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14-3-3λ Affects Production of a Sinapoyl Derivative in Lignin Biosynthesis during Drought Stress in Arabidopsis Thaliana

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Abstract The phenylpropanoid biosynthetic pathways have been targeted for bio-engineering as compounds produced by this pathway are known for anti-oxidant, anti-viral, anti-inflammatory, anti-allergenic, vasodilatory activities as well as response to various stresses in plants. Our interest is to study how drought stress influences biosynthesis of flavonoids and lignin monomers in this pathway and the roles of candidate regulatory enzymes such as 14-3-3 proteins in regulation of these compounds. Our laboratory has identified an isoform (14-3-3 λ) that demonstrates a role in drought stress tolerance and also affects the biosynthesis of flavonoids and lignins. To determine the role of the 14-3-3 λ in the phenylpropanoid pathway, we used a reverse genetics approach, in which the amounts of secondary metabolites produced in a 14-3-3 λ knockout mutant were compared to the wild-type Arabidopsis thaliana (Columbia-0) under normal and drought stress conditions. Using LC-MS, our analyses show that the quantity of sinapoyl derivatives varied significantly between the drought treated 14-3-3 knockouts and the drought treated wild-types. There were significant changes in a sinapoyl derivative and no change in the flavonol glycosides in drought or hydrated samples of the mutants or the wildtype. Gene expression results revealed significant changes under drought conditions in two enzymes involved in lignin biosynthesis:phenyl alanine lyase (PAL) and Caffeoyl Coenzyme A 3-O-methyltransferase 1 (COMT). The metabolite profile and gene expression data indicate that 14-3-3λ is affected genes in the lignin biosynthesis and flavonoid biosynthesis pathways.

Keywords 14-3-3λ, Phenylpropanoid Pathway, Lignin Biosynthesis, Arabidopsis Thaliana

1. Introduction

The 14-3-3 family of proteins is greatly diverse and highly conserved throughout all eukaryotes [1]. Originally purified from bovine brain serum [2], the 14-3-3 proteins were long believed to be specifically localized in nervous tissue and were highly conserved in mammals. It wasn't until the past twenty years that they were found to be fairly ubiquitous and highly conserved among other eukaryotes including plants, insects, amphibians and yeast [3]. By their ability to form homo- or heterodimers, these proteins can serve in critical roles in signaling pathways by forming protein-protein interactions with various enzymes and substrates [4]. The 14-3-3 proteins occur as many isoforms within the same organism, lending them an array of biological functions such as cell cycle regulation, anti-apoptotic function, metabolism control, and gene transcription control [5]. The binding sites are highly conserved throughout all 14-3-3 isoforms; however, their C-termini have 0% homology, and are likely responsible for their various cellular localizations [6-8] and different biological effects [9].

In the past two decades, 14-3-3 proteins have demonstrated roles in a wide variety of cellular processes in plants in order to cope with complex environmental stresses. A study investigating high salinity and low temperature effects on rice callus seedlings discovered an up regulation of a 14-3-3 gene under both conditions [10]. Enzymology studies have also uncovered the activity of 14-3-3 proteins as regulators of nitrate reductase [11], sucrose phosphate synthase and starch synthase [12], indicating a wide range of metabolic regulatory behavior. Further evidence of 14-3-3 proteins' role is their regulation of H^+ ATPase transport proteins in Arabidopsis thaliana guard cells [13]. A valuable function of flavonoids in planta is their response to biotic and abiotic stress by scavenging free radicals. Flavonoid biosynthesis is governed by a variety of genes which are induced under stress conditions to combat the influx of reactive oxygen species (ROS), such as peroxide anions ($O^{2-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$)

