A COMPREHENSIVE, SYSTEMS-BIOLOGY ANALYSIS OF THE RESPONSE OF SOYBEAN ROOT HAIRS AND STRIPPED ROOTS TO HEAT STRESS

A Thesis

presented to

the Faculty of the Graduate School

at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Josef Batek

Dr. Gary Stacey, Thesis Supervisor

December 2015

The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

A COMPREHENSIVE, SYSTEMS-BIOLOGY ANALYSIS OF
THE RESPONSE OF SOYBEAN ROOT HAIRS
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a candidate for the degree of Master of Science,

and hereby certify that, in their opinion, it is worthy of acceptance.

Professor Gary Stacey

Professor David G. Mendoza-Cózatl

Professor Scott C. Peck

ACKNOWLEDGMENTS

I would first like to thank my graduate advisor, Curator Professor Gary Stacey for giving me the opportunity to join the best scientific team I could have wished for. Thank you for leading by example, and being actively involved in every step of my career.

I would also like to thank my thesis committee members, Dr. David Mendoza and Dr. Scott Peck for your guidance, advice and encouragement. I am also very grateful for the support provided by the entire Plant Science and Biochemistry departments at Mizzou, in particular Dr. James Schoelz that always has his door open for students. In addition I would like to thank Dr. Gus T. Ridgel for establishing the Ridgel Fellowship which provided me with financial support throughout my years at Mizzou.

I would like to thank past and current members of the Stacey lab who provided a challenging and enjoyable work environment. I am especially thankful to Dr. Minviluz (Bing) Stacey and Dr. Oswaldo Lopez-Valdez, for their guidance and friendship.

Furthermore, I would also like to thank Dr. Ljiljana Paša-Tolić and the members of Pacific Northwest National Laboratory for making me feel welcome during my research periods.

Finally, I want to extend my deepest thanks to my entire family for their love and support, in particular George and Sharon for their constant encouragement throughout my career.

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Introduction

Heat Stress

Heat stress (HS) is a major abiotic stress in biological systems, and can be defined as a rise in temperature beyond a critical threshold sufficient to cause irreversible damage in plants. Heat stress limits plant growth, metabolism and productivity. High temperatures can negatively affect various stages of plant development at different molecular and biochemical levels such as: growth, reproduction, redox homeostasis, disruption of the photosynthetic apparatus, yield and germination (Hasanuzzaman et al., 2013, Wahid et al., 2007).

Heat stress affects processes like germination and growth. The loss of cell water content associated with HS directly reduces growth rates (Essemine et al., 2010, Kosova et al., 2012). In addition, soybean HS resulted in lower seed numbers, increased abscission and abortion of flowers and decrease pollen production (Thuzar et al., 2010, Tubiello et al., 2007). Like other abiotic stresses (e.g. drought), heat stress may uncouple essential enzymes, and disrupt metabolic pathways leading to a harmful accumulation of ROS (Reactive Oxygen Species), which generates oxidative stress disrupting several crucial biological functions in the plant (e.g. membrane stability). Furthermore, photosynthesis is another physiological process in plants that is highly sensitive to heat stress. For example, HS can alter the photosynthetic rate by reducing the amount of photosynthetic pigments, and altering thylakoid structure (Hasanuzzaman et al**.,** 2013). The negative impact of high temperatures during the reproductive and developmental stages of soybeans has been previously identified as a major threat to yield in different regions around the world (Deryng et al., 2014).

It is forecasted that global temperatures will increase between 2 to 5° C by 2100, which will significantly impact crop production worldwide Intergovernmental Panel on Climate Change (http://www.ipcc.ch). Soybean is the world's most widely grown seed legume, providing a major source of protein and vegetable oil for human and animal consumption. Since soybean oil is the dominant vegetable oil produced in the U.S., extensive efforts are being made to utilize soybean oil as a future source of alternative fuels (Candeia et al., 2009).

Plants are sessile organisms and have developed complex regulatory pathways to recognize and adapt to a wide range of environmental changes. Although plants have several morphological and physical responses to heat stress (e.g. leaf orientation), this study focuses exclusively on the response of soybean root tissues to HS at a molecular level. Plants respond to HS through changes in 'omics' profiles, including changes in the transcriptomic, proteomic and metabolomic. Among the mechanisms that plants employ to cope with heat stress are the expression of genes encoding heat sock transcriptional factors (HsFs), heat shock proteins (HSPs), and production of key metabolites (Perez-Clemente et al., 2013, Hasanuzzaman et al., 2013).

Heat shock transcription factors (Hsfs) play a critical role in heat acclimation by regulating the expression of heat shock proteins (HSP), which increase tolerance against heat stress (Kotak et al., 2007). Hsfs are divided into three classes (A-C) based on their oligomerization domains. Generally, Hsfs consist of a DNA-binding domain, a hydrophobic (HR-A/B) oligomerization region, a nuclear localizations signal (NLS) and a transcriptional activation domain (Nover et al., 2001, Chung et al., 2013). Hsfs recognize binding motifs, categorized as heat stress elements (5-AGAAnnTTCT-3), which are conserved in the promoters of heat-stress inducible genes. For example, in tomato the (*HsFA1*) protein was shown to be a

master regulator of the heat shock response. Moreover, Hsfs may also have other roles besides abiotic responses, such as development (e.g. embryogenesis) (Mishra et al., 2002, Kotak et al., 2007). The expression of heat shock proteins (HSPs) is among the most important adaptive strategies in response to HS. HSPs act as molecular chaperones by protecting proteins from denaturation and preserve cellular stability through protein folding (Feder and Hofmann, 1999, Baniwal et al., 2004). Moreover, HSPs can be segregated into five different families ranging in molecular mass from 20 to 100 KDa (HSP100, HSP90, HSP70, and HSP20) (Swindell et al., 2007). Within the family of HSPs, HSP70 has a fundamental role in heat stress resistance. For example, mutants unable to synthetize HSP70 are more susceptible to heat injury (Burke**,** 2001). Recently, small HSPs (HSP15-30) have also received attention due to their abundance and diversity in plants, and their drastic expression level change (200-fold) in response to heat stress (Wahid**,** 2007). Furthermore, HSPs are also involved in signaling, amino acid biosynthesis and carbohydrate metabolism by regulating the expression of many genes in response to stress (Usman et al., 2014).

Heat stress can create a metabolic imbalance by affecting or altering proteins, membranes, cytoskeleton structures, and enzyme activity (Ruelland and Zachowski, 2010). In order to cope with heat stress, plants alter their metabolism in several ways. For example, a common defensive mechanism is the production of compatible solutes, which are able to maintain cell turgor via osmotic adjustments. Plants also induce antioxidant systems in response to heat stress in order to maintain cellular redox balance (Janska et al., 2010).

Previous studies identified compatible solutes or osmoprotectants (low molecular weight organic molecules) that act as osmolytes in response to osmotic stress (Lang, 2007, Yancey, 2004). Some examples of compatible solutes are sugars (sucrose, threhalose), polyols (mannitol,

sorbitol), and amino acids or derivatives (proline, glutamine) (da Costa et al., 1998). Solutes are termed compatible due to their ability to be present at high concentrations while not disrupting basal metabolism (Davies et al., 2010, Obata and Fernie, 2012). In the present study, I focused on metabolites that were produced in soybean root hairs and stripped roots (roots lacking root hairs) in response to heat stress.

Systems Biology

While advances in next generation sequencing and mass spectrometry based analysis have reduced the cost and time to characterize the plant molecular response to abiotic stresses, several challenges remain before a true 'systems-level' view can be obtained. For example, it remains a formidable challenge to integrate large and disparate datasets (e.g., transcriptomics, proteomics, and metabolomics). Classical 'omics' studies have largely measured the average response to abiotic stress in whole plants, organs and tissues that discount the effect of the dilution of the signal arising from a particular cell or cell type. This signal dilution effect makes it difficult to develop computational models for data integration (Aderem**,** 2005, Libault et al., 2010a). Over the past several years, our laboratory has employed soybean root hairs a single, differentiated, root epidermal cell model for system biology, including transcriptomic, proteomic and metabolomic analysis (Libault et al., 2010b, Libault 2010a, Brechenmacher et a.l., 2012, Brechenmacher et al., 2009a). In the current study, I used root hairs as a model system to profile the metabolomic changes in response to heat stress. My study was part of a larger study that included transcriptomic and proteomic analysis of these same tissues. The distinct stages of root hair development and functions are described below.

Formation of root hairs

Root hair cells develop from the differentiation of specialized epidermal cells (trichoblasts). In Arabidopsis, the development of trichoblasts into root hair cells depends on the position of epidermal cells relative to the underlying cortical cell (Dolan et al., 1994). Trichoblasts can be distinguished from artichoblasts (hairless cells) early in development during their initial formation at the meristematic zone by cytoplasmic structural differences (e.g. reduced vacuolation) (Gierson et al., 2014).

Root hair initiation

The first step in the formation of root hairs is to determine the epidermal initiation site; a process that is regulated by a variety of genes that are sensitive to hormonal and environmental factors (Schiefelbein, 2000, Cho and Cosgrove, 2002). In Arabidopsis, for example, the initiation site is precisely regulated by auxin and ethylene, which both act as positive regulators of root hair elongation (Pitts et al., 1998). Auxin-resistant mutants develop fewer root hair bulges during initiation, and the defect during initiation can be reversed with auxin treatment. Ethylene biosynthesis inhibitors were also shown to inhibit root hair formation (Masucci and Schiefelbein, 1996, Tanimoto et al., 1995). Trichoblasts exhibit polar expansion, which is the first morphological indication of root hair initiation. Microtubule rearrangements are associated with the initial formation of the bulge that will eventually develop into root hairs. The formation of this bulge reflects several physiological changes in the root hair cell. The acidification of the swollen cell wall at the initiation site is one of the physiological changes reported in root hairs (Bibikova et al., 1998, Datta et al., 2011)**.** It was previously documented that the localized decrease in pH facilitates cell wall loosening by inducing cell wall proteins such as EXPANSINS, which interact with cell wall polysaccharides, leading to cell wall modification as a key step in root hair initiation (Cho and Cosgrove, 2002).

Tip growth (Polar Growth)

After initiation, root hair cells undergo a specialized form of cell growth referred to as 'tip growth'. Briefly, tip growth involves the growth and expansion of the cell wall in a highly localized region of the cell. Polarized growth of cell walls can be observed in many biological systems including fungal hyphae, pollen tubes, and algal rhizoids (Bibikova et al., 1999). During tip growth, the cytoplasm is highly polarized and is sustained by exocytosis of vesicles in the root hair apex. These vesicles contain compounds needed for cell biosynthesis and modification such as cell wall polysaccharides, cell wall glycoproteins, integral proteins, and membrane transport proteins (Grierson et al., 2014). Besides membrane trafficking, transmembrane ion gradients have an important role in regulating root-hair tip growth (Ishida et al., 2008). Higher concentrations of cytoplasmic calcium (Ca^{2+}) were observed at the tip region compared to the rest of the root hair cell. The calcium gradient regulates the direction of growth by facilitating vesicle fusion, and subsequently delivering cell wall proteins. Root hair cells will re-orient towards the highest calcium concentration (Monshausen et al., 2007, Bibikova et al., 1999). Potassium is fundamental for root hair tip growth as a major osmotically active ion. Previous studies of plants mutated in specific potassium transporters showed a clear alteration of root hair tip growth (Desbrosses et al., 2003). Interestingly chloride has also been linked to tip growth in root hair cells. Inhibition of Cl^- channels led to a complete block of tip growth. It was also reported that lipids such as phosphoinositides and phospholids are also associated with vesicle transport and tip growth in root hair cells (Zonia et al, 2002, Malhó, 1998**).**

Function of root hairs

Root hairs have important roles in anchoring roots in the soil, nutrient and water uptake, and are also the preferred infection site of nitrogen fixing bacteria. Nutrient or mineral

concentrations in soil water are lower than the concentration of plants. In order to transport vital nutrients via the xylem, plants must overcome larger concentration gradients by energy expenditure to support active transport. The root hair plasma membrane contains several transport channels and proton pumps that mediate nutrient uptake (Raven and Johnson, 2010).

Root hairs increase the root surface area thus facilitating the uptake of water and nutrients from the soil. Plants with a higher density of root hairs facilitate the uptake of nutrients such as phosphorus (P) by increasing the absorptive surface area of the root and allowing the root to explore a greater soil volume. Previous studies showed that wild type Arabidopsis plants are more efficient in phosphorous uptake than the *rhd6* (root hair defective) mutant, which lacks root hairs (Masucci and Schlefelbein, 1994, Bates and Lynch, 2000). Nutrient limitation can affect root hair development. For example, root hair density was up to 5-times greater when roots were grown in low phosphorous conditions (1 µm) relative to high phosphorous (1000 µm) (Bates and Lynch, 1996, Savage et al., 2013). N-compound transporters such as nitrate (N03-) and ammonium (NH_4^+) have also been characterized in tomato root hairs. Studies in Arabidopsis reveal high affinity nitrate (NO_3^-) transporters, which are highly regulated under NO_3^- deficient conditions (Lauter et al., 1996). Other nutrient deficiencies such as iron, zinc and manganese can stimulate root hair production (Bates and Lynch**,** 1996, Müller and Schmidt 2001). Increasing the efficiency of nutrient capture in root hairs should increase crop production and improve agricultural sustainability.

Infection site

Legume plants root hairs are the primary infection site for nitrogen-fixing bacteria (Rhizobia). This symbiotic interaction starts with the secretion of flavonoids either from the root exudates or from the seed coat during germination, which induces nodulation genes in the

compatible rhizobia (Subramanian et al**.,** 2007). The bacterial nodulation genes lead to the production of the Nod Factor (lipochitooligosaccharide). The immediate response to the Nod factor on root hair cells include the depolarization of the membrane potential and changes in calcium concentration (Cardenas et al., 2000). Root hairs then curl promoting rhizobial infection. The rhizobia, once in the root hair, forms an infection thread by which the bacteria moves out of the root hair cell and into the plant cortex. This process is proceeded by cell division in the root cortex leading ultimately to the formation of the nodule, a novel organ which is intracellularly colonized by the rhizobia. Within the mature nodule, the rhizobia convert atmospheric nitrogen to ammonium, which is used as a N source by the plant host (Oldroy and Downie, 2008, Sulieman and Tran, 2014). Previous studies documented the detrimental effects of high soil temperature on both nodule formation and nitrogen fixation (Zahran, 1999). These effects are due, in part, to the detrimental effects of heat stress on photosynthesis, which supplies carbohydrates to the nodules, but also reflects the temperature sensitive nature of rhizobial survival in the soil and the processes involved in nodulation and nitrogen fixation (Michiels et al., 1994, Asadi Rahmani et al., 2009).

The following chapters present the results of a comprehensive systems biology analysis in soybean root hairs (RH) and stripped roots (STR, roots lacking root hairs) in response to heat stress (40°C). My specific focus on the metabolomics analysis of the heat stress response was only one part of a larger study that included transcriptomics and proteomics. For example, our RNA-seq analysis revealed 2,013 genes differentially regulated in response to heat stress in RH. Moreover, a gene regulatory module analysis revealed that transcriptional responses to heat stress are controlled by ten different regulatory modules. Our proteomic analysis identified 244 proteins that specifically responded to heat stress in RH. Finally, my metabolomic analysis

identified 30 compounds differentially regulated in soybean RHs in response to HS. To the best of our knowledge, this is the first time that such an extensive study of the plant HS response was performed on a single plant cell type (i.e., soybean RH). Hence, this study represents an important step toward a comprehensive understanding of the heat stress response in plants at a single cell resolution.

Chapter 1

(Submitted for publication to Frontiers in Plant Science)

Soybean root hairs grown under heat stress show global changes in their transcriptional and proteomic profiles

Oswaldo Valdés-López[1,2*], Josef Batek[1*], Nicolas Gomez'Hernandez[1], Cuong T. Nguyen[1], Mariel Carolina Isidra-Arellano[2], Ning Zhang[3], Trupti Joshi[3, 4,5], Dong Xu[3, 4], Kim K, Hixson[6], Karl K, Weitz[6], Joshua T. Aldrisch[6], Ljiljana Paša-Tolic[6] and Gary Stacey[1**]

[1] Division of Plant Sciences and Biochemistry, National Center for Soybean Biotechnology, C.S. Bond Life Sciences Center, University of Missouri, Columbia, MO. 6511, USA
[2] Laboratorio de Genomica Funcional de Leguminosas, FES Iztacala UNAM, 54090, Mexico
[3] Informatics Institute, C.S. Bond Life Sciences Center, University of Missouri, Columbia, MO 65211, USA.
[4]Department of Computer Science, University of Missouri, Columbia, MO 65211, USA
[5]Department of Molecular Microbiology and Immunology and Office of Research, School of Medicine, University of Missouri, Columbia, MO 65211, USA
[6] Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA 99352, USA

[*]Equal contribution

[**] Correspondence:
Gary Stacey
Division of Plant Sciences
271 E Christopher S. Bond Life Science Center
University of Missouri
Columbia, MO 65211, USA
Phone: 573-884-4752
staceyg@missouri.edu

Abstract

Heat stress is likely to be a key factor in the negative impact of climate change on crop production. Heat stress significantly influences the functions of roots, which provide support, water and nutrients to other plant organs. Likewise, roots play an important role in the establishment of symbiotic associations with different microorganisms. Despite the physiological relevance of roots, few studies have examined their response to heat stress. In this study, we performed genome-wide transcriptomic and proteomic analyses on isolated root hairs, which are a single, epidermal cell type, and compared their response to whole roots. We identified 2,013 genes differentially regulated in root hairs in response to heat stress. Our gene regulatory module analysis identified ten key modules that controlled the majority of the transcriptional response to heat stress. We also conducted proteomic analysis on membrane fractions isolated from roots and root hairs. These experiments identified a variety of proteins whose expression changed within 3 hours of application of heat stress. Most of these proteins were predicted to play a significant role in thermo-tolerance, as well as in chromatin remodeling and post-transcriptional regulation. The data presented represent an in-depth analysis of the heat stress response of a single cell type in soybean.

Introduction

Temperature is a critical factor that controls plant growth and development (Patel et al., 2009). The Intergovernmental Panel on Climate Change (IPCC) has forecasted that global temperatures will increase between 2 to 5^{o}C by the end of this century (http://www.ipcc.ch). In most regions, this global warming will negatively impact plant growth and development. As a consequence, the yields of a variety of important crops, such as corn, wheat and soybean will be

compromised. Thus, it is imperative to understand the physiological and molecular processes that plants use to cope with heat stress as a first step to breed for plants more tolerant to the negative effects of climate change.

Heat stress is considered one of the main factors that negatively affect crop production (Tubiello et al., 2007; Wheeler et al., 2013). This is because high temperatures reduce plant growth, as well as the number of flowers and seeds per pod. At a biochemical level, high temperatures induce protein denaturation, increase membrane lipid fluidity, increase reactive oxygen species production, and inhibit function of the photosynthetic apparatus (Larkindale et al., 2005; Hasanuzzaman et al., 2013; Qu et al., 2013). Plants have developed a variety of adaptations that allow them to cope with heat stress. Some of these responses include changes in leaf orientation, modification of membrane lipid composition, activation of antioxidative mechanisms, accumulation of osmolites and early maturation (Hasanuzzaman et al., 2013). These responses are finely regulated at transcriptional, post-transcriptional and post-translational levels by different transcription factors (TFs), small RNAs and protein kinases, respectively (Chen et al., 2012; Guan et al., 2013; Sullivan et al., 2014).

Soybean is a chief source of protein for human consumption and is grown on about 6% of the world´s arable lands (Hartman et al., 2011). Soybean production in the United States significantly increased over the last ten years with a concomitant increase in the value of the crop. However, as a clear example of the impact of abiotic stress, US soybean production was reduced ~7% during the severe drought/heat period in 2012 (http://www.ers.usda.gov/topics/in-the-news/us-drought-2012-farm-and-food-impacts.aspx#crop).

Useful models to predict the impacts of changing climate on plant productivity will require accurate, quantitative data that predict impacts across broad levels and spatial scales.

"Systems biology is a comprehensive, quantitative analysis of the manner in which all the components of a biological system interact functionally over time and space" (Aderem et al., 2005). The recent explosion of interest in systems biology is the result of the development of new tools for system-level analysis of cellular function and the availability of an increasing number of full genome sequences, which enables the comprehensive application of these new technologies. The ultimate goal is a new, predictive view of biological function, supplanting the older descriptive understanding. Hence, there is a need to integrate system approaches to understand the effects of climate change on molecules, cells, organisms and ecosystems.

However, the promise of this new 'predictive' science has yet to achieve its full potential. A number of challenges remain. For example, although the new tools do indeed provide for a full systems view of cellular function, integration of diverse multi-omics data (e.g., proteomics, metabolomics, transcriptomics, etc.) remains a formidable challenge. Among the issues compounding the problems of data integration is "signal dilution", which results from the fact that most studies average the response of whole tissues, obscuring the actual cellular response. Hence, it is impossible to discern the difference, for example, of a gene that is expressed at a low level in all cells from a gene that is expressed at a very high level, but only in a few cells. Approaches are needed to conduct complete functional systems analyses on single cells. For example, efforts have been made to characterize the transcriptome and metabolome of plant trichomes (Dai et al., 2010). Other papers have sought to use laser-captured cells (e.g., transcriptomics on laser-captured cells) (Aziz et al., 2005; Brady et al., 2007). However, this approach cannot be routinely applied across all functional genomic platforms.

Most studies of plant responses to heat stress have focused mainly on above ground organs. Here, we focus our evaluation of heat stress on root cells and tissues. Roots provide

support, water and nutrients to other plant organs (Khan et al., 2011). Indeed, soil temperature can influence root growth, cell elongation, root length and extension, initiation of new lateral roots and root hairs, and root branching (Pregitzer et al., 2000). These effects are likely manifestations of the variety of physiological effects brought about by temperature on plant roots; including changes in root respiration, nutrient uptake, as well as physicochemical effects on the soil environment (e.g., changes in nitrogen mineralization). Ambient temperature changes on above ground plant organs (e.g., effects on photosynthetic rates) also affect below ground growth and physiology. Despite the physiological relevance of roots, few studies have examined the response of these plant organs to heat stress.

In this study, we analyzed the transcriptional and proteomic responses of soybean roots to heat stress. In order to better understand the root responses to this abiotic stress, we performed genome-wide transcriptomic and proteomic analyses on root hairs, which are a single epidermal cell type. Our transcriptional analysis identified 2,013 genes differentially regulated in root hairs in response to heat stress. These data were used to predict key regulatory modules controlling the heat stress response. We also conducted proteomic analysis on membrane fractions isolated from roots and root hairs. These experiments identified a variety of proteins whose expression changed within 3 hours of application of heat stress. Most of these proteins were predicted to play a significant role in thermo-tolerance, as well as chromatin remodeling and post-transcriptional regulation. The data presented represent an in-depth analysis of the heat stress response of a single cell type in soybean.

Material and Methods

Plant Material and Treatments

Soybean seeds [*Glycine max* L. (Merrill) cv. Williams 82] were surface sterilized and sown on agar plates containing 1X B&D (Broughton and Dilworth, 1971) nutrients. Plates containing seeds were incubated for three days under dark conditions at 25 ^{0}C in a growth chamber. These seedlings were further incubated for various time points (0 h, 3 h, 6 h, 12 h and 24 h) at 25^{0}C (control) or 40^{0}C (heat stress). After specific incubation time, the whole roots were detached from the shoots and frozen in liquid nitrogen. These roots were used to isolate root hairs and corresponding stripped roots (i.e., roots with root hairs removed) according to the methods described in (Brechenmacher et al., 2009). Root hairs (RHs) and stripped roots (STRs) were quick frozen in liquid nitrogen and then stored at -80 ^{0}C until use. Two biological replicates per time point were collected. In each biological replicate, 50 plates (each plate contained 20 seeds, five plates for each time and temperature condition, in total 1000 seedlings were used in each biological replicate) were included.

Protein and RNA extraction

Proteins and total RNA were extracted from 1 g of RHs or STRs using Trizol reagent supplemented with protease inhibitors according to the manufacturer´s instructions. Total RNA was subsequently purified using a chloroform extraction. Total RNA concentration and integrity were analyzed using a Nanodrop (Thermo Scientific, Whilmington, DE) analyzer and a Bioanalyzer (Agilent, Santa Clara, CA), respectively. A Coomassie Plus (Thermo Scientific, Grand Island, NY) protein assay was used to quantify the protein concentration and about 200 µg of protein per sample were obtained.

