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14-3-3 Lambda Protein Affects Anthocyanin Production in *Arabidopsis thaliana* during Drought Stress

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Abstract

Plants evolve to adapt to environmental stresses, including changes at the genetic and molecular levels. For bioengineers to utilize genetic manipulation to build tolerance into crops, a better understanding of the mechanism is needed. Published studies have demonstrated that 14-3-3 lambda (14-3-3λ) protein affect the phenylpropanoid (Pp) biosynthetic pathway and alters production of flavonoids and downstream compounds of importance for stress tolerance. The 14-3-3 family of proteins binds to many different client proteins and serves as signaling scaffolds. In this study 14-3-3λ knockout mutants were used to investigate changes in metabolite accumulation in the downstream Pp pathway. Amongst them are anthocyanins which are important antioxidants involved in a variety of plant functions including stress response. Investigating how drought stress influenced anthocyanin production identified nodes in the Pp pathway affected by 14-3-3λ. A metabolomics analysis employing high resolution mass spectrometry (HRMS) and metabolomics software was used to identify metabolites in 14-3-3 knockout which changed relative to wild-type *A. thaliana* (Columbia-0) during drought stress. The metabolites Cy-3-p-coumaurolysinapoylsophoroside-5-diglucoside, 3-caffeoylferuloylsophoroside-5-succinoylglucoside, 3-caffeoylferuloylsophoroside-5-malonyldiglucoside, 3-feruloylsophoroside-5-succinoyl glucoside, petunidin-3,5-O-diglucoside and malvidin-3-O-p-coumarylmonoglucoside show significant differences in their profiles ranging from 18- to > 500-fold between the Col-0 and 14-3-3λ knockout in wet and dry groups. The findings suggest that 14-3-3λ interacts along the CHS, and CHI nodes, which in turn regulate the downstream production of specific anthocyanins. The interaction of 14-3-3λ with CHS was confirmed using co-immunoprecipitation and co-localization studies. This study supports the hypothesis that manipulation of gene expression of 14-3-3λ can lead to development of drought tolerance in plants.

Keywords: anthocyanin biosynthesis, *Arabidopsis thaliana*, drought stress, phenylpropanoid pathway, 14-3-3λ

1. Introduction

In *A. thaliana* there are several isoforms of 14-3-3 proteins (DeLille, Sehnke, & Ferl, 2001; Ferl, 1996). The 14-3-3 proteins are a highly conserved family of proteins in eukaryotic organisms and have been studied thoroughly in various plant models including *A. thaliana* (DeLille et al., 2001; Lukaszewicz, Matysiak-Kata, Aksamit, Oszmianski, & Szopa, 2002; Muslin & Xing, 2000; Paul, Denison, Schultz, Zupanska, & Ferl, 2012). First purified from bovine brain(Moore, 1967), the 14-3-3 proteins were originally thought to be associated with neural tissue (Ichimura et al., 1988), but later found to be highly conserved among other eukaryotes including plants (Ferl, 1996; Li et al., 2015; Rosenquist, Sehnke, Ferl, Sommarin, & Larsson, 2000). The 14-3-3 proteins are capable of dimerizing and binding to the phosphorylated motif of multiple partners at the same time, bringing proteins together. This allows 14-3-3 to play critical roles in the signaling pathways through interactions with various binding partners (Aitken, 1996; Roberts, 2003). The binding sites which are in the C-terminal regions have little homology among the isoforms and this factor plays a role in their varied cellular localizations (Berg,

Holzmann, & Riess, 2003; Bihn, Paul, Wang, Erdos, & Ferl, 1997; Lapointe, Luckevich, Cloutier, & Séguin, 2001; Martin, Rostas, Patel, & Aitken, 1994) and its functions (Bunney, van Walraven, & de Boer, 2001; Kumar, Muthamilarasan, Bonthala, Roy, & Prasad, 2015). It has been demonstrated that knockout of 14-3-3 in potato plant can result in a major decrease in antioxidant capacity highlighting 14-3-3 importance in the Pp pathway (Lukaszewicz et al., 2002). The 14-3-3 proteins have also been shown to affect a variety of cellular processes in plants in response to environmental stress factors such as dehydration, insect attack and UV (Jahn et al., 1997; Li et al., 2015; Roberts, Salinas, & Collinge, 2002).

From previously published studies, it was suggested that 14-3-3 proteins affect production of anthocyanins which are a family of molecules involved in a variety of functions including defense (Dixon & Steele, 1999; He et al., 2010; Holton & Cornish, 1995). As one example anthocyanin loss of function lines of *A. thaliana* grown under high light conditions, showed no significant growth difference compared to controls, suggesting that anthocyanins major role in the Pp pathway may be focused on other functions such as defense and stress response (Misyura, Colasanti, & Rothstein, 2012). Numerous studies have been conducted on the anthocyanin biosynthesis in *A. thaliana*, but the role of 14-3-3 proteins on anthocyanin production remains unresolved and specific nodes of interaction in the anthocyanin pathway by 14-3-3 proteins has not been fully elucidated.

The focus of this research was to study effect of 14-3-3λ knockout on anthocyanin production through the Pp pathway in *Arabidopsis thaliana*, under drought stress (dry) conditions. Published research has identified the 14-3-3λ mutant as having greater sensitivity to dry conditions (Peethambaran, Chi Li, Dzugan, Xiang, & Balsamo, 2012), and also revealed that 14-3-3λ affects production of synapoyl maleate and lignin biosynthesis under dry conditions (Lindberg et al., 2014). These findings indicate that 14-3-3 proteins are interacting along with flavonoid synthesis in the Pp pathway. This study proves that specific nodes are affected by 14-3-3λ proteins in production of anthocyanins. A reverse genetics approach was applied to screen anthocyanin metabolites under dry and well hydrated (wet) conditions using *A. thaliana* 14-3-3λ homozygous TDNA knockout mutant and WT. The 14-3-3λ homozygous TDNA knockout mutant (SALK_075219) had significantly different amounts of total flavonoid, phenolics and antioxidants compared to Columbia-0 (WT) under dry conditions. This provided the rationale for investigating differentially regulated anthocyanins in 14-3-3λ knockout mutant. Comparing dry and wet conditions to 14-3-3λ knockouts and WT *A. thaliana* we observed significant increases in the metabolites listed in Table 1, for WT, with smaller changes in the 14-3-3λ knockout between the conditions. These data support the hypothesis that 14-3-3λ protein has a significant effect on the Cyanidin and Delphinidin nodes of the Kegg anthocyanin biosynthetic pathway. Moving further upstream of anthocyanins, in the Pp pathway these are metabolites produced by the action of Chalcone Synthase (CHS) and Chalcone Isomerase (CHI) nodes. The CHS gene has been shown to have an elevated transcription rate under environmental and pathogenic stress conditions resulting in accumulation of various flavonoids and anthocyanins (Feinbaum & Ausubel, 1988; Li & Strid, 2005). CHI mutants have been shown to have lower levels of flavonoids and high sensitivity to UV-B damage. When the CHS and CHI knockouts were drought stressed, lower expression of 14-3-3 was observed, suggesting that CHS and CHI interact with 14-3-3 during dry conditions.

