under K^+ deficiency, at 48h. The results showed an increasing trend in *OMT1* for all samples, especially at 48h subjected to treatment, and *2-ODD* for the shoot samples. At the same time, *2-ODD* decreased under drought and was not affected under K^+ deficiency. *SDH* exhibited a decreasing trend in all shoot samples except samples subjected to K^+ deficiency. Simultaneously, all root samples showed an increasing trend (Fig. 8).

Lignans content changes in response to multiple stresses in the L. album

SMs profile corresponds with transcriptome results was mostly showed an increasing trend in lignans under stress. The 6-MPTOX content was more than PTOX content in root and shoot tissues. The highest PTOX content (500.25 µg/g) was in the samples subjected to the combination of both conditions after 48h, likely due to increasing SECO content and upregulation

of *SDH*, *OMT1*, and *2-ODD* genes. 6-MPTOX content extremely increased in all samples at 48h compared with untreated samples with significant differences ($P \leq 0.01$). The highest value of 6-MPTOX was 664.7 and 8077.6 µg/g in shoot and root under drought stress, respectively. In comparison, the PTOX content of samples subjected to drought decreased at 48h after an initial increase. A decrease in *2-ODD* gene expression at the root decreased PTOX, whereas 6-MPTOX content obtained the highest value. The highest values of SECO content were 126.25 µg/g and 14.6 µg/g in the root and shoot subjected to drought stress, respectively. The downregulation of SDH and OMT1 gene expressions was simultaneously accumulated a copious amount of SECO in the roots. SECO content exhibited an initial increase in shoot samples subjected to K^+ deficiency and the combination of both conditions at 12h; however, after 48h decreased, nevertheless was more than in untreated samples (Fig. 9).

Discussion

The *L. album* is a well-known rich source of SMs, particularly lignans. Several studies have been carried out on their physiological traits, biochemical properties, and expression of some key genes involved in the biosynthesis of lignans *in vitro* cultures so far [3,9,20]. This study aimed to recognize a common base of transcriptional changes occurring in different tissues during multiple stresses *in vivo* cultures and genes of PTOX.

First, 20 commonly regulated genes responsive to biotic and abiotic stresses and 38 commonly regulated genes responsive to abiotic stresses were found using transcriptome data analyses in the shoot and root, respectively. Then, only one commonly regulated gene, endochitinase EP3, with a significant increase, was identified in both tissues. The *EP3*, catalyzing the hydrolysis of chitin, is involved in the plant development processes, generation of signal molecules, plant defense responses, and programmed cell death [21,22]. The expression of the *EP3* gene increased in response to drought, salinity treatments of UV light, exogenous elicitor treatment, wounding, and pathogen attack [11,21,22]. Moreover, the β-1,3-glucanase gene increased in our results associated with the shoot that previously was demonstrated as crucial for flax resistance to *Fusarium* spp. [23]. Flax plants with β-1,3-glucanase overexpression, generating pectinase inhibitors, and employing chitinases and peroxidases have increased the accumulation of SMs and changes in cell wall composition, leading to building a barrier to fungal growth [24].

Biological processes, including secondary metabolism, stress, development, cell wall, lipid metabolism, and protein degradation, were commonly altered by all stress. Such common regulation reflects the flexibility of biological systems through the high flexibility of their metabolism in response to stimulants during the evolution of the plant's immobile life [11].

The stress-responsive common GO terms consisted of AP2/ERF, HB-7, and NAC transcription factor families that significantly upregulated in ABA treatment and drought stress and downregulated in *Fusarium* treatment and K^+ deficiency. Individual study of flax under repeated drought has identified 10 top TFs, including bHLH (Basic helix-loop-helix), C2H2, NAC, MYB, ERF, bZIP, WRKY, and MYB, as well as DREB, HSF, and NFYA10 as known main regulators of abiotic resistance pathways [10]. NAC TFs also contribute to stress response to maize, rice, and flax tolerance to aluminum. These TFs, along with MADS-box, adjust plant growth and development and involve cell wall alteration leading to tolerance to aluminum [13]. Most of the TFs related to potassium starvation have been demonstrated to belong to MYB, bHLH, NAC, B3, bZIP, WRKY, and AP2/ERF, participating in physiological plant processes, stress resistance, and secondary metabolism [18]. The stresses signaling pathways share common components comprising ROS, calcium ions, hormones, TFs, and mitogen-activated protein kinase (MAPK) cascades [25]. The root GO terms showed that biological processes, namely TCA / org transformation, oxidoreductase activity, cell wall, transport, hormone metabolism, and receptor kinase signaling, are specifically modulated under abiotic stress. In an individual study of flax under unfavorable pH and Zn deficiency, GO terms of oxidoreductase activity, particularly peroxidases, cell wall, ion homeostasis and response to stress were most changed [12].

The total number of DEGs observed for drought stress was more than the number of DEGs for other stresses. Under drought stress, the differences between tolerance and susceptible genotypes arise in not only gene expression patterns but also

phenotypes [10]. Concerning the fact that the expression level of stress-responsive genes is typically higher in tolerance genotypes [13]. Therefore, these results corroborate the concept that flax has better drought tolerance than other stresses [10].

As already stated, some of our results agreed with the individual studies, while some opposed them. These individual stresses might cause plants a different adjustment response that is shared or different components. When plants simultaneously encounter stress combinations, it could require similar or opposing molecular, physiological, and metabolic responses. The precise choice of which adjustment strategy during multiple stress is presumably to be affected by factors like the intensity of each individual stress, the time course of stress, plant age, and genotype (tolerant or susceptible to any one of the individual stress) [26]. This choice may influence the accumulation of SMs, causing spatial and temporal modulation of the biosynthetic pathways, subsequently, survival improvement under long-term stressful environments [27].

MapMan analysis categorized SMs into 16 groups. Phenylpropanoid and flavonoid pathways were highly affected by all of the stresses. The highest accumulation in transcript abundance was for flavonoids and phenylpropanoids under drought stress. The increase of these compounds is extremely related to the balance of carbohydrates between sources and sinks. Under severe drought stress, the accumulation of flavonoids and phenolics is increased, resulting from the transport of soluble sugars due to decrease water potentials [28,29].

In contrast, outcomes related to drought stress in this work showed a reduced five-fold (-5) transcript abundance in the glucosinolate pathway. Similar to the results of this work, drought stress significantly reduced glucosinolate accumulation in *Boechera holboellii* Hornem. Á.Löve & D.Löve and some *Brassica carinata* A.Braun cultivars, whereas some *B. carinata* cultivars reported a significant increase in glucosinolate accumulation [30,31]. Treatment of potassium sulfate during drought conditions on canola declined glucosinolate accumulation compared to the untreated plants [32]. Root tissue showed a few changes in the transcript abundance of SMs. However, mostly stress-responsive transcripts are altered under adverse conditions. Interestingly, SMs, such as sinapic acid, lignin, and flavanols, which are proposed to have defensive roles, increased in *Sinopodophyllum hexandrum* Royle under water deficit [33].