Microsomal fraction

The microsomal fraction was purified from RH or STR extracts according to (Brechenmacher et al., 2009). Briefly, homogenized RH preparations were sonicated in 0.1 M

Tris-HCl, pH 8, 10 mM EDTA, 0.4% β-mercaptoethanol and 250 mM sucrose. Cell debris was allowed to settle and organelles removed from the suspension by centrifugation at 20,000 x g for 30 min at 4^0C. The microsomal fraction was obtained by centrifugation at 100,000 x g for 1 h at 4^0C. The pellets were solubilized in 0.1 M Tris-HCl, pH 8.5, 8 M urea and 2% dodecyl-β-maltoside. Proteins were precipitated using 25% trichloroacetic acid (TCA), washed in acetone, and resolubilized with 8 M urea and Tris-HCl, pH 8.5. Finally, the supernatants were passed through 5.0 and 0.45-µM polyvinylidene difloride membrane filters (Millipore, Billerica, MA). Proteins were quantified using the bicinchoninic acid protein assay kit (Thermo Scientific, Grand Island, NY). The same procedure was used to obtain microsomal fractions from homogenized STR preparations, except that the roots were ground using a mortar and pestle to extract the protein.

Protein sample preparation and LC-MS/MS analysis

The extracted microsomal proteins were dried with a speed vac, followed by solubilization and denaturation in 150 µL of 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]1-propanesulfonate (CHAPS) and 5 mM Tris(2-carboxyethyl)phosphine (TCEP) in 50 mM ammonium bicarbonate, pH 8. These preparations were vortexed, sonicated and then heated at 60°C for 30 min. Protein concentrations were again verified using the Coomassie Plus Protein Assay with a bovine serum albumin standard. The denatured samples were diluted ten-fold with 50 mM ammonium bicarbonate. $CaCl_2$ was added to a concentration of 2 mM and trypsin was added at a trypsin:sample ratio of 1:50 (w/w). The samples were digested overnight at 37 °C and were alkylated with chloroacetamide at a concentration of 5 mM in the dark for 2 hs at room temperature (RT). The peptides were desalted using SCX SPE resin (SUPELCO Supelclean, 100 mg) using first a 10 mM ammonium

formate, pH 3.0, 25% acetonitrile solution to wash the peptides followed by 80:15:5 methanol:water:ammonium hydroxide to elute the peptides. The SCX SPE resin removed the detergents but the ammonium salts still needed to be removed before iTRAQ labeling. For this latter purpose, the samples were loaded onto a C-18 SPE column (SUPELCO Discovery, 50 mg) followed by a wash using 0.1% TFA in nanopure water and then subsequently 80% acetonitrile/0.1% TFA in water to elute the peptides. Peptides were quantified using a BCA assay with a bovine serum albumin standard.

Peptides were labeled with 8-plex iTRAQ reagents as described below (AB Sciex, Foster City, CA). 30 µg of each sample was placed in a new tube and dried in a speedvac. 13 µg of dissolution buffer (provided in the iTRAQ kit) was added to each sample and vortexed into solution followed by brief centrifugation to concentrate sample at the bottom of the tube. Each iTRAQ reagent (10 µL) was diluted with isopropanol (35 µL) and then added to each sample. The reaction was carried out for 2 hs at RT. 50 mM ammonium bicarbonate (200 µL) was added to quench each reaction tube. After 1 h, the contents from all iTRAQ reactions were added to one tube and the sample was vortexed, followed by drying in a speed vac.

The labeled peptides were separated using an off-line high pH (pH 10) reversed-phase (RP) XBridge C18 column (Waters, Milford MA) (250 mm x 4.6 mm column containing 5 µm particles and a 4.6 mm x 20 mm guard column) using an Agilent 1200 HPLC System (Agilent Technologies, Santa Clara CA). The sample loaded onto the C18 column was washed for 15 min with Solvent A (10 mM ammonium formate, adjusted to pH 10 with ammonium hydroxide). The LC gradient used a linear increase of Solvent B (10 mM ammonium formate, pH 10, 90% acetonitrile) to 5% over 10 min, then a linear increase to 45% Solvent B over 65 min, and then a linear increase to 100% Solvent B over 15 min. This level of Solvent B was held at 100% for 10

min and subsequently dropped to 0% Solvent B, holding the column at 100% Solvent A for 20 min. The flow rate was 0.5 mL/min. A total of 48 fractions (1.98 mL each) were collected evenly over the gradient between 15-110 min into a deep (2 mL/well) 96 well plate throughout the LC gradient. The plate fractions were concentrated using a speed vac. The high pH RP fractions were then combined into 12 fractions using the concatenation strategy reported in a previous study (Wang et al., 2011) which were further dried down and resuspended in nanopure water at a concentration of 0.075 µg/µL. Fractions were stored at -20 ^{0}C until time for LC-MS/MS analysis.

Peptide mixtures were analyzed on a high-resolution, reversed-phase constant flow nano capillary LC system coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose CA). The automated LC system was custom built using two Agilent 1200 nanoflow pumps and one Agilent 1200 Capillary pump (Agilent Technologies, Santa Clara CA), and a PAL® autosampler (LEAP Technologies, Carrboro, NC). Full automation was made possible by custom software allowing for parallel event coordination. Therefore, 100% of the MS duty cycle was used by way of two trapping and two analytical capillary columns. Capillary reversed-phase columns were prepared in-house by slurry packing 3-µm Jupiter C18 (Phenomenex, Torrence, CA) into 35 cm x 360 µm o.d. x 75µm i.d. fused silica (Polymicro Technologies Inc., Phoenix AZ). Trapping columns were prepared similarly, but using 3.6 µm Aeris Widepore XB-C18 resin packed into a 4 cm length of 150µm i.d. fused silica. Electrospray emitters were custom made using 150 µm o.d. x 20µm i.d. chemically etched fused silica (Kelly et al., 2006). Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid acetonitrile (B) operated at 300 nL/min with a gradient profile as follows (min:%B); 0:5, 2:8, 20:12, 75:35, 97:60, 100:85.

Sample injections (5 µL) were trapped and washed on the trapping columns at 1.5 µL/min for 20 min before alignment with the analytical columns.

The LTQ Orbitrap Velos mass spectrometer was operated with a heated capillary temperature and spray voltage of 350 ^{0}C and 2.2 kV, respectively. Full MS spectra were recorded at a resolution of 100K (for ions at *m/z* 400) over the range of *m/z* 400-2000 with an automated gain control (AGC) value of 1e6. MS/MS was performed in the data-dependent mode with an AGC target value of 3e4. The ten most abundant parent ions, excluding single charge states, were selected for MS/MS using high-energy collisional dissociation (HCD) with a normalized collision energy setting of 40%. A dynamic exclusion time of 45 s was used.

Identification of differentially expressed proteins

MS/MS spectra were first converted to peak lists using DeconMSn (version 2.2.2.2, http://omics.pnl.gov/software/DeconMSn.php) (v1) with default parameters. Sequence determination was provided by SEQUEST v27 in conjunction with the soybean genome annotation (Gmax v10.3;Wm82.a2.v1). Both full and partially digested tryptic peptides were considered with two missed cleavages allowed. The mass tolerance for precursor ions was 50 ppm and fragmentation tolerance for HCD (higher energy collisional dissociation) was 0.05 Da. All peptides were identified with <1% False Discovery Rate by using an MS-Generating Function Score (MS-GF) <1E-10 and a decoy database searching strategy (Kim et al., 2008; Kim et al., 2010; Granholm et al., 2014). Modifications were searched looking for static alkylation on cysteine and 8 plex iTRAQ modifications on the N-terminus and lysine residues. Other modifications included in the search were dynamic oxidation on methionine. Relative abundances of peptides were determined using iTRAQ reporter ion intensity ratios from each MS/MS spectrum. Individual peptide intensity values were determined by dividing the base

peak intensity by the fraction associated with each reporter ion. Multiple scans of the same peptide were consolidated into a single peptide value by summation. Log2 transformed peptide abundances were then normalized according to the mean of the 2 pooled references (added to 2 channels of each iTRAQ 8-plex experiment) so that samples from different iTRAQ experiments could be compared. The peptide abundances were further normalized (to remove iTRAQ channel bias) using the central tendency normalization algorithm (which normalizes each proteome dataset to the global population median) available in Inferno (http://code.google.com/p/inferno4proteomics/). The Rrollup function in Inferno was used to roll up peptide values to a protein value. The Rrollup function works by taking log2-transformed data and identifying the peptide which has the most presence and greatest abundance across all samples used for comparison. All peptides were scaled to the most present and abundant peptide and the final protein abundance value used represents the median of the scaled peptide abundances. ANOVA significance testing was performed on each sample time point, determining significance via p-value between samples subjected to either 25^0C or 40^0C. P-values were further corrected for multiple-testing error using Benjamini-Hochberg p-value correction. Fold changes are displayed as log2 fold change of protein values obtained at 40^0C/protein values obtained at 25^0C.

Preparation of RNA-seq library

Total RNA was isolated from 1 g of control- or heat-stressed RHs or STRs as described above. Non-strand-specific mRNA-seq libraries were generated from 4 µg of total RNA from each tissue and prepared using the TruSeq RNA sample Prep Kit (Illumina) according to the manufacturer's instructions.

High-Throughput Transcriptomics Sequencing

Forty non-strand-specific RNA-seq libraries 2 root types (RH and STR) X 2 treatments (Control or Heat stress) X 5 treatments X 2 replicates] were multiplexed and sequenced for 51 cycles using an Illumina HiSeq 2000 (Illumina, San Diego, CA) according to the manufacturer's instructions. Image analysis and base calling were performed using the Illumina pipeline (http://www.illunina.com).

Mapping and Processing of RNA-seq reads

Following base calling, quality filtering was performed on the RNA-Seq reads generated with the Illumina HiSeq 2000 using an in-house custom Perl script including removal of reads with "N" base and trimming of the 3' end of the read for below threshold quality. Additional filtering for removal of bad-quality bases (any base with quality score values lower than 20 percentile was considered as a base with bad quality) and read length size (<50 bp) was performed using the FASTA/Q Trimmer command of the FASTX-toolkit available in FastQC software package (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). RNA-seq reads with good quality were aligned to the soybean reference genome (Gmax v10.3;Wm82.a2.v1 (Schmutz et al., 2010) using Tophat (version 1.4.1) (Trapnell et al., 2009). The genome indexes for Tophat were built using bowtie-build command of bowtie (version 0.12.7) with the reference genome file as the input. Tophat was then run with the default parameters to map the trimmed- and filtered-reads for each library to the reference genome. Tophat was supplied with the reference GTF file using the –G option and replicates of each condition/sample were mapped independently to improve alignment sensitivity and accuracy for further analysis. For analysis of protein-coding genes, only uniquely mapping reads were used. The gene expression abundance was calculated in RPKM using Cufflinks software (Trapnell et al., 2012).

Identification of differentially expressed genes

Low-count reads with a total sum fewer than 10 were removed prior to data analysis (Auer et al., 2011). A Poisson linear mixed-effects model (Blekhman et al., 2010) was applied to the raw read counts separately for each gene using the software R/lme4 package (2.10.0 version) with the library size as the offset value to make the comparison across different samples comparable. Each generalized Poisson linear mixed model includes the cell type effect, treatment effect, and the random biological replicate effect, as well as random plate effect accounting for the correlation between observations that share the same plate. The likelihood ratio tests were then conducted to identify differentially expressed genes between the treatment and control groups for each of the cell types (i.e., $RH_{control}$ *vs.* RH_{heat}). P-values for the likelihood ratio tests were obtained, and an adjusted-P value (Storey et al., 2003) was then computed to produce lists of differentially expressed genes with an estimated FDR of 1%. Among these significantly differentially expressed genes, genes with a fold change above 2 were further considered.

Gene Regulatory Networks Analysis

A gene regulatory module analysis was performed using the method described by (Zhu et al., 2012). Briefly, based on the differentially regulated genes between the treatment and control groups for each exposition time (i.e. $RH_{control}$ and RH_{heat}), the expression levels of transcription factors (TF) were clustered into two or three categories (1: highly expressed; 0: normally expressed; -1: lowly expressed) using the K-means clustering algorithm, where the number of categories equals the number of types (>3, <-3, or in between) of expression. A specific set of TFs was assumed to regulate the expression of genes in a module through a path in the binary decision tree composed of TFs as internal nodes and condition subgroups as leaf nodes. A regulatory path from the root node to the leaf node was interpreted as a series of binary queries on the expression level (up-regulated or not, or down-regulated or not) of internal nodes (i.e.,

TFs) under treatment conditions leading to the observed expression levels of the genes in the leaf node under the same treatment conditions. Therefore, the regulatory decision tree represents the combinatorial logic by which the TFs regulate the expression of the genes in the module under different treatment conditions. In order to construct the gene regulatory module, the differentially expressed genes were clustered using the K-means algorithm, aiming to assign genes exhibiting similar expression patterns across all the treatment conditions into the same cluster. Once the genes were clustered, the modules were constructed in an iterative two-step manner, including: 1) constructing a binary tree consisting of several TFs that can best interpret the expression of the genes in a cluster, and 2) re-assigning into clusters those genes whose regulatory tree can explain their expression pattern best. The two steps were alternated until the likelihood of the gene expression data was maximized. After a gene regulatory tree was constructed for every gene cluster, a gene re-assignment procedure was used to assign each gene to a cluster whose regulatory tree best explained its expression values over all the treatment conditions.

Gene Functional Classification

The biological relevance of the differential regulated genes and proteins was assessed by a gene function enrichment analysis using the method Singular Enrichment Analysis (SEA) available in the web-based tool AgriGo (Du et al., 2010). (http://bioinfo.cau.edu.cn/agriGO/analysis.php). Briefly, Gene Ontology (GO) terms enriched in each individual set of genes and proteins were compared to a default gene reference background. P-values for the GO terms were obtained through Fisher's exact test, and a q-value was computed to produce lists of significant GO terms with an estimated FDR of 5%. Among these significantly enriched GO terms, terms with q-values > 0.05 were further considered. Additionally, MAPMAN gene functional classification was used (Thimm et al., 2004; Usadel et

al., 2009). For MAPMAN analysis an in-house custom soybean mapping file, which allows a survey of all the functional categories included in the software MAPMAN, was used.

RNA extraction and qRT-PCR

Total RNA was isolated from stressed or control RHs and STR using Trizol Reagent (Invitrogen), according to manufacturers' specifications. Genomic DNA (gDNA) was removed from purified RNA by using TURBO DNAse (Ambion) according to manufacturer's instructions. Two μg of gDNA-free RNA were used to synthetize cDNA as described in (Libault et al., 2010).

qRT-PCR was performed as described in (Libault et al., 2008) using the housekeeping gene *cons6* to normalize the expression levels of the analyzed genes (Libault et al., 2008). Primer design was performed as described in [53]. The sequences are reported in Supplementary Table 3.Expression levels of each candidate gene were calculated according to $E=P_{eff}^{(-\Delta Ct)}$, where P_{eff} is the primer efficiency calculated using LinRegPCR (Ramakers et al., 2003). Fold changes were calculated between the ratios of the expression levels of heat-treated (40°C) and control (25°C) samples, and expression levels were calculated for 3 different time points (3h, 12h, and 24h) for two biological replicates.

Results

RNA-seq analysis

Most studies of the transcriptional responses to heat stress used entire organs (e.g. roots or leaves) (Barah et al., 2013; Johnson et al., 2014). Thus, the values obtained from these studies represent an average of the response of all the different cell types in the tissue analyzed. In order to reduce the 'tissue dilution' effects inherent to such studies, we conducted an RNA-seq analysis using a single-type of soybean cell, the root hair.

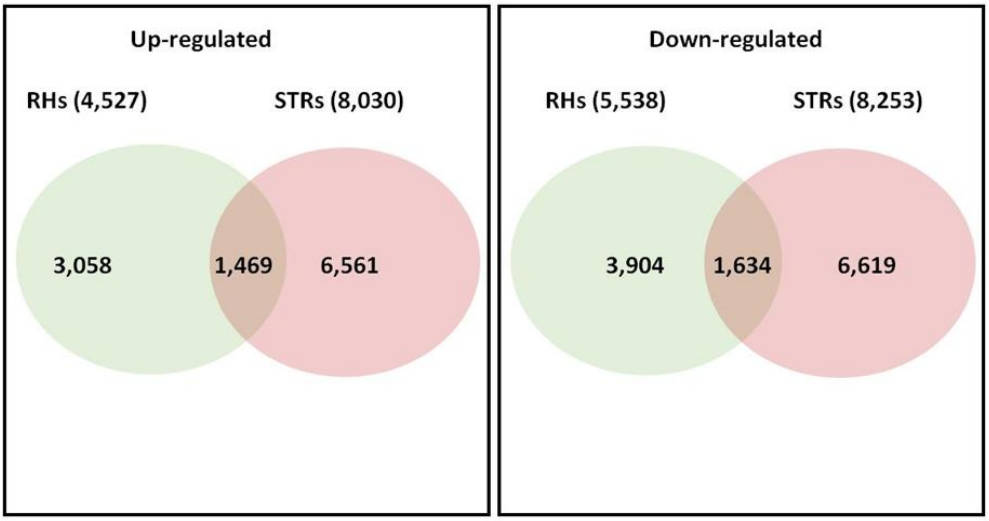
A total of 40 cDNA libraries were derived from control RHs (RH_C) and RHs exposed to 40^0C (RH_H), as well as from control STRs (STR_C) and STRs exposed to 40^0C (STR_H). These libraries were sequenced using the Illumina HiSeq2000 platform. After filtering low quality reads, a total of 1,053,532,578 reads (50 bp in size) were aligned to the soybean genome reference sequence (Gmax v10.3;Wm82.a2.v1; Schmutz et al., 2010) (Supplemental Table 1) using Bowtie and Tophat software (Trapnell et al., 2009). Of these, 997,923,404 reads were uniquely mapped to the soybean genome and were used for further analysis. Of the 56,044 predicted protein-coding genes in the soybean genome (http://www.phytozome.net), 46,366 genes were deemed to be expressed in this study based on the occurrence of at least one read in all two biological replicates (Supplemental Table 1). The RNAseq gene expression, proteomic expression and differential expression datasets are all available for browsing in the Soybean Knowledge Base (SoyKB) (Joshi et al., 2012; Joshi et al., 2014).

Transcriptional responses to heat stress at single cell resolution

In order to identify genes that were differentially regulated in response to heat stress, the RNA-seq data were analyzed using a generalized Poisson linear mixed-effects model with an additional cutoff of 2-fold in pairwise comparisons (e.g., RH_H/RH_C). On average, 2,013 regulated genes were identified in RHs, with a maximum of 3,450 (3 h treatment). In contrast, on average, 3,257 regulated genes were identified in STRs, with a maximum of 5,007 (6 h treatment). Across all four-exposure time points (3 h, 6 h, 12 h and 24 h) to the heat stress, a total of 10,065 and 16,283 differentially regulated genes were identified in RHs and STRs, respectively (Supplemental Figure 1). A comparison across the four-treatments revealed 4,527 genes that were up-regulated in RHs, whereas 8,030 were up-regulated in STRs (Supplemental Figure 1). Subsequently, a comparison between the differentially regulated genes in both RH and

STR samples was performed to identify genes commonly or uniquely regulated in these tissues. This analysis revealed that 3,103 genes (1,469 up-regulated and 1,634 down-regulated) were commonly regulated in both RHs and STRs (Figure 1 and Supplementary Table1). In contrast, 6,962 (3,058 up-regulated and 3,904 down-regulated) and 13,180 (6,561 up-regulated and 6,619 down-regulated) were uniquely regulated in RHs and STR, respectively (Figure 1). These numbers attest to the strong impact that heat treatment has on transcription.

Figure 1 Number of overlapping and non-overlapping heat-responsive genes among soybean root hairs (RHs) and stripped roots (STRs).
Differentially regulated genes in each cell type were identified by linear mixed models at FDR < 0.01, with additional cutoff of 2-fold in pairwise comparison (heat-stressed vs control). Over- and non-over-lapping genes were identified after a pairwise comparison between treatments. Numbers in parenthesis indicate all the regulated genes across the four exposure times.



To confirm these RNAseq results, the expression of 15 randomly selected genes was analyzed via qRT-PCR (Supplemental Figure 2). The pattern of expression obtained by qRT-PCR showed the same trend observed by RNAseq in response to heat stress. We found some differences in the fold-change measured by the two methods, which we explain by the relative sensitivity of each method, as well as technical aspects (e.g., efficiency and specificity of qRT-PCR primers).

We also compared the transcriptional response in RH and STR across the four treatment time points (3 h, 6 h, 12 h and 24 h). This analysis revealed that only 15% (689 genes: 362 up-regulated and 327 down-regulated) of the differentially regulated genes in RHs were regulated across all four-treatment time points. In the STR samples, only 13% (1,026 genes: 530 up-regulated and 496 down-regulated) of the differentially regulated genes were regulated at all four treatment time points (Supplemental Figure 1). Collectively, these data indicate that each root-tissue type responded differently to the heat stress. As one might expect, the RH tissue appeared to respond faster to the heat treatment than the STR tissue, in that the maximum differential gene expression was seen at 3 h in RHs and 6 h in STRs.

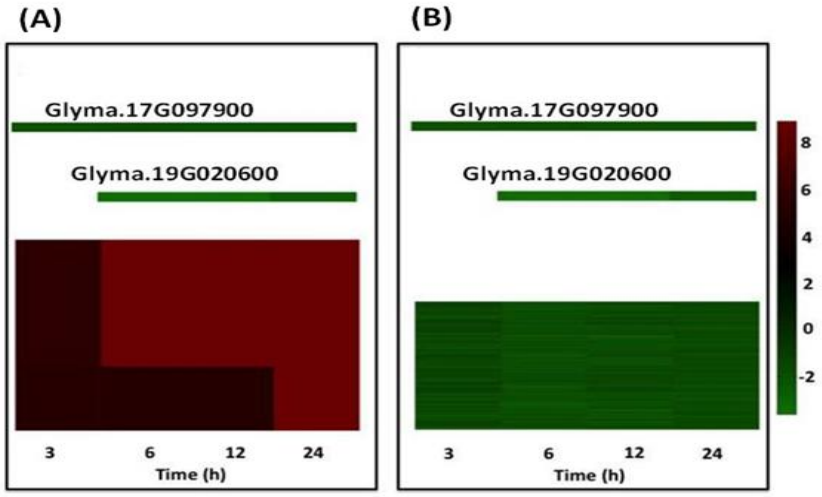
Transcriptional responses to heat stress are controlled by ten different regulatory modules in RHs.

Recently, we developed a new algorithm that can predict gene regulatory modules from either DNA microarray or RNA-seq transcription data (Zhu et al., 2012; Zhu et al. 2013). This allows prediction of: 1) transcription factors (TFs) that control a specific regulatory module; and 2) genes that participate in a specific regulatory module (Zhu et al., 2012; Zhu et al. 2013). We used this algorithm to analyze the differentially regulated genes that responded across all four-treatment time points (i.e., 689 commonly regulated genes). This analysis predicted ten different regulatory networks (Supplemental Figure 3). These modules are regulated in a combinatorial manner by five TFs: Heat Stress Factor (HSF; Glyma.03G157300), AP2/EREBP (Glyma.13G152000), MAD-box (Glyma.07G181600) and two WRKYs TFs (Glyma.17G097900 and Glyma.19G020600). With the exception of the HSF, the expression of the TFs that control the ten gene regulatory modules was down-regulated across the four treatments. Furthermore, we found that five of the ten regulatory networks contain down-regulated genes, whereas the other

five networks have either highly- (fold-change ≥ 3) or mildly (fold-change ≤ 2) up-regulated genes. Interestingly, the TF WRKY encoded by Glyma.17G097900, regulates eight of the ten modules, which indicates that this TF may be a master regulator of the heat stress response in soybean RHs.

