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Gene Expression and Physiological Analysis to Study Differences Between Oryza Sativa Cultivars Susceptible and Resistant to Chalky Grain Formation Subjected to High Nighttime Temperatures.

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Gene Expression and Physiological Analysis to Study
Differences Between *Oryza Sativa* Cultivars Susceptible and Resistant to Chalky Grain
Formation Subjected to High Nighttime Temperatures.

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Abstract

Starch composition and grain quality of rice is greatly influenced by genotype and environmental factors. The detrimental effects of high nighttime temperatures on rice yield and quality has recently become apparent, with some of the warmest average nighttime temperatures being recorded in the past few years. One of the most notable effects of this stress, an increase in grain chalk formation, correlates with a decrease in quality. This effect varies greatly between cultivars as some show less temperature-sensitive quality reduction than others. The goal of this research is to elucidate fundamental changes that occur in developing plants and grains as they respond to high nighttime temperatures. Two cultivars were used: Cypress, considered to have greater tolerance to high temperatures, and LaGrue, considered more susceptible. To assess physiological differences between cultivars, gas exchange measurements were collected from field-grown plants to determine photosynthetic rates. Gene expression analysis was carried out using DNA gene strip arrays using endosperm tissue isolated from plants grown in temperature-controlled conditions, identified genes differentially expressed in these cultivars. Differential gene expression was seen between cultivars regardless of nighttime temperature. There were however, few genes showing differential expression in response to temperature treatment within a cultivar. Among the genes differentially expressed between cultivars, no differences were observed in genes encoding enzymes in the starch biosynthesis pathway. Furthermore, no significant differences were found among genes encoding enzymes involved in amyloplast packaging or starch breakdown. Cypress endosperm had higher levels of expression in many genes involved in cell wall organization. LaGrue had a significantly higher photosynthetic rate during grain filling stages of development. The increased rate of photosynthesis may be related to the larger panicle on LaGrue leading to a stronger sink demand for carbon. This work adds to

understanding the combined effects that genotype and environment have on rice quality. There were few differences observed in endosperm gene expression but a difference was seen in physiological measurements, therefore future research can focus on what influence these physiological differences play in rice quality.

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Table of Contents

List of Tables

List of Figures

I. Introduction

Rice (*Oryza sativa)* is one of the most favored cereal crops in the world it is grown on almost every continent and sustains almost two-thirds of the world's population (Zhou et al., 2002). Whole grain rice is relatively nutrient rich providing 15 vitamins and minerals. Rice is also sodium, cholesterol, and gluten free. It is also a heart healthy food. Like consumption of other whole grains, eating rice can help reduce risk of heart disease, diabetes, and certain cancers (Middleton et al., 2000). China and India are the world's largest rice producers (Mohanty et al., 2013). In the United States, Arkansas is the number one rice-producing state, growing approximately half of the rice in the U.S. (Counce et al., 2015). The state of Arkansas depends on quality rice-production. Rice is the second-highest value commodity crop and the second largest agricultural export. A sustainable increase in rice production will be integral in feeding the increasing human population.

The world's population is predicted to grow from the current seven billion to 9.6 billion by the year 2050, which means we will need to produce 70% to 100% more food with decreasing land available for agricultural production (Godfray et al., 2010). Although decreasing availability of arable land is an important issue for food production, a changing global climate is also a critical challenge affecting agricultural production of many crops. Current Intergovernmental Panel on Climate Change (IPCC) models project mean global surface temperature increases between 1.8 °C and 4 °C over the 21st century, at a rate of 0.2°C increase per decade. Analysis of historical data indicates that every 1 ºC increase in average temperature lowers yields by up to ten percent (Lobell et al., 2011). The impacts of climate change are not limited to gradual increases in average temperatures, because the intensity and magnitude of extreme temperature events are also likely to increase substantially (Peterson et al., 2012). Global warming trends

have shown an asymmetric change in daily maximum and minimum averages (Easterling et al., 1997). Peng et al. (2004) reported that average nighttime temperatures at the International Rice Research Institute (IRRI) from 1979 to 2003 increased three times greater than average daytime temperatures. This paper was salient in brings to light the important correlation between a decrease in rice yields and an increased nighttime temperature during the growing season. Rice, being a tropical plant, thrives in a warm daytime temperature. In a report from Welch et al., (2010) historical data from farmer managed fields all over Asia was evaluated and showed that yields increased with an increase in daytime temperature. However, these increases were negated when they took into account the reduction in yield resulting from high nighttime temperatures (HNT). Another important factor to consider is the plant growth stage when the HNT occur. Analyses of recent historical data has revealed that HNT during the kernel setting stage can result in the biggest decreases in head rice yield (HRY) compared to HNT occurring during other plant life stages (Lanning et al., 2012).

The developmental stage in which a plant is exposed to heat stress determines the extent of possible damage to the rice crop (Wahid et al., 2007). HNT that occur during the flowering stages of rice have been shown to decrease pollen viability and spikelet fertility, which in turn decreases yield (Mohammed et al., 2013). Pollen swelling is an important occurrence that drives premature anther dehiscence (Matsui et al., 1999), and exposure to high temperature decreases the pollen grain's ability to swell (Matsui, et al., 2000). Endo et al. (2009) found that high temperatures negatively affected some tapetum functions such as pollen adhesion to the stigma. Even after anthesis and grain setting, high temperatures can have detrimental effects on rice production.