2. Materials and Methods

2.1 Plant Growth Conditions, Drought Stressing and Sampling

Wild-type (Columbia-0), 14-3-3 T-DNA mutant Lambda (SALK_075219), CHS T-DNA mutant (SALK_077592) and CHI T-DNA mutant (SALK_ 034145) were purchased from Arabidopsis Biological Resource Center (ABRC, Ohio State University). Seeds were grown in a Sunshine Mix soil (Sun Gro Horticulture, Quincy, MI) and hydrated with Scotts peters professional water soluble fertilizer 20-20-20 (Scotts, Marysville, OH) as previously described (Lindberg et al., 2014). Genotypting of these knockout lines were conducted using polymerase chain reaction and western blot for the 14-3-3 T-DNA mutant Lambda (SALK_075219). Drought treatment was carried out on 5 week old plants, half of the plants were watered normally and the other half was not watered, soil moisture was monitored daily and leaf mass harvested from all plants when the drought treated soil moisture level reached ~30%, at which point wilting of the leaves was observed. Harvested leaves were immediately frozen in liquid nitrogen and freeze dried (LabConco Corporation, Kansas City, MO). The dried leaf samples were stored at -80 ºC until extraction.

2.2 Metabolite Extraction and Isolation

Metabolites were extracted using a modification of the method described by (Lindberg et al., 2014). Briefly, frozen tissue was ground with a mortar and pestle, then suspended in extraction solvent (Methanol:Acetone; 1:1 v/v), followed by addition of 1 µg of apeginin (10 µg mL^{-1}) as an internal standard. The supernatant was

transferred to a BD Falcon tube (ThermoFisher Scientific, Waltham, MA). The pellet was extracted two more times with extraction solvent and dried using a Savant SpeedVac centrifugal evaporator (Savant Instruments Inc., Farmingdale, NY). Chlorophyll was precipitated by addition of acetone then water at a 70:40 v/v ratio and centrifugation at 13,000 × g. The supernatant was dried using the Savant SpeedVac, samples were reconstituted in a 10:90 ratio of methanol/0.1% formic acid in water and analyzed by LC-MS.

2.3 LC-MS Conditions

Samples were analyzed by Liquid Chromatography/Mass Spectrometry using an Accela UHPLC interfaced to an Exactive Plus ion trap mass spectrometer with a HESI source (ThermoFisher Scientific, San Jose, CA). Chromatographic separations were achieved employing a 2.1 × 150 mm, 5 μm, Imtakt Flavonoid RP18 column (Portland, OR) with gradient elution at 0.6 mL/min. The column temperature was maintained at 65 °C. Mobile phase A was water with 0.1% formic acid and mobile phase B was 98:2 acetonitrile:water with 0.1% formic acid. Mobile phase A was held at 100% for 0.5 min and then a three step linear gradient was formed from 0% to 20% mobile phase B over 5.5 min, to 60% mobile phase B in 2 min and then to 95% phase B in 4 min. The final composition was held for 1 min before returning to the initial conditions. Positive and negative electrospray ionization (ESI) data were acquired (separate injections) from *m/z* 200 to *m/z* 1200 with a mass accuracy within 5 ppm at 35 000 resolutions. A single 10 μL injection was used for each ionization mode. Instrumental settings follow: maximum injection time 10 msec, capillary temperature 320 °C; tube lens voltage 175 V; ESI spray voltage 4.3 kV for positive ion mode, 3.6 kV for negative ion mode; sheath gas 2 arbitrary units (arbs).

2.4 Total Flavonoid, Phenolic, Antioxidant and Free Radical Scavenging Analysis

Analysis was performed using modifications of the methods described by Kiranmai et al., and Mahboubi et al., (Kiranmai et al., 2011; Mahboubi, Kazempour, & Boland Nazar, 2013), the response from each assay was calculated using 4PL curve fitting and expressed as μg mL^{-1}.

2.4.1 Total Phenolic

Total phenolic contents in the sample extracts was determined using the Folin-Ciocalteu's reagent (Folin & Denis, 1912) and the method described by Mahboubi et al. We use 25 μL aliquot of each sample extract and diluted calibrator solution (Gallic acid 15.6 to 1000 μg mL^{-1} in methanol) was mixed with 0.125 mL of Folin-Ciocalteu's reagent (10%). After approximately 5 minute, 0.1 mL of 7.5% (w/v) sodium carbonate solution was added and mixed. That was followed by incubation for 1 hour and measure absorbance at 765 nm.

2.4.2 Total Flavonoid

For total flavonoid contents we use a 25 μL aliquot of each sample extract and diluted calibrator solution (Quercetin 15.6 to 1000 μg mL^{-1} in methanol) and mix with 75 μL of ethanol (95%), 0.5 μL of aluminum chloride (10%), 0.5 μL potassium acetate (1 M) and 140 μL deionized water. That was followed by incubation at RT for 30 minutes and measure absorbance at 415 nm.

2.4.3 Total Antioxidant

For total antioxidant activity was determined using the method described by Kiranmai et al., we use 100 μL of each sample and diluted calibrator solution (Ascorbic acid 78 to 5000 μg mL^{-1} in methanol) and mix separately with 1.0 mL of a mixture of (0.6 M Sulfuric Acid/28 mM Sodium phosphate/4 mM Ammonium Molybdate). That was followed by incubation at 95 °C for 90 minutes, measure absorbance of the reaction mixture at 695 nm.

2.5 Protein Extraction, Quantification and Western Blot

Protein extraction was carried out on freshly collected 0.1 gram plant leaf weight, frozen in Liquid Nitrogen and maintained frozen while samples were pulverized to a fine powder and mixed Laemmli buffer and boil at 95 °C for 10 min. Centrifugate at 1000 × g for 5 minutes at 4 °C.

2.5.1 Protein Quantitation

Protein was quantitated with a Thermo Scientific Pierce 660 nm Protein Assay kit (cat #22662), in a ready-to-use format (Thermo Fisher Scientific, Waltham, MA). A 10 μL aliquot of the kit pre-made Albumin standard solutions ranging from 0.125 to 2000 μg mL^{-1} and 10μL aliquots of the unknown protein samples extracts were added to wells in a clear flat bottom 96 well plate. An aliquot of 150 μL of the kit developing reagent mixture was placed in the well of the microtiter plate containing samples and the plate incubate for 1 min at room temperature, then read at 660 nm on a Spectramax 386 plus plate reader (Molecular Devices, Sunnyvale, CA). Protein concentration was calculated using a linear curve fit in Softmax Pro ver 5.0 (Molecular Devices, Sunnyvale, CA) and unknown sample concentration read of the calibration curve.

2.5.2 Western Blot

For the gel electrophoresis the Bio Rad Mini Protean TGX precast gel was used 4-15% (Bio Rad, Raleigh, NC). 20μg of protein was mixed with 1μL of Bromophenol blue and volume completed to 15 μL of with Laemmli buffer containing β-mercaptoethanol. Running buffer was 1X Tris-HCL-Glycine at 120 V for 60 min. Blot with 1X transfer buffer Tris HCL-Glycine with 10% methanol at 70 V for 60 min. Blocked with 5% non-fat milk in 1x TBST 1 hr at RT. Incubated with the Santa Cruz anti-14-3-3 rabbit polyclonal antibody as a primary antibody (Santa Cruz Biotechnology Inc, Dallas, TX), 1:1000 dilution in 1x TBST with 5% non-fat milk overnight at 4 °C. Incubate blot in cell signaling HRP-anti-rabbit antibody (Cell Signaling Technology, Danvers, MA) as the secondary antibody at 1:10 000 dilution in 1x TBST with 5% non-fat milk 1 hr at RT. Mix Thermo Scientific chemi substrate solutions in a 1:1 v/v ratio and apply to blot, incubate for 1 min and Image blot.