By way of an example, we describe in detail the regulatory modules 7 and 9 in Figure 2. Module 7 contains two signal-transduction related genes (Glyma.14G100800: Receptor kinase; Glyma.15G048500: MAPKKK) whose expression was up-regulated across the four treatments (Figure 2 A). These genes are predicted to be under the control of two WRKY TFs (Glyma.17G097900 and Glyma.19G020600) whose expression was down-regulated across the four treatments. Module 9 contains 84 down-regulated genes controlled by the same two WRKY TFs that control module 7. Some of the genes belonging to this network are likely involved in cell wall degradation, flavonoid biosynthesis and transcriptional regulation by different TFs, like MYB and C2H2 (Figure 2 B). Collectively, the gene network analysis indicates that many heat-stress induced genes are significantly regulated by only five different TFs. Furthermore, four (i.e., Glyma.17G097900, Glyma.19G020600, Glyma.13G152000, and Glyma07G181600) of these five TFs likely act as repressors of gene expression.

Figure 2 Gene regulatory modules controlling the transcriptional responses of soybean root hairs to heat stress. Panels A and B show two significant modules controlled by two different TFs. Individual genes are represented by small squares. Transcript abundance values were false color-coded by using a scale of -2 to ± 8. The intensity of green and red colors indicates the degree of expression of the corresponding genes. Data are the average of two biological replicates.



Heat stress induces changes in the root hair proteome

Changes in transcriptional activity do not always reflect changes in expression of the encoded proteins (Stevens and Brown, 2013; Ponnala et al., 2014). Therefore, we also undertook an analysis of the RH and STR proteome in response to heat stress. We specifically focused on the microsomal (membrane) fraction in order to reduce the complexity of the protein profile and to specifically determine how membrane function (e.g., transporter expression) was affected by heat stress. Proteins were isolated from the same RHs and STRs used for the RNA-seq transcriptome analysis. LC/MS-MS analysis of these samples identified 244 and 113 differentially expressed proteins in RHs and STRs, respectively (Table 1). To identify commonly or uniquely regulated proteins, we performed a comparative analysis among the proteins detected in both cell types. Our analysis revealed that only 49 proteins were commonly expressed in response to heat in both RHs and STRs (Table 2 and Supplementary Figure 4). In contrast, 195 proteins were expressed only in RHs whereas 64 were exclusively expressed in STRs (Table 2).

We observed that 3 h of heat treatment was sufficient to trigger significant changes at protein levels in response to heat stress. Additionally, we detected more differentially expressed proteins in RHs than in STRs. For instance, 135 differentially regulated proteins (73 induced and 62 repressed) were detected in RHs after 24 h of treatment, whereas 43 (23 induced and 20 repressed) were detected in STRs. This is likely due to a reduction in the effects of tissue dilution (that averages the signal over many cell types) in the STR samples, relative to the RHs. The proteomic expression and differential expression data are available for browsing in the Soybean Knowledge Base (SoyKB) (Joshi et al., 2012; Joshi et al., 2014).

Table 1 Differentially regulated protein in soybean heat-stressed root hairs and stripped roots identified by LC/MS/MS.

Time (Hrs)	Root Hairs			Stripped Root		
	Induced	Repressed	Total	Induced	Repressed	Total
0	0	0	0	0	0	0
3	14	2	16	16	0	16
6	36	20	56	18	0	18
12	27	10	37	22	14	36
24	73	62	135	23	20	43

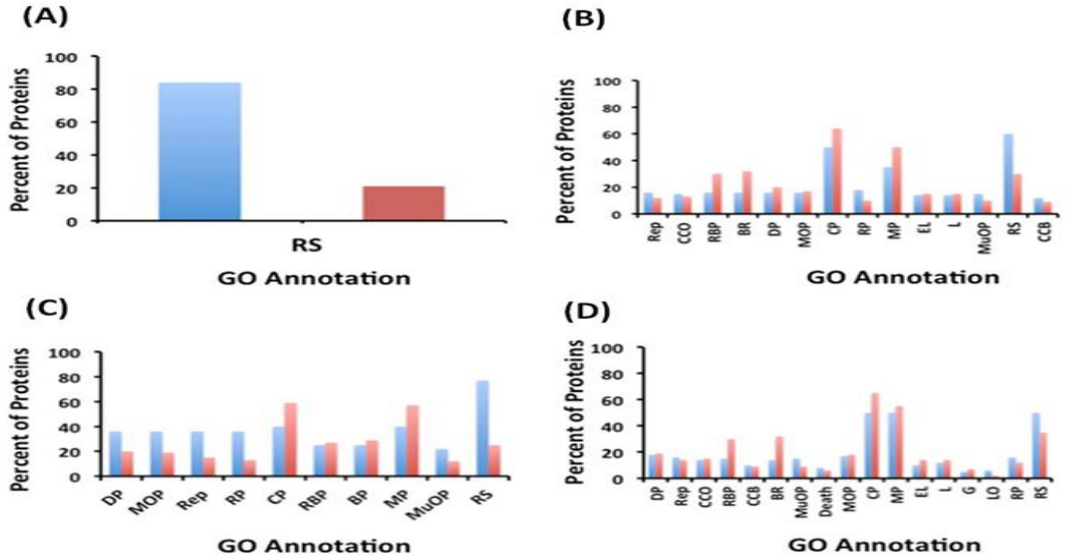
Table 2 Number of overlapping and non-overlapping heat-stress responsive proteins among soybean RHs and STRs.

Exposition time (h)	Proteins detected in both cell types			Proteins detected only in RHs			Proteins detected only in STRs		
	Induced	Repressed	Total	Induced	Repressed	Total	Induced	Repressed	Total
3	10	0	10	4	2	6	6	0	6
6	12	0	12	24	20	44	6	0	6
12	13	1	14	14	9	23	9	13	22
24	13	0	13	60	62	122	10	20	30

Heat-stress related proteins play some role in the chromatin remodeling and post-transcriptional regulation in RHs

A functional enrichment analysis, as well as a functional classification by using MAPMAN software, was performed on the regulated proteins identified in each heat treatment (Figure 3 and Supplemental Figure 5). This analysis revealed that proteins known to respond to environmental stimuli that includes response to light, temperature and heat stress, were those most enriched among the proteins responding to the heat treatment. This category was followed by proteins involved in cellular and metabolic processes, for instance in cell wall formation, amino acids and lipid biosynthesis as well as in the elimination of reactive oxygen species (Figure 3). Interestingly, 84% of the identified proteins responding after 3 h of treatment were related to the heat stress response. Although proteins with a potential role in adaptation to heat stress were identified at the other treatment time points, proteins with a potential role in chromatin remodeling, post-transcriptional and post-translational regulation were also identified in these time points (Figure 3).

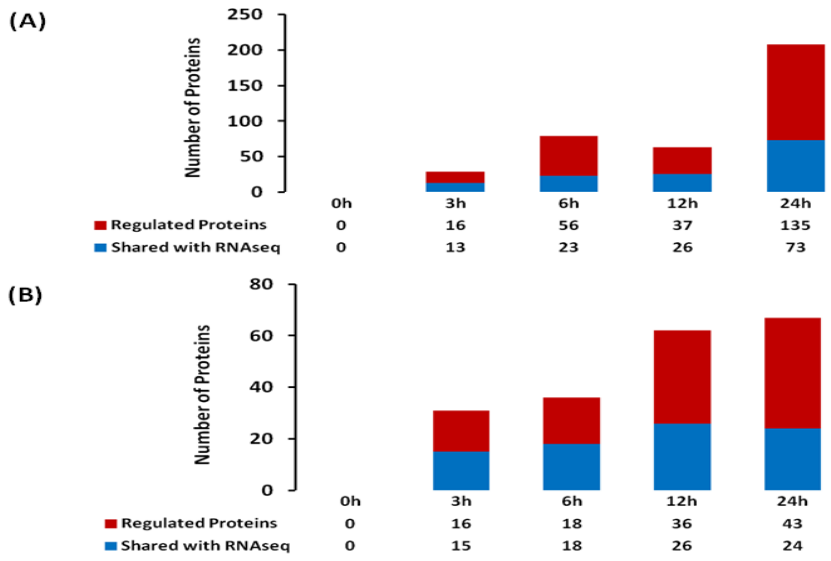
Figure 3 Gene Ontology (GO) enriched terms of the differentially regulated genes identified in soybean root hairs.



The GO annotation is: RS: Response to Stimulus; Rep: Reproduction; CCO: Cellular Component Organization; RBP: Regulation of Biological Process; BR: Biological Regulation; DP: Development Process; MOP: Multicelluar Organismal Process; CP: Cellular Process; RP: Reproductive Process; MP: Metabolic Process; EL: Establishment of Localization; L: Localization; MuOP: Multi-organism Process; CCB: Cellular Component Biogenesis; G: Growth; LO: Locomotion.

In order to assess the relationship between the RNA and protein expression profiles, a pairwise comparison was made between the proteins and mRNA levels at the various treatments. This analysis gave a relatively low correlation value (-0.2-0.79) between the mRNA and protein expression levels. However, on average, expression values for the mRNAs of 61% and 79% of the expressed proteins in RHs and STRs, respectively, were present in the transcriptome data set (Figure 4 and Supplemental Table 2). Together, our proteomic data indicates that the majority of the expressed proteins have some, predicted role in coping with the heat stress, but also likely function to reprogram the transcriptional activity during heat stress conditions.

Figure 4 Relationship between protein- and mRNA levels in heat-stressed soybean root hairs (A) and stripped roots (B). Pairwise comparison between expressed proteins (Red) and mRNA levels (Blue) at various exposure times, on average, expression values for the mRNAs of 61% and 79% of the expressed proteins in RHs and STRs, respectively, were present in the transcriptome data set.



Discussion

The predicted effects of continued climate change are complex but include effects on air and surface temperature, with coincident effects on water availability. In most regions, these effects are expected to significantly impact crop yields. Thus, it is important to understand the molecular mechanisms that allow plants to adapt and tolerate climate change induced stresses, including heat stress. Most of the studies to understand the plant responses to heat stress have focused on the leaf responses. For example, in different plant species both transcriptomic and proteomic analysis indicates that most of the molecular leaf responses are to protect the photosynthetic apparatus and to acquire general thermo-tolerance (Sullivan et al., 2014; Barah et al., 2013; Johnson et al., 2014; Liu et al., 2014). Despite the physiological relevance of roots, as well as the obvious effects that above ground processes have on root physiology, less attention

has been placed on understanding how roots also respond to heat stress. Therefore, we undertook a detailed transcriptomic and proteomic study of the heat stress response in soybean roots. An important and unique aspect of our study was the examination of the effects on soybean root hairs, a single, differentiated, root epidermal cell.

Significant transcriptional and translational reprograming has been observed in different plant species grown under heat stress conditions (Sullivan et al., 2014; Johson et al., 2014; Zeller et al., 2009; Li et al., 2013). Likewise, it was reported that this reprograming occurs very quickly, for instance after 10 min of treatment (Matsuura, 44). Somewhat similar results were observed in our transcriptional analysis. Interestingly, we did observe that RHs showed a faster (e.g. 3 h) transcriptional and translational reprograming to heat stress than STRs. This observation suggests that studies of the response in single cells may reveal very different response kinetics and gene/protein expression responses than one can measure by studies of whole organs.

Over 2000 and 3000 genes were differentially regulated by heat in the RH and STR samples, respectively. A gene function enrichment analysis of these regulated genes suggest that heat has a strong effect on cellular metabolism, impacting genes involved in metabolic processes, response to environmental stimuli, transcriptional regulation, protein folding, chromatin remodeling, lipid and ATP biosynthesis. It is important to note that these transcriptional responses are somewhat different from those reported for heat-stressed leaves, where the majority of the regulated genes are involved in thermo-tolerance and protection of the photosynthetic apparatus (Sullivan et al., 2014; Usadel et al., 2009; Barah et al., 2013; Zhu et al., 2013). The data indicate that there is a significant and early remodeling of the root transcriptional program in response to heat stress, presumably to maintain vital biological processes.

Under continuing predicted climate change conditions, it is important to develop stress-resistant crops. It was proposed that transcriptional network analysis can significantly contribute to the identification of new maker genes for potential use in plant breeding programs (Gehan et al., 2015). For example, previous regulatory network analysis in Arabidopsis and rice plants identified the TFs HSF, NF-X1, NF-Y, ZIM, bHLH, MYB and DREBP as key to the transcriptional response to heat stress (Barah et al., 2013, Sarkar et al., 2014). Additionally, it was demonstrated that the rice transcriptional response to heat stress is mainly controlled by three gene regulatory modules (Sarkar et al., 2014). Similarly, our gene regulatory module analysis indicates that relatively few TFs are the main regulators of the heat stress response in soybean roots. However, these TFs are predicted to act in a combinatorial manner to control ten different regulatory modules. Specifically, these TFs are HSF (Glyma.03G157300), AP2/EREBP (Glyma.13G152000), MAD-box (Glyma.07G181600) and two WRKYs (Glyma.17G097900and Glyma.19G020600). Interestingly, previous studies in other plant species support the participation of these TFs in the plant response to different abiotic stresses, including heat stress (Lata et al., 2011; Rushton et al., 2010; Lenka et al., 2011). However, further research focused on these specific TFs and the genes belonging to the identified modules that they control should provide additional mechanistic details to aid efforts to develop more heat tolerant soybean.

Although useful in predicted genes for further study, transcriptome analysis does not predict the level of expression of the encoded proteins. Hence, our proteomic analysis identified 357 proteins whose expression level was significantly affected by heat treatment. Interestingly, in contrast to the enrichment analysis of the transcriptome, the majority of proteins responding to heat are predicted to play a role in thermo-tolerance. For instance, we did identify heat-shock, class I and II, proteins, as well different peroxidases. Furthermore, it was reported that plants can

modify membrane fluidity in response to heat stress (Hasanuzzaman et al., 2013). Consistent with this, we observed that different fatty acid desaturases were down-regulated. Other proteins which cause a significant downward expression of proteins in heat-stressed RHs include histones, which contribute to chromatin structure (Berger, 2007). Previous research also implicated histone modifications as playing an important role in plant adaptation to abiotic stresses (Pecinka et al., 2012). For instance, it was reported that the occupancy of the histone H2A.Z, which tightly wraps the DNA, is reduced in heat-stressed Arabidopsis plants (Kumar and Wigge, 2010). This reduction in H2A.Z occupancy has a positive impact on the expression of heat-stressed induced genes (Kumar and Wigge, 2010). Thus, down-regulation of the soybean histone H2A by heat could contribute to an activation of chromatin regions supporting the expression of different genes in heat-stressed RHs.

Finally, coupling transcriptomic and proteomic analysis of the same samples provides the opportunity to directly compare the data obtained. As seen in a number of previous studies (Petrica et al., 2012; Stevens and Brown, 213; Ponnala et al., 2014), there was a relatively low, overall correlation between the protein and mRNA expression levels. This is not unexpected since translation is governed by a variety of regulatory mechanisms, independent of transcription rate. These include the impacts of miRNA, mRNA half-life, translational rates, as well as protein turnover (Petrica et al., 2012).

In conclusion, our results clearly demonstrated that roots respond strongly to heat stress and that the response of the single cell RHs is quite distinct from that of the remaining root tissue. The datasets generated provide a rich resource for further study and efforts to develop crop plants that can withstand the impacts of a changing climate.

Chapter 2

Identification of Metabolites in Soybean Root hairs and Stripped Roots in Response to Heat Stress

Josef Batek[1], Nicolas Gomez Hernandez[1], Cuong T. Nguyen[1], , Ning Zhang[2], Trupti Joshi[2, 3,4] Jennifer Kyle[5], Kim K, Hixson[5], Karl K, Weitz[5], Joshua T. Aldrisch, Young-MO Kim[5], Ljiljana Paša-Tolic[5] and Gary Stacey[1**]

[1] Division of Plant Sciences and Biochemistry, National Center for Soybean Biotechnology, C.S. Bond Life Sciences Center, University of Missouri, Columbia, MO. 6511, USA
[2] Informatics Institute, C.S. Bond Life Sciences Center, University of Missouri, Columbia, MO 65211, USA.
[3]Department of Computer Science, University of Missouri, Columbia, MO 65211, USA
[4]Department of Molecular Microbiology and Immunology and Office of Research, School of Medicine, University of Missouri, Columbia, MO 65211, USA
[5]Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA 99352, USA

[**] Correspondence:
Gary Stacey
Division of Plant Sciences
271 E Christopher S. Bond Life Science Center
University of Missouri
Columbia, MO 65211, USA
Phone: 573-884-4752
staceyg@missouri.edu

Abstract

Soybean is the most important legume crop worldwide and, therefore, will be among the most important crops to be affected by the predicted increase in global temperature due to climate change. Heat stress may adversely affect various stages of soybean process such as germination, growth, reproduction and yield. A number of previous studies documented the effect of elevated temperature on soybean, above ground organs, but few focused on the response of roots to heat stress. Therefore, in the current study, we focused on characterizing the response of soybean root hairs, a single cell model system, and the corresponding stripped roots (i.e., roots lacking root hairs). Mass-spectrometry was used to characterize the soybean root metabolome with specific focuses on changes in response to heat stress. These studies identified 54 differentially regulated metabolites and seven classes of lipid species that were differentially accumulated in response to heat stress. Among the metabolites identified whose abundance showed a significant response to heat stress were members of the Raffinose Family of Oligosaccharides (RFOs) and lipid species such as glyerolipids and glycerolphospholipids. The data presented represents a detailed study of the soybean root response to heat stress with a specific focus on a single differentiated cell type, the root hair.

Introduction

Soybean (*Glycine max* L.) is the most important commercial legume crop worldwide and a major source of protein and vegetable oil for both human and animal consumption. Plant metabolism, growth, and productivity are negatively affected by various environmental stresses such as drought, cold, salinity and high temperature, drastically affecting crop yield (Hasanuzzaman et al., 2013). Climate model forecasts predict significant increases in mean

global temperature, which will likely negatively affect worldwide crop production Intergovernmental Panel on Climate Change (http://www.ipcc.ch). Heat stress may adversely affect various developmental stages of plant process such as germination, growth and reproduction. Hence, in order to breed for more tolerant plants it is crucial to understand the molecular and biological process that plants employ to cope with adverse climate changes.

Heat stress can trigger a metabolic imbalance by affecting or altering proteins, membranes, cytoskeleton structures, and enzyme activity (Bita and Gerats, 2013). Since plants are sessile organisms, they have developed complex regulatory pathways to recognize and adapt to a wide range of environmental changes such as changes in leaf orientation, early maturity, and modification of membrane lipids. Another common defense mechanism is the accumulation of compatible solutes that are able to maintain cell turgor via osmotic adjustments. Stressed plants also produce antioxidant systems to maintain cellular redox balance (Hasanuzzaman et al., 2013, Janska et al., 2010).

Most previous studies of plant response to heat stress have exclusively measured the response of whole plants or organs with a bias toward above ground tissues. These studies suffer from signal dilution, which disregards the response contribution of single cells within the plant. Hence, the data obtained from such studies cannot distinguish, for example, the expression of a gene expressed at a low level in all cells from that expressed at a high level but only in a few cells or specific cell type. In order to overcome this signal dilution effect, there is a need for methods to conduct functional genomic studies of single cells or single cell types. In the present study we utilized soybean root hairs, which represent an established single cell model for studies of root system biology (Libault et al., 2010a). Root hairs are single cell extensions of the root epidermis that function primarily in water and nutrient uptake. Therefore, these cells would be

expected to be among the first to recognize and to respond to environmental changes (Libault et al., 2010a, Brechenmacher et al., 2010).

Metabolites such as carbohydrates, amino acids, organic acids and lipids are the end product of gene expression and their corresponding levels can be interpreted as the ultimate response to specific environmental responses. Hence, the knowledge of transcript and protein levels involved in a specific response cannot alone facilitate a deeper biological understanding. Therefore, establishing a direct relationship between transcriptomic, proteomic, and metabolomic levels will allow us to gain a deeper insight in the actual physiological response to environmental changes (Lu et al., 2013, Zivy et al., 2015). Metabolomic approaches have been successfully employed to study environmental changes (Rodziewicz et al., 2013). However, most previous studies focused on above ground plant organs (e.g. leaves) in response to heat stress. In our study, we focused on below ground tissues; roots hairs (a single cell model) and stripped roots (i.e., root lacking root hairs) due to their crucial role in plant nutrient uptake and metabolism

Root metabolites were extracted from soybean seedling root hairs (RH) and stripped roots (STR, roots without root hairs) either mock treated or subjected to heat stress treatment (40°C). Metabolite extracts of soybean root hairs and stripped roots were profiled by gas chromatography-mass spectrometry (GC-MS) and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). Analysis of the data identified 43 metabolites whose abundance was significantly impacted by heat stress. Among the compounds showing the most significant response to heat stress were carbohydrates from the raffinose family (Galactinol and Raffinose), glycerophospholipids, and phospholipids. The metabolomic profile presented represents an in-depth analysis of a single cell type in soybean.

1. Material and Methods

Growth, Root Hair, and Stripped Root Isolation

Soybean (*Glycine max* 'Williams 82) seeds were surface sterilized by soaking twice in 20% (v/v) bleach for 10 min each, followed by treatment for 10 min with 0.1 N HCl. The seeds were subsequently washed five-times in sterile water and then dried in air for 20 min. Sterilized seeds were placed on nitrogen-free B&D agar medium (Brought and Dilworth, 1971) in 20-cm-diamerter glass plates, and grown for 3 days in a dark growth chamber (25°C, 80% humidity). About 1,000 soybean seeds were used per treatment/time point.

In order to apply heat stress, 3 days old seedlings were incubated at 40°C for 3, 6, 12 and 24 h, respectively. These conditions were previously shown to result in a maximal response as measured by transcriptome and proteome analysis (Valdez-Lopez, et al., submitted) As controls, seedlings were grown similarly but not subjected to heat stress (i.e., maintained at 25˚C). After the heat treatments, root hairs were isolated using a method previously described (Wan et al., 2005; Brechenmacher et. al., 2009a). Briefly, the roots were separated from shoots and collected in liquid nitrogen. The roots were gently stirred for 20 min allowing the separation of the root hairs cells from the root. The liquid nitrogen was filtered through a wire mesh (~250 um) to separate root hair cells from stripped roots (roots with no root hair). The tissues were weighed (e.g., average of 1g of root hair tissue) and stored at -80°C until metabolite extraction. Three biological replicates were generated for each time point, in each biological replicate a total of 1000 seeds were used.

1.1 GC-MS

Gas chromatography-mass spectrometry (GC-MS) analysis of soybean root hairs and roots were carried out as described previously (Brechenmacher et al., 2010a). Root hairs and

stripped roots were lyophilized until dry. The dried roots were homogenized using mortar and pestle, and 1 mg of homogenized tissue was transferred to a 4-mL glass vial. Metabolites were extracted by adding 1 mL of chloroform, vortexed, and incubated for 45 min at 50°C in HPLC-grade water (1.5mL). The two phases were separated by centrifugation at 2,900 x g for 20min at 4°C. One mL of each layer was collected into a vial and dried in a speed vac vacuum separately. The non-polar layer was saved for lipid analysis. For the dried polar layer residue, chemical derivation was performed with methoxyamine hydrochloride (50 uL at 15 mg mL^{-1}). Compounds were sonicated until dissolved and incubated for at least 1 h for methoximation. Silylation was performed by incubating the samples for 1 h at 50°C with 50uL of N-methyl-N-trimethylsilyltrifluoroacetamide and 1% (w/v) of trimethylchlorosilane (Thermo Fischer, San Jose, CA). The final reaction mixture was split into two vials for duplicated injections on the GC-MS. The samples were equilibrated at room temperature and transferred to a glass inserts, and analyzed using Agilent 7890A GC apparatus equipped with a 30 m Agilent DB-5MS column was coupled to a 5975C MSD detector (Agilent Technologies, Inc.; Santa Clara, CA, USA).

1.2 UPLC-ESI-MS

Ultra-performance liquid chromatography/electrospray-mass spectrometry (UPLC-ESI-MS) was performed as previously described by (Gao et al., 2012). Briefly, as above lipids were extracted from soybean root hairs and stripped roots using cold (-20°C) chloroform/methanol (2:1, *v/v*) in a 5:1 ratio over the sample volume. The mixtures were vortexed and chilled in ice for 10 min, samples were then centrifuged at (13,523xg from 10min). After centrifuge separation 200 uL of the chloroform layer was removed and further dried in vacuum (Thermo Fischer Scientific, San Jose, CA). Lipid extracts were reconstituted in 200 µL of isopropanol containing 10mM ammonium acetate.