and singlet oxygen ($^1O_2$) [14].The effects of 14-3-3 on phenylpropanoid pathway have been studied in potato plants (Solanumtuberosum L.) where repression of 14-3-3 proteins showed a 65-fold decrease in antioxidant capacity. In contrast, over-expression of 14-3-3 resulted in a 2-fold increase in the antioxidant capacity of potato tubers in the same study [15]. It was also demonstrated that the transgenic lines with the least antioxidant activity had the lowest anthocyanin (flavonoid) content. The most abundant phenolic acid present in the over-expressing transgenic potatoes was chlorogenic acid (about 40%) and the most abundant anthocyanin was putinidin (about 50%), indicating that both phenylpropanoid compounds were affected in the same way. Another study found that the biosynthesis of legume-specific isoflavonoids is also regulated by 14-3-3 proteins [16]. But, the results of either of these studies have not identified the nodes of the phenylpropanoid pathway which are affected by 14-3-3 proteins. The objective of this research was to determine the effect of 14-3-3λ (At5g10450) on the flavonol glycosides and a sinapoyl derivative, produced via the phenylpropanoid pathway, during drought stress in Arabidopsis thaliana. Previous research in our laboratory identified an isoform of 14-3-3 called lambda (λ) which was shown to contribute to drought tolerance [17], therefore it is of interest to investigate the role of this proteins on the biosynthesis of secondary metabolites that are synthesized via the phenylpropanoid pathway. Using a colorimetric total flavonoid assay, it was observed that each of the 14-3-3λ homozygous TDNA knockout mutants (SALK_075219 and SALK_129554) had the same accumulation of flavonoids (results not shown here), and therefore this research only involved the analysis of a single mutant (SALK_075219). Also, both the T-DNA alleles were shown in our earlier work to behave the same under drought conditions [17]. Using Arabidopsis thaliana 14-3-3 λ homozygous TDNA knockout mutant (SALK_075219) and wild-type (Columbia-0), a reverse genetics approach was taken in which the flavonol glycoside composition of each genotype was screened by Liquid Chromatograph – Mass Spectrometry (LC-UV-MS). Using this method, five phenylpropanoid compounds that showed differential accumulation during drought were separated by the same extraction and chromatographic parameters. The identification of one of the secondary metabolites that showed a significant change was confirmed by NMR spectroscopy. The quantity of the five phenylpropanoid compounds was determined relative to the identified compound. Gene expression analysis of eight key enzymes in flavonol biosynthesis and in lignin biosynthesis was also monitored.

2. Materials and Methods

2.1. Plant Growth Conditions and Harvest Procedure

Wild-type (Columbia-0) and T-DNA mutant (14-3-3λ2 SALK_075219) were purchased from Arabidopsis Biological Resource Center (ABRC, Ohio State University) and grown and watered regularly on Sunshine Mix (Sun Gro Horticulture, Quincy, MI) in an environmental growth chamber at 24 ºC with 70% relative humidity and a 12 hour photoperiod. Seeds were suspended in 0.1% Agar solution and refrigerated for 48 hours prior to planting to ensure simultaneous germination. Drought treatment was carried out using mature plants (3 weeks old) by continuing to water only half of them, and harvesting the entire crop when the drought treated soil moisture level reached 30% which was determined to be the correct drought simulation condition [27]. The age of the plants during harvest time was usually 4 weeks old. Soil moisture was measured using a Wescorpsychrometer/hygrometer (Wescor Biomedical Systems, Logan, UT). Leaves from individual plants were placed in labeled aluminum foil and immediately frozen in liquid nitrogen. After each harvest was completed, lead samples were stored at -80 ºC until extraction.

2.2. Flavonol Extraction and Isolation

Arabidopsis thaliana leaf tissue was frozen at -80 ºC and freeze dried (LabConco Corporation, Kansas City, MO). An aliquot (0.1 g) was frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle, and extracted with 0.5 mL extraction solvent (Methanol:Acetone; 1:1 v:v). The extract was then transferred to a 2 mL eppendorf tube and stored on ice. The mortar and pestle were then washed with 1 mL extraction solvent which is then added to the 2 mL eppendorf tube. The extracts were then centrifuged 12,000 rpm for 5 minutes. The supernatant was transferred to a 15 mL BD Falcon conical centrifuge tube (ThermoFisher Scientific, Waltham, MA). The plant pellet was then extracted twice more with 1 mL extraction solvent. All of the extracts were combined (3.5 mL total volume) and dried using a Savant SpeedVac SVC200H centrifugal evaporator (Savant Instruments Inc., Farmingdale, NY). The dry extracts were reconstituted in 1 mL acetone. Water was added drop-wise to precipitate chlorophyll and centrifuged at 12,000 rpm for 5 minutes. The supernatant was transferred to a new 15 mL BD Falcon conical centrifuge tube and dried using the Savant SpeedVac SVC200H centrifugal evaporator. The dried extract was reconstituted in 0.2 mL methanol and 0.8 mL 0.1% formic acid in water and transferred to an HPLC autosampler vial using a fitted filter tip. The extracts were separated and quantified using a Shimadzu LC-MS 2010EV (Shimadzu Corporation, Kyoto, Japan) liquid chromatograph – mass spectrometer. Using a modified method [23], separation and quantification of phenylpropanoid compounds was carried out. A binary gradient elution was employed using water (0.1% formic acid) and acetonitrile (0.5% formic acid) as mobile phases. HPLC conditions: Agilent Zorbax 300SB-C18 (5 µm, 4.6 x 50 mm), solvent system: A – Water (0.1% formic acid), B – Acetonitrile (0.5% formic acid); 10-30% B over 15 min, 30-60% B over another 10 min, 60-70% B over another 2

min, hold at 70% B for another 2 min, 10% B for the final 12 minutes for reequilibration of the column; flow rate: 0.2 mL/min; injection volume: 20 µL; detector wavelength: 320 nm. Using Kaempferitrin (Kaempferol-3,7-O-dirhamnoside, Chromadex, Irvine, CA) as an internal standard, quantitative analysis was carried out by the development of a calibration curve (Table 1S, Figure 2 and 2S, Figure 3S).