Several phenolic compounds originating from phenylalanine, including flavonoids, monolignols, lignans, lignins, coumarins, and hydrolyzable tannins, are formed through the phenylpropanoid pathway [34]. The *Linum* genus, exclusively *L. album*, contains the highest levels of lignans, especially PTOX. In contrast to the notable amount of experience in the lignans pathway, the pathway of this compound until the end product PTOX has not been completely clarified.

The beginning of this pathway starts with the deamination of phenylalanine and synthesizes 4-coumaroyl-CoA by 4CL. Then, 4-coumaroyl-CoA is converted to caffeoyl-CoA through several reactions by HCT, which catalyzes two different steps, followed by methylation via CCoAOMT and synthesizes feruloyl-CoA [1,35]. Feruloyl-CoA is converted to coniferyl alcohol through two reduction reactions by CCR and CAD [8,36]. Since coniferyl alcohol has been known as a critical precursor in the biosynthesis of PTOX, the above-stated steps were considered upstream [37]. In the present study, CAD contains the highest number of transcripts in upstream steps showing spatial expression patterns and may have different functional roles in specified tissues; for example, *OsCCR10* involve in response to drought in rice root tissues [38]. knockout of *OsCCR10* with the CRISPR/Cas9 system revealed reduced drought tolerance due to a decline in lignin accumulation in root [39]. The later specified steps of PTOX and its derivative synthesis after coniferyl alcohol were considered downstream. Downstream steps begin with coupling two molecules of coniferyl alcohol to get pinoresinol enantiomers by an oxidase (LAC11) or peroxidase (POD) with the aid of dirigent proteins, depending on plant species [40–42]. *L. usitatissimum* generates both enantiomers (-)- and (+)-pinoresinol, followed by the stepwise reductive conversion to lariciresinol and then SECO through PLR (1–4) [43]. (-) SECO, resulting from (+)-pinoresinol, is catalyzed to matairesinol by the action of SDH. Subsequently, matairesinol is converted to deoxypodophyllotoxin by several enzymatic reactions in *P. hexandrum*, such as PhOMT3, CYP71CU1, PhOMT1, and 2-ODD. Although the genes encoding enzymes associated with steps between matairesinol to deoxypodophyllotoxin are not yet identified in *Linum*, This study realized some of these genes [8,44,45]. The transcriptomic analysis of *L. usitatissimum*

revealed that the expression level of *LAC11*, *POD*, *4CL*, and *SDH* genes was highest under drought stress. In contrast, root tissues subjected to abiotic stress demonstrated the downregulation of the expression level of these enzymes. Conversely, quantitative expression with qRT-PCR of *SDH* genes in *L. album* was an increasing trend in root tissues subjected to stress and differing in shoot tissues. Furthermore, *2-ODD* excluding drought stress and *OMT1* showed a rising trend in *L. album*, conforming to previously reported studies in *P. hexandrum*, but this pathway evolved independently in the two species [19]. Quantitative expression of genes related to PTOX biosynthesis, studying in different tissues of *P. hexandrum* exhibited that *SDH*, *CAD*, *CCR* and *cinnamate 4-hydroxylase* genes increase in rhizomes more than roots [35]. A study reported increased gene expressions of growth and development and PTOX biosynthesis at 15°C, while decreased gene expressions and content accumulation of PTOX and dominated genes responsive to stress at 25°C [1]. In the study carried out by Kumari et al. (2022), genes of phenylalanine ammonia lyase (*ShPAL*), *Sh4CL*, *ShC3H*, *ShCCoAOMT*, *ShCOMT*, *ShCAD*, *ShDPO*, *ShPLR*, and *ShSDH* demonstrated upregulation, as well as, an increase in PTOX accumulation content in root subjected drought stress [33]. however, PTOX was not evident in the leaf.

High-performance liquid chromatography (HPLC) analysis, consistent with qRT-PCR results, demonstrated the combination of both conditions in the root with increased SECO content and upregulation of three selected genes (*SDH*, *OMT1*, and *2-ODD*) led to producing the highest PTOX at 48h. At the same time, shoot tissues subjected to such conditions formed PTOX by consuming the precursor of SECO. Under drought stress in the root at 12h, a copious amount of SECO and overexpression of *2-ODD* caused the conversion of 6-MPTOX and PTOX during stepwise reactions [1]. However, PTOX content declined because of the downregulation of *2-ODD* after 48h, according to qRT-PCR. Probability involving other enzymes related to lignans biosynthesis toward 6-MPTOX generation, the PTOX subject to drought stress decreased. Despite the high SECO amount and overexpression of *2-ODD* (in the shoot after 48h) significantly decreased PTOX because of the downregulation of *SDH* and *OMT1*. The K^+ deficiency and drought stress often have opposite accumulation patterns of three selected lignans. The differences in response to individual and combined stresses in *L. album* suggest the complex regulatory mechanism of lignans biosynthesis, which needs further investigation. The expression of *PLR* in *S. hexandrum* contradicts the PTOX content in the different tissues, proposing that the PTOX-producing tissue cannot necessarily be its pool [46].

Application of abiotic and biotic elicitors, including chitosan, methyl jasmonate, salicylic acid, yeast extract, and Ag^+, has mostly established that the expression of genes associated with lignan biosynthesis and lignan content enhanced in *Linum* spp. *in vitro* [47–50]. Also, a study carried out on different accessions of *L. album* under drought stress presented different patterns based on physiological and biochemical responses [51].

In general, the results from qRT-PCR and HPLC analysis and transcriptomic analysis are consistent, corroborating with previous studies, although they may be different in some cases due to species or genotype differences, type of culture, and stress intensity. The K^+ deficiency and drought stress often had opposite up/down-regulation patterns, which with the synergistic negative effects, put a lot of expenses on the plant. While fusarium's up/down-regulation pattern was similar to K^+ deficiency, drought stress was similar to ABA treatment.

Therefore, strategies based on transcriptome for species that likely accumulate lignans would aid in identifying common features between species and environmental cues to clarify the PTOX biosynthesis pathway.

Conclusion

This study evaluates the common core of gene expression changes using comprehensive transcriptome data of the flax. The results showed that the EP3 gene increased in all stresses. In the analysis of SMs pathways, the most alterations were observed in the biosynthetic pathways of flavonoids and phenylpropanoids. In the pathway of phenylpropanoid biosynthesis, 12 gene-encoding enzymes of the PTOX biosynthetic pathway had the highest expression increase for *LAC11*, *POD*, and *4CL*. The results of HPLC and qRT-PCR were consistent with the *in silico* results, and all revealed that K^+ deficiency

and drought had opposite patterns. This work suggests new insights into the response of *Linum* species to abiotic stresses that can be employed to improve PTOX for commercial goals.