High nighttime temperatures that occur during grain filling stages of growth have detrimental impact on grain quality. When rice plants are in the R8 growth stage a majority of the grains are in the midst of the grain filling process (Ambardekar et al., 2011). HNT lead to improper assembly of amyloplasts (Lisle et al., 2000), which creates empty pockets in the center of the rice grain, and gives the grains an opaque, chalky appearance. Furthermore, the empty pockets in loosely packed starch reduces milling quality by weakening the grains making them prone to breakage or pulverization during the milling and polishing processes (Swamy & Bhattacharya, 1982). Together, rough rice yield and milling quality determine the economic value of rice from the field to the mill and in the market. Broken rice is worth only 50-60% of the value of whole rice (Cooper et al., 2008). Chalky grains that aren't pulverized during the milling process lower the value of the rice in all markets (Lyman et al., 2013). By focusing on overall yield and neglecting milling yield in rice breeding programs, breeders have inadvertently selected for high yielding varieties that tend to produce poor quality grain. Taking into account data for raw paddy yield versus milled rice yield, in combination with warm nighttime temperatures, data suggests that cultivars which have a lower yield but consistently produce quality grain should be the cultivars of choice. Current high yielding cultivars produce a lower quality grain that fissures and pulverizes during milling, may significantly reduce a farmer's profit (Lyman et al., 2013).

Rice grain composition and quality are not only affected by environmental stress but also influenced enormously by genotype. Rice is more biodiverse than other cereal grains. From an industrial point of view, this large biodiversity is important as it allows isolation of rice starches with different functionalities (Yu & Wang, 2007). Differences in physical behavior and functionality of rice starches are related to starch granule ultrastructure, which is determined by

the genetics of the cultivar (Hsu et al., 2014; Tian et al., 2009). The inheritance of starch quality is complex. There have been many studies that have attempted to look at major quantitative trait loci (QTL) involved in final starch microstructure. To this day, over 100 QTLs have been reported for the chalkiness trait located across all twelve chromosomes (Liu et al., 2012b; Peng et al., 2014; Wan et al., 2005; Zhou et al., 2009). Using a set of chromosomal segment substitution lines made by crossing a HNT tolerant *indica* variety 9311 and *japonica* variety Nipponare, Zhang et al. (2014a) were able to identify two QTL on chromosome 8 (*qHAC8a, qHAC8b*) and one on chromosome 4 (*qHAC4)* that reduced negative effects of high temperature on grain quality. Many QTL have been reported to be involved in chalky phenotype in rice, however they are found on many different chromosomes; how the genes located in these QTL interact to produce the undesirable phenotype needs more investigation.

Similar to rice cultivars being tolerant or susceptible to certain pathogens, HNT effects on grain quality varies between rice cultivars. Experimental data from Arkansas Rice Performance Trials 2007 through 2010 revealed that US rice cultivars can vary greatly in both milling quality and chalk accumulation (Ambardekar et al., 2011; Lanning et al., 2011). A number of reports have identified long grain cultivar Cypress as being less susceptible to chalk formation, whereas long grain cultivar LaGrue showed increased chalk formation when exposed to elevated nighttime temperatures (Ambardekar et al., 2011; Cooper et al., 2008; Counce et al., 2005). LaGrue originated from a cross made between the parent that originated from cv. Bonnet 73 crossed with cv. Nova 76, and another parent that originated from cv. Bonnet 73/3 crossed with cv. Newrex, at the Rice Research and Extension Center, Stuttgart, AR, in 1985 (publically released in 1993). Cypress originated from a cross between the parent cv. L-202 and parent cv. Lemont, made at the Louisiana Agricultural Rice Research Station in 1983 (publically released

in 1992). Therefore, each are high yielding long-grain varieties that were developed for production in the southeastern U.S.

Starch is the main component of rice endosperm. Its biosynthesis is a complex process dependent on multiple enzymes, many of which have protein isoforms encoded by different genes (Counce et al., 2005). Starch biosynthesis occurs in plastids. A temporary storage form of starch is produced in chloroplasts during photosynthesis and long-term storage starch is produced in amyloplasts. Unlike chloroplasts that proliferate via binary fission, small amyloplasts in the endosperm divide simultaneously at multiple sites (Yun & Kawagoe, 2010). In cereals such as maize, wheat, and barley, each amyloplast forms one granule of starch. Rice amyloplasts in contrast, form multiple grains of starch with a polyhedral shape (Yun & Kawagoe, 2010). Starch granules are composed of two different arrangements of D-glucose polymers, amylose and amylopectin, that make up repeated amorphous and crystalline lamellae (Jeon et al., 2010). Amylose is a linear polymer chain of 1,4-linked α-D-glucan (Jeon et al., 2010). Amylopectin is made of 1,4-linked α-D-glucan chains that are highly branched with α-(1-6)-glycosidic bonds (Jeon et al., 2010).

The starch of rice endosperm is a complex structure made up of a combination of amylose and amylopectin. Different rice varieties have differential amylose content (AC) and can be classified as follows: waxy (0-2%), very low (3-12%), low (13-20%), intermediate (21-25%), high (≥26%; (Pandey et al., 2012). The conversion of sucrose into amylose and amylopectin occurs via a complex metabolic pathway, the regulation of which is very poorly understood. Several different enzymes catalyze the process: sucrose synthase, adenosine diphosphate glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching enzymes (SBE), and starch debranching enzymes (DBE; Counce et al., 2005, Wang et al., 2014).

Before glucose-1-phosphate can be added to an amylose or amylopectin chain, it is first converted to adenosine 5' diphosphate-glucose (ADP-glucose) by AGPase (Vandeputte & Delcour, 2004; Wang et al., 2014). This is the first committed step in the starch biosynthetic pathway (Green & Hannah, 1998). In cereal endosperm AGPase is located mainly in the cytosol of the cell, and ADP-glucose molecules transported into amyloplasts are committed to starch biosynthesis (James et al., 2003). AGPase enzyme is made up of two small catalytic subunits and two large regulatory subunits (James et al., 2003; Nakamura, 2002). The smaller subunit is thought to be responsible for the catalytic activity of the enzyme, whereas the larger subunit is responsible for modulation of regulatory properties of the enzyme complex (Tetlow et al., 2004). AGPase activity is regulated by 3-phosphoglycerate (3-PGA) and inorganic phosphorus (Sikka et al., 2001). In rice, the AGPase gene family consists of two isoforms of the small subunit genes and four isoforms of the large subunit genes (James et al., 2003; Nakamura, 2002; Ohdan et al., 2005). Therefore, it is likely that there can be multiple isoforms of this enzyme complex, because of its heterotetrameric structure and the presence of multiple subunit forms. Once formed, ADP-glucose is transported into the amyloplasts via a transporter encoded by the rice OsBT1 gene (Toyota et al., 2005).