Table 1S. spectral data for Kaempferitrin

Position	^{1}H NMR (Chemical Shift, Multiplicity)
2	-
3	-
4	-
5	-
6	6.46 (d)
7	-
8	6.79 (d)
9	-
10	-
1'	-
2', 6'	7.79 (d)
3', 5'	6.92 (d)
4'	-
5-OH	12.61 (s)
4'-OH	10.26 (s)
1''	5.30 (d)
2''	3.99 (s)
3''	3.48 (m)
4''	3.16 (m)
5''	3.12 (m)
6''	-
2''-OH	5.00 (d)
3''-OH	4.67 (d)
4''-OH	4.77 (d)
5''-CH3	0.80 (d)
1'''	5.55 (d)
2'''	3.84 (s)
3'''	3.64 (m)
4'''	3.29 (m)
5'''	3.41 (m)
6'''	-
2'''-OH	5.16 (d)
3'''-OH	4.82 (d)
4'''-OH	4.93 (d)
5'''-CH3	1.13 (d)

2.3. Fraction Collection by HPLC

Fractions were collected using a Bio-Rad Model 2128 Fraction Collector (Bio-Rad Laboratories Inc., Berkeley, CA) in 30 second intervals. High Performance Liquid Chromatography experiments were executed using an Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA) equipped with a photodiode (PDA) array detector. HPLC conditions: Waters Symmetry Shield RP18 (2.1 x 50 mm; 5 µm), solvent system: A – Water, B – Acetonitrile; 10-15% B over 18 min, 18-20% B over 4 minutes, 10% B for the final 8 minutes for reequilibration of the column; flow rate: 0.2 mL/min;injection volume: 50µL. Calculation of the delay time between the detector and fraction collector was performed by purging the plumbing from the detector module to the fraction collector with air and then monitoring the time required for a drop to reach the fraction collector at the flow rate used for analysis (0.2 mL/min).

2.4. Structural Elucidation by NMR

All NMR experiments were conducted on a BrukerAvance III 400 MHz NMR Spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 400.13 MHz. Spectra were recorded at 25 º C using standard Bruker 1D and 2D sequences. For structural characterization of Kaempferitrin, various nuclear magnetic resonance (NMR) experiments were employed including 1H, 1D-TOCSY, 2D-COSY and 1D-NOESY with presaturation.

2.5. Real Time PCR

Gene expression of eight genes that are important in the flavonol biosynthesis and in the lignin biosynthesis was monitored using Real time PCR. The gene expression of chalcone synthase (CSH, At5g13930), chalcone isomerase (CHI, At3g55120), dihydroflavonone reductase (DFR, At5g42800), phenylalanine ammonia-lyase (PAL, At2g37040), 4-coumarate:CoA ligase (4CL, At1g51680), and leucoanthocyanidin dioxygenase (LDOX, At4g22880), Flavonol 3 hydrase (F3H, At3g51240), Caffeoyl Coenzyme A 3-*O*-methyltransferase 1 (COMT, At5g54160). Three biological replicates of 4 week old wild-type and knockout plants grown in the growth chamber. RNA was extracted using trizol method and converted to cDNA using Ambion kit. The primers for these genes are shown in the following table 1.

Table 1. Real time primers

NAME	Forward primer	Reverse primer
CSH	TCGGCAGGCTCTTT TCAGT	GGAGATGGAAGGTG AGACCA
CHI	TCATCGATCCTCTTC GCTCT	AGGTGACACACCGTT CTTCC
DFR	GACGACTTATGCA ACGCTCA	TCCGTCAGCTTCTTG GAACT
PAL	ATCGAAGTGATCC GTTACGC	ACTCCGATTGGTGTT CCTTG
4-CL	CGCAAACCCTTTCT TCACTC	ACTCCGTCGTCGTTT TGAAG
LDOX	CGATGAAAAAGAT CCGTGAGAA	CACTCCCCAATCCAA AGATG
F3H1	TCAGATCGTTGAG GCTTGTG	ATGTCGAAACGGAGC TTGTC
COMT	GTCATGCTCGACC GTATCCT	CTAGGGTCAGTCCCG TGGTA

2.6. Statistical Analysis

The differences between drought-treated versus untreated plants were analyzed using Wilcoxin rank sum test. P values obtained from one tailed test were used to conclude significance (α = 0.05).