Four uL of reconstituted lipids were loaded to a small capillary column (180 um i.d.x2 cm) packed with reverse-phase particles (Symmetry C18, 5 µm; Waters, Milford, MA) under the following isocratic conditions: (solvent A) 93% acetonitrile/water (40:60, v/v) containing 10 mM ammonium acetate and (solvent B) 7% acetonitrile/isopropanol (10:90, v/v) containing 10 mM ammonium acetate. The lipids retained on the trapping column were then forward-flushed to the analytical column using gradient elution as follows: t = 0 min, 10% B; t = 2min, 30% B; t = 10 min, 40% B; t = 20 min, 55% B; t = 40min, 60% B; t = 70 min, 99.5% B; t = 90 min, 99.5% B; t = 95 min, 60% B; t = 97 min, 60% B; t = 98 min, 99.5% B; t = 100 min, 99.5% B; t = 102 min, 10% B; t = 130 min, 10% B (Gao et al. 2012). An analytical column was slurry packed as previously described by (Ding et al. 2008) with (HSS T3, Jupiter C18, C8) (5 µm; Waters Corporation, Milford, MA), connected to a Waters NanoAcquity UPLC system. The columns (150 um x 20 cm) were maintained at 40°C in a column oven.

The LC system was interfaced to a LTQ-Velos Orbitrap mass spectrometer (Thermo Fisher, San Jose, CA) coupled to a chemically etched ESI (electrospray ionization) emitter. The heated capillary temperature was 350°C and the spray voltage was 2.2 kV. Data-dependent MS/MS scan events were carried out in the ion-trap (collision-induced disassociation; CID) or Orbitrap (Higher-energy collisional disassociation; HCD) using a normalized collision energy (NCE) of 35 and 40 arbitrary units, respectively. CID and HCD were both set with a maximum charge state of 2 and an isolation width of 2 *m/z* units The activation q value of 0.18 was employed for CID , the full scan mass ranges for both positive and negative ESI modes were 300-2,000 *m/z* and 200-2000 *m/z* for each mode respectively. Each sample was analyzed in both ESI modes (positive and negative) (Gao et al., 2012).

Data was acquired under the control of Thermo Xcalibur software (Thermo Fisher). The raw MS data files were subjected to deisotoping, yielding monoisotopic mass, charge state, and intensity of major peaks using Decon2LS (Jaitly et al., 2009). Data files were further analyzed using an in-house software LIQUID (Lipid informed Quantification and Identification (Pacific Northwest National Laboratory, WA) allows users to conduct informed and high-throughput global liquid chromatography-tandem mass spectrometry (LC-MS/MS). LIQUID is user friendly software that enables users to quickly identify and quantify lipids from LC-MS/MS datasets. In addition to plots, LIQUID provides information such as intensity, mass measurement error, and elution times.

1.3 Metabolomic Data Analysis

GC-MS raw data files from each experiment were processed using metabolite detector software version 2.0.6 beta (Hiller et al., 2009). Metabolites were initially identified by matching experimental spectra to an augmented version of FiehnLib (Kind et al., 2009) that contains spectra and validated retention indices for over 700 metabolites. Finally, unidentified and identified features were transferred to DAnTeR (Taverner et al., 2012) for further visualization and statistical analysis after logarithmic transformation. Raw data were log transformed and globally normalized with Central Tendency Method around the median. Fold-change was calculated as the difference of the logarithmic means between treatments. Statistical analysis was done via ANOVA, to identify metabolites significantly regulated in root hairs and stripped root in response to heat stress. P values were adjusted with Benjamini & Hochberg method (Benjamini and Hochberg, 1995). All metabolites having $P < 0.05$ were considered significantly regulated.

For the UPLC-MS data sets an initial filter was applied, lipids were removed if more than 50% of the values were missing while comparing two conditions [25°C (control) and 40°C(treatment)], the missing intensity values were estimated using a small value (half of the minimum positive values in the original data). The data was further normalized with the Central Tendency Method around the median. The normalized data was then transformed to logarithm base of 2. The significantly differentially expressed lipids were determined using a one sample t-test to identify metabolites significantly regulated in root hairs and stripped roots in response to heat stress (40°C), using a combined cutoff of fold change ≥ 2 or $\leq 1/2$ and p-value ≤ 0.05.All metabolites having $P< 0.05$ were deemed significantly regulated.

Results and Discussion

In an effort to identify metabolites that significantly respond to heat stress in soybean root hairs and stripped roots (roots without root hairs) a metabolomic profile of both tissues was performed. The metabolomic changes were compared after heat exposure at various time points (3h, 6h, 12h, and 24h). Heat stress was imitated by subjecting samples to 40°C heat treatment with a control temperature at 25°C. Three biological replicates for each time point were collected and extracted metabolites were analyzed via GC-MS and UPLC-MS. Principal component analysis (PCA) was conducted for data overview in MetaboAnalyst 3.0 (Xia et al., 2015), clearly segregating metabolites that were significantly regulated in response to heat stress in both tissues. These data depicts metabolites and lipids that were segregated by time points, heat treatment and tissue (Supplementary Figure 6).

Metabolic Response to Heat Stress

GC-MS analysis revealed a total of 201 different compounds detected in root hairs and stripped roots. However, only 93 of these compounds could be tentatively identified to the

corresponding metabolomic libraries and/or available literature (Supplementary Table 4). Metabolites identified in at least one exposure time include sugars, amino acids, organic acids and nucleotides present in both tissues.

A lipidomic (UPLC-MS) profile analysis was performed in order to identify classes of lipids that responded to heat stress. A total of 63 and 56 lipids were detected in root hair and stripped roots, respectively. Classes of lipids that were deemed significantly regulated by heat stress include glyerolphospholidipds (Triacylglycerolipids, TG; Diacylglycerolipid, DG) and glyerolphospholipid (PA; Phosphatic Acid, PC; Phosphatidylcholine, PE; Phosphatidylethanolamine, and PG; Phosphatidylglycerol).

Collectively a total of 320 compounds were identified from GC-MS and UPLC-MS in at least one time point, 93 were identified as known compounds compared to Fiehns metabolomic library, and 7 classes of lipids were identified in response to heat stress in both tissues. Each compound was detected according to its unique retention time and mass-to-charge ratio and matched to a known metabolomic library (Supplementary Table 4, Supplementary Table 5).

Significantly Regulated Metabolites in Response to Heat Stress

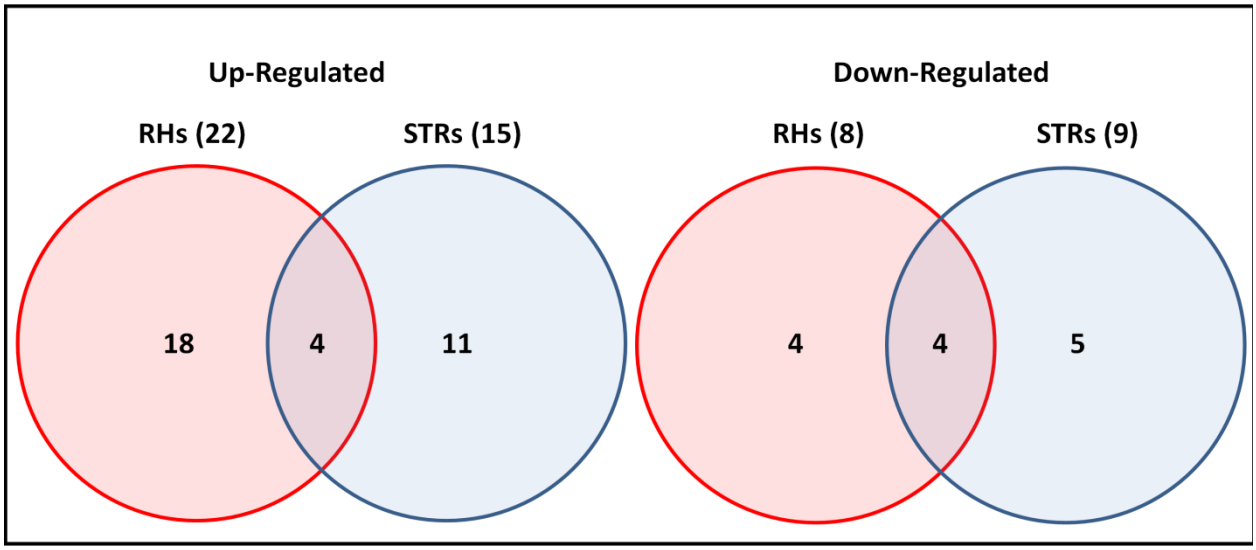
Statistical analysis was performed to identify metabolites that were significantly regulated in root hairs and stripped roots in response to heat stress. After GC-MS/MS statistical analysis, heat treatment (40°C) suggested that the expression levels of 30 metabolites in root hairs and 24 in stripped roots compared to control (25°C) were differentially accumulated across all time points (Table 3).

Table 3. Metabolites significantly regulated in soybean root hairs in response to heat treatment (40°C) at four exposure times (3, 6, 12, and 24 h). (Fold change >log2, Adjusted P value <0.05 deemed statistically significant)

Treatment	Metabolite	FC	Pval	Metabolomic Pathway	Classification
3H	Cellobiose	-1.2282	0.00042	Starch and Sucrose Metabolism	Sugar
	Galactinol	2.8861	8.42E-10	Galactose Metabolism	Sugar alcohol
	Glycerol 3-phosphate	1.7347	0.000523	Glycerophospholipd Metabolism	Glyerophospholipid
	Raffinose	2.2789	2.79E-08	Galactose Metabolism	Sugar/trisaccharide
6H	Galactinol	3.0711	5.88E-16	Galactose Metabolism	Sugar alcohol
	Glycerol 3-phosphate	1.4035	0.003404	Glycerophospholipd Metabolism	Glyerophospholipid
	N-methylalanine	1.4751	0.017347	Biosynthesis of Amino Acids	Amino Acid
	Raffinose	3.1621	8.69E-15	Galactose Metabolism	Sugar/trisaccharide
12H	Galactinol	3.6162	4.09E-20	Galactose Metabolism	Sugar alcohol
	Glycerol 3-phosphate	2.1997	0.000523	Glycerophospholipd Metabolism	Glyerophospholipid
	Glycine	2.7434	0.006914	Biosynthesis of Amino Acids	Amino Acid
	Malonic acid	-1.61	3.00E-07	Pyrimidine metabolism	Organic Acid
	Myo-inositol	1.045	5.55E-07	Inositol phosphate metabolism	Carbohydrate
	Raffinose	4.0732	1.72E-13	Galactose Metabolism	Sugar/trisaccharide
24H	2-aminoadipic acid	1.2713	0.024934	Amino Acid Biosynthesis	Amino Acid
	3-hydroxy-3-methylglutaric acid	1.903	6.69E-11	Biosynthesis of Amino Acids	Amino Acid
	4-guanidinobutyric acid	1.0394	2.40E-05	Biosynthesis of Amino Acids	Organic Acid
	Adenine	-1.2694	0.003037	Biosynthesis of Amino Acids	Nucleotide
	Aspartic acid	-1.4682	1.82E-11	Biosynthesis of Amino Acids	Amino Acid
	Cycloleucine	1.3239	0.026568	Biosynthesis of Amino Acids	Amino Acid
	D-glucose-6-phosphate	-1.0759	5.81E-11	Glycolysis/Glucogenesis	Sugar/Phosphorylated
	Galactinol	4.7879	4.91E-25	Galactose Metabolism	Sugar alcohol
	Glutaric acid	1.5987	7.82E-05	Biosynthesis of Amino Acids	Organic Acid
	Glyceric acid	1.7135	3.77E-15	Glyerolipid metabolism	Sugar/acid
	Glycerol 3-phosphate	1.7211	0.001808	Glycerophospholipd Metabolism	Glyerophospholipid
	L-glutamic acid	-1.3814	6.31E-07	Biosynthesis of Amino Acids	Amino Acid
	L-tyrosine	1.9579	1.34E-11	Biosynthesis of Amino Acids	Amino Acid
	Malonic acid	-2.2448	1.56E-07	Pyrimidine metabolism	Organic Acid
	Maltose	-1.1363	1.78E-07	Starch and Sucrose Metabolism	Sugar/dissacharide
	Raffinose	6.5466	6.51E-14	Galactose Metabolism	Sugar/trisaccharide

A comparison analysis between differentially regulated metabolites was performed to identify metabolites that were unique or commonly expressed in both tissues. This analysis yielded a total of 54 significantly regulated metabolites detected among both tissues, 4 of these metabolites were commonly regulated in response to heat stress in both RH and STR. In contrast 22 metabolites were regulated only in RH whereas 16 were specifically regulated in STR (Figure 5). A total of 37 metabolites were significantly up-regulated and 17 were down-regulated. Out of the 36 up-regulated metabolites, 4 were commonly shared between tissues and 4 were commonly shared among down-regulated metabolites (Figure 5). Across the 4 exposure times, 70% of the metabolites were significantly up-regulated whereas 30% was down regulated in both tissues.

Figure 5. Total Number of overlapping and non-overlapping heat-responsive metabolites between soybean root hairs (RH) and stripped roots (STR). *(Fold change >log2; 2-fold pairwise comparison [heat-stressed vs. control], Adjusted P value(FDR) <0.05 deemed statistically significant)*

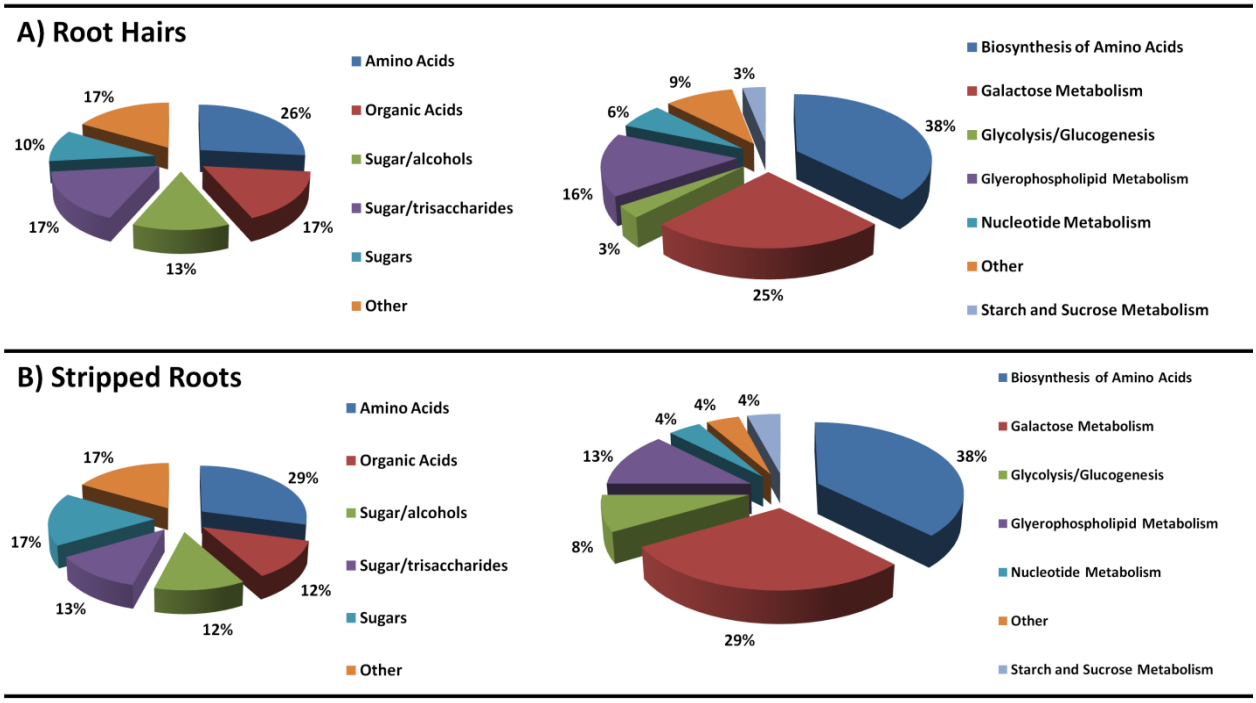


Moreover, we identified a higher number of differentially regulated metabolites in RH than in STR (30 in RHs and 24 STRs, respectively), most likely this resulted from reduced signal dilution in the RH samples relative to the STR samples. The highest metabolomic changes were observed at 24h in both tissues, 46% of the significantly regulated metabolites were identified at this time point (Table 3). Since heat stress can create metabolomic imbalance by altering or affecting genes, proteins, membranes, and enzymatic activity, it is likely that the stronger response at 24h reflects the cumulative effect of a longer exposure time (24h) (Shabrawi et al., 2010, Phang et al., 2008, Grant, 2004). A comparison analysis was performed across the four-treatment times (3, 6, 12 and 24h) for RHs and STRs (Supplementary Figure 7). In RHs, three metabolites were commonly shared among four time points, including two members of the Raffinose Family of Oligosaccharides (RFOs), galactinol and raffinose. RFOs have been previously been assigned important function in plants in response to stress, including

osmoprotection, desiccation tolerance in seeds, transport and signaling (Sengupta et al., 2015**,** Downie et al., 2003, Nishizawa et al., 2008). Glycerol 3-phosphate, an essential component of energy-producing pathways, was also found to be significantly regulated at all time points in root hairs.

All significantly regulated metabolites were classified into various functional categories and metabolic pathways. The metabolites deemed to be statistically significantly, such as galactinol and raffinose, were identified using online databases e.g. KEGG (Kyoto Encyclopedia of Genes and Genomes), Lipidmaps, Plantcyc (Figure 6). Several compounds, including carbohydrates, amino acids, organic acids, and various lipid classes were found to be regulated in response to heat stress in soybean root hairs and stripped roots. Their putative functions and metabolomic implications in relation to heat stress are discussed below in detail.

Figure 6. Classification and metabolic pathway distribution of identified metabolites in root hairs and stripped roots in response to heat treatment. *(Fold change >log2, Adjusted P value <0.05 deemed statistically significant)*



Heat Stress Induces Changes in Carbohydrates Levels

The metabolomic profiling of soybean root hairs and stripped roots revealed that heat stress triggered metabolomic changes of soluble sugar concentrations at various exposure times. In root hairs 40% of the metabolites identified were classified as carbohydrates (e.g. galactinol, raffinose, cellobiose, maltose), and 42% in stripped roots at the different time points. Heat stress triggered the induction in root hairs of galactinol and raffinose (3, 6, 12, and 24 h) and myoinositol (12 h). In contrast, the accumulation of cellobiose (3 h), glucose-6-phosphate (24 h) and maltose (24 h) was reduced in root hairs in response to heat stress (Supplementary Figure 7; Table 3). Galactinol and raffinose were also identified in stripped roots (3, 6, 12, 24 h), while glucose-6-phospahte, fructose-6-phospahte and maltose accumulation was repressed (24 h).

The regulation of carbohydrates observed such as raffinose, and galactinol can be attributed to a common defense mechanism triggered in plants subjected to abiotic stresses were the production and accumulation of compatible sugars function as osmoprotectants. Carbohydrates maintain cellular turgor through osmotic balance regulation defending the plant from cellular degradation (Krasensky et al., 2012, Banu et al., 2010, El-Shabrawi et al., 2010). Previous RFOs studies reported that carbohydrates have multiple functions, in addition to osmotic adjustment, including expressing or altering antioxidant systems to maintain cellular redox balance, and acting as signaling molecules that may have a crucial role in response to abiotic stresses (Sengupta et al., 2015).

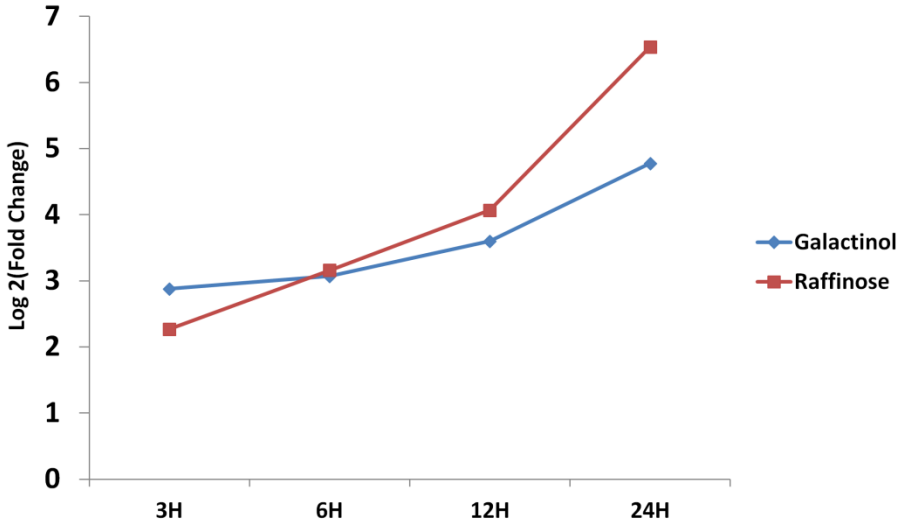
Raffinose Family Oligosaccharides (RFOs) *Galactinol and Raffinose*

Raffinose family oligosaccharides (RFOs) have a broad range of putative functions. RFOs have been implicated in membrane protection, desiccation tolerance of seeds, several

cellular functions such as membrane trafficking, transport, storage of carbon, radical scavenging, and accumulating in response to various abiotic stresses in vegetative tissues (Sengupta et al., 2015, Nishizawa et al., 2008, Peters et al., 2010).

Initially galactinol and raffinose accumulation was triggered at 3 h of heat exposure in RHs and STRs. After fold change were converted to log2, galactinol and raffinose increased significantly across all time points in root hairs (Figure 7). In stripped roots, both compounds were also detected at all time points. However, at 6 h galactinol and raffinose at 12 h, showed no significant differences between control and treatment (Supplementary Table 4). This may be due to a reduction in the signal due to tissue dilution resulting from measurements across all cell types in stripped root samples. In RHs, both compounds demonstrated a gradual linear increase across exposure times, with a maximum response at 24 h. Galactinol and raffinose accumulation correlated with the increase of heat exposure time (Figure 7).

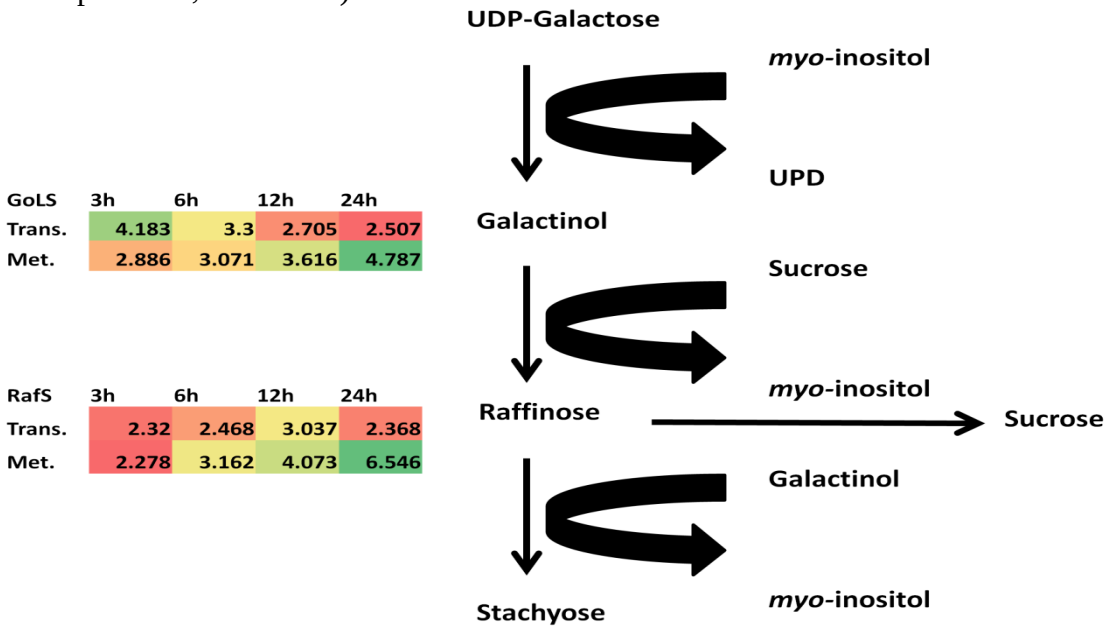
Figure 7. Raffinose Family Oligosaccharides (RFOs) in response to heat treatment. Blue; Galactinol; Red; Raffinose. *(Fold change >log2, Adjusted P value <0.05 deemed statistically significant)*



As previously stated RFOs have been characterized in several organisms and implicated in crucial physiological functions. Metabolic pathway analysis was performed using KEGG

database (http://www.genome.jp/kegg/) revealing RFOs enzyme activity. The first committed step in the biosynthesis of RFO is the formation of galactinol from myo-inositol (substrate in the biosynthesis of RFOs), and UDP-galactose catalyzed by galactinol synthase (GolS, E.C. 2.4.1.123) an obligatory enzyme for RFOs biosynthesis. The formation of RFOs is followed by the sequential addition of galactose units contributed by galactinol, to sucrose leading to the formation of raffinose and stachyose mediated by two other major enzymes raffinose synthase (RafS, EC 2.4.1.82) and stachyose synthase (StaS, EC 2.4.1.67) (Hincha et al., 2003, Peterbauer and Richeter, 2001). We modified the metabolic pathway output from KEGG in order to perform a comparison between the metabolomics data and previous transcriptomic data generated from soybean root hairs in response to heat stress (40°C) (Valdez-Lopez et al., Submitted). In order to assess the correlation between transcriptomic and metabolomic profiles, a pairwise comparison was performed between the mRNA levels and significantly regulated metabolites (Figure 8).