Methods

RNA-Seq data collection and analysis

Public available RNA-Seq datasets for six stress treatments in *L. usitatissimum* were obtained from NCBI GEO (www.ncbi.nlm.nih.gov/geo). The datasets were related to whole-plant, leaf and root tissues at the seedling stage (Table 1). Adapter sequences and low-quality reads were trimmed using Trimmomatic v0.39 based on its adaptive trimming algorithm, maximum information (MAXINFO), to balance the benefits of retaining longer reads against the drawback of having low-quality bases [52]. The other trimming criteria were TRAILING: 20, MAXINFO: 80: 0.8, MINLEN: 80, changed based on the length of the reads. The clean reads were mapped to the *L. usitatissimum* reference genome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Lusitatissimum) using HISAT v2-2.1.0, and the number of mapped reads was counted using python script HTSeq-count v0.12.4 according to GTF file (phytozome-next.jgi.doe.gov/info/Lusitatissimum_v1_0) [53,54]. For each dataset, a gene expression matrix was created and subjected to DEG analysis. DESeq2 R package was applied to identify DEGs. Also, DEGs were found using the edgeR R package for the datasets without any biological replications [55,56]. These packages provide statistical routines for determining differentially expressed genes using a model based on the negative binomial distribution. Up and down-regulated genes were considered differentially expressed if the FDR-corrected p-values were ≤ 0.05.

Functional Enrichment Analysis

DEGs were subjected to further functional analysis. They were mapped to each functional category with the GO database. Then, a Venn diagram was conducted to identify common significant DEGs of each stress type using Venny 2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/index.html). In other words, for each stress type, common DEGs among the different datasets were defined as the final DEG lists and were considered for further downstream analysis.

TAIR (The Arabidopsis Information Resource) IDs related to the common DEGs were subjected to GO enrichment analysis using AgriGO [57]. The common DEGs identified across the studies analyzed were classified functionally and MapMan category. MapMan analysis was primarily performed upon TAIR gene listing by the *A. thaliana* genome mapping files to classify functional categories [58]. The second MapMan analysis gave an overview of gene expression data onto diagrams of the main pathways of SMs biosynthesis for all the transcriptomic datasets individually.

Ptox Biosynthetic Pathway Genes And Ppi Network Analysis

Annotation and enrichment test of the transcriptome data of *L. usitatissimum* for pathways of PTOX was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Phytozome database. The log2 (fold-change) values of DEGs of each dataset were used to plot the heatmap in R (version 3.5.0). The log2 (fold-change) values of enzyme-related DEGs of the lignan biosynthesis pathway were considered if the FDR-corrected p-values were ≤ 0.05, at least in one of the six stresses. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (http://string-db.org/cgi/input.pl) was used to identify known PPI among the enzyme-related DEGs of the lignan biosynthesis pathway [59]. Networks were visualized by Cytoscape software [60].

Plant Materials

The plant materials were collected from wild habitats at Imam Khomeini International University, Qazvin, Iran (36.15°N, 117.15°E), then identified by Dr. Ahmadreza Mehrabian (Department of Plant Sciences and Biotechnology, Shahid Beheshti University). A voucher specimen has been deposited in the Herbarium of Shahid Beheshti University (HSBU-2019980).

The plant seeds were sown after germination in 10 cm diameter plastic pots filled with a mixture of peat moss and perlite in a 1:1 ratio under the natural light condition in the greenhouse. Seedlings were then irrigated with Hoagland's nutrient solution (KH_2PO_4, KNO_3, $Ca(NO_3)_2$ $4H_2O$, $MgSO_4.7H_2O$, H_3BO_3, $MnSO_4$ H_2O, $ZnSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, $NaMoO_4{\cdot}2H_2O$, Na-EDTA, $FeSO_4$ $7H_2O$) [61]. Seedlings with three replications were treated with Hoagland's nutrient solution containing − 9 bar of PEG-6000, Hoagland's nutrient solution without potassium, and a combination of both treatments at 12 and 48h [18]. Untreated seedlings were used as controls. The shoot and root samples harvested were instantly frozen in liquid nitrogen before storing at −80°C. Plant study complies with relevant institutional, national, and international guidelines and legislation.

Qrt-pcr Assay

Total RNA was isolated from shoots and roots of *L. album* during the vegetative stage using a column RNA Isolation Kit (DENA Zist, Asia) following the manufacturer's instructions. The extracted RNA was treated with DNase I (Sigma, USA), and the synthesis of the first strand of cDNA was then performed using Easy cDNA Synthesize (ParsTous, Iran). Primers of candidate genes were designed by OLIGOTECH software (OligoTech. Inc., Wilsonville, OR, USA) and Vector NTI software package (Invitrogen, Carlsbad, CA, USA). The qRT-PCR was performed using RB SYBR qPCR Kit (RNAbiotech, Iran). PCR amplification was conducted at 95 C for 1 min, followed by 40 cycles of desired Tm (See additional file) for 1 min and 72°C for 15 s. The relative expression of each gene of interest was calculated with REST using the formula $2^{-\triangle\triangle Ct}$ and expressed relative to GAPDH as the internal control.

Hplc Analysis

The shoot and root samples of *L. album* (1gr) were prepared using a pestle and mortar in liquid nitrogen, followed by extraction in methanol (80% v/v). The samples were then sonicated at room temperature for 1h. Dichloromethane (4.0 ml) and water (4.0 ml) were added to obtain a partition of compounds between two layers, followed by centrifugation at 5,000 rpm for 15 min. The dichloromethane fractions were then collected, dried, and dissolved in 1.0 ml of HPLC grade methanol for HPLC analysis [62].

The Lignan content was determined using a Waters liquid chromatography apparatus consisting of a 2695 Separations Module (USA) and a 2487 Dual Absorbance Detector (USA). Data acquisition and integration were carried out with Millennium32 software. The chromatographic separations were performed on a 250 × 4.6 mm Eurospher 100-5 C18 column (KNAUER company, Berline, Germany) with a reversed-phase matrix in a gradient system using acetonitrile (solvent A) and distilled water (solvent B) with a 1 mL/min flow rate. A UV detector was set at 280 nm. The presence of PTOX, 6-MPTOX, and SECO was identified on the basis of retention time and comparison of UV spectra with the authentic PTOX, 6-MPTOX, and SECO standards, purchased from Sigma-Aldrich (Taufkirchen, Germany). Different concentrations of the three compounds (25, 50, 75, and 100 µg/ml) were used for the calibration curves.

Statistical analysis

One-way ANOVA analysis was conducted using R statistical software v.3.6.0 (freely available at http://www.r-project.org), followed by Duncan's multiple comparisons at a significance threshold of 0.05. Data were presented as means ± standard deviations (SD).