3. Results

The results reported herein were compiled in triplicate, from separate harvests. Flavonol glycoside accumulation was profiled in both the 14-3-3 λ knockout (SALK_075219) and wild-type under wet and drought conditions (Fig. 1, Fig.7, Table 2).

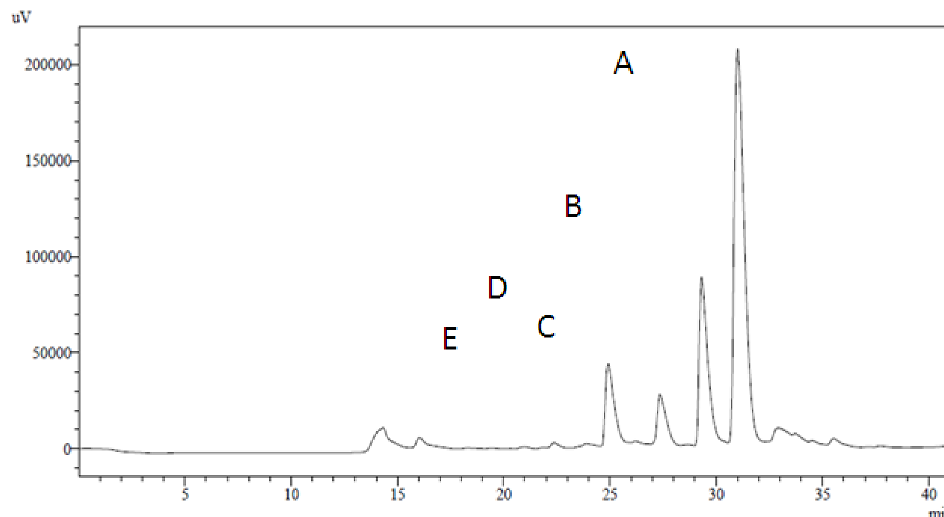

Figure 1. Sample LC-MS chromatogram of drought treated wild-type (Columbia-0) Arabidopsis thaliana, showing separation of a sinapoyl compound (A), Kaempferitrin (B), Kaempferol-3-O-Glucoside-7-O-Rhamnoside (C),Kaempferol-3-O-Rhamnosyl(1→2)Glucoside-7-O-Rhamnoside (D) andQuercetin-3-O-Glucoside-7-O-Rhamnoside (E)

Table2. Phenylpropanoid compounds isolated in LC-MS method with their correlated retention times and mass-to-charge ratios (*m/z*) based of methods in[23]

Peak	Compound	Retention Time (min)	m/z
A	Sinapoyl derivative	31.13	339
B	Kaempferitrin	29.49	577
C	Kaempferol-3-O-Glucoside-7-O-Rhamnoside	27.45	593
D	Kaempferol-3-O-Rhamnosyl(1→2)Glucoside-7-O-Rhamnoside	25.04	739
E	Quercetin-3-O-Glucoside-7-O-Rhamnoside	23.55	609

The amounts of the four flavonol glycosides and the sinapoyl derivative were calculated relative to accumulation of Kaempferitrin. (Table 3).

Table 3. Quantification of the compounds using the Kaempferitrin standard curve. Numbers represent average of three values with standard deviation in ug/mL. *p<0.05

	Drought-Treated		**Untreated**	
Compound	*Wild-Type*	*Knockout*	*Wild-Type*	*Knockout*
A	463.75± 0.36	564.81± 24.02 *	446.658 ± 3.99	434.606 ± 1.70
B	58.72 ± .030	63.522± 16.07	74.132± 1.16	52.015± 0.35
C	38.281± 0.60	64.613± 16.33	53.383± 1.49	60.245± 0.56
D	24.487± 0.59	32.259± 9.12	31.877± 0.48	30.506± 0.39
E	5.768± 0.05	10.003± 6.41	13.141± 0.54	13.438± 0.19

The peak in the extract which had retention time similar to Kaempferitrin standard was used for quantification of the compounds. The Kaempferitrin standard used as reference was confirmed using nuclear magnetic resonance experiments (Fig 1S, 2and 3S)