Figure 8. Biosynthetic pathway of Galactinol, Raffinose, Stachyose in Plants. Correlation between transcriptomic and metabolomic data (Trans: Log2 fold change, of transcripts; Met: Log2 fold change of metabolites regulated in response to heat stress. Transcriptomic data from (Valdez-Lopez et al., Submitted).



The comparison was made by obtaining the putative enzyme or KEGG accession/ EC numbers (GolS, E.C. 2.4.1.123; RafS, EC 2.4.1.82; RafS, EC 2.4.1.82) that were closely associated with our significantly regulated metabolites galactinol and raffinose. The enzyme sequences were extracted and compared against Phytozome Version 2.2 (http://www.phytozome.net), database yielding the corresponding GlymaIDs (GolS: Glyma.10G145300.1; RafS: Glyma.06G179200.1; STS: Glyma.19g217700.1). Interestingly the mRNA levels of GolS: Glyma.10G145300.1 and RafS: Glyma.06G179200.1 were significantly regulated in response to heat stress in our previous transcriptomic data sets.

The analysis yielded a low correlation value (-0.2-0.69) between mRNA and metabolomic expression levels. The integration of transcript and metabolite data revealed the variation in metabolite flux and transcript abundance in response to heat stress in the pathway. Furthermore, although metabolites are the end product of gene expression levels, the low correlation suggest that changes in the transcriptome do not always reflect changes in the metabolome that are triggered by environmental stresses. This can be accounted for the fact that metabolomic regulation also takes place in other cellular levels such as posttranscriptional regulation, RNA processing, translational or posttranslational (Hamanishi et al., 2015, Urano et al., 2009, Kaplan et al., 2007). However, metabolite-transcript relationships from the same metabolic pathway were identified and may be useful for a deeper understanding of critical heat response mechanisms in soybean root hairs.

Heat Stress Regulates Amino Acid Abundance

Amino acids showed a variable response to heat stress across all time points in both root hairs and stripped roots. In RHs and STRs, amino acids accounted for 26% and 29% respectively, of significantly regulated metabolites responding to heat stress. In RHs, 75% of the

amino acids that were regulated in response to heat stress were induced, in contrast 57% that were down regulated in STRs. Data analysis showed that the highest amino acid abundance level changes triggered by heat stress in RH and STRs, were at 12 h after heat exposure. (Table 3) N-Methylalanine (6h), methylglutaric acid (24 h), cycloleucine (24 h), and tyrosine (24 h) were induced specifically in RH, whereas L-homoserine (12 h) and L-proline (12 h) were induced specifically in STRs. Glycine, aspartic acid, and L-glutamic acid showed similar abundance levels in both tissues (Table 3, Supplementary table S4).

Soybean accumulates compatible solutes (low-molecular weight) such as amino acids, which do not interfere with basal physiological functions even at high concentration levels. Amino acids maintain cellular turgor by osmotic adjustment, detoxification of reactive oxygen species, and by intracellular pH regulation (Hasanuzzaman, et al., Lu et al., 2013, Zhifang et al., 2003, Silvente et al., 2012) Amino acids have a crucial central role in metabolism at various cellular levels. Heat stress may stimulate rapid conversion from amino acids to sugars that accumulate in response to stress (Krasensky and Jonak, 2012).

The negative response of glutamic and aspartic acid to heat stress maybe due to a decline in protein concentration, which is the result of the accumulation of higher molecular weight compounds in response to heat stress (e.g. Carbohydrates and Lipids). Glutamic acid (Glu) is a precursor of 4-aminobutyric acid (GABA), whose abundance was previously shown to change in response to a wide range of abiotic stresses, including, heat, drought, cold, salt, and mechanical damage (Kinnersley and Turano, 2000). For example, Mayers et al., 1990 showed that cowpea cells exposed to heat stress doubled GABA concentration within 15 minutes with an even greater increase by at 24 h. GABA accumulation was correlated with an increase in cytoplasmic pH

levels and cytoplasmic calcium; and a decrease of L-Glu concentration was observed in both tissues after 24 h of exposure (Bown and Shelp, 1997).

The Aspartate Family (Asp-family) of amino acids lead to the biosynthesis of amino acids Lys, Thr, Met, Ile and Gly and, hence this family impacts numerous metaboloic pathways. Moreover, aspartate is closely linked with exposure to environmental stresses via the connection/linking with tricarboxylic acids (TCA), which play crucial roles in response to abiotic stresses. Since heat stress is associated with a reduction of biomass accumulation, a decrease of both compounds can be attributed to heat exposure (Misyura et al., 2014, Kirma et al., 2012).

Heat stress alters the metabolomic profile of soybean RHs and STRs, including a decrease in protein synthesis. Since, amino acids are chemical building blocks their tendency to accumulate upon stress onset might be directly related to growth recovery (Obata and Fernie, 2012). Glycine accumulation in response to stress was observed at 12 h in both RH and STR tissues. Previous studies indicate that glycine accumulated in response to a wide range of environmental stresses across various crop species and tissues (Lu et al., 2013, Thakur and Rai, 1982, Galili and Less, 2008).

Proline abundance is well documented to change in response to various abiotic stresses (e.g. drought). Therefore, it is not surprising to see that heat stressed affected proline levels in soybean roots. Proline acts as an osmoprotectant by stabilizing protein structure, and scavenging free radicals. However, the role of proline in response to heat stress remains controversial. Some authors reported that proline accumulated in response to drought stress but does not accumulate during a combination of drought and heat stress (Hayat et al., 2012, Versules and Sharma, 2015). The low levels of proline expression suggest that it is rapidly synthesize to generate larger

compatible solutes such as carbohydrates, which in turn become the major contributors for osmoregulation in response to heat stress in RH.

Our results suggest that the amino acid concentrations of soybean RHs and STRs were altered after heat treatment at various time points. However, the response of individual amino acids is complex, likely reflected their role in a variety of metabolic processes.

Organic Acids

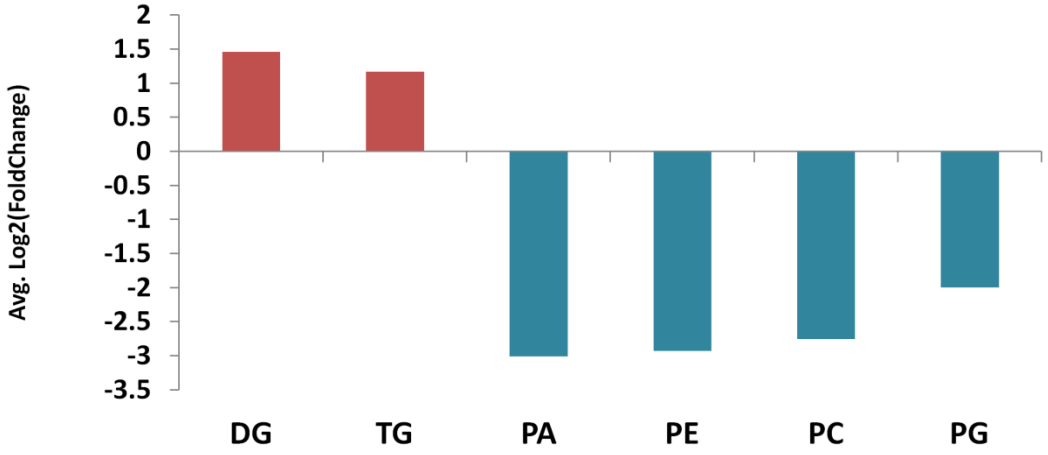
Organic acids showed a variable response to heat stress. The content of malonic acid (12 h, 24 h) decreased in RHs, while an increase of 4-guanidobutryic, glutaric and glyceric acids was observed at 24 h in RHs. In STRs fumaric acid (24 h) was reduced, while an increase in glycolic (12 h) and hydroxylglutaric acid (24 h) was detected (Table 3) .Organic acids have crucial roles including photorespiration, transport, oxidative defense, and as osmoprotectants in response to environmental stresses (Sicher, 2015, Wahid et al., 2007). Previous studies reported a decline in dicarboxylic acids (malonic and fumaric acids) in response to elevated temperatures in soybeans leaflets, which correlates with the observations in this study (Yu et al., 2012). Malonic and fumaric acid are involved in several metabolic pathways and their ionized form (malonate and fumarate) are indirectly or directly involved in the Tricarboxylic Acid Cycle (TCA). Fumarate is a crucial intermediate of the TCA cycle, while malonate may act as a major competitive inhibitor of succinate (TCA intermediate), and it may also act as an osmoprotectant against environmental stresses (Sicher, 2015, Yu et al., 2012, Li and Copeland, 2000). The study suggests that heat stress impairs the synthesis of TCA cycle intermediates/compounds, which in turn create a negative energy flux in RHs and STRs.

Other organic acids identified in response to heat stress were glycolic, glutaric, and glyceric acids that are primarily involved in the biosynthesis of amino acids, and act as compatible solutes in response to stress. Interestingly, Glycerol 3-phosphate (G3P) was induced at all time point in RHs and at (6h; 24h) in STRs. G3P is an obligatory component of high energy metabolic pathways such as glycolysis and glycerolipid biosynthesis and has been implicated in signaling and defense against biotic and abiotic stresses (Xia et al., 2009).

Lipids

The response of specific lipid species was profiled in RHs and STRs in response to heat stress. A total of seven lipid classes were identified to be significantly regulated in response to heat exposure. The changes in lipid species are presented as the average fold-change response to heat stress detected at all time points for both tissues (Figure 9, Supplementary Table 5). Four classes of glycerolphospholipids (PA; Phosphatic Acid, PC; Phosphatidylcholine, PE; Phosphatidylethanolamine, and PG; Phosphatidylglycerol) and two glycerolipids (DG: Diacylglycerol and TG: Triacylglycerol) showed significant changes in response to heat stress in root hairs. A same pattern was also observed in stripped roots, with the exception of the accumulation of a sphinglolipid observed exclusively in roots (Ceramide).

Figure 9. Average expression levels of lipid class composition in response to heat stress at (3,6,12 and 24h). A) RHs B) STRs *(Fold change >log2; 2-fold pairwise comparison [heat-stressed vs. control], P <0.05 deemed statistically significant)*



Lipid profiles are influenced by plant species, genotype, agricultural practices and environmental stresses such as temperature. Moreover, they also perform several key physiological functions in plant growth and development such as energy reserves, structural components of cellular membranes, and intra- and intercellular signaling (Bita and Gerats, 2013, Zhang and Xiao, 2015). Heat stress triggers several physiological changes that can disrupt membrane composition and functions. Thus, maintaining the overall integrity and fluidity of membranes is crucial for the ability of the plant to tolerate heat exposure (Ahmad et al., 2013, Tsvetkova et al., 2002).

The main components of membrane lipids are classified as glyerophospholipids such as (PA, PC, PE, and PG). Since the plasma membrane is an important target for heat stress the identification of these lipids in response to environmental stresses has been extensively studied (Balogh et al., 2013, Janda et al., 2013). In the present study, glycerophospholipids (PA, PC, PE and PG) were repressed during heat stress, while glyerolipids (DG, TG) were induced (Figure 9). Glycerolipids (DG, TG) constitute the most important seed storage reserve in several plant

species including soybean (Graham, 2008). Previous untargeted metabolomic analysis in Arabidopsis seedlings revealed that levels of polyunsaturated triacyglycerols were increased rapidly and dramatically in response to heat stress, which is consistent with our findings (see Supplemental Table 2) (Mueller 2015). Moreover, DGs and TGs were shown to accumulate in soybean leaf in response to water stress (Wilson et al., 1985). One proposed metabolitic pathway for TG biosynthesis is the Kennedy pathway, G3P $\longrightarrow$ lysophophatidic acid (LPA) phosphatidic acid (PA) $\longrightarrow$ diacylglycerol (DG) $\longrightarrow$ triacylglycerol (TG), which is the direct incorporation of fatty acids into G3P to yield TGs (Bates et al., 2012, Tjellstrom et al., 2012). Both glycerolipid and GP biosynthesis share common intermediates such as PA which is a universal intermediate for GP synthesis. Furthermore, once PA is dephosphorylated it may yield DGs which in turn will be converted to TG. PA may also act as a substrate for membrane phospholipids. In addition, PCs with modified acyl chains may also be converted to DG that can be acylated to yield TGs under the regulation of DGAT (acyl-CoA:DAG acyltransferase (Nakamura et al., 2007, Bates and Browse, 2012). Previous studies identified phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) to be key precursors for lipid biosynthesis and their abundance was shown to respond to environmental stress (Welti et al., 2002).

Heat-induced glycerolipid accumulation may be due to an increase in TG biosynthesis or a reduction in TG breakdown. The accumulation of glycerolipids in response to heat stress may reflect decreased degradation and turnover of the cellular TG pool created by limited fatty acid consumption that may be triggered by heat stress. Moreover, accumulation of TG may also reflect alternative lipid remodeling such as lipid channeling or other posttranscriptional mechanisms (e.g. mRNA, RNA binding, feedback mechanisms) and may not be driven by

massive de novo fatty acid biosynthesis (Mueller 2015). In this study, we identified seven lipid classes that were significantly regulated in response to heat stress. The accurate quantification of lipid species may provide important insights into their response to abiotic stresses.

Conclusion

In an effort to gain a deeper understanding of the actual physiological response to heat stress in soybean root hairs and roots, metabolomic profiling of root tissues was performed. Most of the organic compounds identified such as galactinol, raffinose, glycerolipids and phospholipids were reported in previous studies of above grown tissues to respond to heat stress (Egert et al., 2013, Nishizawa et al., 2008, Mueller et al., 2015). However, the current study is the first time that such compounds were identified in soybean root hairs (single cell) in response to heat stress.

The results presented in this study are the completion of a larger comprehensive systems biology analysis performed in our laboratory to analyze the response of soybean [*Glycine max* L. (Merrill) cv. Williams 82] root hairs and roots in response to heat stress (transcriptomic and proteomic) (Valdez-Lopez et al., Submitted). The transcriptomic analysis revealed that on average 2,013 genes were differentially regulated in response to heat stress. Moreover, a computational gene regulatory module analysis established that ten different regulatory modules regulate transcriptional response to heat stress in soybean root hairs. Interestingly, one of the ten modules predicted TF WRKY encoded by (Glyma.17G097900.1) regulates 80% of the ten modules identified, suggesting that it may act as a master regulator of heat stress response in soybean root hairs. We also performed a microsomal fraction proteomic analysis that identified 244 proteins differentially accumulated in response to heat stress in root hairs. A functional

enrichment analysis depicted that most proteins identified were predicted to play a role in thermo-tolerance, at our earliest treatment time (3h) 84% of them were involved in response to environmental stimuli (Valdez-Lopez et al., Submitted).

Furthermore, in order to integrate our multi-omics studies, we performed pairwise comparisons. Although, the analysis gave a relative low correlation values (-0.2-0.79) between mRNA and protein expression levels, on average, 61% of the mRNAs expression values were identified in the significantly regulated proteins. In order to reduce the complexity of the protein profile and to determine how membranes respond to heat stress, we targeted our proteomic analysis to microsomal (membrane) fraction. In an effort to correlate the proteomic and metabolomic data, we performed a pairwise comparison that identified no common shared components. Since about 75% of proteins in plants are synthesized in the cytoplasm, the absence of proteins shared between our proteomic and metabolomic analysis can be explained by the loss of key cytoplasmic proteins necessary for the production of metabolites (Spremulli, L. L, 2000). For a complete integration of our omics profiles in soybean root hairs and roots in response to heat stress, a global proteomic analysis would be ideal. Moreover, we performed the same comparison for our transcriptomic and metabolomic data, and found two key regulators in the biosynthesis of RFOs, that were up-regulated at all exposure times GolS: Glyma.10G145300.1 and RafS: Glyma.06G179200.1. These enzymes could be key targets for breeding stress tolerant plants.

Advances such as network modeling are necessary for accurate multi-omics data integration (Aderem, 2005). It is hoped that parallel advances between bioinformatics and biotechnology will provide an accurate integration of the data sets presented, providing us a deeper understanding of the responses of soybean root hairs and roots in respect to heat stress.

Appendix

Supplementary Information

Table S1: mRNA-seq analysis of 40 libraries generated from soybean control and heat-stressed root hairs and stripped roots.

Table S2: Correlation values for trancriptomic and proteomic expression levels measured in root hairs and stripped roots.

Table S3: Primer sequence used for real-time PCR.

Table S4: Normalized GC-MS data detected on metabolites extracted from root hairs (RH) and stripped roots (STR) in response to heat stress at (3h,6h,12h,and 24h) after heat exposure (40C).

Table S5: Diffrentially regulated lipids detected by LC-MS/MS extracted from root hairs (RH) and stripped roots (STR) in response to heat stress at (3h,6h,12h,and 24h) after heat exposure (40°C).

Figure S1: Number of overlapping and non-overlapping heat-responsive genes among the different exposure time points in soybean root hairs.

Figure S2: qRT-PCR validation of heat stress-responsive genes. A total of 15 randomly selected genes were used for qRT-PCR validation. Log2 fold change values (Control 25°C/Treatment 40°C) from the qRT-PCR data were plotted against Log2 (Control 25°C/Treatment 40°C) RNAseq values. Data are the average from two biological replicates.

Figure S3: Gene Regulatory Modules identified in soybean heat-stressed root hairs.

Figure S4: Number of overlapping and non-over-lapping heat-responsive proteins among the different exposure-time points in soybean root hairs.

Figure S5: MapMan Classification of the regulated proteins in heat-stressed root hairs and stripped roots.

Figure S6: Principal component analysis generated using MetaboAnlayst 3.0 for metabolites identified via GC/MS and LC/MS in RHs and STRs at 24h were 46% of the metabolomic changes were observed. A) GC/MS intensity values analysis between tissues; **RH:Green/STR:Red** at 24h between *[heat-stressed vs. control]* B)LC/MS intensity values analysis in RH between *[heat-stressed vs. control]* at 24h C)LC/MS intensity values in STR between *[heat-stressed vs. control] (LC/MS analysis was separated by tissue for clarity)*(△ control 25°; + Heat treatment 40°C).

Figure S7: Number of overlapping and non-overlapping heat-responsive metabolites between soybean root hairs (RHs) and stripped roots (STRs). Number in parenthesis indicates all the regulated metabolites at various exposure-times (3h, 6h, 12h, and 24h). *(Fold change >log2; 2-fold pairwise comparison [heat-stressed vs. control] between, P value <0.05 deemed statistically significant)*

Supplementary Table 1. mRNA-seq analysis of 40 libraries generated from soybean control and heat-stressed root hairs and stripped roots.

Cell type	Raw reads[a]	Good quality reads[a]	Mapped reads[a]	Uniquely mapped reads[a]	Multi-mapping reads[a,b]
Root Hairs	673,819,026	585,811,470	509,022,644	482,471,224	26,551,420
Stripped Root	754,723,285	613,110,894	544,509,934	515,452,180	29,057,754

Table S2: Correlation values for root hairs and stripped roots trancriptomic and proteomic expression levels. (A) Correlation values for each time point and tissue. (B) Correlation values for each gene expressed at all time points. RH: 8; STR: 10.

(A)

Time points and RH tissue	**Correlation values**
3H	0.005
6H	0.594
12H	0.639
24H	0.798
Time points and STR tissue	**Correlation values**
3H	-0.571
6H	0.040
12H	-0.192
24H	0.427

(B)

RH all time point, 8 genes	**Correlation Values**
Glyma04g05720	-0.860
Glyma06g05740	0.014
Glyma07g32050	-0.598
Glyma13g24440	-0.536
Glyma13g24480	-0.562
Glyma13g24490	-0.082
Glyma18g43430	-0.929
Glyma20g01930	-0.179
STR all time point, 10 genes	**Correlation Values**
Glyma04g05720	-0.61
Glyma06g05740	-0.26
Glyma07g32050	-0.45
Glyma08g07330	-0.03
Glyma08g07340	0.08
Glyma08g07350	-0.11
Glyma08g22630	-0.09
Glyma12g01580	0.76
Glyma13g24490	-0.89
Glyma20g01930	0.60

Table S3: Primer sequence used for real-time PCR.

Primer ID	Root Hairs Glyma ID	qRT-PCR forward primer	qRT-PCR reverse primer	Putative Gene Function
1	Glyma.04G054400.1	TCCGTCTCTGCTTATGGTCA	GCTGTGATCGGTAGGTGACA	HEAT-SHOCK PROTEIN 17
2	Glyma.07G200200.1	TGTCGTGCGTTGGTGAGATA	CTGCAGCTTAAAATTTGTTCTCAGT	HSP20 family protein
3	Glyma.09G131500.1	GTTAAATGGTACAGGATGCATAAGT	TGCAATACACAGACACATTTTCACA	Molecular chaperone HtpG
4	Glyma.14G063800.1	CTGGTGGCAAGAGTTAGAGCA	TGGGGAATGTTTCGGCATGA	HEAT-SHOCK PROTEIN 17
5	Glyma.16G178800.1	TCTGTCAGTCTGTGTGTTGCAT	AGGAAGGTATAAGAGGCAAAGTCA	Molecular chaperone HtpG
6	Glyma.19G011400.1	TGTCATCATCAGCCAATCCAA	ACACAAACAACATCACATCATCACT	HSP20 family protein
7	Glyma.02G205600.1	ACACAAACAACATCACATCATCACT	CCACTTTGCATCTTGTCCTTCG	Heat shock 70kDa protein
Primer ID	**Stripped Roots Glyma ID**	**qRT-PCR forward primer**	**qRT-PCR reverse primer**	**Putative Gene Function**
1	Glyma.01G106800.1	AGAAGGACTCCGAATCCAAGA	GGTGAAGTTGCAAGCTCGATT	Alpha crystallins
2	Glyma.01G238600.1	TGGTGTTGTGATGCCTGGTT	CTATTTAGAGCCTCGGCGCA	HEAT SHOCK PROTEIN 70KDA // SUBFAMILY NOT NAMED
3	Glyma.02G252800.1	GCCTCGTCTAGTAAACCCAGT	TCCCACACGTCCAGAGAAAC	Alpha crystallins
4	Glyma.05G022200.1	TTACAGCTGAGGTGGAGAGC	CATTTTACGGCTTCGCCAAGTA	ATP-DEPENDENT CLP PROTEASE
5	Glyma.08G069000.1	TTGGTGGCAGAAGGAGCAAT	TCCAATCCACTCGTGTGCTC	HSP20 family protein
6	Glyma.08G106700.1	TCGTCTCAAGTCTTGGTCCC	GCTTGAATGACAAGACAACTGACA	PEPTIDYL-PROLYL CIS-TRANS ISOMERASE
7	Glyma.15G077700.1	GGGAGAGGCAATGGTATGACA	ATGCAGTCAACCCATTTTCAG	Molecular chaperone (DnaJ superfamily)
8	Glyma.07G200300.1	TCTCTCGGTCTTGTGTTCCAA	ACACCTGACTCATGCAGCAA	HSP20 family protein

Table S4: Normalized GC-MS data detected on metabolites extracted from root hairs (RH) and stripped roots (STR) in response to heat stress at (3h,6h,12h,and 24h) after heat exposure (40C).