2.6 Real time PCR to Determine Expression of 14-3-3λ in CHS and CHI Mutants

The RNA was isolated using Trizol methods (Chomczynski & Sacchi, 1987), 0.1 gm of tissue was flash frozen in liquid nitrogen and grounded into fine powder, 0.5 mL of Trizol added. After thawing, the mixture was transferred to an Eppendorf tube and centrifuged at 13,000 RCF for 5 minutes, add 0.2 ml of chloroform and vortex briefly and incubate at room temperature for 10 minutes before centrifuging at 13,000 RCF. The top layer was moved into a new tube and 0.25 ml of RNA precipitation solution (0.8 M Sodium citrate/1.2 M NaCl) and 0.25 ml of isopropanol added, centrifugate at 13,000 RCF. The RNA pellet washed twice with 75% ethanol before air drying. RNA pellets dissolved in RNAse free water. The RNA was converted to cDNA using a RETRO Script kit (Ambion, Foster city, CA). The primers were designed specific to Arabidopsis 14-3-3λ cDNA (AT5G10450) and a PCR was conducted for 25 cycles. Primers targeting a specific region of 14-3-3λ were amplified using the forward primer 'TGCTGGAGCGAGTGAGTCTA' and reverse primer 'AGCCTGTTT GGCCATGTTAC'. An actin primer for gene ACT2 (AT3G18780) 'TCCAGTGTTGTTGGTAG GCCA' and TCTCAGCACCAATCGTGATGAC' was run at the same time to control for loading differences.

2.7 Protein Immuno Precipitation and Co-Immuno Precipitation with Magnetic Beads

Immuno precipitation (IP) of 14-3-3 and co-IP of binding partners was carried out with a Thermo IP/co-IP magnetic bead kit, catalog #88805 (Waltham, MA) as described in the kit method. Briefly an antibody specific to 14-3-3λ was conjugated to the magnetic beads then crosslinked to permanently bind the antibody to the beads. The beads were exposed to 0.5 mL of protein extracts from wild type and KO plants for 1 hour at room temperature (RT) with gentle shaking to keep beads suspended. The captured protein along with binding partners was eluted with 100 μL the low pH elution solution and 5 minutes of incubation at RT with gentle shaking. The eluate solution was transferred to a clean tube and neutralized with 10 μL of neutralizing buffer as described in the kit manual. A 50 μL aliquot of the eluate was diluted with 100 μL 0.5M bicarbonate buffer digested with 50 μL of 1 mg mL^{-1} trypsin and 37 °C overnight, then analyzed by LC-MS.

2.8 Co-Localization of 14-3-3λ with CHS Using Confocal Microscopy

Microscopic imaging was performed on a spinning disk (Yokagawa CSU-X1; Andor Technology) confocal microscope using a 60x 1.4 NA oil immersion objective lens on a TiE microscope equipped with Perfect Focus System (Nikon) equipped with an electronic shutter (Sutter Instrument) for transmitted illumination, a linear encoded X and Y, motorized stage (ASI Technologies), and a multi-bandpass dichromatic mirror (Semrock) and bandpass filters (Chroma Technology Corp.) in an electronic filter wheel for selection of BFP, GFP, or RFP emission. 405-, 488-, and 561-nm laser illumination was provided by a high-powered (20 mW 405-nm; 50 mW 488- and 561-nm) monolithic laser combiner module (MLC 400B; Agilent Technologies) that were shuttered with electronic shutters and directed to a fiber-coupled output port with an Acousto optic tunable filter and to the confocal scan-head via a single mode polarization-maintaining fiber coupled delivery system (Agilent Technologies). The exposure times for the images were 500 milliseconds for 405 and 488 nm and 600 ms for 561 nm. The primary antibody for 14-3-3λ was anti-mouse kindly gifted by Dr. Robert Ferl, University of Florida. A final dilution of 1:20 was followed according to the protocol of Pasternak et al. (2015). The secondary antibody for 14-3-3λ was tagged with Cy5 and a final dilution of 1:500 was used. The primary antibody used to probe chalcone synthase was from Agrisera (AS 12 2615). The secondary antibody for chalcone synthase was tagged with Cy3. Primary and secondary dilutions were 1:20 and 1:500 respectively (Pasternak et al., 2015).

2.9 Statistical Analysis

For total Flavonoid, Phenolic, Antioxidant and Free radical scavenging capacity, the differences between drought-treated versus untreated plants were analyzed with a paired t-test, in Microsoft Excel. Metabolite profiling analyses were carried out with MetaboAnalyst. Data was first transformed by log normalization and

analyzed using t-test statistics in Microsoft Excel. P values obtained from the t-test were used to conclude significance ($\alpha = 0.05$).

3. Results & Discussion

Analyses of total flavonoids, total phenolic, total antioxidant and free radical scavenging capacity in 14-3-3λ between wet and dry conditions, suggest a differential response in the Pp pathway regulating these molecules (Figure 1). The Col-0 wild-type showed no significant changes in total flavonoids and antioxidants, between wet and dry conditions; however, significant change in total phenolic was observed. In the 14-3-3λ knockout, significant differences were observed for total flavonoids, phenolics, and antioxidants, demonstrating that 14-3-3λ knockout affect the flavonoid branch of the Pp pathway.

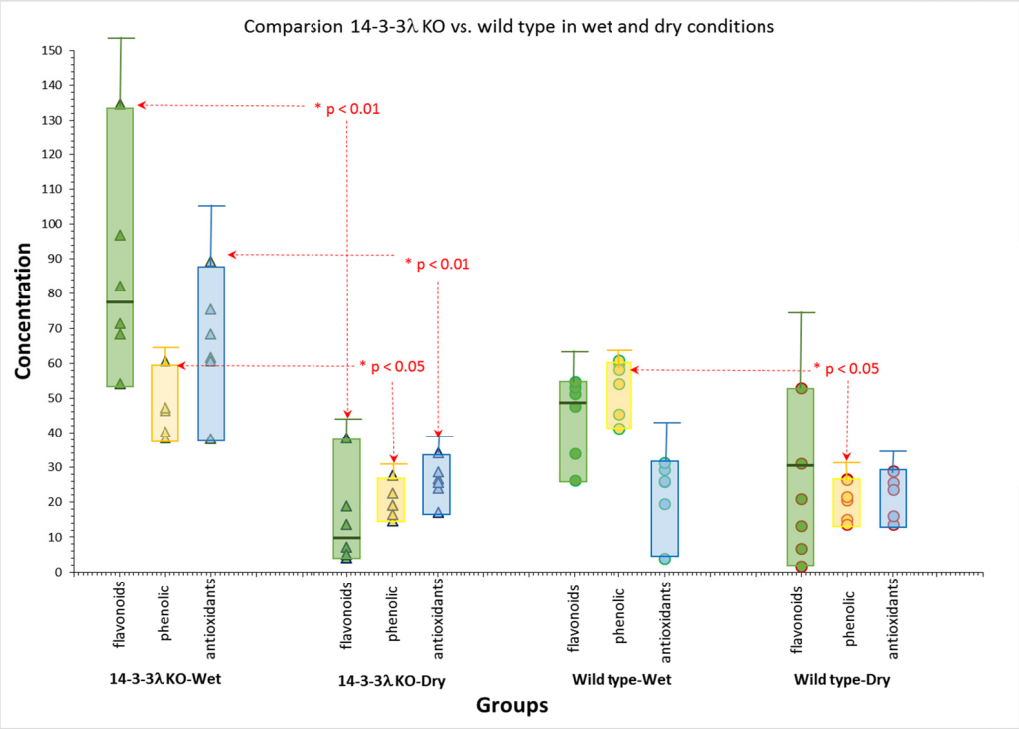

Figure 1. Spectrophotometric analysis of total Flavonoid, total Phenolic and total Antioxidant in *A. thaliana* Columbia-0 and 14-3-3λ mutant under normal hydration and drought conditions