Declarations

Data Availability

All raw sequence data used to identify genes involved in the PTOX biosynthesis pathway for Linum is accessible from NCBI, the National Center for Biotechnology Information, as described in Table 1. Files are available for download through the NCBI website (https://www.ncbi.nlm.nih.gov/bioproject/) with accession number of PRJNA598287, PRJNA515812, PRJNA414507, PRJNA504749, PRJNA343117, and PRJNA497472.

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Author contributions

Z.D. contributed to the conception of the study, collection of plant materials, analysis and interpretation of data, and wrote the manuscript with the supervision of M.T. G.G. supervised the study, collection of plant materials, and review & editing. M.T. contributed to the conceptualization, interpretation of data, and review & editing. M.R.B.Z. contributed to the data analysis and review & editing. M.H.M. hosted Z.D. at the Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, for a short research stay, supervised the work, carried out lignans analysis, and review & editing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

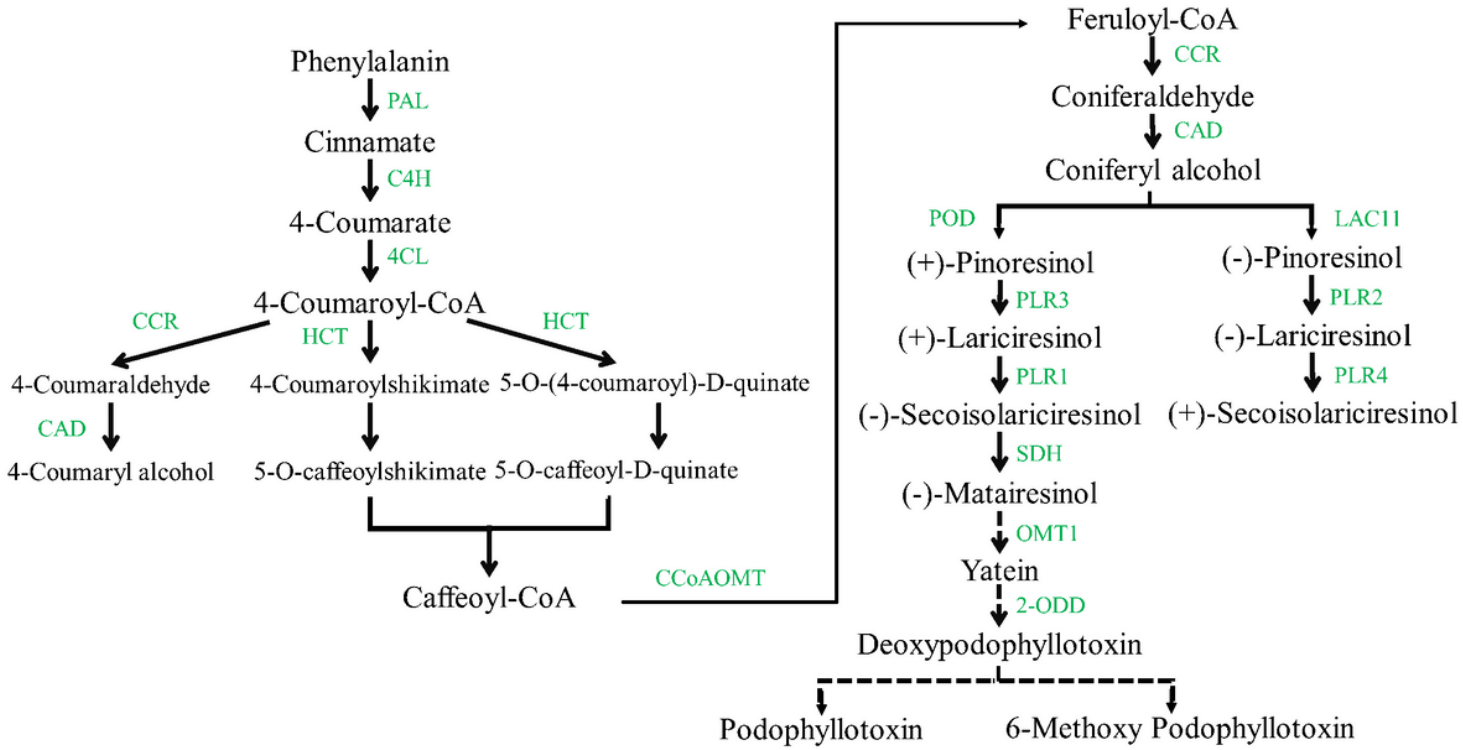

Figure 1

Putative biosynthetic pathway of podophyllotoxin (from KEGG and Phytozome database). Enzyme abbreviations are as follows: PAL: phenylalanine ammonia lyase; C4H: cinnamate 4-hydroxylase; 4CL: 4-coumarate-CoA ligase; HCT: shikimate O-hydroxycinnamoyltransferase; CCoAOMT, *trans*-caffeoyl-CoA 3-O-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD: coniferyl-alcohol dehydrogenase; POD: lactoperoxidase; LAC11: laccase; PLR: pinoresinol/lariciresinol reductase; SDH: secoisolariciresinol dehydrogenase; OMT1: 5'-desmethyl-yatein O-methyltransferase; 2-ODD: deoxypodophyllotoxin synthase.