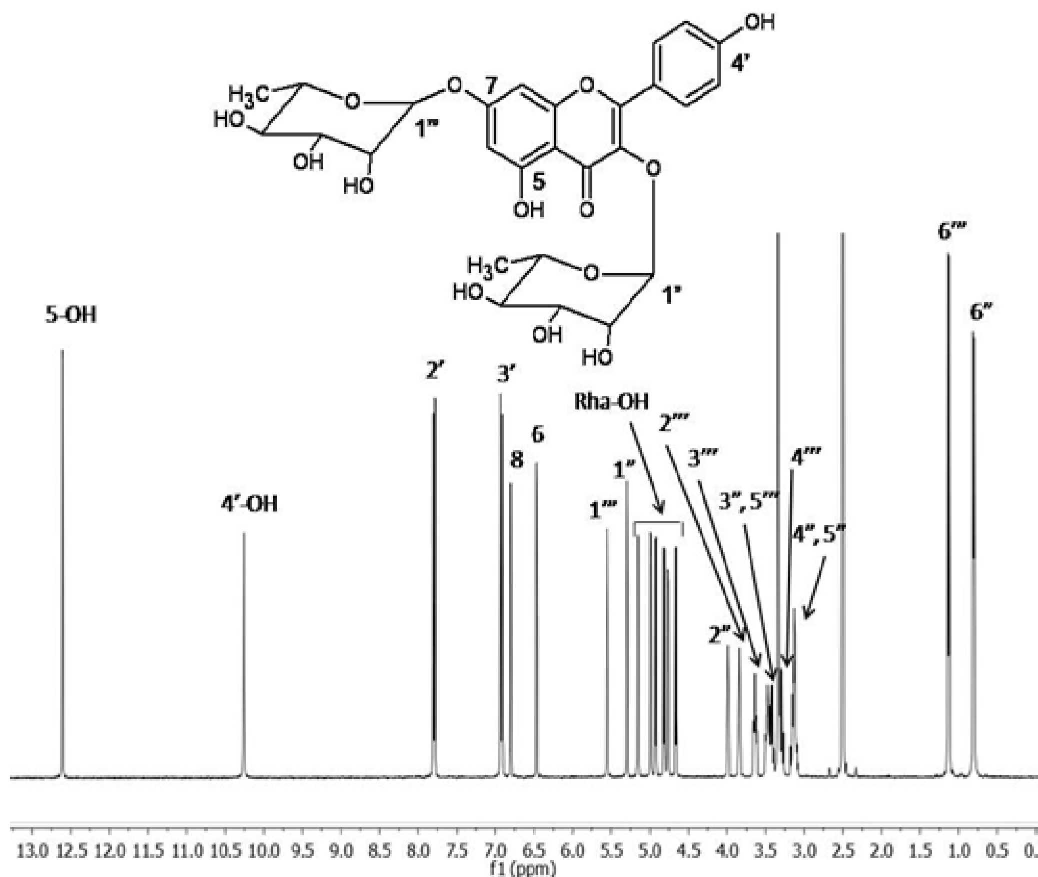

Figure 2. 2 ^{1}H NMR spectrum of Kaempferitrin standard. Solvent (d6-DMSO) peak displays a strong signal at 2.5 ppm and absorbed water displays a strong peak at 3.3 ppm

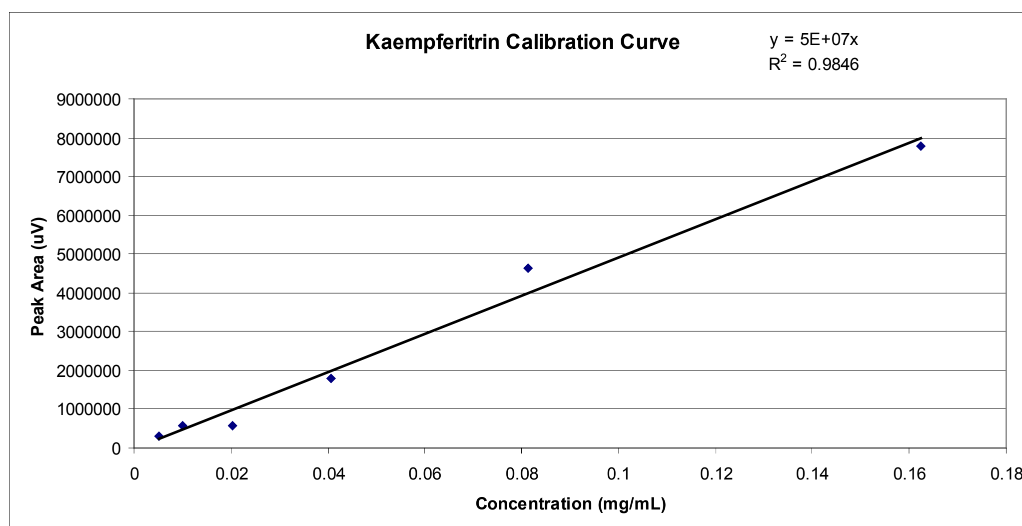

Figure 2S. Standard Calibration Curve of Kaempferitrin which was used to calculate the quantity of the 4 compounds Sinapoyl Malate (A),Kaempferol-3-O-Glucoside-7-O-Rhamnoside (C),Kaempferol-3-O-Rhamnosyl(1→2)Glucoside-7-O-Rhamnoside (D), and Quercetin-3-O-Glucoside-7-O-Rhamnoside(E) and Kaempferitrin (B)