Metabolomics profiling is a well-accepted methodology for analysis of large numbers of metabolites and MetaboAnalyst has demonstrated utility in metabolite profiling (Xia, Sinelnikov, Han, & Wishart, 2015). Metabolite profiling of anthocyanins in 14-3-3λ knockout and comparing to wild-type under wet and dry conditions by high resolution accurate mass Mass Spectrometry, representative chromatography (Figure 2) shows good peak resolution of the metabolite peaks across the run, a necessity for reliable metabolite profiling.

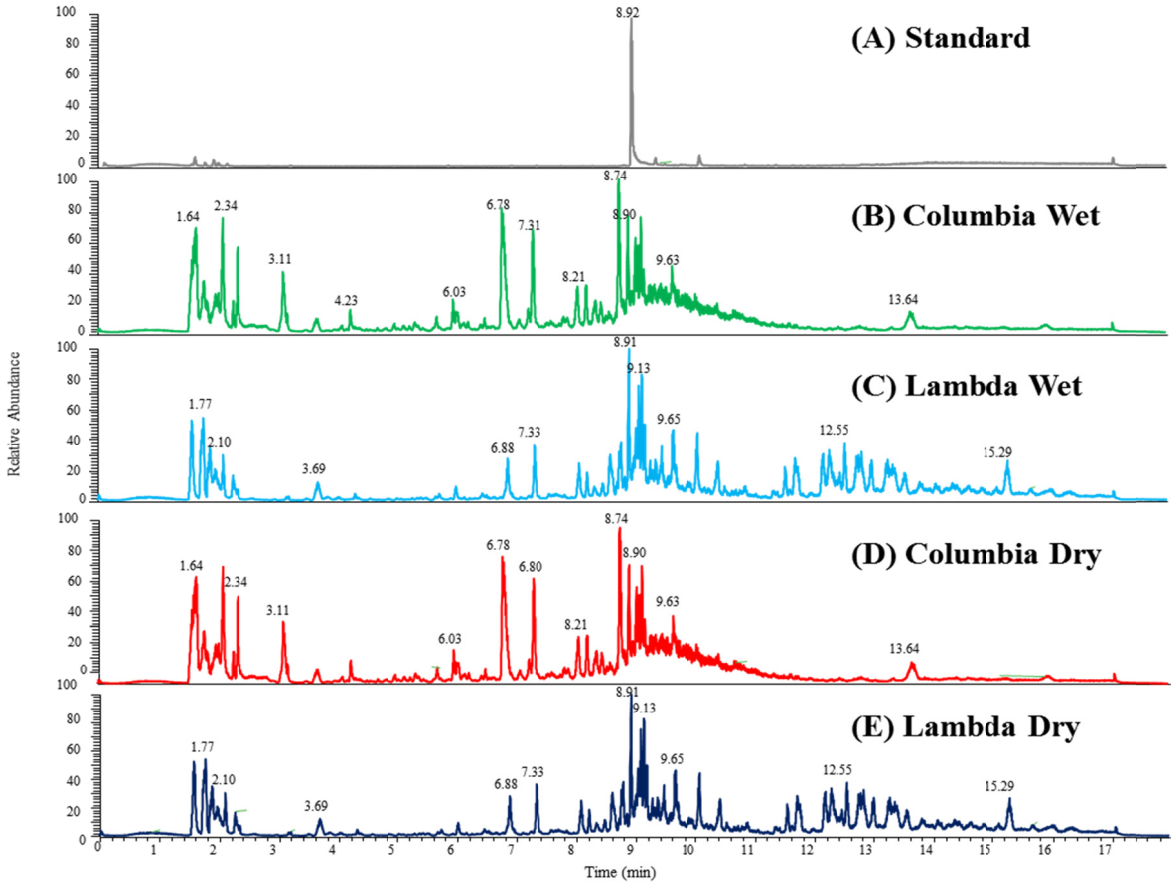

Figure 2. LC/MS total ion chromatograms for calibration standard and *A. thaliana* extracts, depicting typical separation of metabolites in the extracts. Calibration standard Kaempferitrin 32 µg mL^{-1} (A), Columbia-0 extract (Col) from normal hydrated plants (B), 14-3-3λ extracts (Lam) from normal hydrated plants (C), Columbia-0 extracts from drought treated plants (D), and 14-3-3λ extracts from drought treated plants (E)

Principal Component Analysis (PCA) of all metabolites (Figure 3) shows that 45.2% of all variance in the analysis is accounted for in the model, providing confidence in the metabolite profiling data (Xia et al., 2015).

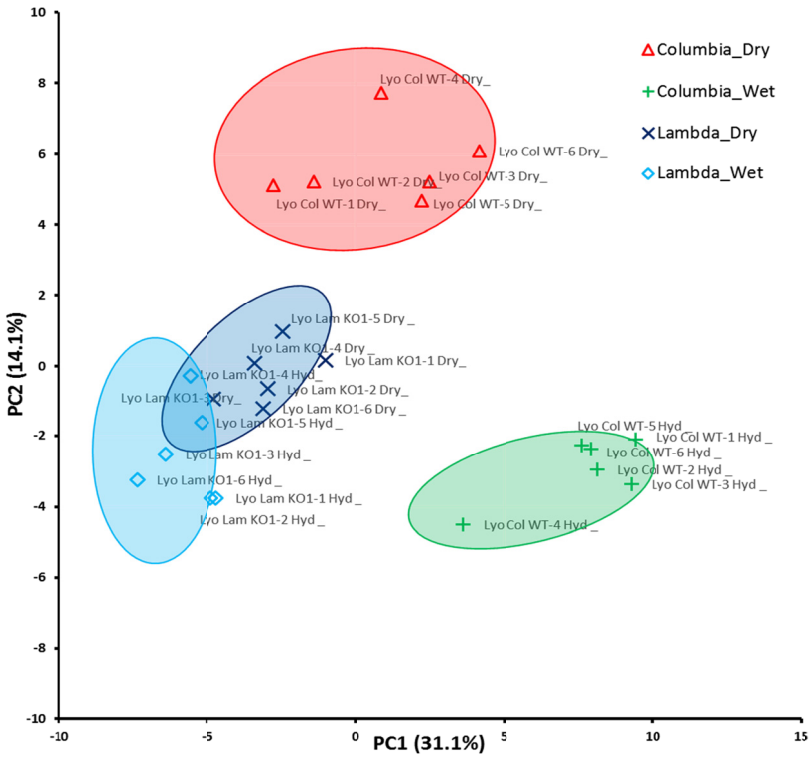

Figure 3. PCA analysis comparing wet and dry groups showing that 45.2% of the variance is accounted for in the model

Using a metabolomics approach several anthocyanin metabolites were identified as showing significant differences between the wet and dry groups (Table 1).