Root Hairs Metabolites	3h log2(fold change)	FDR	6h log2(fold change)	FDR	12h log2(fold change)	FDR	24h log2(fold change)	FDR
1,3-propanediol								
2,3-dihydroxybutanoic acid								
2-aminoadipic acid	-0.37528	0.031801	-0.72437	0.000368	0.018752	0.93837	-1.0064	0.10905
2-hydroxyglutaric acid	0.077095	0.058166	0.23006	2.62E-06	0.71519	2.04E-06		
2-hydroxypyridine	0.77822	0.12827	-0.21329	0.47591	0.068663	0.74443	-0.85035	0.022135
2-methyl-3-hydroxybutyric acid	-0.3237	0.77011	0.009301	0.81625	-0.35621	0.94977	0.34242	0.000637
2-methylsuccinic acid	-0.19404	0.005998	-0.47836	6.03E-08	-0.2005	0.015466		
3-hydroxy-3-methylglutaric acid	0.31498	0.10241	0.62855	1.19E-05	0.73154	0.000146	0.22957	0.59379
3-hydroxybutyric acid	0.16993	0.19443	0.60709	0.092485	-0.21988	0.16004	0.11019	0.91188
3-hydroxypentanoic acid	0.1691	0.6251	0.61073	0.61895	0.12925	0.7551	0.13437	0.13104
4-guanidinobutyric acid	-0.07714	0.70471	-0.33667	0.02866	-0.31152	0.018729	-1.4779	1.97E-06
4-hydroxy-3-methoxybenzoic acid	-0.13633	0.71284	-0.4031	0.001321	0.1577	0.32675	0.75747	0.003101
4-hydroxybutyric acid	0.44726	0.072455	-0.23361	0.63227	-0.39602	0.91712	-0.22935	0.084439
aconitic acid	-0.26074	0.6705	0.35663	0.74502	0.18097	0.87382	-0.63098	0.084439
Adenine	0.23588	0.77011	-0.39571	0.084277	-0.3709	0.16027	-0.4677	0.061714
Adenosine	-0.29866	0.015758	-0.54303	0.000497	-0.53572	0.14147	0.21935	0.61909
Allantoin	0.12547	0.74388	0.007272	0.99351	0.27261	0.21318	-0.22062	0.24611
allo-inositol	0.052436	0.52659	0.0996	0.50209	0.5416	2.04E-06		
Arabitol	0.092531	0.10483	0.10421	0.073788	0.32731	0.002087	-0.91022	1.31E-05
aspartic acid	-0.53444	0.000107	-0.92638	8.79E-14	-0.97582	1.40E-08	-0.53085	5.09E-05
benzoic acid								
Beta- alanine	0.15387	0.26041	-0.24171	0.01494	-0.42059	0.031105	-1.1072	0.028038
beta-cyano-L-alanine	0.44744	0.047513	0.32203	0.20909				
carbonate ion	0.24769	0.15574	-0.32315	0.091374	-0.14437	0.99925		

Cellobiose	-1.2282	0.002558	-0.40438	0.27522	0.004039	0.91553	0.069673	0.79853
citric acid	-0.19524	0.012121	-0.49553	3.37E-05	-0.58062	1.49E-06	-2.0549	0.084822
Cycloleucine	0.018582	0.92332	0.2652	0.20909	-0.07192	0.99735	-2.2448	1.14E-06
Daidzein	-0.80826	0.28776	0.28008	0.99873	0.20914	0.72295	-0.35353	0.092123
dehydrolalanine	0.058181	0.8622	0.037176	0.90995	-0.35812	0.42452	-0.95253	0.011694
D-glucose-6-phosphate	-0.22584	0.10768	-0.46909	0.00015	-0.63935	0.003357	-0.30861	0.26891
D-malic acid	-0.24243	0.081458	-0.23786	0.47591	-0.12041	0.22942		
D-mannitol	0.40894	0.33787	0.30019	0.94946	-0.07254	0.75387	-0.03954	0.90993
D-threitol	0.12956	0.49925	0.080084	0.62545	-0.13218	0.62818	-0.60473	0.020247
ethanolamine	0.032097	0.80308	-0.46163	0.099035	-0.27446	0.23168	-0.92704	0.031148
Fructose	0.37687	1.44E-05	0.57402	1.38E-07	0.002961	0.99925		
fructose-6-phosphate	-0.27049	0.11518	-0.31988	0.02866	-0.61381	0.003436	-0.64612	0.11268
fumaric acid	-0.48742	2.27E-06	-0.71357	7.52E-09	-0.75225	3.75E-05	-0.62276	6.76E-05
Galactinol	2.8861	9.48E-08	3.0711	8.79E-14	3.6162	7.72E-18	-1.2433	0.14911
galactonic acid	-0.32965	0.001284	-0.47331	5.40E-09	-0.26336	0.000374	-0.9733	0.038643
Galactose	-0.45147	0.17762	-0.43919	0.066537	0.70823	0.1758	0.11538	0.57538
gluconic acid	-0.6961	0.081624	-0.44526	0.044306	0.48926	0.37934	-0.67011	1.96E-05
Glucose	0.34757	0.000154	0.49268	2.42E-06	0.215	0.030371	-0.25754	0.14435
glutaric acid	0.026819	0.75892	-0.06228	0.61569	0.6207	0.001006	1.2961	0.49052
glyceric acid	-0.02999	0.68869	0.111	0.013855	0.78584	0.00025	0.60799	0.18084
glycerol 3-phosphate	1.7347	0.003086	1.4035	0.011489	2.1997	0.002672	0.84337	0.068829
glycerolphosphoinositol	0.70929	8.67E-05	0.45779	2.42E-06	0.66792	0.028289	-0.49219	2.88E-05
Glycine	-1.0428	0.05415	0.50483	0.13041	2.7434	0.02339	1.7135	3.57E-13
glycolic acid							0.66727	0.10905
inositol (undefined)	-0.28596	0.000833	-0.10384	0.24745	0.46251	0.003042	-0.49674	0.05355
L-(+) lactic acid	-0.04108	0.80308	0.024662	0.87495	-0.36453	0.58188	-0.70801	0.035783
Lactulose	-0.51671	0.23495	0.68413	0.23183	-0.15127	0.80179		
L-alanine	0.54448	0.001338	0.36346	0.000229	-0.47454	0.70019	0.29807	0.59049
L-asparagine	0.34735	0.009098	0.33622	0.005918	0.008607	0.90152	-0.22012	0.022491
L-cysteine							1.3239	0.043261
L-glutamic acid	-0.17222	0.058162	-0.44995	1.72E-08	-0.47134	0.001606	0.32833	0.005814

L-glutamine	-0.01352	0.95713	-0.21681	0.16879	-0.03563	0.7551	-1.0055	0.004707
L-homoserine	-0.46394	0.21616	0.38499	0.50209	-0.79459	0.074147	-0.48755	0.018552
L-isoleucine	-0.11685	0.17762	-0.24763	0.002404	-0.46646	0.003436	-0.49239	1.80E-07
L-leucine	-0.03725	0.77011	-0.16721	0.084019	-0.75284	0.018729	1.5895	0.020247
L-proline	-0.27028	0.038824	-0.41233	0.000527	-0.58381	0.000149	1.5987	0.000235
L-serine	-0.00397	0.98249	-0.08311	0.44238	-0.06295	0.65255	0.23621	0.83771
L-threonine	-0.04024	0.71284	-0.012	0.87832	-0.48348	0.00183	-0.30958	0.041891
L-tryptophan	-0.39067	0.000858	0.045044	0.62545	-0.02596	0.91041	-0.22321	0.093158
L-tyrosine	0.30306	0.083012	0.32767	0.032487	0.82792	0.000284	-0.24196	0.28789
L-valine	0.19221	0.026932	0.14191	0.012663	-0.3753	0.000587	0.10093	0.4607
maleic acid	-0.07282	0.95023	-0.35517	0.086556	0.075722	0.74443	-0.38603	2.26E-05
malonic acid	-0.62452	0.002133	-0.74591	0.000696	-1.61	5.66E-06	-0.80944	0.030176
Maltose	-0.21894	0.49371	-0.61806	0.003762	-0.57749	0.083658	-1.0981	0.004668
mucic acid	-0.60817	9.48E-08	-0.64305	3.72E-07	-0.80959	0.002229	-0.19544	0.25216
myo-inositol	-0.32744	0.000607	0.03209	0.66717	1.045	9.53E-06	-0.85536	0.043315
N-acetyl-D-mannosamine			0.55735	0.002404	0.081109	0.75496	-0.60477	0.83771
N-methylalanine	1.1591	0.16014	1.4751	0.044306	0.005277	0.99925	0.48354	0.000255
O-phosphocolamine	-0.0332	0.74701	0.73575	0.41881	0.41347	0.6802	-0.15092	0.31523
oxalic acid	0.20657	0.1718	-0.08736	0.50209	-0.47918	0.006196	0.69093	0.001032
Palatinol	-0.20277	0.33904	-0.19103	0.31231	-0.1726	0.65255	-1.4682	4.90E-10
Palatinose	0.45578	0.77011	0.044837	0.81625	0.49437	0.91712	-0.8764	2.57E-09
palmitic acid	0.53452	0.015915	-0.10241	0.60459	-0.16544	0.99925	1.0394	8.33E-05
phenylalanine	0.046382	0.49925	0.1104	0.078592	-0.1565	0.23168	-0.61887	0.000527
phosphoric acid	0.02927	0.63361	-0.25021	0.23183	-0.26762	0.077094	-0.92273	2.96E-05
Porphine	0.48422	0.064686	-0.03686	0.95967	-0.103	0.80992	-0.3974	0.093312
Putrescine	-0.32053	0.25524	0.58977	0.31231	-0.15943	0.52373	0.61628	7.20E-06
pyroglutamic acid	-0.05445	0.57233	-0.37492	5.12E-05	-0.33291	0.001994	-0.42628	0.001454
pyruvic acid	-0.0611	0.80308	-0.20527	0.24669	-0.31931	0.74443	0.81959	0.004238
Raffinose	2.2789	1.76E-06	3.1621	5.48E-13	4.0732	1.09E-11	0.70251	2.09E-08
ribonic acid	-0.74614	0.025988	0.51707	0.66822	0.21297	0.95611	0.28009	0.31523
Ribose	-0.04379	0.96008	-0.28821	0.18213	-0.14307	0.62613		

shikimic acid	0.16437	0.032977	0.029646	0.61569	0.28601	0.000838	-0.46562	0.008291
succinic acid	-0.08787	0.16014	-0.0931	0.09298	0.49335	0.003724	-0.57936	0.004025
sucrose	0.38449	0.010498	0.034605	0.77658	0.42845	0.064633		
threonic acid	-0.19357	0.099741	-0.27374	0.004421	-0.46921	5.44E-05	1.903	1.41E-09
Unknown 001	-0.24501	0.30751	-0.26661	0.71766	-0.30759	0.02339	-0.51594	0.029561
Unknown 002	0.37782	0.032977	-0.141	0.60459	-0.30731	0.99925	0.27112	0.048818
Unknown 003	0.53578	0.15141	0.042545	0.60967	-0.27183	0.90152	-1.3814	3.61E-06
Unknown 004	0.25001	0.53704	0.027994	0.96919	0.23371	0.91712	-0.346	8.33E-05
Unknown 005	0.33216	0.27174	-0.93929	0.23598	-0.03362	0.91712	-0.18894	0.87183
Unknown 006	0.38207	0.078017	-0.19574	0.43137	-0.3122	0.96952	-0.90988	2.39E-05
Unknown 007	-0.19956	0.21282	-0.77109	1.68E-07	-1.0705	2.86E-06	-0.6741	8.09E-08
Unknown 008	0.13338	0.49925	0.25963	0.62545	0.26749	0.54875	0.57892	4.96E-07
Unknown 009	0.047396	0.80308	-0.00838	0.90995	0.33626	0.78482	1.2713	0.041337
Unknown 010	0.22964	0.36682	0.1827	0.81625	0.41152	0.52734	-0.37595	0.031148
Unknown 011	-0.01257	0.85541	-0.8234	0.035867	0.28783	0.85721	0.63454	0.097547
Unknown 012								
Unknown 013	-0.03728	0.77011	0.222	0.81625	-0.53973	0.24817	-0.89142	0.000116
Unknown 014	-0.25008	0.80308	-0.23847	0.79365			-0.5328	0.002054
Unknown 015	0.47728	0.032977	-0.0768	0.94166	-0.09764	0.96952	-0.15888	0.058654
Unknown 016	-0.01402	0.92332	-0.50835	0.023693	-0.36134	0.91712	1.7211	0.004167
Unknown 017	-0.38445	0.56193					0.44564	0.96073
Unknown 018	0.52139	0.33904	0.086218	0.60967	0.203	0.6802	-0.67398	2.42E-07
Unknown 019	-2.4012	0.36225	-0.7598	0.23707	-0.13432	0.91712	0.21772	0.084822
Unknown 020	0.29359	0.746	0.87722	0.066994	1.0281	0.038335	-0.83085	2.57E-09
Unknown 021	0.074212	0.8284	-0.5633	0.23687	-0.72057	0.14147		
Unknown 022	-0.32306	0.012282	-0.56261	1.28E-07	-0.43895	0.00055	1.1782	0.000144
Unknown 023	1.7049	0.53704	0.84977	0.77658	-0.61843	0.20767	0.10978	0.28043
Unknown 024	-0.27137	0.74701	-0.05325	0.94799	0.20666	0.99925	2.0272	1.14E-06
Unknown 025	0.69855	0.33507	1.1438	0.034391	-0.08092	0.65255	0.89437	0.032396
Unknown 026	0.003689	0.92332	0.45693	0.10034	0.12081	0.99925	-0.85081	4.00E-08
Unknown 027	0.30184	0.032977	-0.04082	0.63728	0.031249	0.95202	0.86306	0.020407

Unknown 028	-0.02358	0.95713	-0.41988	0.000409	-0.20787	0.74443	-1.3302	0.045078
Unknown 029	0.4306	0.29747	0.38014	0.54105	-0.03249	0.94977	0.089711	0.60657
Unknown 030	0.065104	0.76727	0.16321	0.60459	0.15615	0.29026	-0.09923	0.44119
Unknown 031	-0.56673	0.80308	-1.6891	0.18704	0.2731	0.84175	-1.2694	0.006308
Unknown 032	0.53522	0.002546	0.48519	0.002043	0.23842	0.48538	0.10652	0.90019
Unknown 033			-0.29631	0.42871	-1.3327	0.016297	0.043648	0.59358
Unknown 034	-0.10469	0.43297	-0.17997	0.18704	0.015608	0.96952	-1.0337	0.00417
Unknown 035			-0.7356	0.000143	-0.39435	0.074147	0.69468	0.032396
Unknown 036	0.013277	0.98249	-0.11036	0.26093	-0.1254	0.11618	-0.02489	0.83771
Unknown 037	0.21851	0.74003	-0.02967	0.6809	0.33298	0.65255	2.0359	1.19E-05
Unknown 038	0.045743	0.71284	0.12898	0.81625			1.9579	4.21E-10
Unknown 039							0.92758	0.001027
Unknown 040	-0.4024	0.16014	-0.4728	8.07E-06	-0.41357	0.001471	-0.76242	3.96E-06
Unknown 041	-0.51958	3.63E-05	-0.74849	7.40E-08	-0.61974	0.001006	-0.31193	0.16208
Unknown 042	0.37979	0.043508	0.35388	0.16081	0.19438	0.70216	-0.99341	1.11E-05
Unknown 043	-0.03423	0.81961	-0.43805	0.000923	-0.41741	0.32089	-0.06827	0.33117
Unknown 044	0.2247	0.018981	0.30202	0.004421	0.061305	0.70216	0.70562	0.043261
Unknown 045	-0.12239	0.8284	-0.42751	3.27E-05	-0.33816	0.020421	-1.7798	9.50E-08
Unknown 046	-0.11286	0.52944	-0.11293	0.37431	-0.05313	0.91712	0.53309	0.44119
Unknown 047	0.18705	0.081624	0.044995	0.71766	0.35624	0.006591	-1.0812	2.49E-06
Unknown 048	-0.26156	0.008911	-0.4158	4.18E-07	-0.3896	0.003724	-0.35676	0.000118
Unknown 049							0.068047	0.38739
Unknown 050	-0.00212	0.8284	-0.58461	0.048061	-0.09624	0.73027	-0.59164	0.24467
Unknown 051							-0.69374	0.000699
Unknown 052	-0.54139	0.11518	-0.16617	0.37761	0.52366	0.45055	-0.85765	2.73E-06
Unknown 053	-0.9171	0.04879	-0.15958	0.29483	0.43723	0.65255	0.001436	0.9342
Unknown 054	-0.26825	0.81961	-1.1332	0.093782	-0.71613	0.24731	-0.96229	0.000223
Unknown 055	-0.08681	0.66443	-0.01021	0.77658	-0.18032	0.19486	-1.5065	0.000355
Unknown 056	0.17441	0.56615	0.17035	0.40116	0.05116	0.91041	0.73906	5.33E-05
Unknown 057	-0.22061	0.02512	-0.19865	0.035867	0.40449	0.21319	0.44813	9.40E-05
Unknown 058	0.83097	7.17E-06	0.12338	0.33375	-2.5252	0.13138	0.46469	4.75E-06

Unknown 059	-0.35483	8.67E-05	-0.39741	0.000281	-0.36488	0.091442	-0.5281	9.44E-06
Unknown 060	-0.04292	0.81961	-0.67514	0.002476	-0.30976	0.45055	-1.1534	0.003494
Unknown 061	0.29008	0.37892	0.34227	0.71766	0.093139	0.62818	0.20507	0.43757
Unknown 062	0.60634	0.018981	-0.35395	0.003762	-0.514	0.13575	-1.292	0.006598
Unknown 063	-0.47968	0.20217	-0.65837	0.031262	0.42358	0.65255	-0.3153	0.9342
Unknown 064	0.22465	0.42216	0.24365	0.70681	-0.56445	0.008521	-0.75573	2.20E-08
Unknown 065	-0.41255	0.000163	-0.15538	0.01181	-0.0448	0.99925	-0.74638	3.74E-05
Unknown 066	-0.43244	0.80308	-0.7869	0.3777	-1.1133	0.46123	0.80577	1.14E-06
Unknown 067	-0.72007	4.40E-06	-0.86946	1.62E-05	-0.93755	0.002229	0.73463	6.20E-06
Unknown 068	-0.01697	0.91242	-0.47765	0.044306	-0.07567	0.95928		
Unknown 069	0.016208	0.91242			0.19346	0.010948		
Unknown 070	-0.28242	0.007643	-0.28914	0.003051	-0.52682	0.020311	-0.09701	0.6714
Unknown 071	-0.05132	0.83079	-0.14971	0.31996	-1.858	0.030371	0.099017	0.16543
Unknown 072					-0.10837	0.96952	0.065903	0.3931
Unknown 073	-1.1766	0.20083	-0.02134	0.89195	-1.6674	0.14243	-0.62012	8.43E-05
Unknown 074	-1.1483	0.17629	0.28318	0.96951	-2.7538	0.035263	-1.0759	1.37E-09
Unknown 075	-0.53967	0.002126	-0.37872	0.013855	-0.11963	0.7397	0.35645	0.00617
Unknown 076	-0.16125	0.26191	-0.05395	0.66673	-0.33996	0.11655	-0.50294	3.90E-06
Unknown 077	0.06256	0.71284	0.65009	0.005034	0.83576	0.001006	-0.14397	0.27739
Unknown 078	-0.13277	0.27174			-0.2712	0.42592	-0.84511	0.004319
Unknown 079							0.095152	0.17102
Unknown 080	-0.01167	0.75892	-0.17589	0.11883	0.14786	0.65255	-1.1256	1.14E-07
Unknown 081	-0.26331	0.001514	-0.14407	0.0685	-0.0367	0.91712	-0.8731	3.15E-06
Unknown 082	-0.17047	0.11518	-0.08782	0.47591	0.06899	0.62613	0.23848	0.001702
Unknown 083	0.1361	0.62704	0.19372	0.29137	-0.01221	0.91712	0.1636	0.16543
Unknown 084	-0.19992	0.002546	-0.13544	0.014431	-0.23192	0.18554	0.17146	0.23237
Unknown 085	-0.26477	0.30751	-0.39027	0.00219	0.026063	0.99925	0.39884	3.61E-06
Unknown 086	-0.52786	0.20549	-0.49138	0.13935	-0.2359	0.71272	-0.5502	0.057852
Unknown 087	-0.35057	0.038492	-0.29643	0.00269	-0.18231	0.2178	-0.47875	0.010926
Unknown 088	-0.49764	0.10184	-0.59068	0.008943	-0.62229	0.37824	-0.90781	0.000611
Unknown 089	-0.14407	0.26191	-0.33986	0.002095	-0.50484	0.000149	-1.3959	2.92E-05

Unknown 090	0.30529	0.17629	0.09766	0.50209	0.12636	0.14769	0.33305	0.035498
Unknown 091	0.11118	0.40806	0.040059	0.78864	-0.0097	0.91712	-1.3071	4.65E-11
Unknown 092	0.25189	0.30931	0.25295	0.060327	0.55969	0.064633	-0.83849	9.01E-09
Unknown 093	-0.03038	0.77011	0.013701	0.81625	0.38986	0.014919	-0.36358	0.033459
Unknown 094	-0.49503	0.079981	-0.06187	0.94522	-0.1375	0.74658	0.30451	0.12136
Unknown 095	-0.28146	0.23495	-0.34129	0.003783	-0.47015	0.04511	-1.0382	0.00015
Unknown 096	-0.26114	0.14372	-0.26527	0.1143	0.2026	0.14243	-0.51766	0.31919
Unknown 097	-0.01554	0.8284	-0.09742	0.56678	0.33848	0.14904	-0.23688	0.83771
Unknown 098	-0.3639	0.021792	-0.41665	2.98E-06	-0.65195	0.16027	0.19269	0.044375
Unknown 099	0.07427	0.51187	0.13474	0.16324	0.58899	0.000301	0.7354	0.000587
Unknown 100	0.050335	0.74701	0.24577	0.29732	-0.47249	0.24817	-1.1363	1.24E-06
Unknown 101	0.4053	0.001892	0.1136	0.16435	0.26029	0.064633	3.3236	6.02E-11
Unknown 102	0.23211	0.38537	0.30595	0.22799	0.3606	0.18498	-0.05817	0.68379
Unknown 103	1.5343	0.001088	1.7587	6.81E-12	2.1858	6.75E-08	0.59004	0.000405
Unknown 104	0.38864	0.005562	0.31636	0.18473	0.25087	0.96952	1.4492	3.72E-09
Unknown 105	0.4053	0.001047	0.53795	7.30E-06	1.2525	3.87E-12	-0.25244	0.90486
Unknown 106	-0.28856	0.002214	0.00717	0.87216	0.044567	0.7295	0.1542	0.035498
Unknown 107	0.47801	0.27144	-0.65823	0.032482	0.017909	0.99925	-0.41904	0.044375
Unknown 108	0.90059	0.016764	1.1814	0.005887	1.107	0.00055	0.33428	0.004707
uracil	-0.693	8.68E-05	-0.66717	0.004673	-0.81908	0.000883	1.3083	0.000428
urea	-0.05112	0.91361	0.62761	0.19295	0.25021	0.9051	4.7879	9.28E-23
xylitol	-0.07893	0.57233	-0.12006	0.2013	-0.10092	0.66562	6.5466	4.10E-12

Table S4(Continued): Normalized GC-MS data detected on metabolites extracted from root hairs (RH) and stripped roots (STR) in response to heat stress at (3h,6h,12h,and 24h) after heat exposure (40C).