The four other phenylpropanoid compounds (A, C, D and E) were quantified with reference to the amounts of kaempferitrin as the standards for the four compounds were unavailable (Table !s, Figure 2 and 2S). Comparison of wild-type drought treated versus untreated showed no significant accumulation of flavonol glycosides or sinapoyl derivative (Table 3). However, a greater accumulation of the sinapoyl derivative was seen in the drought-treated knockout plants compared to hydrated knockout mutants (Table 3). This accumulation was also significantly increased in the drought treated mutants compared to the drought treated wild-type (Table 3, $p<0.05$*).

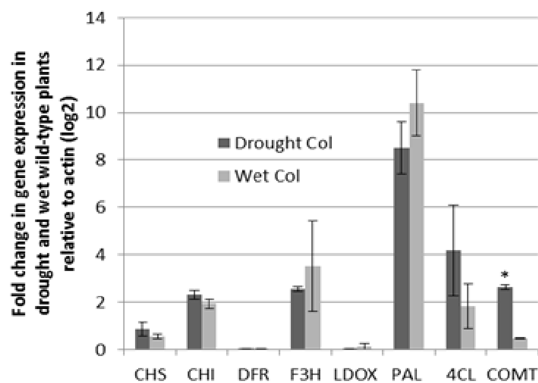

Figure 3. Gene expression analysis of eight phenylpropanoid and lignin biosynthesis genes in wild-type (Col-0) in wet and dry conditions using Real Time PCR.*$p<0.01$.Error bars represent standard deviation The values expressed are $\log_2$

Knocking out 14-3-3λ resulted in increased biosynthesis of lignin precursor. It is highly likely that this protein is interacting with the early enzymes in the phenylpropanoid pathway after the divergence of flavonoid biosynthesis to lignin biosynthesis. To provide support for this hypothesis, gene expression of five enzymes (CHS, CHI, DFR, F3H, LDOX) involved in flavonoid biosynthesis and three genes from lignin biosynthesis (PAL, 4CL, COMT) was monitored in wet and dry wild-type and knockout plants.

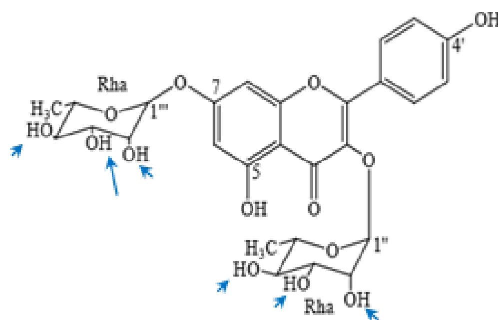

Figure 3S. Structure of kaempferitrin with proton assignment

The gene expression profiles of all the selected genes were first determined in wet and dry wild-type plants to analyze the differential changes due to drought stress. None of the gene except COMT1 showed a two fold up-regulation after drought treatment (Figure3). It was hypothesized that the genes affected by 14-3-3 λ knockout will express differently from the profile observed in wild-type.

Two of the enzymes, CHS and 4CL showed up-regulation by more than 2 fold under drought conditions in the 14-3-3 knockouts (figure 4). Comparison of the basal gene expression in wet conditions in the wet wild-type and knockouts showed PAL to significantly different (more than 3 fold) (figure 5). All the other genes were not expressed differentially under wet conditions. However, in drought conditions PAL expression decreased 1.5 fold in wild-type but increased 3 fold in the knockout plants (figures 5 and 6). CHS gene did not show any change in expression in wild-type under drought conditions but significantly increased in the 14-3-3 knockout plants (figure.5). The COMT involved in monolignols biosynthesis [18] also showed a significant increase in its expression in wild-type during drought conditions but remains unchanged in the 14-3-3λ knockout mutants (figure. 6). These results further substantiates the claim that 14-3-3λ affects the phenylpropanoid and lignin biosynthesis pathway and hence plays an important role in drought tolerance.