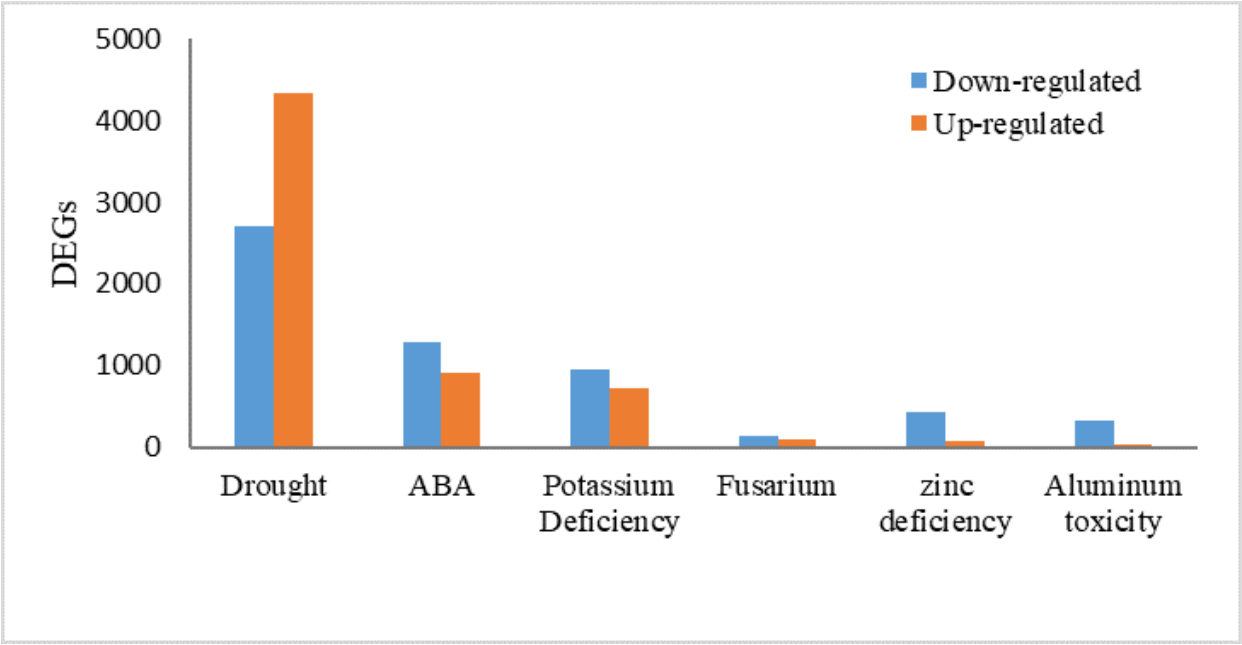

Figure 2

DEGs identified under abiotic and biotic stresses in *L. usitatissimum*

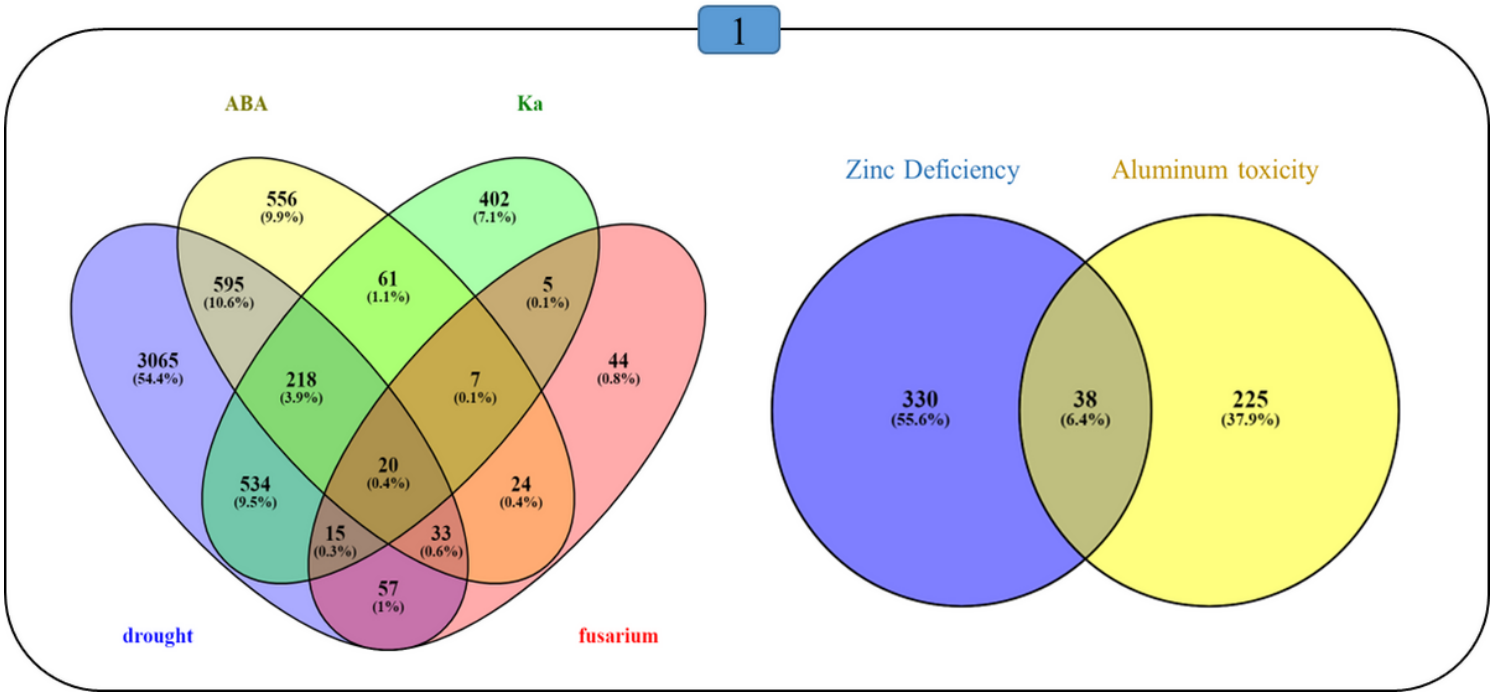

Figure 3

Comparison between transcriptomic responses to abiotic and biotic stresses in *L. usitatissimum* Venn-diagram showing the number of commonly regulated and unique genes responsive to drought, ABA, K^+ deficiency, *Fsarium* treatment (left), Aluminum toxicity and Zinc deficiency (right).