Table 1. Summary of LC/MS metabolite profiling for normal hydration and drought treated wild-type (Columbia-0) *Arabidopsis thaliana,* and 14-3-3λ mutant. Compounds showing significant changes amongst the group are shown. Positive ionization mode LC/MS data (top frame, panel 1 and 2). Negative ionization LC/MS data (bottom frame, panel 3 and 4)

Panel 1		Wild Type Wet vs Dry		Knockout Wet vs Dry	
Metabolite Name	IonMZ	Col_Wet/Col_Dry		Lam_Wet/Lam_Dry	
		Fold Change	P Value	Fold Change	P Value
3-p-coumaroylsinapoylsophoroside-5-diglucoside	644.1847	1.5	1.22E-01	-5.0	4.79E-03
3-caffeoylferuloylsophoroside-5-succinoylglucoside	604.1428	18.6	3.21E-03	0.0	3.41E-01
3-caffeoylferuloylsophoroside-5-malonyldiglucoside	678.1614	18.1	4.59E-03	-1.7	5.08E-01
Panel 2		Wet Wild Type vs Knockout		Dry Wild Type vs Knockout	
Metabolite Name	IonMZ	Col_Wet/Lam_Wet		Col_Dry/Lam_Dry	
		Fold Change	P Value	Fold Change	P Value
3-p-coumaroylsinapoylsophoroside-5-diglucoside	644.1847	> 500	3.72E-04	65.9	6.34E-07
3-caffeoylferuloylsophoroside-5-succinoylglucoside	604.1428	> 500	2.04E-03	128.9	2.41E-01
3-caffeoylferuloylsophoroside-5-malonyldiglucoside	678.1614	70.8	3.50E-03	2.3	1.50E-01
Panel 3		Wild Type Wet vs Dry		Knockout Wet vs Dry	
Metabolite Name	IonMZ	Col_Wet/Col_Dry		Lam_Wet/Lam_Dry	
		Fold Change	P Value	Fold Change	P Value
Peonidin-3-O-monoglucoside	461.1089	13.9	1.10E-03	-1.7	2.64E-02
Cyanidin-3,5-O-diglucoside	609.1465	1.4	2.12E-01	-2	2.28E-02
Peonidin-3,5-O- diglucoside	623.1617	0.8	4.64E-01	-2.5	2.33E-03
Delphinidin P-Coumaoroyldiglucoside	678.1614	1.1	3.01E-01	-1.67	1.37E-02
Panel 4		Wet Wild Type vs Knockout		Dry Wild Type vs Knockout	
Metabolite Name	IonMZ	Col_Wet/Lam_Wet		Col_Dry/Lam_Dry	
		Fold Change	P Value	Fold Change	P Value
Peonidin-3-O-monoglucoside	461.1089	0.6	3.55E-02	-43.5	7.75E-05
Cyanidin-3,5-O-diglucoside	609.1465	13.3	7.11E-05	4.4	1.08E-02
Peonidin-3,5-O- diglucoside	623.1617	2.6	5.94E-04	1.2	5.42E-01
Delphinidin P-Coumaoroyldiglucoside	678.1614	2.2	5.12E-05	1.2	1.96E-01

When specific metabolites were observed to have high fold change differences between the groups, and were compared against the Kegg anthocyanin pathway nodes, we find that the likely nodes of interaction for production of these metabolites would be in the cyanidin and delphinidin nodes (Table 2).

Table 2. Anthocyanin metabolites and associated genes, location on Kegg Anthocyanin Biosynthesis pathway

Metabolite	Locus	Kegg Pathway *A. thaliana* Gene(s)	Function	Anthocyanin Node	Location
3-p-coumaroylsinapoylso phoroside-5-diglucoside	BAA74428, AT4G14090	AT4	anthocyanin-containing compound biosynthetice procsee, phenylpropanoid metabolic process	Cyanidin	Chloroplasts
3-caffeoylferuloylsopho roside-5-succinoylglucoside	BAA74428, AT4G14090	AT4	anthocyanin-containing compound biosynthetice procsee, phenylpropanoid metabolic process	Cyanidin	Chloroplasts
3-caffeoylferuloylsopho roside-5-malonyldiglucoside	BAA74428, AT4G14090, AT3G29590	AT4, AT3	anthocyanin-containing compound biosynthetice procsee, phenylpropanoid metabolic process	Cyanidin	Cytoplasm
Peonidin-3-O-monoglucoside	AT4G14090	AT4	anthocyanin-containing compound biosynthetice procsee, phenylpropanoid metabolic process	Cyanidin	Chloroplasts
Cyanidin-3,5-O-diglucoside	AT4G14090	AT4	anthocyanin-containing compound biosynthetice procsee, phenylpropanoid metabolic process	Cyanidin	Chloroplasts
Peonidin-3,5-O-diglucoside	AT4G14090	AT4	anthocyanin-containing compound biosynthetice procsee, phenylpropanoid metabolic process	Cyanidin	Chloroplasts
Delphinidin p-coumaroyldiglucoside	AT4G14090	AT4	anthocyanin-containing compound biosynthetice procsee, phenylpropanoid metabolic process	Delphindin	Chloroplasts

Anthocyanin metabolites such as 3-p-coumaurolysinapoylsophoroside-5-diglucoside and 3-caffeoylferuloy lsophoroside-5-succinoylglucoside when compared in wild-type and 14-3-3λ knockout plants, were 500-fold different between Col and 14-3-3λ wet and 69-fold change between Col and 14-3-3λ dry (Table 1, panel 2). The fold changes between wet wild-type and the 14-3-3λ knockout suggests that 14-3-3 is important in production of specific anthocyanins such as 3-p-coumaurolysinapoylsophoroside-5-diglucoside, 3-caffeoylferuloylsopho roside-5-succinoylglucoside, 3-caffeoylferuloylsophoroside-5-malonyldiglucoside, 3-feruloylsophoroside-5-succinoylglucoside, Petunidin-3,5-O-diglucoside and Malvidin-3-O-p-coumarylmonoglucoside (Table 1, panel 1 to 4). The genes that regulate the metabolites shown in Table 1 are in the synthetic pathway from 4-coumaryl-coA to naringenin chalcone which is then converted to naringenin. These two steps in Pp pathway are regulated by chalcone synthase (CHS) and chalcone isomerase (CHI). Figure 4 represents a heat map distribution of the top 25 anthocyanin metabolites showing that the groups are clustered, demonstrating up or down regulation of the metabolites with consistency amongst the groups and providing confidence in the data which are listed in Table 2.