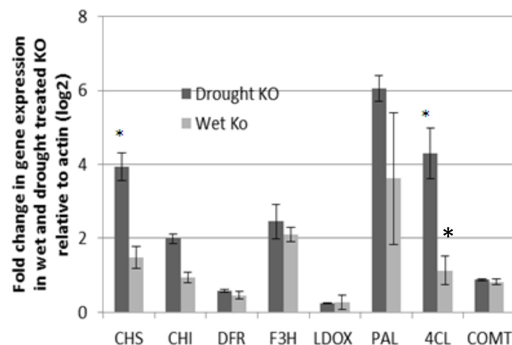

Figure 4. Gene expression changes in wet and dry 14-3-3 λ knockout in selected phenylpropanoid and lignin biosynthesis genes using real time PCR. Error bars represent standard deviation. The values expressed are *$p<0.01$

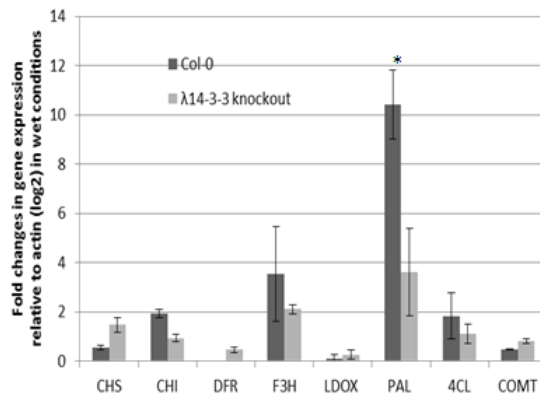

Figure 5. Gene expression analysis of eight phenylpropanoid and lignin biosynthesis genes in wild-type (Col-0) and 14-3-3λ knockout in wet conditions using Real Time PCR. Error bars represent standard deviation. The values expressed are $\log_2$. *$p<0.01$

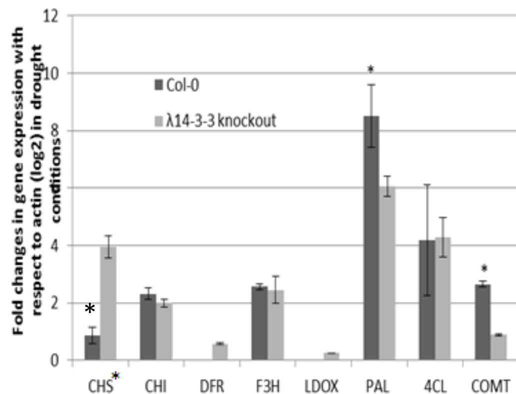

Figure 6. Gene expression analysis of some of the phenylpropanoid genes and lignin biosynthesis genes in wild-type (Col-0) and 14-3-3λ knockout in drought conditions using Real Time PCR. Error bars represent standard deviation. The values expressed are $\log_2$. *$p<0.01$