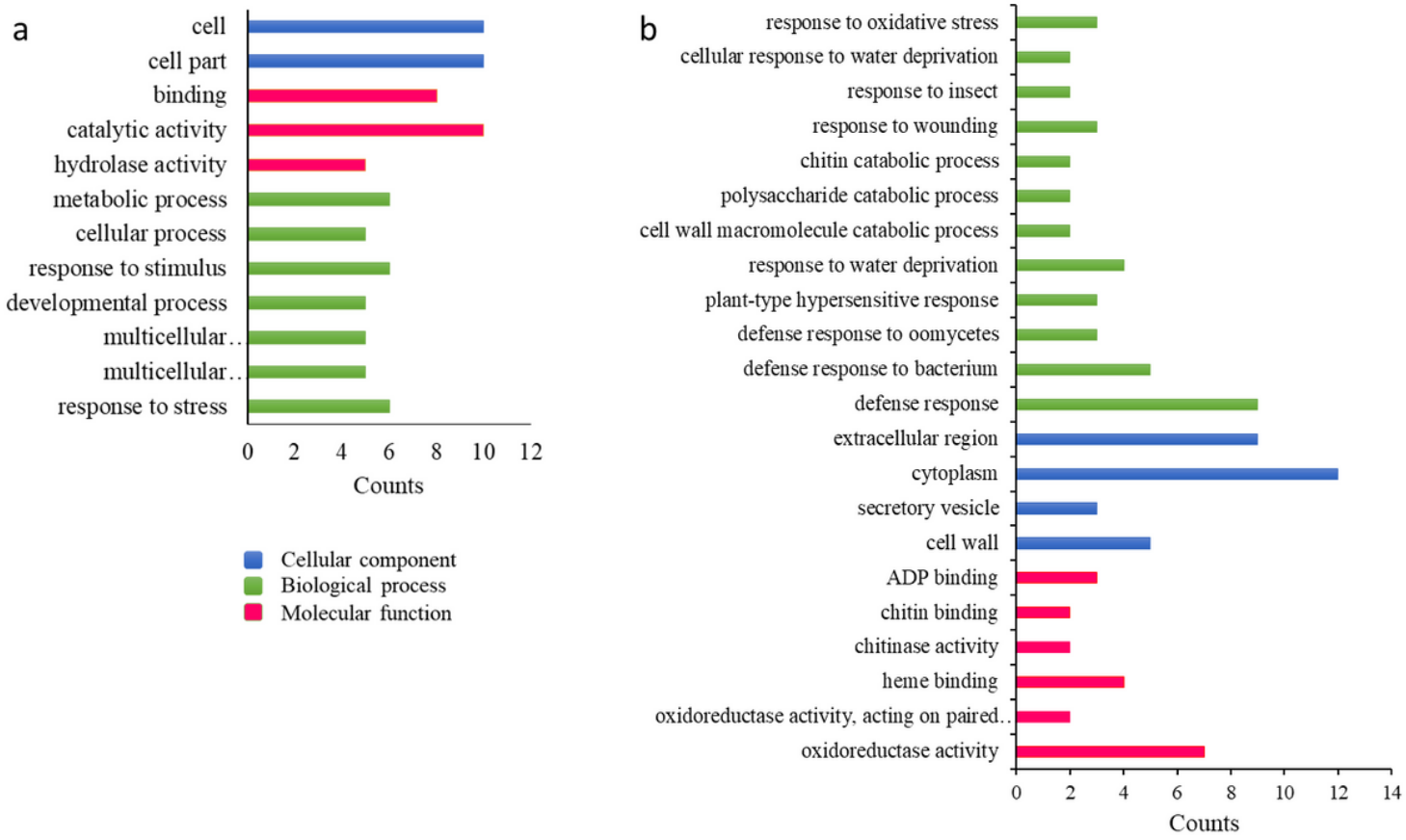

Figure 4

Gene function prediction by gene ontology terms in *L. usitatissimum*; red: Molecular function; green: Biological process; blue: Cellular component. A: Shoot; B: Root.

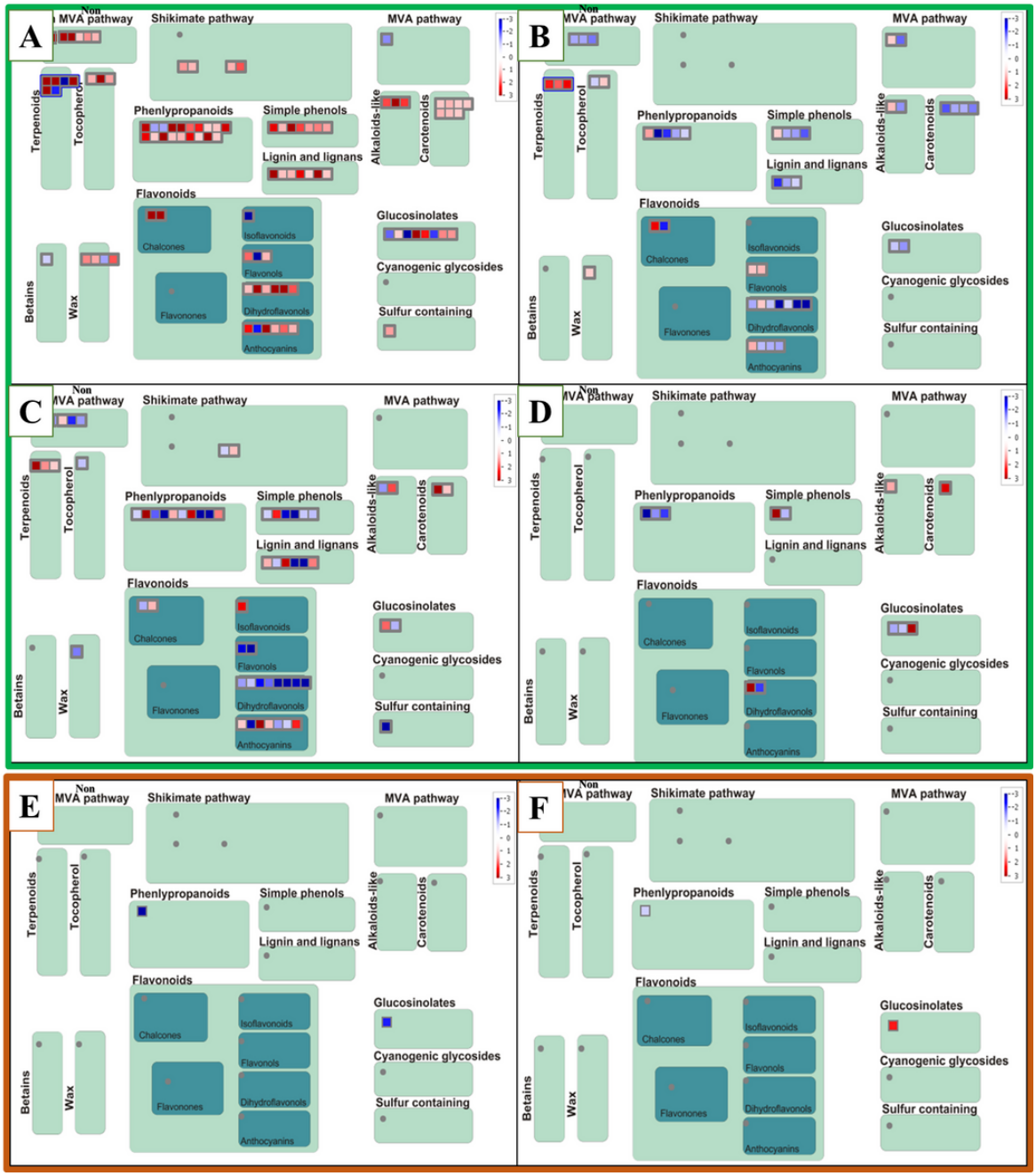

Figure 5

Overview of DEGs of *Linum* species under different stresses those are involved in the biosynthesis pathways of specialized metabolites. A) drought stress, B) K^+deficiency stress, C) ABA treatment, D) *Fusarium* treatment, E) Zinc deficiency, and F) Aluminum toxicity. Red represented upregulated genes, and Blue downregulated genes.