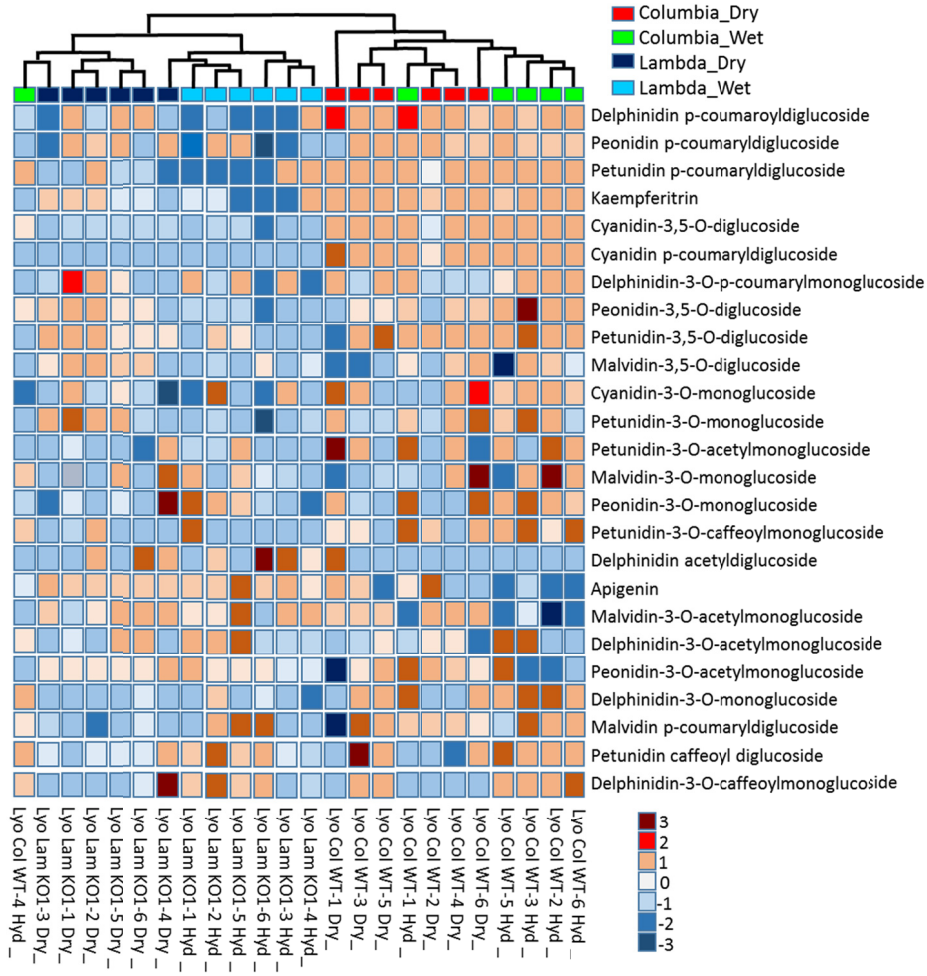

Figure 4. Heat Map for all metabolites analyzed under normal hydration and drought in 14-3-3 λ and Columbia. Top 25 metabolites shown (distance measure using euclidean, and clustering algorithm using ward)

To further assess whether these branches of the Pp pathway were affected by 14-3-3λ, knockouts of CHS (SALK_077592) and CHI (SALK_034145), which are known to affect anthocyanin production (Huang et al., 2010), were analyzed for the transcript and protein levels of 14-3-3λ. Real time analysis of the CHS and CHI mutants compared to the wild-type in wet and dry conditions showed > 2 fold change in expression of 14-3-3λ in the wet conditions but decreased more than 4 fold under drought conditions (Figure 5).

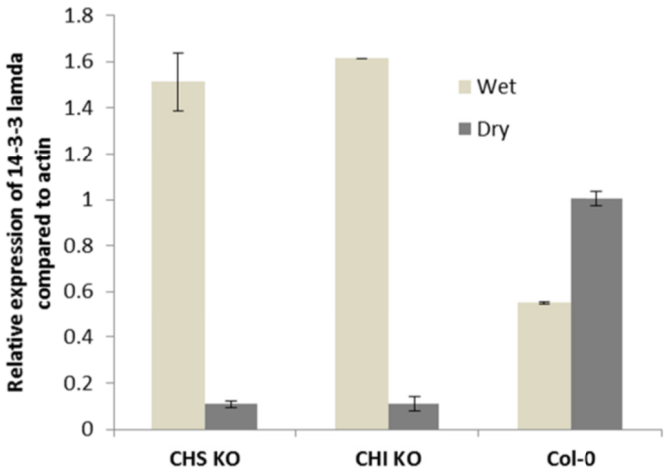

Figure 5. Relative expression levels of 14-3-3λ in CHI and CHS knockout plants, compared to Col-0 wild type under wet and dry conditions (14-3-3 expression relative to Actin expression)

Western blot analysis of CHS and CHI knockout mutants show a decrease in 14-3-3λ protein under dry conditions while in wet conditions 14-3-3λ protein increases supporting that 14-3-3 interacts along both nodes (Figure 6).

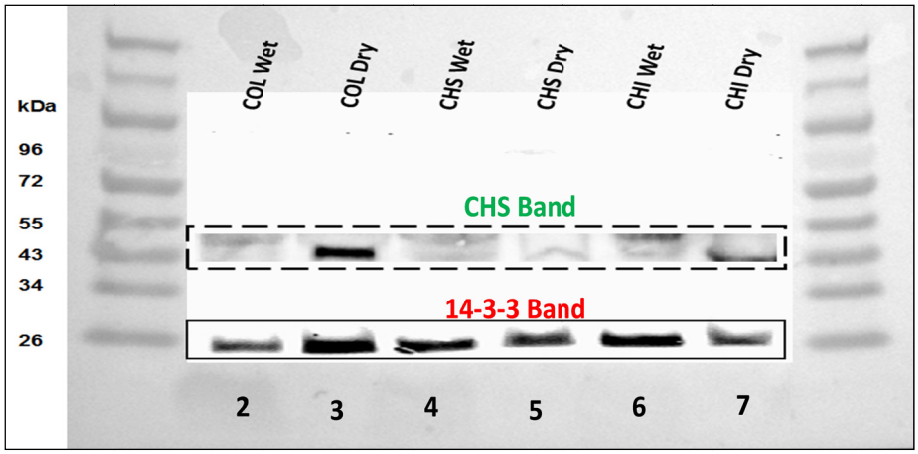

Figure 6. Western blot probed with 14-3-3λ antibody showing protein levels in14-3-3λ, CHI and CHS knockout plants, compared to Col-0 wild type under wet and dry conditions. The 14-3-3 band is shown by the solid line box and the CHS band is shown by the dashed line box

The 14-3-3λ protein levels in wildtype (Col-0) samples increase during drought, while the CHS and CHI knockout mutants shows no change. This shows that knocking out CHS and CHI affects 14-3-3 expression. Published studies (Chunthaburee et al., 2016; Dao, Linthorst, & Verpoorte, 2011; Wang et al., 2016) and a review on CHS (Dao et al., 2011), demonstrates that CHS and CHI have increased expression during stress in plants. This suggest that CHS and CHI are likely also increased in expression under drought stress in *A. thaliana* and supports the observation of differential accumulation of anthocyanins observed in this study.

To investigate if 14-3-3λ and CHS are co-localized during wet and dry conditions, immunolabeling followed by confocal microscopy was carried out on wet and dry Col-0 leaf mass. The image in Figure 7 shows three color images representing a 14-3-3λ specific antibody with fluorescent labeled secondary antibody (red), DAPI stain to see the nucleus (blue) and CHS detection with a secondary antibody (green), were merged into a single image, showing that 14-3-3λ and CHS are co-localized and expressed around the guard cells and cell membrane only in the epidermal cells during wet conditions. The green dots within the cells are CHS in the layer below epidermal

which is the mesophyll cells. There is no expression of 14-3-3λ in the mesophyll cells and the expression is limited to only the guard cells and the cell membrane in the epidermal cells. The yellow arrow in the merged panel shows line scan analysis of the pixel intensity of the red signal for 14-3-3λ and green signal for CHS. Line scan data is quantified and presented as the fluorescence of each of the three colors in the graph panel at the bottom of the figure. The peaks of each color are right above each other for the spot marked with the yellow arrow representing co-localization of CHS and 14-3-3. Under dry conditions 14-3-3 (red) and CHS (green) signals are similar in intensity and is co-localized in the guard cells and cell membrane.