Stripped Roots	**3h**		**6h**		**12h**		**24h**	
Metabolites	**log2(fold change)**	**FDR**	**log2(fold change)**	**FDR**	**log2(fold change)**	**FDR**	**log2(fold change)**	**FDR**
1,3-propanediol	-0.54223	0.75115	-0.42491	0.44073	-0.29741	0.089825	-1.7897	0.043721
2,3-dihydroxybutanoic acid	-1.0175	0.75115	0.37639	0.48952	0.26941	0.93995	-0.19016	0.92475

2-aminoadipic acid	-0.79898	0.75115	0.064602	0.73713	1.1757	2.27E-05	0.87353	0.076053
2-hydroxyglutaric acid	-0.42914	0.72889	-0.34529	0.53604	2.5639	0.79219	-0.75214	0.90299
2-hydroxypyridine	0.041321	0.90639	1.1427	0.62243	-0.24164	0.91203	-0.04307	0.89973
2-methyl-3-hydroxybutyric acid	-1.3631	0.75115	0.28656	0.52256	1.053	0.10921	-0.55132	0.069272
2-methylsuccinic acid	0.17644	0.75115	0.64344	0.37769	1.5414	0.42629	-0.33252	0.72828
3-hydroxy-3-methylglutaric acid	-0.40293	0.72889	1.8948	0.6849	0.89238	0.001071	0.024029	0.90615
3-hydroxybutyric acid	0.26288	0.72889	0.27535	0.49856	0.4728	0.006063	0.75963	0.022079
3-hydroxypentanoic acid	-0.08468	0.90639	0.44218	0.73954	-0.62403	0.04288	-0.61878	0.10065
4-guanidinobutyric acid	-0.33103	0.75115	-0.90469	0.26563	0.069401	0.91203	0.054687	0.90421
4-hydroxy-3-methoxybenzoic acid	-0.79046	0.72889	1.9081	0.29594	0.70482	0.24993	-0.26288	0.24457
4-hydroxybutyric acid	-0.48907	0.75115	1.0891	0.75488	-0.90379	0.098827	-1.7009	0.8629
aconitic acid	-0.66241	0.97155	-0.36716	0.44811	-0.0989	0.94539	-1.346	0.016602
adenine	-0.03625	0.97155	-0.48671	0.36932	-0.63803	0.06961	0.099911	0.67538
adenosine	0.33492	0.72922	-0.08496	0.44955	-0.31859	0.073193	-0.02269	0.91776
allantoin	0.37788	0.75115	0.058527	0.62243	-0.29874	0.24151	-0.38453	0.48748
allo-inositol	0.27859	0.82968	-0.33409	0.37769	-0.25023	0.99114	-1.1301	0.52555
arabitol	-0.09014	0.97155	-0.27048	0.54966	1.0312	0.11367	0.82998	0.19876
aspartic acid	0.12484	0.75115	-0.60346	0.36932	0.77098	0.98688	-0.02501	0.90049
benzoic acid	-0.46543	0.82968	-0.41705	0.37769	1.7185	0.018997	-0.28483	0.86149
Beta- alanine	0.11155	0.88123	0.44351	0.7692	-0.16489	0.42629	-0.11284	0.67538
beta-cyano-L-alanine	-0.12814	0.90639	1.5074	0.76264	0.30978	0.58903	-0.24862	0.70125
carbonate ion	-0.79067	0.13814	0.28874	0.37769	0.41452	0.017667	-0.1106	0.80958
cellobiose	-0.41827	0.84828	-0.11045	0.53604	1.6111	0.26844		
citric acid							0.20562	0.98666
cycloleucine	-0.42798	0.84828	0.47221	0.76264	0.97694	0.35558	0.057538	0.91776
daidzein	0.27404	0.72889	-0.37707	0.35607	0.2287	0.9175	-0.06798	0.73381
dehydrolalanine	0.17443	0.75115	-0.04677	0.62243	-0.22291	0.69639	-0.78909	0.80958
D-glucose-6-phosphate	-0.10623	0.97155	0.69368	0.82615	0.043122	0.73899	0.67382	0.13622

D-malic acid	-0.10993	0.90639	1.1451	0.44811	0.31096	0.41881	0.10962	0.82289
D-mannitol	0.34727	0.75115	0.007851	0.44811	-0.25647	0.12983	-0.06649	0.97524
D-threitol					0.003	0.84093	0.25671	0.71478
ethanolamine	0.27433	0.81663	0.57879	0.37769	-0.62635	0.88045	-1.2511	0.19876
fructose	0.32788	0.82083	-0.18545	0.44466	-0.33587	0.14638	-0.06501	0.8297
fructose-6-phosphate	-0.56741	0.82968	-0.42301	0.3576	0.95805	0.14638	0.12837	0.98742
fumaric acid	-0.68914	0.97155	0.65867	0.44073	0.96755	0.18905	-0.93574	0.003429
galactinol	0.001398	0.75115	0.77878	0.72821	2.0023	0.57804	2.3492	0.41123
galactonic acid	-1.5718	0.4485	0.58883	0.74943				
galactose	0.1262	0.94394	-0.36692	0.37769	0.32564	0.77273	-0.64204	0.53423
gluconic acid	-0.16988	0.75115	-0.1498	0.50559	0.017694	0.95383	-0.10658	0.71833
glucose	0.041679	0.90639	-0.07436	0.89146	-0.62736	0.42721	-0.31536	0.80492
glutaric acid	0.43348	0.72889	0.76169	0.86877	0.36615	0.20305	0.64201	0.10065
glyceric acid	0.14404	0.75115	-1.5681	0.34118	0.34953	0.80158	0.057548	0.95934
glycerol 3-phosphate	-1.2173	0.99439						
glycerolphosphoinositol	0.25191	0.72922	-0.56805	0.34073	-0.07864	0.52929	-0.35414	0.003268
glycine	0.30263	0.99439	0.18935	0.81718	1.4635	0.00118	-0.04178	0.90615
glycolic acid	-0.6875	0.76523	0.46996	0.3661	3.4617	6.69E-05	1.2731	0.58998
inositol (undefined)	0.064387	0.9552	2.7102	0.36932	-0.32037	0.88079	-0.46967	0.97524
L-(+) lactic acid	-0.64094	0.72889	0.68309	0.67484	-1.2287	0.018997	-0.95881	0.001492
lactulose	0.15818	0.75115	-0.16131	0.66179	0.059284	0.90764	0.28099	0.44012
L-alanine								
L-asparagine	-0.23619	0.75115	2.8892	0.53513	0.002315	0.91203	0.7462	0.024265
L-cysteine	-0.18673	0.75115	3.6179	0.38332	0.10566	0.88079	-0.09415	0.97524
L-glutamic acid	0.17453	0.75115	-0.50433	0.20246	0.12948	0.49858	0.0176	0.97524
L-glutamine	-0.87705	0.11581	-1.6727	0.017405	-1.5703	1.84E-06	-1.0057	0.062495
L-homoserine	-0.28764	0.75115	3.3264	0.37769	0.25412	0.45106	-0.11369	0.73695
L-isoleucine	-0.08064	0.97155	0.24124	0.82975	0.1799	0.69639	0.058278	0.73381
L-leucine	-0.0492	0.99439	-0.7443	0.34073	0.032946	0.98688	-0.0639	0.94537
L-proline	0.091302	0.81663	-0.20244	0.6298	-0.44166	0.87452	-0.09075	0.91776
L-serine			0.20993	0.44811	-0.44638	0.21672		

L-threonine	0.31928	0.90639	1.6621	0.62755	0.81256	0.005152	-0.11192	0.78025
L-tryptophan	0.74148	0.72889	-0.26327	0.44641	-0.00039	0.88079	-0.13315	0.80958
L-tyrosine	-0.08983	0.75115	2.048	0.89389	-0.30406	0.46968	-0.1292	0.53423
L-valine	-0.30273	0.75115	0.26809	0.6849	0.58028	0.26844	-0.29101	0.90615
maleic acid	0.038563	0.97482	-0.39708	0.20246	-0.26094	0.16741	-0.01026	0.97408
malonic acid	-0.04869	0.99044	0.45776	0.44641	0.048321	0.93995	0.5383	0.26046
maltose	-0.61461	0.75115	0.41753	0.48689	-0.02969	0.88079	-0.0735	0.80958
mucic acid	0.55438	0.72889	-0.22854	0.38249	0.14815	0.90764	-0.42397	0.8297
myo-inositol	0.043854	0.97155	-0.08923	0.89469			-0.1731	0.70443
N-acetyl-D-mannosamine								
N-methylalanine	-0.35009	0.91162	-0.62098	0.62243	0.48838	0.5061	1.1897	0.062495
O-phosphocolamine	-0.46189	0.75115	0.55256	0.77049	-0.95714	0.078643	0.81449	0.78025
oxalic acid	0.19309	0.81663	2.1628	0.72443	1.6116	0.25313	-0.04947	0.78025
palatinol	-0.32088	0.75115	-1.0101	0.30747	0.071852	0.95441	0.021686	0.74842
palatinose	0.62785	0.72889	1.2016	0.017405	0.8106	0.2721	1.1596	0.007638
palmitic acid	-0.03796	0.99439	-0.20089	0.43374	-1.0746	0.013214	-0.69779	0.13553
phenylalanine	0.096116	0.84315	-0.5011	0.34073	0.8215	0.078643	-0.02929	0.89973
phosphoric acid	-0.63836	0.97155	-0.75529	0.34118	0.005317	0.88083	0.007472	0.91776
porphine	-0.39607	0.75115	0.78394	0.44811	0.059045	0.98688	0.17317	0.63364
putrescine	-0.00431	0.91162	0.48476	0.44811	-0.10034	0.64802	-0.22721	0.78025
pyroglutamic acid	-0.90284	0.99439	0.23014	0.59762	-1.6841	0.014155	-1.9026	0.12807
pyruvic acid	-0.04909	0.75115	-0.331	0.36932	0.44989	0.9263	-0.20599	0.91776
raffinose	-0.67993	0.4316	-1.6194	0.084476	-0.6828	0.054899	-1.2811	0.001492
ribonic acid	-0.01041	0.91162			-0.4874	0.99762	1.0016	0.1117
ribose	-0.03645	0.88123	-0.4636	0.19499	0.45859	0.88079	-0.08318	0.78025
shikimic acid	0.51199	0.84828	0.051115	0.97713	0.14816	0.91203	-0.99324	0.53423
succinic acid	-0.32125	0.97155	-0.5773	0.36932	0.97621	0.092774	0.12046	0.97524
sucrose	0.025747	0.99439	0.20327	0.73713	-0.25894	0.17517	-0.50527	0.069272
threonic acid	-0.027	0.99439	3.6771	0.43374	0.24223	0.42629	0.13683	0.91776
Unknown 001	-0.18779	0.75115	5.0659	0.37769	-0.01033	0.98688	0.08235	0.71478
Unknown 002	0.29047	0.72889	0.17269	0.59876	0.347	0.88083	-0.17545	0.71833

Unknown 003	0.11887	0.84315	0.59622	0.98916	1.0013	0.062383	-0.27979	0.74842
Unknown 004	0.11518	0.84828	0.65307	0.36932	0.28139	0.65691	-0.32841	0.18728
Unknown 005	-0.68142	0.81663	-0.84491	0.44811	-0.97695	0.002013	-0.65926	0.049555
Unknown 006	-0.11533	0.89682	-1.6216	0.084184	0.002114	0.98688	0.3862	0.18412
Unknown 007	-1.3986	0.84315	0.32493	0.72287	-1.6028	0.011144	-0.4841	0.059518
Unknown 008	0.15655	0.88123	1.1686	0.59474	0.41592	0.17517	0.1951	0.67538
Unknown 009	0.002989	0.75115	-1.2883	0.37769	-0.29174	0.26844	0.1871	0.90615
Unknown 010	-0.00983	0.94298	0.88239	0.36932	0.40788	0.479	1.1488	0.00863
Unknown 011	0.65109	0.82083	0.52212	0.37769	0.18804	0.98688	-0.459	0.24457
Unknown 012	0.088782	0.82968	-0.1992	0.49856	1.6968	0.067698	0.29992	0.13553
Unknown 013	0.16595	0.76302	-0.51655	0.34118	0.87775	0.55162	0.001609	0.90615
Unknown 014	-0.63224	0.98452	1.0664	0.084476	0.12533	0.93995	0.16178	0.74842
Unknown 015	-0.80105	0.40445	-0.01219	0.85932	-0.14133	0.83005	-0.79072	0.28798
Unknown 016	0.70182	0.57664	-0.50144	0.36932	-1.6155	0.20563	1.1679	0.13553
Unknown 017	0.36355	0.72922	-0.41395	0.37769	0.54775	0.9232	0.80274	0.062495
Unknown 018	-0.09197	0.99439	2.1194	0.36932	0.34578	0.72675	0.049433	0.90615
Unknown 019	0.098802	0.84315	0.32309	0.64984	-1.1999	0.078643	0.32391	0.78025
Unknown 020	0.34155	0.72889	0.14027	0.59474	-0.0606	0.80158	-0.20487	0.73695
Unknown 021	-0.00655	0.99439	2.076	0.36932	0.70615	0.000448	-0.14866	0.89973
Unknown 022	0.087684	0.99044	0.7087	0.084184	-0.12613	0.93995	-0.07405	0.97408
Unknown 023	0.2312	0.75115	-0.16679	0.43528	0.36197	0.95441	-1.1598	0.030654
Unknown 024	-0.26283	0.91162	-0.40642	0.34073	-2.4788	1.06E-05	-1.3147	0.076053
Unknown 025	0.32624	0.97155	-0.20739	0.49856	0.002729	0.98688	-1.3732	0.63835
Unknown 026	0.7575	0.045926	0.91648	0.24484	-0.09712	0.80288	0.032021	0.89973
Unknown 027	-0.38837	0.81663	-0.1983	0.44073	0.70813	0.20755	0.38884	0.48748
Unknown 028	-0.03796	0.98452	-0.52989	0.36932	-0.06921	0.73899	-0.16326	0.48748
Unknown 029	-0.84029	0.75115	0.97886	0.44811	2.0924	0.42508	0.17065	0.91776
Unknown 030	0.010722	0.97482	0.80782	0.85932	-1.0475	0.9232	0.92306	0.90049
Unknown 031	-0.91872	0.3605	-0.0324	0.44073	-0.08316	0.45246	-1.6263	7.42E-07
Unknown 032	0.28627	0.72922	-0.00936	0.69747	-0.27377	0.42629	-0.29426	0.91507
Unknown 033	-0.44658	0.72889	0.63848	0.76264	-0.77332	0.001445	-0.31824	0.16234

Unknown 034	-1.2567	0.72922	-0.46883	0.37769	-1.1387	0.018997	-0.40185	0.18869
Unknown 035	-0.16808	0.75115	3.6117	0.38249	0.094755	0.91591	-0.16644	0.90615
Unknown 036	-0.14845	0.75115	-0.32017	0.36932	-0.07862	0.88079	0.064285	0.94401
Unknown 037	-1.5888	0.72889	1.9628	0.37769	0.20147	0.80288	-0.31526	0.15825
Unknown 038	0.16825	0.75115	0.92861	0.44811	0.32431	0.16025	0.33257	0.98666
Unknown 039	-1.6717	0.86911					0.07732	0.34554
Unknown 040	0.052853	0.98452	2.714	0.62243	0.048203	0.88079	0.10311	0.98666
Unknown 041								
Unknown 042	0.17941	0.84828	-0.04983	0.59235	-0.35666	0.049115	-0.07242	0.95934
Unknown 043	-1.0596	0.72889	2.1789	0.06872	3.3937	0.17236	0.4358	0.97721
Unknown 044	-0.06553	0.97155	-0.4239	0.34118	0.66631	0.017667	0.25354	0.21095
Unknown 045	-0.34798	0.94298	2.1659	0.37769	0.47774	0.80288	0.41437	0.53423
Unknown 046	0.18964	0.75115	-0.6732	0.24092	-0.38156	0.20755	-0.19978	0.46575
Unknown 047	0.29319	0.86096	1.2833	0.36932	0.30366	0.36102	-0.0537	0.92475
Unknown 048								
Unknown 049	-0.9847	0.72889	0.017695	0.73713	1.1311	0.31572	0.15792	0.83836
Unknown 050	0.60776	0.81663	1.7918	0.34118	1.8614	0.02942	2.8215	0.022941
Unknown 051								
Unknown 052			3.6809	0.37769	0.14918	0.63501	-0.35052	0.53106
Unknown 053	-0.41229	0.99439	1.0996	0.34073	0.26715	0.63443	-0.09357	0.92964
Unknown 054	-0.01015	0.99439	0.15034	0.8904	0.25929	0.88079	-0.42621	0.48748
Unknown 055	0.19199	0.76302	-0.60833	0.36932	0.72231	0.98079	0.26001	0.91776
Unknown 056	-0.26612	0.99044	-0.97047	0.6849	-0.01887	0.98688	0.10021	0.90615
Unknown 057					0.082972	0.9309	-0.41123	0.88262
Unknown 058	-1.7145	0.99439	-0.56257	0.54491	3.1181	0.014155	0.44136	0.82289
Unknown 059	0.57037	0.40445	-0.54649	0.40793	0.54089	0.06961	0.84892	0.044437
Unknown 060			-1.3784	0.37769				
Unknown 061	0.27326	0.9078	-0.0429	0.54887	-0.29527	0.089825	-0.00041	0.92964
Unknown 062	-0.36436	0.97155	-0.23153	0.59419	0.21605	0.62977	0.19567	0.91776
Unknown 063	0.097277	0.82968	0.4677	0.94967	-1.2622	0.1914	0.33872	0.87269
Unknown 064	-0.13909	0.88123	-0.87681	0.067772	0.091577	0.94539	0.023144	0.91776

Unknown 065	0.27303	0.75115	0.63078	0.02206	-0.32443	0.26813	-0.70568	0.004785
Unknown 066	-0.31433	0.95294	0.1305	0.76264	0.003994	0.88079	-0.37045	0.53423
Unknown 067	-1.3963	0.045926					-0.10405	0.89973
Unknown 068	0.28545	0.88123	0.71094	0.44811	-0.59128	0.98079	-0.52584	0.33663
Unknown 069	3.7486	0.000111	3.1527	0.37769	3.5729	0.018997	3.5594	8.42E-05
Unknown 070	-0.44402	0.79418	1.2482	0.36932	1.1066	6.69E-05	-0.01932	0.89973
Unknown 071	-0.25037	0.84828	3.1002	0.44073	1.8153	0.88079	0.50077	0.32053
Unknown 072	-0.21027	0.86911	-0.16304	0.55866	0.68853	0.98688	0.23269	0.91776
Unknown 073	0.26787	0.75115	0.045076	0.62243	1.1936	0.017667	0.22217	0.46575
Unknown 074	-0.35024	0.75115	2.759	0.44811	0.32511	0.58684	0.59	0.70125
Unknown 075	-0.23142	0.81663	0.14962	0.6849	0.51704	0.98688	1.1636	0.029243
Unknown 076	-0.39155	0.76411	1.662	0.38249	0.3042	0.41881	0.006221	0.98666
Unknown 077	0.0977	0.87071	0.25969	0.84634	0.22629	0.94172	-0.76327	0.004785
Unknown 078	-0.38438	0.91975	-0.08324	0.73713	0.97313	0.20755	0.2859	0.82289
Unknown 079	0.29821	0.72889	0.14284	0.97265	0.33173	0.9175	-0.94205	0.52555
Unknown 080	-0.71243	0.72889	1.2698	0.7692	-0.20016	0.88079	0.09864	0.78025
Unknown 081	-0.85202	0.11581	-1.8122	0.084184	-0.62715	0.020099	-1.7677	2.77E-05
Unknown 082	0.39055	0.99439	-1.531	0.37769	1.0477	0.073193	1.319	0.003268
Unknown 083	-0.65278	0.82083	0.37248	0.64984	1.1255	0.41881	0.9346	0.12807
Unknown 084	-0.51652	0.99439	-1.7234	0.20246	-2.2002	0.000725	-1.8426	0.14058
Unknown 085								
Unknown 086	4.9259	0.004088	4.8452	0.017405	5.5904	0.054899	7.8716	0.000218
Unknown 087	0.55295	0.72889	-0.43381	0.38249	-0.23829	0.42427	0.61104	0.049453
Unknown 088			0.097383	0.82925				
Unknown 089	-0.19169	0.98452	-0.47529	0.38249	1.7246	0.078643	0.88436	0.18056
Unknown 090	-0.18338	0.85131	3.4308	0.36932	0.37829	0.36102	0.502	0.10441
Unknown 091	-0.39612	0.72889	2.4678	0.4495	0.80881	0.050832	-0.1807	0.73381
Unknown 092	0.12957	0.97155	-1.2847	0.19499	-0.31113	0.91203	-0.64976	0.52555
Unknown 093	-2.358	0.72889	-0.78676	0.093246	0.63895	0.9263	-1.2294	0.079477
Unknown 094	0.11302	0.84315	1.9803	0.44811	0.44702	0.18861	0.18126	0.48748
Unknown 095	-0.42328	0.72889	1.9199	0.44811	-0.53386	0.45108	-1.4579	0.029243

Unknown 096	0.1844	0.75115	0.42213	0.53787	-1.13	2.77E-05	-0.13317	0.78025
Unknown 097	-0.29692	0.75115	0.40175	0.37769	-0.0509	0.98688	-0.13981	0.70443
Unknown 098	0.38682	0.72889	0.64313	0.59474	-0.43897	0.14638	-0.2773	0.68845
Unknown 099	1.5072	0.75115	4.1767	0.084476	-1.6886	0.80158	0.11613	0.74842
Unknown 100	-0.84523	0.75115	-0.56565	0.37769	1.0171	0.13264	-0.20351	0.73381
Unknown 101	-0.28429	0.75115	1.3973	0.44811	-0.55525	0.092774	-0.38189	0.45514
Unknown 102	-0.10777	0.75115	-0.73705	0.36932	-0.01691	0.91203	-0.40238	0.2705
Unknown 103	0.35055	0.75115	-0.78483	0.49856	0.33243	0.78432	0.49652	0.63364
Unknown 104	0.15177	0.99726	-0.71613	0.20246	1.7742	0.092774	-0.53203	0.29166
Unknown 105	0.15722	0.75115	0.08984	0.73713	-0.65005	0.054899	0.53043	0.36365
Unknown 106	0.35086	0.72889	1.3386	0.19499	-0.5965	0.045065	0.15496	0.71478
Unknown 107	0.14758	0.83871	0.19493	0.68134	-0.13205	0.50434	-0.04782	0.91507
Unknown 108	-0.05302	0.84828	1.523	0.6849	0.14864	0.79219	-0.36837	0.44012
uracil	-1.1613	0.72889	0.85807	0.57446	-0.21145	0.21026		
urea	-1.6271	0.95294	-0.65308	0.36932	2.4689	0.090021	0.59295	0.90049
xylitol	0.44782	0.75115	-0.1667	0.49856	-0.03306	0.98688	-0.35876	0.74842

Table S5: Diffrentially regulated lipids detected by LC-MS/MS extracted from root hairs (RH) and stripped roots (STR) in response to heat stress at (3h,6h,12h,and 24h) after heat exposure (40°C).

Root Hairs

ID	p.value	log2(fold change)	Time Point
DG(14:0/18:2/0:0)	0.003885	1.728262	24h
DG(14:0/18:3/0:0)	0.024406	1.449639	24h
DG(15:0/18:2/0:0)	0.027652	1.1432	24h
DG(18:3/18:3/0:0)	0.027588	1.520639	6h
PA(18:3/18:3)	0.01223	-3.00755	24h
PC(18:2/18:3)	0.017141	-1.66119	24h
PC(18:2/18:3)	0.007118	-4.02355	3h
PC(18:3/18:3)	0.000541	-2.55962	24h
PE(16:0/18:3)	0.006498	-1.83	24h
PE(18:0/18:2)	0.021566	-2.21362	24h
PE(18:1/18:2)	0.022993	-3.96088	24h
PE(18:2/18:3)	0.037234	-2.79327	24h
PE(18:3/18:3)	0.000365	-3.81923	24h
PG(16:0/18:2)	0.043947	-1.0251	12h
PG(16:0/18:2)	0.009537	-1.69772	24h
PG(16:0/18:3)	0.009831	-3.00736	24h
PG(16:0/18:3)	0.008732	-1.14435	6h
PG(18:2/18:3)	0.004406	-2.40363	24h
PG(18:3/18:3)	0.003679	-2.67038	24h
TG(14:0/15:0/16:0)	0.048646	1.017798	24h
TG(14:0/16:0/18:2)	0.041709	2.112245	6h
TG(14:0/18:2/18:3)	0.049435	-2.27226	6h
TG(15:1/16:0/18:2)	0.035154	2.17649	24h
TG(16:0/18:3/18:3)	0.042744	1.041599	3h
TG(16:1/18:3/18:3)	0.038965	1.152987	12h
TG(18:2/18:3/18:3)	0.037018	1.173548	24h

Stripped Roots

ID	p.value	log2(fold change)	Time Point
DG(18:3/18:3/0:0)	0.015299	2.727237	1.44744 3H
DG(18:1/18:2/0:0)	0.020524	3.171677	1.665246 6H
DG(18:2/0:0/18:2)	0.006967	2.994163	1.582153 6H
PA(18:3/18:3)	0.036307	2.030705	1.02198 12H
PA(18:2/18:2)	0.028537	2.114904	1.080592 6H
PC(16:0/16:0)	0.003319	5.157603	2.366701 12H
PC(18:2/18:2)	0.007332	0.305518	-1.71067 12H
PC(18:2/18:3)	0.023801	0.12052	-3.05265 12H
PC(16:0/18:1)	0.002332	4.595248	2.200143 24H
PC(18:0/18:1)	0.030253	6.060893	2.59953 24H
PC(18:2/18:2)	0.031744	0.327185	-1.61182 24H
PC(18:2/18:3)	0.002231	0.129448	-2.94956 24H
PC(18:3/18:3)	0.019377	0.15279	-2.71037 24H
PC(16:0/16:0)	0.026231	3.348158	1.743368 3H
PC(16:0/18:2)	0.009046	2.047487	1.033854 3H
PE(18:2/18:2)	0.013655	0.28961	-1.78782 12H
PE(18:2/18:3)	0.018606	0.402762	-1.312 12H
PG(16:0/18:2)	0.019793	0.417999	-1.25843 24H
PG(16:0/18:3)	0.030221	0.411891	-1.27967 24H
PG(16:0/18:0)	0.049883	5.37515	2.426305 3H
TG(14:0/14:0/16:0)	0.042881	4.061669	2.022073 12H
TG(14:0/16:0/16:1)	0.019264	2.192891	1.132834 6H
TG(16:0/17:1/18:1)	0.016273	5.328966	2.413856 6H
TG(16:1/16:1/16:1)	0.048722	2.215416	1.147578 6H
Cer(d18:1/24:0)	0.038081	2.555599	1.353661 24H

Figure S1: Number of overlapping and non-overlapping heat-responsive genes among the different exposure time points in soybean root hairs.