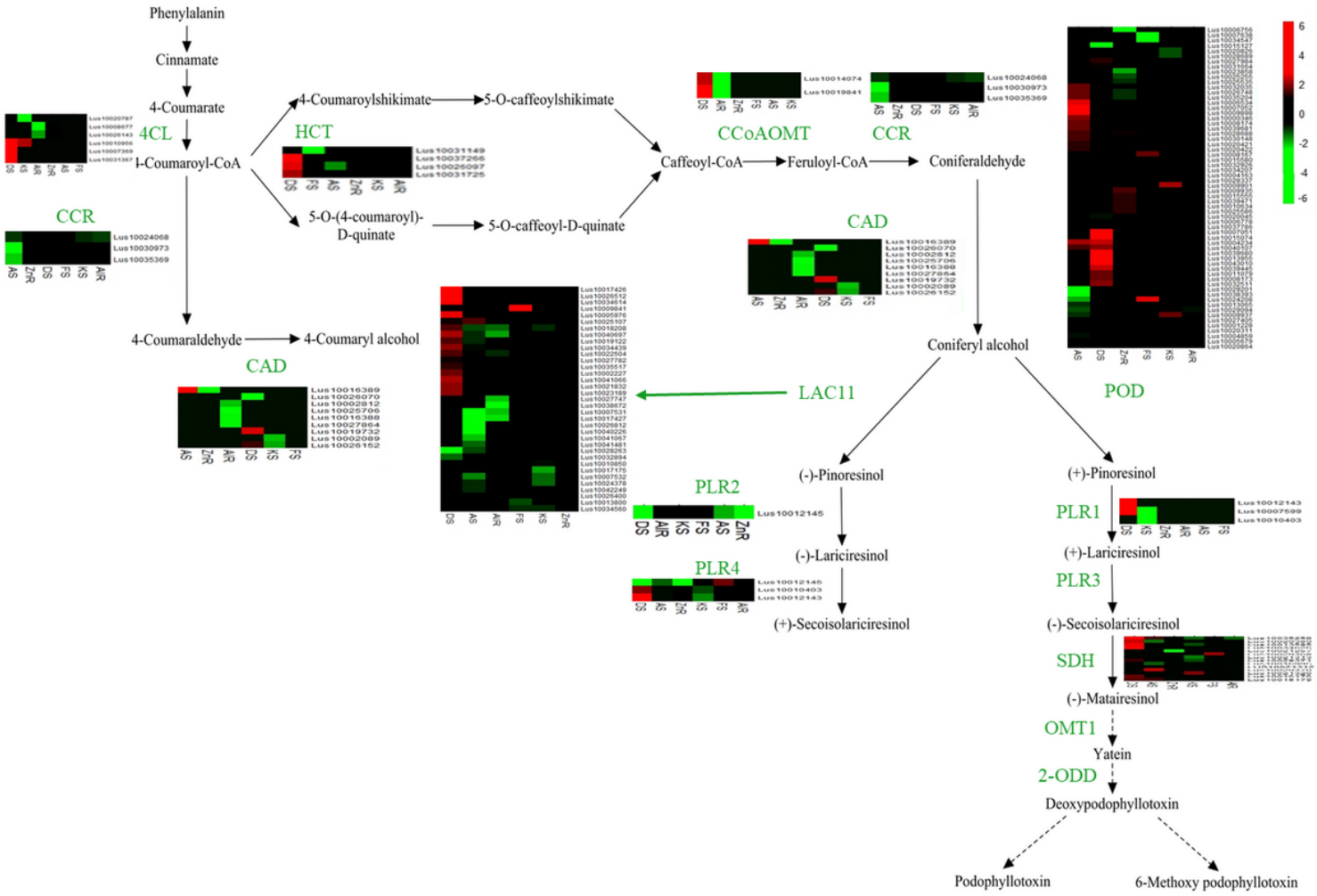

Figure 6

Transcription profiling of genes in podophyllotoxin biosynthesis pathway originating from phenylalanine: Transcriptome data analyzed under different stress. The biosynthetic pathways and Transcription profiles of genes affected in the biosynthesis of podophyllotoxin are demonstrated at studied stress: DS: drought-shoot; AS: ABA-shoot; KS: K^+ deficiency-shoot; FS: Fusarium-shoot; ZnR: zinc deficiency-root; AlR: aluminum toxicity-root. Expression levels of genes encoding enzymes related to the podophyllotoxin biosynthetic pathway are represented; Red: upregulated; Green: downregulated.

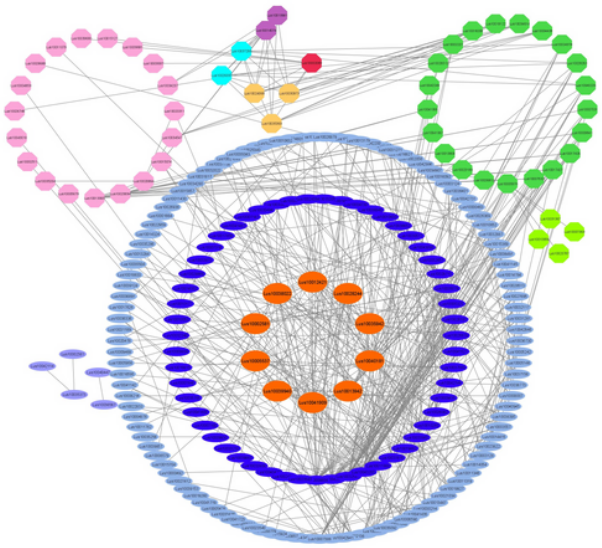

Figure 7

Interaction networks of ID transcriptions in the biosynthetic pathway of podophyllotoxin. ID transcriptions showed a significant difference in at least one stress. Transcription IDs that did not connect to any node were removed and displayed ten hub genes and the first neighbor. Green:LAC11, pink: POD, red:CAD, orange: CCR, dark orange:TFs, light green: 4CL, blue:HCT, purple: CCoAOMT.