Once inside the amyloplasts, starch synthase enzymes catalyze the attachment of the ADP-glucose to the non-reducing end of the glucan chain (Jeon et al., 2010). There are many isoforms of starch synthase (SS) proteins within a rice plant, and it appears that all are encoded by different genes. The isoforms are grouped into five classes; SS-I, SS-II (a, b, c), SS-III(a, b), SS-IV(a, b) and granule bound SS (GBSSI, GBSSII; (Hirose & Terao, 2004; Nakamura, 2002; Ohdan et al., 2005; Tetlow et al., 2004). Soluble starch synthase enzymes are primarily responsible for amylopectin biosynthesis (Tetlow et al., 2004; Vandeputte et al., 2004).

However, the SS-I isoform is important in short amylose chain elongation, and elongates exterior short chains with a degree of polymerization (DP) of six to seven units, and adds only a few glucan residues (Fujita et al., 2006). Rice has only one isoform for the SS-I class. In rice *OsSSI* knockout lines, seed morphology and starch structure is very similar to wild type and no chalky phenotype is observed (Fujita et al., 2006). There are three isoforms for SS-II in rice, however SS-IIa and SS-IIc are the major isoforms expressed in the endosperm (Ohdan et al., 2005; Zhang et al., 2011). SS-II is involved in elongation of medium size glucan chains and competes with SS-I to produce intermediate chain lengths that are then added to by SS-III (Zhang et al., 2011). SS-III enzymes catalyze the elongation of long amylopectin chains. There are two isoforms for this class, SS-IIIa and SS-IIIb and expression occurs primarily in the seed endosperm and leaf, respectively (Hirose & Terao, 2004; Ohdan et al., 2005; Zhang et al., 2011). Rice also possesses two isoforms of SS-IV (Dian et al., 2005). This class of starch synthase has not been functionally characterized in rice, however SS-IVb is primarily expressed in the endosperm (Dian et al., 2005; Ohdan et al., 2005; Tetlow et al., 2004). The combination of multiple enzymes ultimately controls catalytic rates, structure of starch crystal, and the early steps in starch biosynthesis.

Granule bound starch synthase is involved in amylose chain elongation and there are two genes that encode GBSS in rice (James et al., 2003; Jeon et al., 2010; Ohdan et al., 2005; Wang et al., 2014). The name of the enzyme is indicative of its role and function. This enzyme is centrally bound in the starch granule where it catalyzes the attachment of ADP-glucose to the non-reducing end of amylose. Ohdan et al., (2005) describes GBSSI as being expressed primarily in the endosperm of rice, while GBSSII is expressed primarily in leaf tissue and other non-photosynthetic tissue and involved in biosynthesis of transient starch. GBSSI, encoded by the *Waxy* (*Wx*) gene, is responsible for the amylose synthesis linking it directly to final AC in

developed endosperm. Because AC is a key determinate on cooking quality of rice there has been a lot of research on this gene. Ayres et al., (1997) demonstrated that a, single nucleotide polymorphism (SNP) in the untranslated region of the first exon of GBSSI is associated with variation in AC in rice endosperm. The waxy locus contains two alleles W_a *and* W_b , and the SNP in the W_b allele maybe temperature sensitive (Wang et al., 1995). The W_b allele can be further sub-divided into eight alleles and one of these alleles differs by a single G-to-T polymorphism at the 5′ leader intron splice site (Larkin & Park, 1999). At high temperatures this SNP causes is a reduction in the efficiency of the pre-mRNA processing, decreasing transcript abundance leading to a decrease in AC (Larkin & Park, 1999; Wang et al., 1995). Cultivars Cypress and LaGrue both contain the W_a the non-sensitive allele (Cooper et al., 2008).

Complex starch architecture originates from the varying activity levels of the isoforms of starch branching enzymes (BE). After the SS enzymes have elongated a glucan chain to a suitable length, approximately degree polymerization 50, BE generate an α-1,6 linkage by cleaving internal α-1,4 bonds and attaching the released reducing end to C6 hydroxyls to form the branch in the amylopectin molecule (Nakamura et al., 2010). There are three isoforms of BE encoded in the rice genome, BE-I, BE-IIa, and BE-IIb. BE-I and BE-IIa are both expressed in all plant tissue, however BE-IIb in monocots is only expressed in non-photosynthetic tissue such as the endosperm (Yamanouchi & Nakamura, 1992). Branching enzymes have different affinities for different DP. Branching enzyme-I preferentially transfers longer chains that eventually link multiple amylopectin clusters, whereas BE-IIb primarily transfers short chains that help create the border between amorphous lamellae and the crystalline lamellae (Abe et al., 2014). Recent studies have provided some evidence of protein-protein interaction that may occur between SS-I, SS-IIa, and BE-IIb (Abe et al., 2014).

Debranching enzymes were once thought to only be involved in the degradation of starch but recent studies have provided evidence they play an important role in amylopectin biosynthesis (Nakamura, 1996). Plants have two classes of debranching enzymes, pullunases (PUL) and isoamylases (ISA). These names refer to the enzyme substrates, ISA mainly debranches phytoglycogen and amylopectin, while PUL mainly debranches pullulan and amylopectin (Nakamura, 2002). Plants only have one known gene for pullulanase and at least three subclasses of amylases, some with multiple isoforms (Fujita et al., 2009). Isoamylase I is coded for by a gene located on chromosome 8 and is preferentially expressed in the endosperm during development (Kubo et al., 1999).