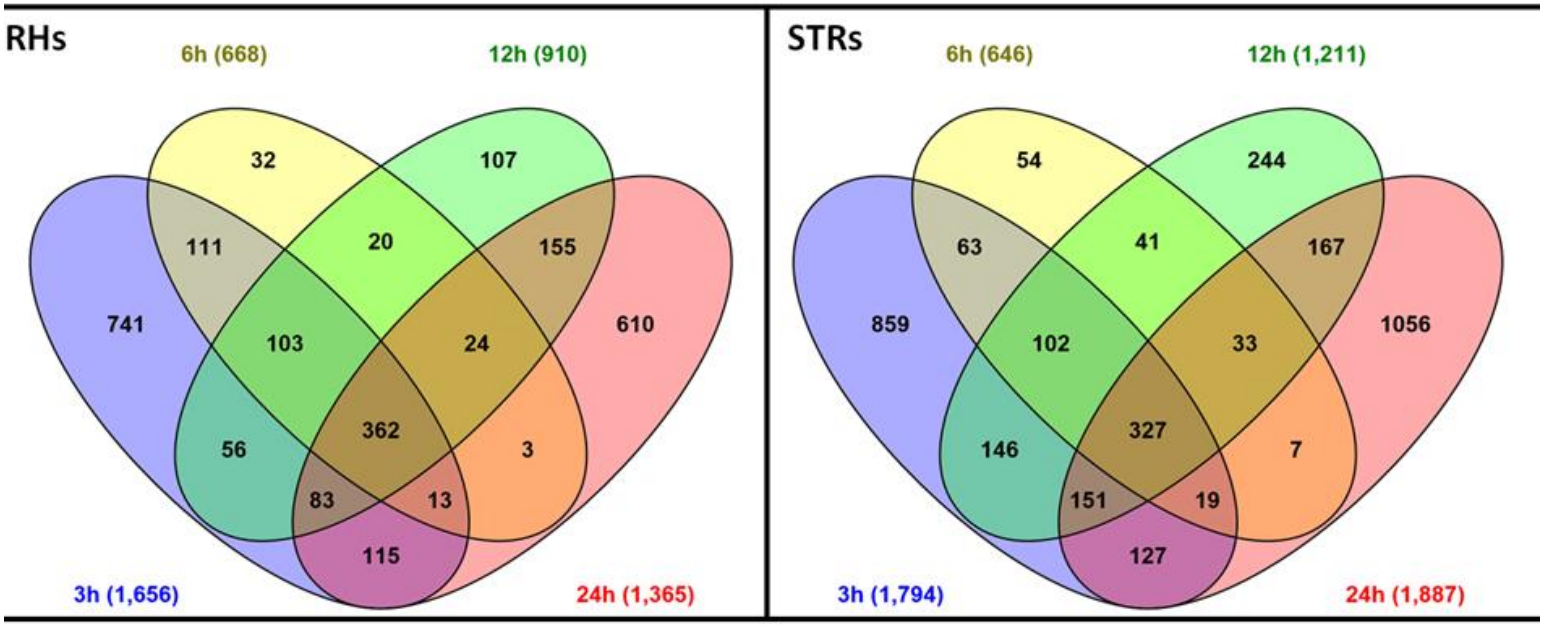


Figure S2: qRT-PCR validation of heat stress-responsive genes. A total of 15 randomly selected genes were used for qRT-PCR validation. Log2 fold change values (Control 25°C/Treatment 40°C) from the qRT-PCR data were plotted against Log2 (Control 25°C/Treatment 40°C) RNAseq values. Data are the average from two biological replicates.

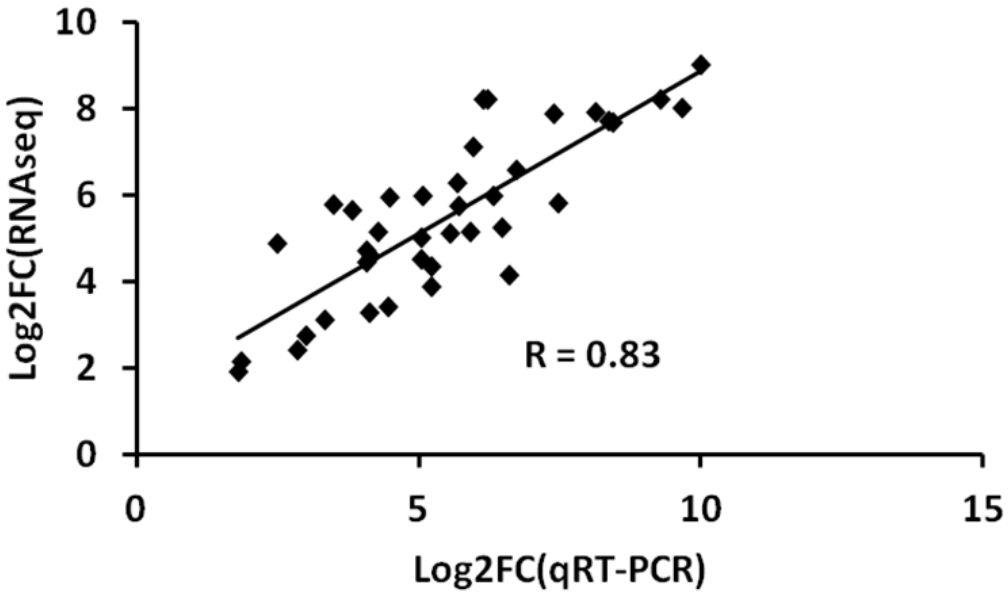


Figure S3: Gene Regulatory Modules identified in soybean heat-stressed root hairs.

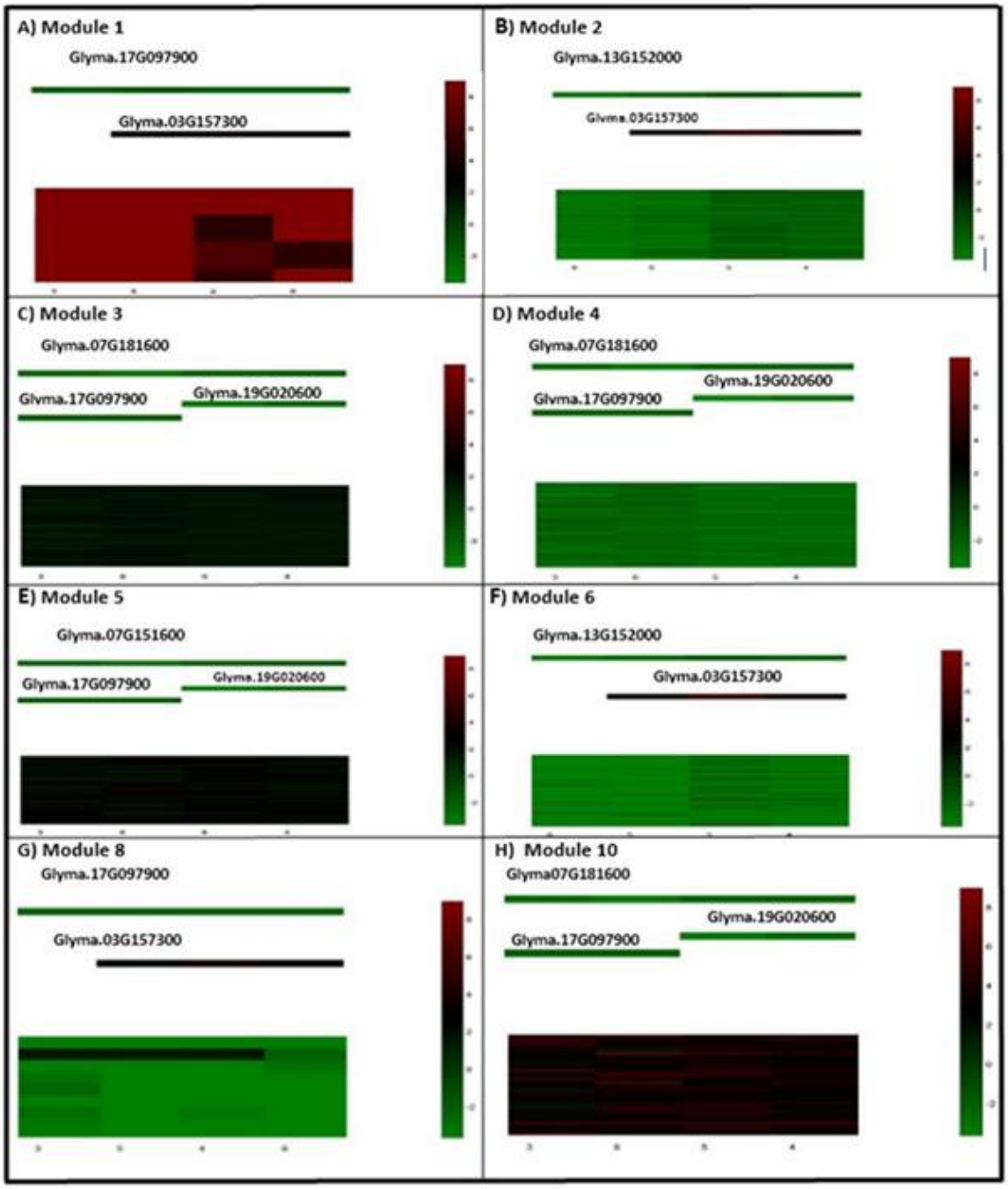


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Figure S4: Number of overlapping and non-over-lapping heat-responsive proteins among the different exposure-time points in soybean root hairs.

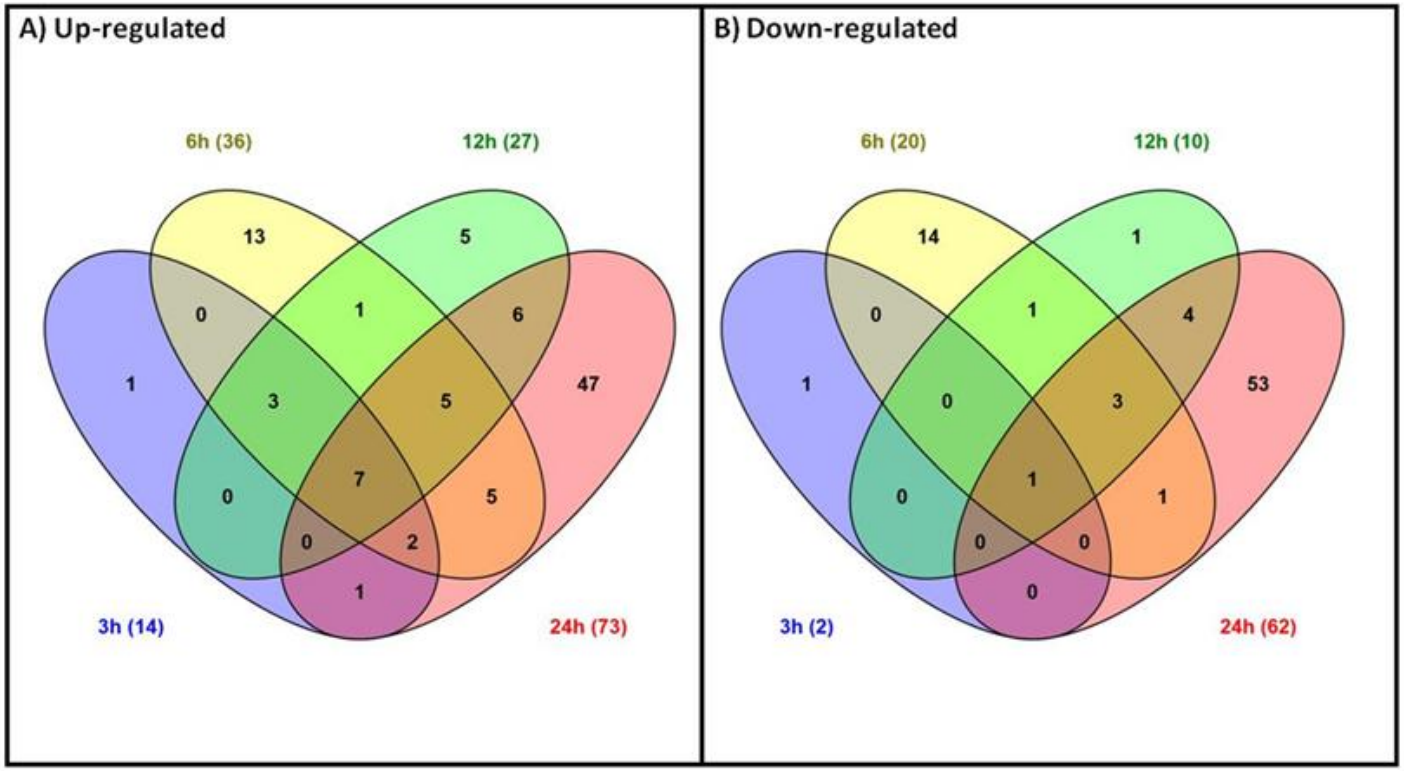


Figure S5: MapMan Classification of the regulated proteins in heat-stressed root hairs and stripped roots.

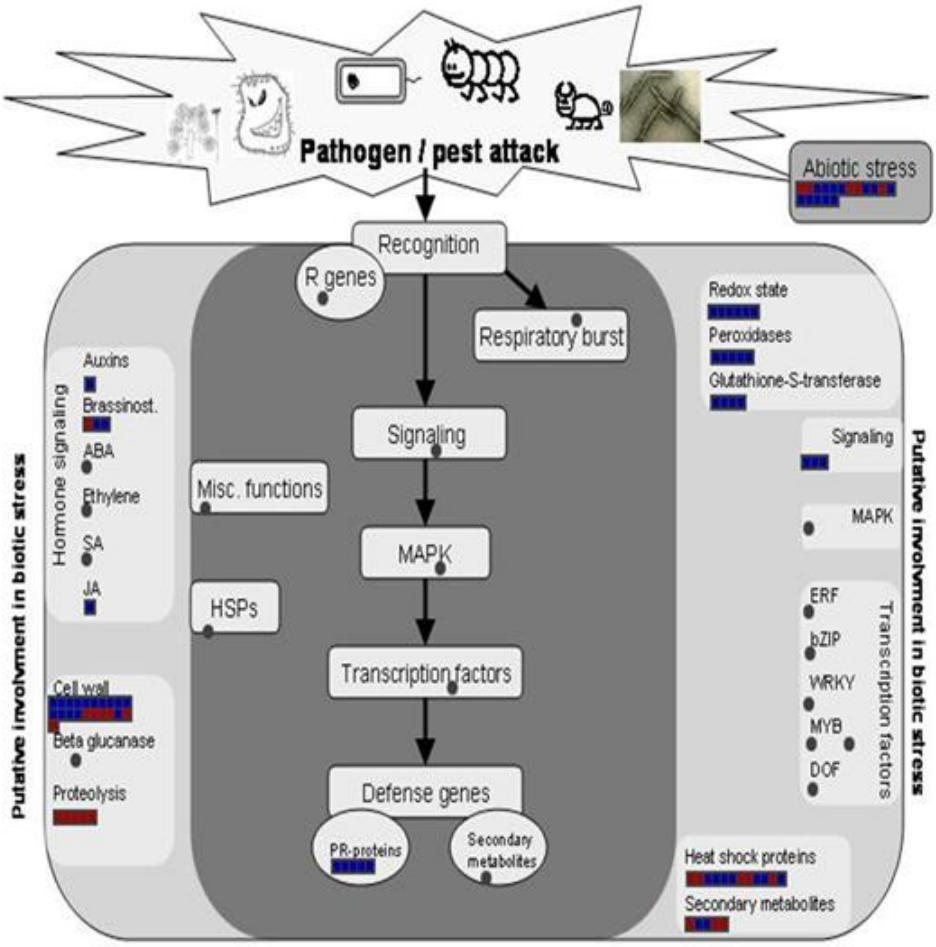


Figure S6: Principal component analysis generated using MetaboAnlayst 3.0 for metabolites identified via GC/MS and LC/MS in RHs and STRs at 24h were 46% of the metabolomic changes were observed. A) GC/MS intensity values analysis between tissues; **RH:Green/STR:Red** at 24h between *[heat-stressed vs. control]* B)LC/MS intensity values analysis in RH between *[heat-stressed vs. control]* at 24h C)LC/MS intensity values in STR between *[heat-stressed vs. control] (LC/MS analysis was separated by tissue for clarity)*(△ control 25°; + Heat treatment 40°C).

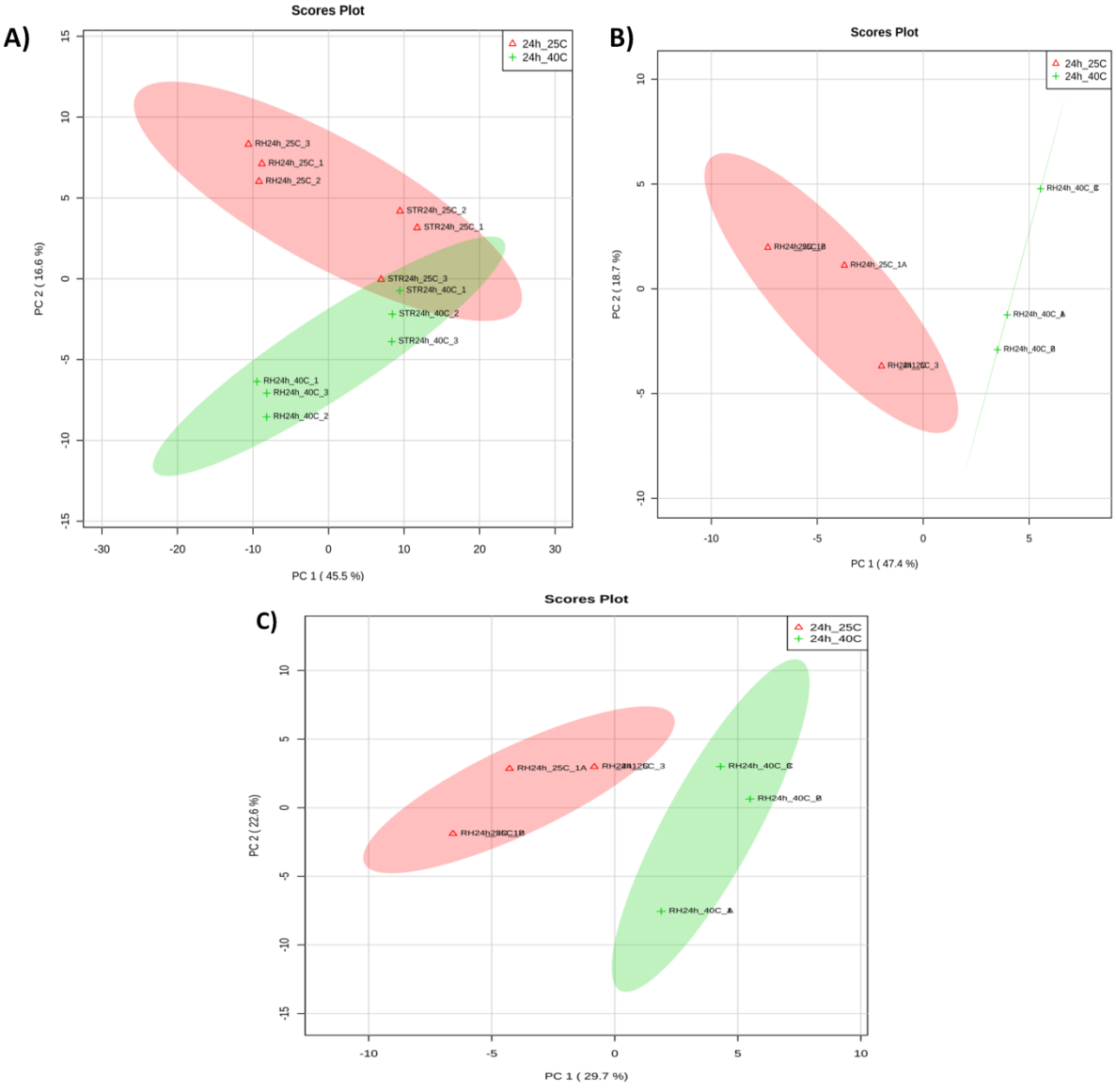
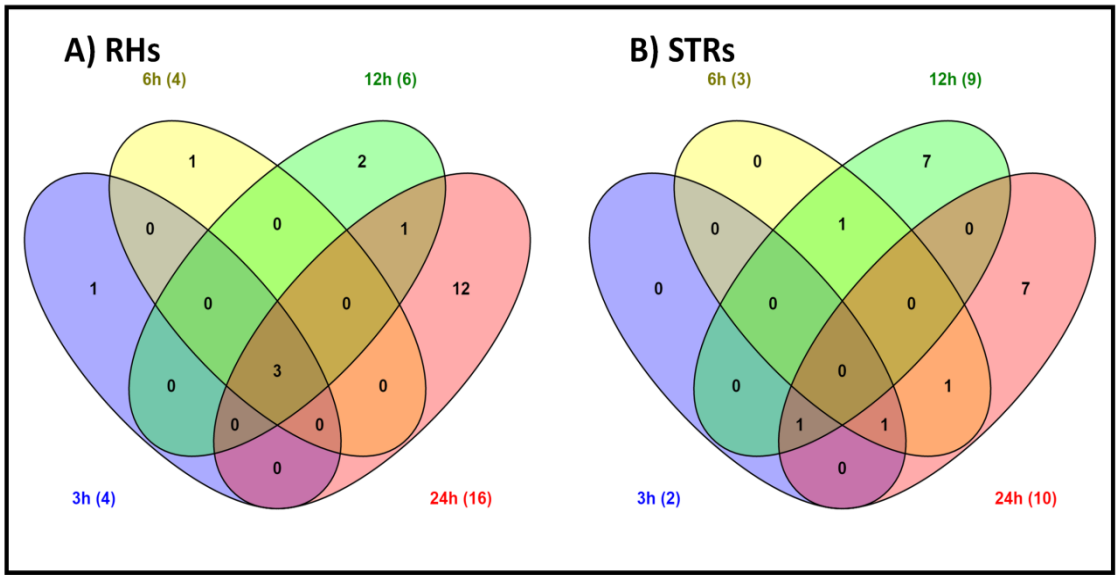


Figure S7: Number of overlapping and non-overlapping heat-responsive metabolites between soybean root hairs (RHs) and stripped roots (STRs). Number in parenthesis indicates all the regulated metabolites at various exposure-times (3h, 6h, 12h, and 24h). *(Fold change >log2; 2-fold pairwise comparison [heat-stressed vs. control] between, P value <0.05 deemed statistically significant)*



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