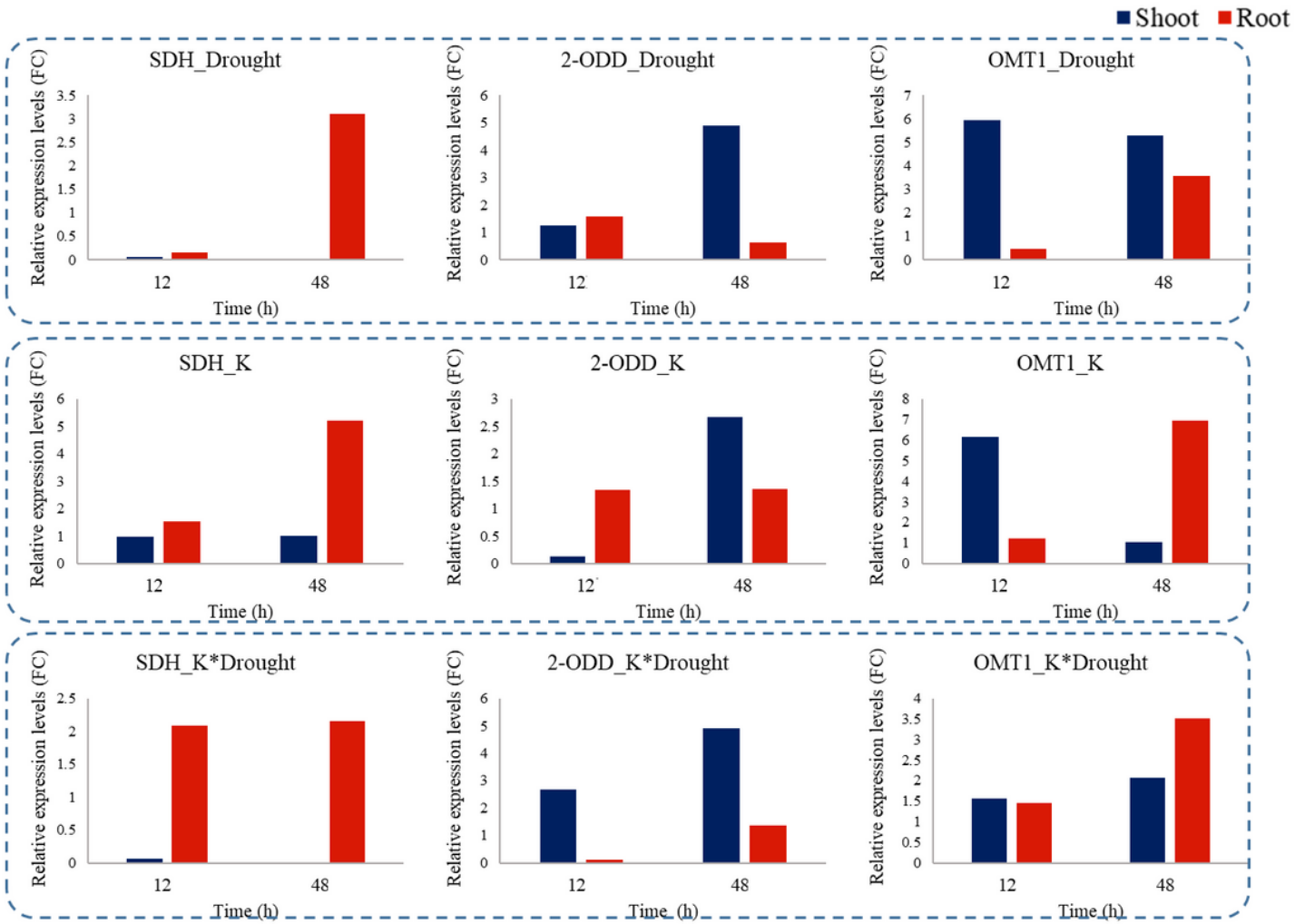

Figure 8

Verification of differentially expressed genes by qRT-PCR in *L. album.* The *GAPDH* gene was used as an internal control to normalize the expression data. The relative expression levels of each genes were expressed as the fold change between control and other treatment. SDH: Secoisolariciresinol dehydrogenase; 2-ODD: deoxypodophyllotoxin synthase ; OMT1: 5'-desmethyl-yatein O-methyltransferase.

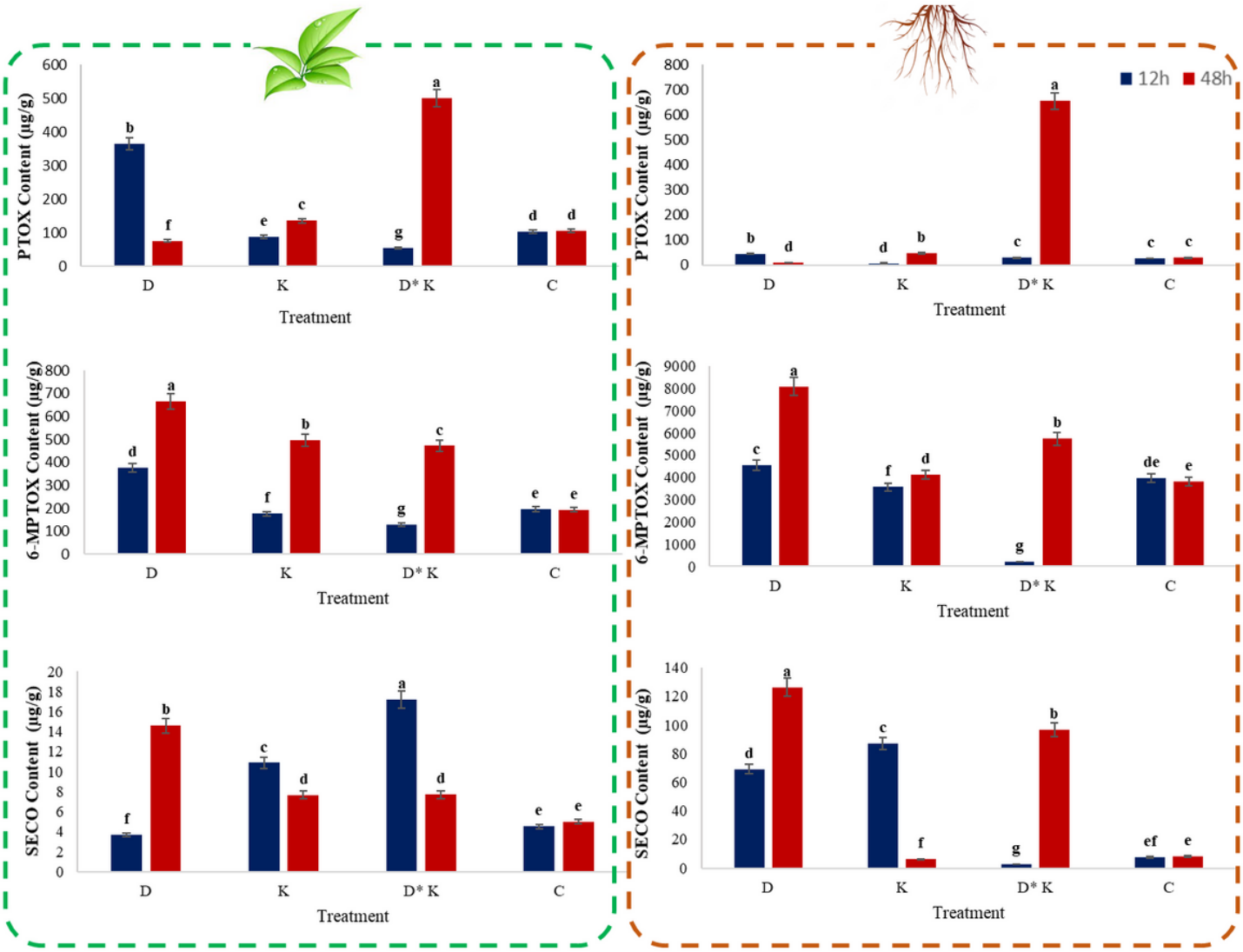

Figure 9

HPLC analysis of lignan contents in *L. album*. The podophyllotoxin, 6-methoxypodophyllotoxin, and secoisolariciresinol content in shoots and root of *L. album* at 12 and 48h. PTOX: podophyllotoxin; 6-MPTOX: 6-methoxypodophyllotoxin; SECO: secoisolariciresinol.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile.xlsx