The starch biosynthesis pathway is complex. There have been many studies attempting to explain how all the starch synthase genes and/or enzymes work in concert to create the complex starch granule (Fu & Xue, 2010). Functional characterization of several regulators of rice starch accumulation suggests that the rate of starch synthesis and deposition can strongly impact chalk formation. Fu & Xue (2010) investigated chalky grain formation by knocking out a transcription factor (TF), Rice Starch Regulator1. Many starch biosynthesis genes increase in expression just after pollination in these gene-silenced lines, when compared to the WT lines. Early high expression of starch biosynthesis genes led to faster starch accumulation and chalky grain. Wang et al. (2008) isolated and described the function of a gene coding for a cell-wall invertase. They found that disruption of this gene, termed 'slowed starch accumulation in the endosperm' increased the chalkiness of the endosperm (Wang et al., 2008). Through the generation of T-DNA tagged mutants, Kang et al., (2005) showed that disruption in a pyruvate orthophosphate dikinase gene leads to a chalky phenotype. Interestingly, these results showed that starch content was similar between rice endosperms, but also that the mutant kernels had increased protein and

lipid contents (Kang et al., 2005). A maize mutant *opaque-2* shows a phenotype of soft and chalky endosperm that is highly susceptible to kernel breakage similar to chalk in rice (Hasjim et al., 2009). The *O2* gene encodes a bZIP transcription factor that binds to the promoter regions mainly in genes that encode storage proteins (Li et al., 2015). *O2* mutant maize lines had lower protein and starch content suggesting early termination of starch biosynthesis (Hasjim et al., 2009). This indicates that chalk formation may result from an imbalance between starch, protein, and lipid accumulation at the core of the rice endosperm.

Before starch can be synthesized, carbon must first be fixed from airborne CO_2. Leaf photosynthesis is the main source of reduced carbon for the grains that the plant is going to produce. Studies have revealed strong positive correlations of either leaf- or canopy-photosynthesis and seed yield in soybean (Jin et al., 2010; Thompson et al., 1995; Wells et al., 1986). In rice, the top three leaves, especially the flag leaf, are the most important sources of carbon for starch production (Fitzgerald & Resurreccion, 2009; Zhang et al., 2015). Fitzgerald & Resurreccion, (2009) found that starch substrate supplies were limited under high temperature treatment. When comparing two *indica* varieties, IR8 and IR60 that were chosen based on anecdotal differences in chalk, subjected to high temperature, IR60 had decreased proportions of filled spikelets along with a lower percent of chalky grains compared to IR8. These findings suggest that IR60 is able to adjust the size of its carbon sink in response to temperature. A team of Chinese researchers investigating a QTL for increase in yield found it to be correlated with an increase in capacity for starch temporary storage in the sheath (Ishimaru, 2003). However no leaf morphological differences were associated with the higher starch in the sheath. These findings indicate that higher starch accumulation may result in superior sink capacity. Other source-sink relationship studies have showed that chalky endosperm development can be overcome by

increasing assimilate supply (Tsukaguchi & Iida, 2008). This previous work on source-sink relationships led to the decision to analyze photosynthetic differences between cultivars, considering that different amounts of sucrose from source to sink could influence varietal differences in chalk formation.

Rice has a compact genome size measuring 389 Mb, making it a fraction the size of the maize genome and about forty times smaller than the wheat genome (Jung et al., 2008). Because of the comparatively small genome size rice is regarded as a reference for cereal genomic research and genetic improvement. The latest, error-corrected genome assembly for rice covers about 97.1% of the entire Nipponbare rice genome (Kawahara et al., 2013). Gene prediction revealed 55,986 genes in the rice genome. The genome along with most recent annotation can be found on Michigan State University's Rice Genome Annotation Project Website: http://rice.plantbiology.msu.edu/index.shtml. Because of the extensive genomic information and the characterized genetic variation in rice varieties, rice is an excellent model for this study. Although previous transcriptome studies have been reported for rice tissues exposed to high temperatures, very few have compared phenotypically distinct varieties and their responses to HNT. The work described here focuses on two long-grain U.S. varieties that differ in chalk formation in response to HNT.

B. Hypothesis and Research Objectives

Hypothesis:

We hypothesize that there are differential expression patterns of genes that encode enzymes involved in the starch biosynthesis process in rice endosperm. This differential gene expression along with differential photosynthetic capacity is influenced both by environment and genotype. Investigation of differentially expressed genes will lead to gene promoter regions and genetic markers for improved rice quality in breeding programs.

Objective 1:

To assess differences in endosperm gene expression in two rice cultivars, tissue was collected from LaGrue and Cypress plants exposed to high nighttime temperatures and transcript profiles were measured.

Objective 2:

To understand the physiological differences between rice cultivars prone to, or resistant to chalky endosperm formation under HNT, the rate of CO_2 gas exchange was examined and compared in flag-leaves of plants grown in the field.

C. Materials and Methods

Plant conditions and maintenance

Two long-grain, pureline rice cultivars Cypress and LaGrue were selected. Round 10-inch-diameter pots were planted with fifteen seeds per pot in Sunshine LC1 potting medium (SunGro Horticulture Distribution, Inc., Bellevue, WA). Pots were placed in the greenhouse, in trays with at least 2.5 cm of water at all times. When plants were at vegetative growth stage V2 (collar formation on leaf 2 on main stem) fertilizer (Miracle-Gro®) water soluble all-purpose plant food, Scotts Miracle-Gro Products, Inc., Marysville, OH) was administered weekly at a rate of one teaspoon per gallon of water.