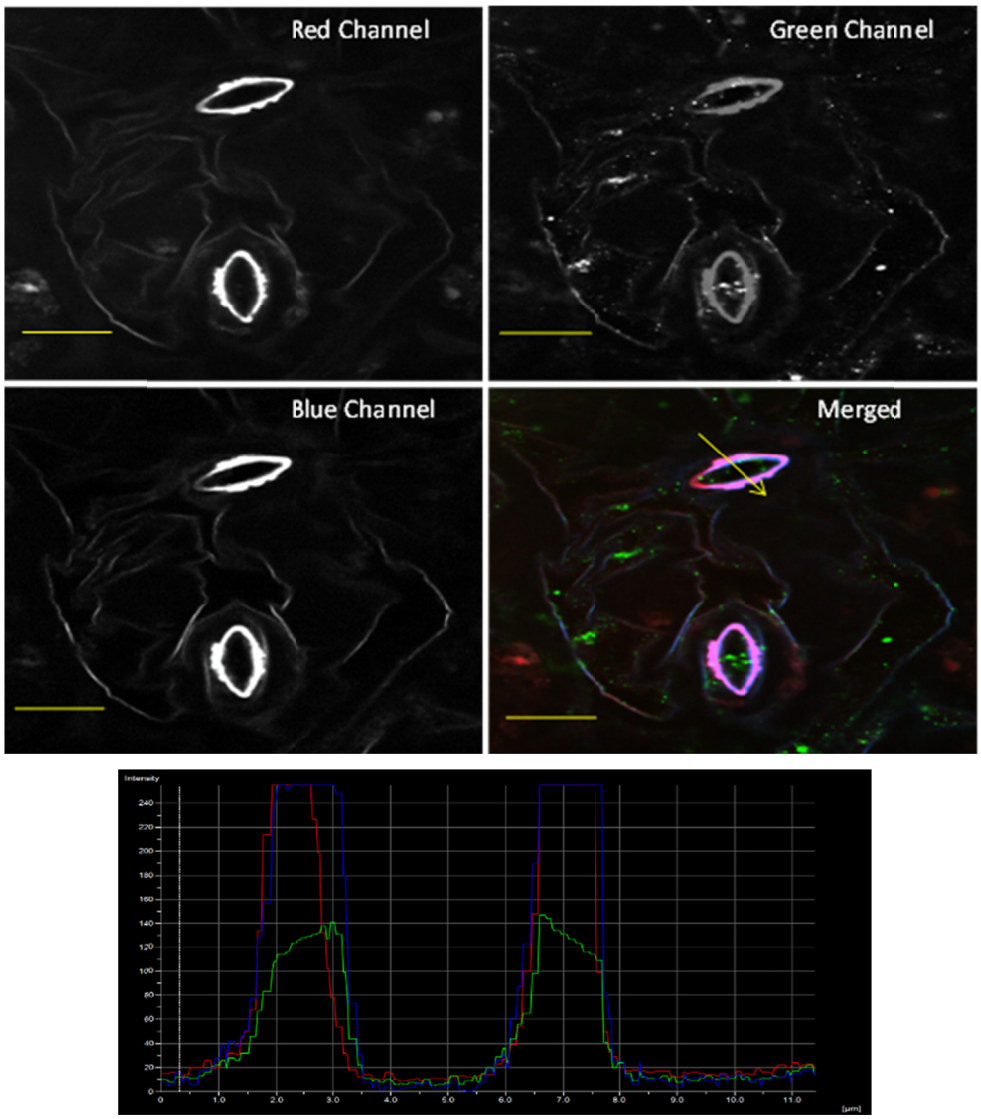

Figure 7. Co-localization analysis of leaf mass from *A. thaliana* under wet conditions. The fluorescence intensity from 14-3-3λ (red), Chalcone synthase (green) and DAPI stain (blue signals) shown in the panels along with the merged image and an intensity graph for the area under the yellow arrow shown in the lower right panel. Yellow scale bar = 10 μM

The yellow arrow in Figure 8 shows co-localization in guard cell membrane. The bottom graph panel in 8 shows that the green signal intensity is same as red indicating presence of CHS and 14-3-3λ in the same spot. These finding further support the role 14-3-3λ in drought tolerance, as closure of the guard cell is critical to reducing loss of moisture.