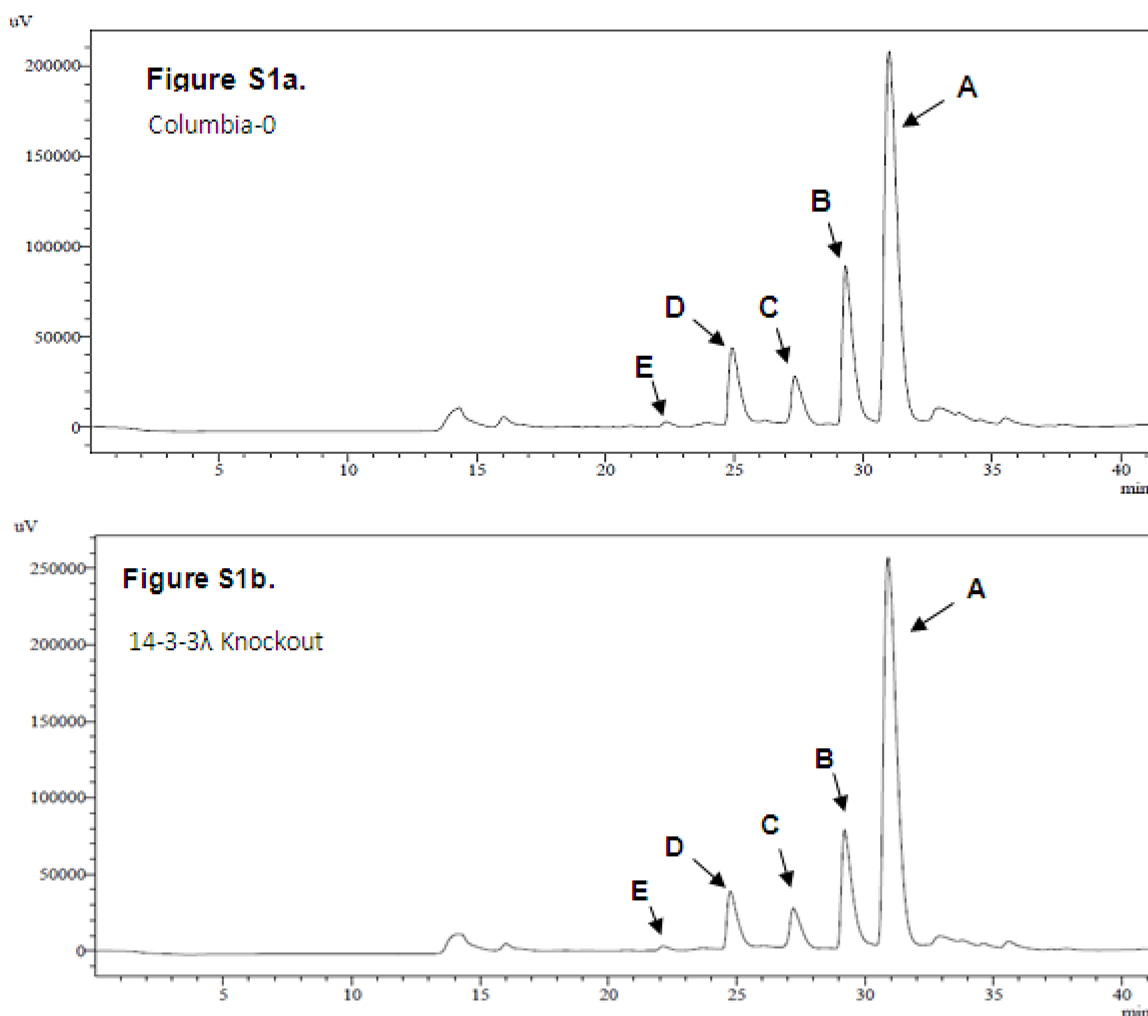

Figure 7.LC-MS Chromatogram of three week old Columbia-0 (S1a) and three week old 14-3-3λ knockout (S1b), showing separation of Sinapoyl Malate (**A**),Kaempferitrin (**B**),Kaempferol-3-*O*-Glucoside-7-*O*-Rhamnoside (**C**),Kaempferol-3-*O*-Rhamnosyl(1→2)Glucoside-7-*O*-Rhamnoside (**D**), and Quercetin-3-*O*-Glucoside-7-*O*-Rhamnoside(**E**)

4. Discussions

Comparing the quantity of sinapoyl derivative accumulated in the wild-type and the 14-3-3λ knockout in drought conditions, a significant increase was noticed (Table 3). Using the m/z and retention time data, we assume that this derivative may be similar to sinapoyl malate [26]. Due to lack of standard the compound could not be confirmed. In literature, sinapoyl malate which is also a sinapoyl derivative is shown to be important for lignin production [18].In a previous publication the total lignin content was measured in dried stems and it was found to be significantly decreased in 14-3-3 λ mutants compared to wild-type [17]. Toludine staining also showed that 14-3-3 λ mutants had a collapsed cell wall and vascular bundles in drought conditions [17]. Silencing 14-3-3λ resulted in increased biosynthesis of lignin precursor under this abiotic stress (Table 3).Lignin is composed of three different monomers and certain ratios of the three determine the strength of the plant cell wall [18]. It is possible that knocking out 14-3-3λ results in disruption of one or more of the lignin monomers. However, this was not confirmed in this study. This finding provides support for the idea of a potential role of 14-3-3λ as a modulator of phenylpropanoid metabolism in wild-type (Columbia-0) *Arabidopsis* thaliana, specifically the lignin polymer built from S units [18]. Gene expression analysis of genes involved in lignin biosynthesis versus flavonol glycosides also showed significant changes in PAL and COMT. Transgenic *Arabidopsis* where COMT expression is down regulated have shown a reduction in lignin content and accumulation of G units and 5-hydroxyguaiacyl subunits, giving rise to benzodiozane substructures in the lignin polymer [25]. This result was also accompanied with loss of S units [25]. There have been other studies such as Zhang et al studies [25] that have demonstrated 14-3-3 λ interacts with proteins such as caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT1) which is responsible for the biosynthesis of sinapyl alcohol, the precursor of syringyl lignin units [25]. COMT1 works with another protein Caffeoyl Co A 3-omethyltransferase (CCoAOMT) to

methylate the phenolic ring of monolignols in *Arabidopsis* [25]. The PAL genes also showed differential accumulation in the 14-3-3 knockout lines. This supports our hypothesis that the 14-3-3λ gene is affecting phenylpropanoid biosynthesis in the early stages of the pathway. There is also a significant change in CHS expression during drought conditions in the knockout plants compared to the wild-type plants. This provides a potential support for our hypothesis that 14-3-3λ is regulating the biosynthesis of structurally supportive lignin monomers and PAL, CHS enzymes in the flavonoid biosynthesis pathway. However, further work is required to characterize the molecular mechanism of 14-3-3λ in causing these changes in the phenylpropanoid pathway.

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