Temperature treatments

Plants were maintained in the greenhouse until they reached reproductive growth stage R5 (at least one caryopsis on the main stem panicle is elongating to the end of the hull), and then plants were moved to two identical growth chambers (Adaptis, Conviron, Pembina, ND). For one week the plants were allowed to acclimate to growth chamber conditions of 15:9 (light: dark) hour photoperiod. The temperature settings were the same for both growth chambers during the one-week acclimation period, 0600 h to 1200 h at 25 °C, 1200 h to 1600 h at 27 °C, 1600 to 2100 h at 25 °C, and nighttime (dark) temperature was 18 °C from 2100h to 0600h. After one week the high temperature treatment chamber was changed so the day temperatures were the same but nighttime temperature was set at 30 °C from 2100 h to 0600h.

Percent chalk measurements

Chalk measurements were taken in duplicate, 3 replications per cultivar x temperature treatment combination, on 100-kernel brown rice sets. Grains were hand dehusked carefully using two large rubber stoppers. Brown rice kernels were placed on a tray so that no kernels touched another. An image of the kernels was taken using a flatbed scanner and analyzed via WinSeedle Pro 2005™ software (Regent Instruments Inc., Sainte-Foy, Quebec, Canada). Statistical analyses using a Student's T-test was performed using Graphpad Prism software.

Photosynthesis measurements

For gas-exchange experiments, field plots of Cypress and LaGrue from the Arkansas Rice Performance Trial were sampled. Gas exchange measurements were performed using a Li-Cor 6400XT Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA) to determine photosynthetic rates. For each data-gathering period, ten measurements at two different plots of each cultivar were collected. Measurements were taken independently at three different growth stages. The first set of measurements was taken on July 25th and 26th; on rice plants at reproductive growth stage R2 (collar formation of the flag leaf). For these measurements the photosynthetic active radiation (PAR) was set to 1300 μmol m^{-2} s^{-1}, air flow was set to 400 μmol s^{-1}, reservoir CO_2 was set at 375 ppm, and chamber block temperature was set to 24° C. Both temperature and CO_2 levels were chosen to approximate ambient levels at the time. The 6400-02B LED light source was used to provide the same light intensity to all samples measured during the first set of measurements. Because the area of the chamber was 2 x 3 cm the rice leaves did not fill the chamber completely. The widths of the leaves were measured before insertion into the testing chamber, and the leaf area measured was entered into the instrument at

the time of the reading. A second set of measurements was taken on August 15th and 16th; measuring rice plants at reproductive growth stage R5 (at least one grain on the main stem panicle is elongating to the end of the hull). The 6400-40 leaf chamber fluorometer was installed for this and a subsequent measurement. The chamber for this source is a smaller circular chamber with an area of only 2 cm^2. With this chamber all leaves completely filled the space so measuring leaf width was not needed. During the second set of measurements PAR was set to 1200 μmol m^{-2} s^{-1}, flow rate was 400 μmol s^{-1}, reference CO_2 level was 375 ppm, and chamber block temperature 25°C. The final measurements were taken on August 29th and 30th: measuring plants that were at reproductive growth stage R8 (at least one grain on the main stem panicle has a brown hull). During these measurements PAR was set at 1200 μmol m^{-2} s^{-1}, flow rate was 400 μmol s^{-1}, reference CO_2 level was 375 ppm, and chamber block temperature 26°C.

Tissue collection for RNA analysis

When the growth chamber treated plants reached R7 to R8, individual grains at the soft to hard dough stages were collected. Panicles were removed from the plants wrapped in aluminium foil and placed in a cooler that contained dry ice and brought to the lab. At a clean lab bench hulls were trimmed at the very tip and endosperm fractions squeezed out and placed into eppendorf tubes frozen in liquid nitrogen. Total RNA was isolated from endosperm fractions using a Masterpure Plant RNA purification kit (Epicentre Inc., Madison, Wis.).

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

For RT-PCR cDNA was synthesized with iScript cDNA synthesis kit (Bio-Rad, Hercules, Calif.). The cDNA was diluted 1:5 by adding 80 μL nuclease-free H_2O to the 20 μL cDNA

synthesis reaction. Gene specific primers (Table 10) were used in standard PCR reactions with cDNA dilutions as template (1 µl/reaction) for each reaction performed with the following conditions: 2 min at 95ºC; followed by 25 cycles of 30 sec each at 95ºC, 56ºC, 72ºC; followed by 5 min at 72ºC. RT-PCR products were separated on 1.2% TAE agarose gels stained with gel-red dye.

Affymetrix® Gene Chip analysis of gene expression

Total RNA from three independent samples from each treatment (Cypress 18°C, Cypress 30°C, LaGrue 18°C, and LaGrue 30°C) was quantified with a Biospec nano spectrophotometer (Shimadzu, Kyoto, Japan). Concentration of the RNA was adjusted to be greater than 50 ng/µL but less than 250 ng/µL and sent to the University of Michigan where transcript levels were analyzed via Affymetrix® US Rice Gene 1.1 ST Array Strips. Expression values from each strip were normalized using robust multi-array average. Before analysis was run probesets with a variance across all samples of 0.05 were filtered out. The expression values were log2-transformed data, fit to linear models designed for microarray analysis, and contrasted. Statistical analysis was done using the Affy and Limma packages of Bioconductor™ software implemented in the R statistical environment.

qPCR primer design

A subset of genes identified as having differential expression between cultivars via array measurements were chosen as targets for qPCR analyses to investigate the reproducibility of expression levels observed in the microarray experiment. Nucleotide sequences for the coding regions as well as the 5' and 3' un-translated regions for specific genes were obtained from the

Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp). Oligonucleotide primers were designed using Primer3web version 4.0.0 (http://bioinfo.ut.ee/primer3/). Primers were checked for absence of substantial hairpin, self-dimer and hetero-dimer formation using OligoAnalyzer 3.1 on the Integrated DNA Technologies website (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/) (Table 10).