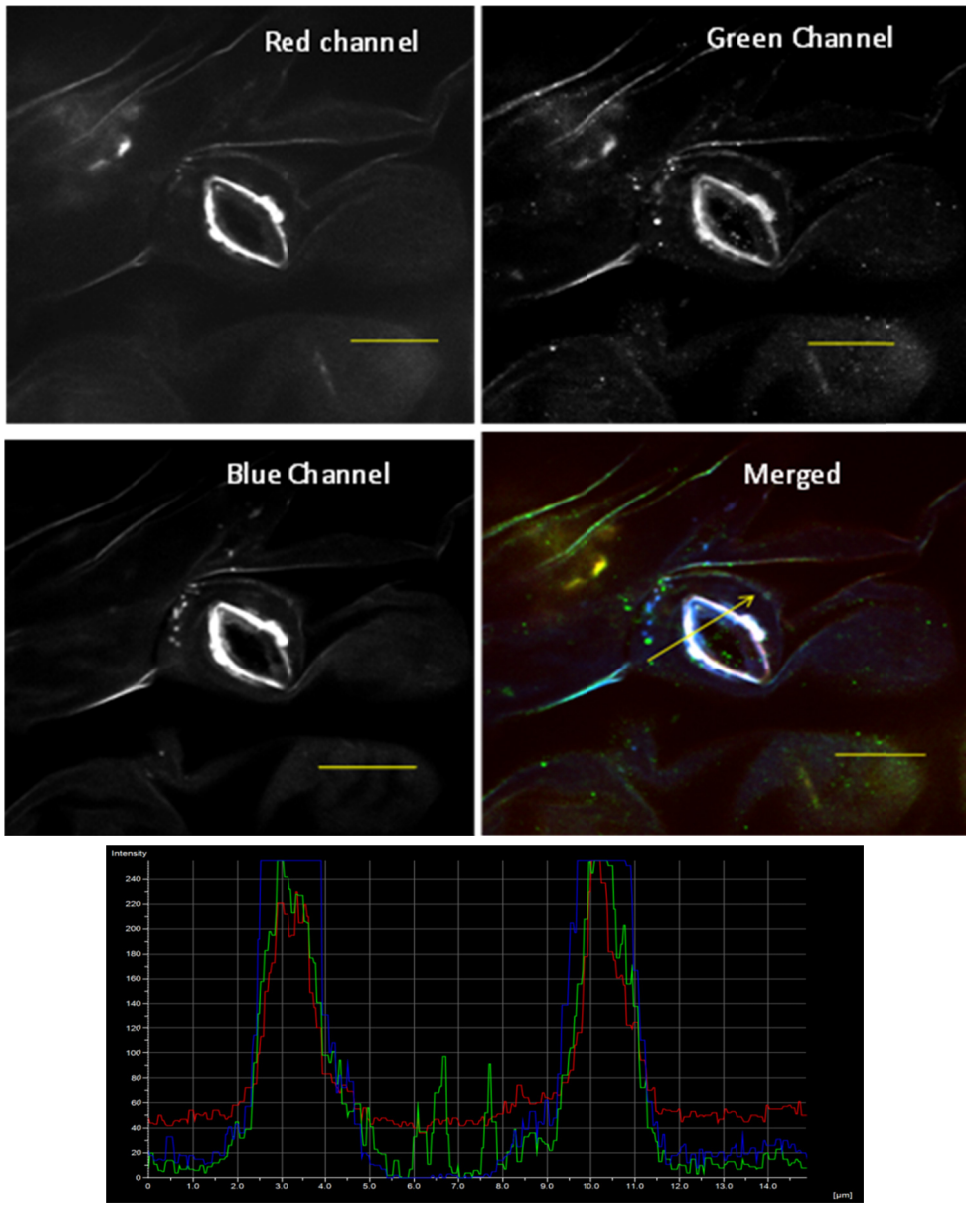

Figure 8. Microscopy analysis of leaf mass from *A. thaliana* under dry conditions. The fluorescence intensity from red, green, blue signals along with merged image and an intensity graph for the area under the yellow arrow shown in the lower right panel. Yellow scale bar = 10 µM

These results support earlier findings that 14-3-3λ interacts with CHS in the Pp metabolism. The stick and box diagram in Figure 9 shows the Pp pathway and highlights the locations along the known pathway where CHI and CHS enzymes effect production of anthocyanins.

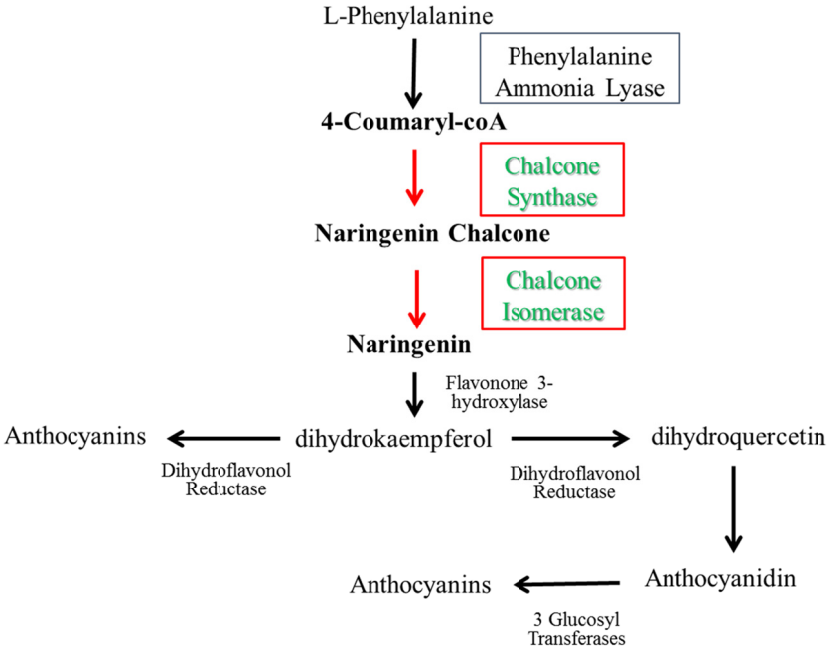

Figure 9. Box and stick pathway for the metabolites with highest fold changes. Knockout plants of CHS and CHI pathway tested under wet and dry conditions to study expression of 14-3-3 under the conditions

Zhao et al., demonstrated that many phospho-peptides, from derived libraries, bind to multiple isoforms of 14-3-3 (Li, Tang, & Guo, 2016) and the PAL and CHS enzyme can be phosphorylated, which would facilitate binding to 14-3-3λ (Allwood, Davies, Gerrish, Ellis, & Bolwell, 1999; Ramprasad, Rakesh, Rani, Vanisri, & Gajula, 2016). A published study investigating CHS and CHI interaction show that both can bind to each other and co-precipitate, this would suggest that CHI or CHS can be co-precipitates if either is bound to 14-3-3 (Burbulis & Winkel-Shirley, 1999). To determine if PAL, CHS or CHI binds to 14-3-3λ, co-immunoprecipitation of 14-3-3λ was carried out on protein extracts from Col-0, CHS KO, CHI KO, 14-3-3λ KO and transgenic 14-3-3λ overexpressor plants followed by tryptic digestion and LC/MS analyses of peptides from 14-3-3λ, PAL-1, PAL-2, CHS and CHI. Responses were observed for the selected peptides suggesting that these partners were co-precipitated along with 14-3-3 (Table 3). The MS ion current from the 14-3-3λ KO was background subtracted from the ion current for the other samples, then converted to (+) or (-) value based upon presence or absence of a signal for the peptide. The 14-3-3λ overexpressor response was not higher than Col-0 as was expected, but this may be due to extraction differences. For Col-0 and the 14-3-3λ overexpressor, responses were observed for peptides arising from 14-3-3λ, PAL-1, PAL-2, CHS, and CHI. Whereas the CHS and CHI knockouts, responses for 14-3-3λ, PAL-1 and PAL-2 peptides was seen but no response was seen for CHS and CHI peptides, since this was expected, it provided additional confidence in the analyses.

Table 3. LC/MS analyses of peptides from Co-immunoprecipitation of 14-3-3 binding partners. Insilico tryptic digestion was used to identify peptides for the analysis. The most abundant peptides were selected for analysis

		14-3-3λ	PAL-1	PAL-2	CHS	CHI
Peptide sequence		NLLSVAYK	FLNAGIFGSTK	FLNAGIFGNTK	DLAENNR	YFQDYC[CAM]GK
SRM transition (m/z)		454.3 to 680.4	577.8 to 894.5	591.3 to 921.5	416.2 to 603.3	540.7 to 770.3
Sample Name	14-3-3λ_KO	-	-	-	-	-
	CHS_KO	+	+	+	-	+
	CHI_KO	+	+	+	-	-
	COL-0	+	+	+	+	+
	14-3-3λ_OE	+	+	+	+	+

Note. KO: knockout; COL-0: wild type; OE: transgenic *A. thaliana* overexpressing 14-3-3λ. (+): presence of a signal for the peptide; (-): absence of a signal for the peptide.

4. Conclusion

These results demonstrate that 14-3-3λ plays a role in the Pp pathway in production of anthocyanins and shows that knockout of CHS and CHI diminishes expression of 14-3-3λ during drought conditions. These data also suggest that PAL-1, PAL-2, CHS and CHI are interacting with 14-3-3. These observations demonstrate that knocking out 14-3-3λ results in decreased 14-3-3 expression under drought conditions, consequently resulting in differential accumulation of anthocyanin metabolites in the knocked out line compared to wild-type. Over-expressing 14-3-3λ leads to increased tolerance to drought in these transgenic plants. The plants showed increased accumulation of anthocyanins in the over-expressing transgenic plants providing additional evidence that 14-3-3 is interacting with the chalcone synthase gene and is involved in anthocyanin production. Hence, 14-3-3λ does play an important role in drought tolerance in *A. thaliana*.

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