Quantitative Real-time Polymerase Chain Reaction

For quantitative real-time PCR (qPCR), cDNA was synthesized with iScript cDNA synthesis kit (Bio-Rad, Hercules, Calif.). The cDNA was diluted 1:5 by adding 80 µL nuclease-free H_2O to the 20 µL cDNA synthesis reaction. Real-time reverse transcription PCR reactions were performed using the *Power* SYBR® Green PCR master mix (Applied Biosystems) with 20 µL reaction volumes. Applied Biosystems StepOnePlus™ real time PCR system with the following protocol was used: 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15s and 61°C for 1 min. Three technical reps were run for each biological rep.

To calculate relative gene expression, the $2^{-\Delta\Delta Ct}$ method Livak & Schmittgen (2001) was used were the $\Delta\Delta C_T$ is calculated as:

$$\Delta C_T = C_{T\ \text{Target Gene}} - C_{T\ \text{Endogenous Control Gene}} \qquad \text{Equation 1}$$

$$\Delta\Delta C_T = \Delta C_{T\ \text{Cypress Tm}} - \Delta C_{T\ \text{LaGrue Tm}} \qquad \text{Equation 2}$$

Using the average function in Microsoft Excel the mean C_T was calculated for each biological rep (Cypress at 18°C, Cypress at 30°C, LaGrue at 18°C, and LaGrue at 30°C). Experiments performed by Jain et al., (2006) showed that ubiquitin 5 (UBQ5) was a good endogenous control to use with rice tissue when performing qPCR analysis, because UBQ5 had the most stable

expression level across many different treatment backgrounds. To normalize for variation of transcript levels in the cDNA, the C_T of the endogenous control gene, in each treatment combination, was calculated as the mean of the mean. For the gene of interest in each treatment-combination the mean C_T of the technical reps minus the control C_T this resulted in three ΔC_T values for that gene in each treatment-combination.

D. Results

Measurement of chalky grain production from growth chamber treatment

Exposure to high nighttime temperatures in growth chamber experiments led to an increase in chalky rice phenotype. Loose packing of amyloplasts results in visible chalkiness (Figure 1). Scanning rice grains with visible light can provide a quantifiable image of the opacity of the individual grains. This is routinely used as a measure of chalkiness via WinSeedle Pro 2005™ Pro software. LaGrue produced a significantly higher percentage of chalky grains, approximately a two-fold increase, at the high temperature treatment (HNT) compared to LaGrue grains grown at the control nighttime temperature (CNT; Figure 2). Cultivar Cypress retained the same level of chalk regardless of nighttime temperature treatment. There was no significant difference in levels of chalk between cultivars grown at the CNT 18 ºC, the average level of chalk measured in LaGrue grains was twice the average level of chalk in Cypress subjected to HNT. These results are in agreement with field data collected for the Arkansas rice performance trails and in growth chamber experiments (Ambardekar et al., 2011; Cooper et al., 2008; Lanning et al., 2011). In the current study, the percent of chalky LaGrue grains was lower than in a previous study (Cooper et al., 2008), however chalk levels significantly increased following HNT treatment.

Scanning Electron microscopy of rice starch granules

To more closely investigate the chalky center of grains that ripened under elevated temperatures, starch morphology was analyzed using scanning electron microscopy (SEM). In LaGrue grains that were allowed to mature at the control temperature, 18 ºC, amyloplasts had a uniform size and a polygonal shape. These amyloplasts showed tight packing leaving no room for air pockets between them. The flat angular edges that remain when the starch granules separate during dissection, are visible using SEM (Figure 3). Starch located in the central part of the grains exposed to a HNT had an opaque chalky appearance. In the grain that developed during HNT, amyloplasts located in the opaque center were smaller and round in shape compared to the amyloplasts that matured at CNT. These rounded amyloplasts do not pack together tightly and allow spaces between the amyloplasts. This loose packing of granules is what gives the opaque, chalky appearance. Loose packing also results in a brittle rice grain that is much more prone to fissure and pulverization during the milling process. This improper formation of starch granules in response to HNT is a strong indication that sucrose/starch synthesis, transport, and/or deposition might play an important role in chalk formation.

Comparison of starch biosynthesis gene expression between cultivars

For a preliminary examination of expression of starch biosynthetic enzyme genes, reverse transcription-PCR (RT-PCR) was conducted on cDNA synthesized from RNA of endosperm from four different cultivars. The cultivars in this experiment were Cypress and Jupiter, which both consistently produce quality rice grains, and cultivars LaGrue and Wells, which both are high yielding but of inconsistent quality especially when exposed to high nighttime temperatures.

Results from RT-PCR indicated the possibility of small differences in expression of starch biosynthetic enzymes when comparing temperature treatments within a cultivar (Figure 4).

To examine whole transcriptome expression patterns in rice endosperm in cultivars known to be sensitive or tolerant to HNT, RNA was collected from rice endosperm and analyzed with Affymetrix® Rice (US) Gene 1.1 ST Array Strips (ssp. Japonica). Rice endosperm were collected from Cypress and LaGrue treated with a HNT of 30 ºC and a CNT of 18 ºC. Multiple endosperm were collected, while in the soft-doughy stage of grain filling, and used for analysis. The array strips contain 816,815 probes with a median of nineteen probes per gene making the total gene-level probe sets 45,207. After transcript profiles were determined, the data for select genes encoding components of starch synthesis were analyzed in detail. Expression levels of starch biosynthesis genes did not display differential expression between cultivars regardless of nighttime temperature. However, expression levels of endosperm-specific isoforms of enzymes in the starch biosynthesis pathway were higher than levels of leaf-specific isoforms agreeing with previous research by Ohdan et al. (2005). This indicates that between the two cultivars, the differences in chalk accumulation do not arise from a difference in transcript accumulation of starch biosynthetic enzyme encoding genes. The improper starch crystal structure may be a result of differences in post-translational modification of starch synthesis enzymes, differences in how the multiple starch granules fill the amyloplasts, or possibly even the over- or under-production of proteins that make up protein bodies found in rice endosperm.

The first important step in the biosynthesis pathway of starch is catalyzed by ADP-glucose pyrophosphorylase (AGPase). This enzyme is a heterotertamer and catalyzes the combination of ATP with glucose-1-phosphate to create ADP-glucose, which is then added to the non-reducing end of an α-1,4-glucosidic chain. Expression patterns in Cypress and LaGrue

were not different between temperature treatments and expression levels were higher for the isoforms that show preferential endosperm expression (Figure 5). Non-differential expression between cultivar or temperature treatment strongly suggests that the chalky grain formation is not due to a lack or abundance of transcripts encoding subunits of AGPase.

Once glucose-1-phosphate has been formed, starch synthase (SS) enzymes catalyze its attachment to the glucan chains. Expression for all of the SS genes was similar across all cultivars and temperature treatments (Figure 6). Granule bound starch synthase I had a high level of expression, double that of GBSSII, and when compared to housekeeping gene expression such as tubulin or actin. The higher expression of GBSSI agrees with previous reports (Ohdan et al., 2005). Soluble starch synthase-I is highly expressed in all tissue indicating the importance of the enzyme for starch biosynthesis in all plant tissue. Our data showed a high level of expression of SS-I, SS-IIa, and SS-IIIa when compared to other SS genes (Figure 6). Among the starch synthases, GBSSI, SS-I, SS-IIa and SS-IIIa are reported to have preferential expression in rice endosperm (Ohdan et al., 2005). These data suggest that at this stage of grain filling, transcriptional accumulation for starch synthase encoding genes is likely not responsible for the chalky phenotype.

Once polyglucan chains in rice become long enough, starch branching enzyme enzymes (BE) cleave a 1,4-linkage and attach it to the C-6 hydroxyl group of another glucan chain. There are three BE isoforms in rice, BE-I, BE-IIa, and BE-IIb. Transcription levels for all forms of BE were very similar across both cultivars and temperature treatments (Figure 7). Both BE-I and BE-IIb showed high levels of expression, at a similar magnitude of the SS genes.

Rice plants express many enzymes that allow for the fine structural modification of the developing starch granule. The final enzyme class involved in starch biosynthesis, starch

debranching enzyme, helps rice and other plants in this process. Debranching enzymes directly hydrolyze α-1,6-glucosidic linkages (Nakamura, 1996). The gene family for debranching enzymes is large, however most of the isoforms show low levels of expression during endosperm development. Isoamylase–I, a debranching enzyme, is encoded by a gene located on chromosome 8 and is critical for starch granule initiation in rice endosperm (Kawagoe et al., 2005). As with the other starch biosynthesis enzymes transcription levels, transcript levels for all debranching enzymes were similar across both cultivars and temperature treatments. Analysis of transcription accumulation for enzymes involved in the starch biosynthesis pathway showed no significant variation between cultivars or temperature treatments (Figure 8). From this we can conclude that differential expression levels of these genes in developing rice grains are not responsible for the formation of chalky grain. Coordinated regulation of the starch biosynthesis pathway is very complex, and analyzing up- or down-regulation of starch biosynthesis alone does not give a clear idea of what other transcripts are accumulating during starch accumulation in the endosperm. To identify genes that may be affected by HNT and could play an important role in chalky grain phenotype, a whole transcriptome analysis on rice endosperm tissue was performed.

Transcripts higher in Cypress regardless of nighttime temperature

Many genes were differentially expressed in the one-way comparison between cultivars. Analysis revealed 34 genes that were expressed higher by two-fold or more in Cypress compared to LaGrue (Table 1). Eighteen genes from this group have an annotated proposed function and sixteen genes have evidence of being expressed or encoding a hypothetical protein, but no function associated. None of the genes are obviously associated with sucrose breakdown or

starch synthesis. A gene encoding a domain for a plant protein of unknown function (LOC_Os09g12970), was among those with the highest levels of differential expression between cultivars, being twenty-fold higher in Cypress. Because this gene has higher expression in Cypress regardless of treatment, it is an unlikely candidate as being directly or indirectly responsible for chalky endosperm.

Of the genes more highly expressed in Cypress, two encode isoforms of fatty acid desaturase (FAD) (LOC_Os11g01340, LOC_Os12g01370). One of these genes codes an omega-3 FAD (LOC_Os11g01340), and was eight-fold up regulated. The omega-3 fatty acid desaturases are membrane bound enzymes that catalyze the conversion of linoleic acid to α-linolenic acid (Liu et al., 2012a), which are components of plant membranes. A gene with transcripts five-fold up regulated encodes a protein that is a putative, peroxidase precursor (LOC_Os01g18910). Plant peroxidases oxidize a large number of components and have been reported to function in the biosynthesis and degradation of lignin in cell walls (Yoshida et al., 2003).

Also found to be up regulated in Cypress regardless of temperature were transcripts encoding seed storage protein glutelin (LOC_Os02g16820). In the most current gene prediction of the rice genome there are fifteen genes identified that encode glutelin proteins, which together make up 80% of the total seed storage protein content found in rice grains (Chen et al., 2013). Another pair of genes that were identified as significantly higher expressed in Cypress (LOC_Os01g18920, LOC_Os08g34830) both contain a hAT dimerization domain. One hAT gene, LOC_Os01g18920, was six-fold up regulated, and the other gene, LOC_Os08g34830, was four-fold more highly expressed in Cypress at both nighttime temperatures. The hAT

dimerization domain is found at the C terminus of the transposases of elements belonging to the *Activator* superfamily (Huang et al., 2009).

Transcripts higher in Cypress versus LaGrue after high nighttime temperature

Taking the two-way interaction of temperature and cultivar into account, statistical analysis identified twenty genes that are expressed significantly higher, two-fold or more, in Cypress over LaGrue when plants were treated with a HNT (Table 2). Annotation of these genes indicates fourteen that have a predicted associated function and six genes that are expressed or encode a hypothetical protein with no known function ascribed. The gene with the highest differential expression, three-fold higher, and an associated function encodes a protein with a transmembrane BAX inhibitor motif (LOC_Os11g37200). This motif is present in a class of proteins known to suppress programmed cell death induced by the BAX protein (Matsumura et al., 2003). There was a small subset of genes highly expressed in Cypress HNT that are usually involved in plant defense. A gene that encodes a protein with a double-stranded RNA binding motif (LOC_Os08g29530), and a gene that encodes a protein with leucine-rich repeat-containing protein kinase family (LOC_Os02g13850) were both expressed two-fold higher in Cypress.

Transcripts higher in Cypress versus LaGrue after control nighttime temperature

A small set of genes showed significantly higher expression levels, two fold or more, in Cypress compared to LaGrue, only during CNT treatments of 18 ºC (Table 3). The only genes identified in this set with an associated function are OsMADS69 (LOC_Os08g20440), OsFBO15 (LOC_Os11g36450), and a putative CHD-3 type chromatin-remodeling factor (LOC_Os01g65850). MADS-box gene family is a large group of transcription factors containing

many genes involved in floral organ identity (Arora et al., 2007). OsFBO15 is an f-box domain containing protein. F-box domains are generally located at N-terminus of proteins and confer interaction with s-phase kinase associated protein 1, which is a major class of plant E3 ubiquitin ligases (Jain et al., 2007). Thus, both these genes encode proteins with important roles in plant cell signaling and/or gene expression.

Transcripts higher in LaGrue versus Cypress regardless of nighttime temperature

Thirty genes had significantly higher expression levels two-fold or greater in LaGrue versus Cypress regardless of nighttime temperature (Table 4). Sixteen of these genes had an associated function and fourteen have evidence of being expressed or a hypothetical protein with no function associated. A small subset of genes identified are plant defense response related genes. These genes include: NBS_LRR disease resistance protein (LOC_Os09g16000), piwi domain-containing protein (LOC_Os02g40280), and an ankyrin repeat domain-containing protein (LOC_Os08g23590). A majority of R genes encode proteins that contain a nucleotide-binding site (NBS) and a series of leucine rich repeats (LRR) (Zhou et al., 2004). Proteins that contain a piwi domain are involved in RNAi and contribute to base pairing of argonaute machinery (Zhang et al., 2014b), which is important for battling virus infections. Analysis also indicated a gene with twenty-fold higher expression in LaGrue over Cypress encoding a protein described as containing a 'no apical meristem' (NAM) domain (LOC_Os09g38010). Characterization of proteins that contain this functional domain in other plants suggests these proteins may act as a transcription factor and are important for flower and embryo development (Souer et al., 1996). Another gene that may be related cell signaling was an F-box/LRR domain containing protein (LOC_Os01g65920), this gene was up-regulated eight-fold in LaGrue over

Cypress. F-box proteins play a critical role in controlled degradation of cellular proteins (Jain et al., 2007), higher expression of this protein and other genes involved in protein metabolism may be a result of need for greater protein recycling due to damage from high temperatures.

Transcripts higher in LaGrue versus Cypress after high nighttime temperature

There were twenty-two genes expressed significantly higher, two-fold or more, in LaGrue compared to Cypress after HNT treatment. This subset includes nine genes with a predicted function associated and thirteen genes containing domains without an associated function. Genes that encode proteins involved in protein processing were enriched in this subset: nucleotide pyrophosphatase/phosphodiesterase (NPP; LOC_Os12g38770), 40S ribosomal protein S9 (LOC_Os07g43510), OsMADS55 (LOC_Os06g11330), ribosomal protein L7Ae (LOC_Os01g70010).

Transcripts higher in LaGrue versus Cypress after control nighttime temperature

There were a relatively large number of genes expressed significantly higher, two-fold or more, in LaGrue versus Cypress with the control nighttime temperature (CNT) treatment. Thirty-four genes with a known function and twelve with no predicted functions were identified. Many genes identified in this analysis encode intracellular components: ribosomal protein L27 (LOC_Os08g31219), RNA polymerase III RPC4 domain containing (LOC_Os01g665800, repressor of RNA polymerase III transcription MAF1 (LOC_Os04g56730), and a MYST-like histone acetyltransferase 1 (LOC_Os07g43360). These gene products are also involved in various ways with protein metabolism. The ability to transport electrons is vital for all living organisms. Electron relay systems in plants are found in the photosystems in chloroplasts, but are

also found in the mitochondrial respiratory chain. In the current study three genes that encode proteins involved in electron transport were found to be expressed higher in LaGrue over Cypress when treated at the CNT: SCO1 protein homolog, mitochondrial precursor (LOC_Os02g06480), ferredoxin--NADP reductase, chloroplast precursor (LOC_Os06g01850), and bifunctional thioredoxin reductase/thioredoxin (LOC_Os07g46410).

Differential expression within cultivars after nighttime temperature treatment

Statistical analysis comparing temperature treatments on Cypress only revealed one gene whose expression was different at a p-value lower than 0.05. Even lowering the critical p-value to 0.5 gave us a small list of genes (Table 7). With this lower critical value HNT led to up regulation of four genes with an associated function and one hypothetical predicted gene. Temperature treatment was only associated with down regulation of one gene, which is predicted to be a retrotransposon (LOC_Os08g39810). Of the four genes showing up regulation one is predicted to be a glycosyl hydrolase (LOC_Os08g40680). This gene family is large and the enzymes are responsible for catalyzing the hydrolysis of a β-O-glycosidic bond on the anomeric carbon of a glucose moiety at the non-reducing end of a carbohydrate or glycoside molecule (Opassiri et al., 2006).

Even at a relatively loose stringency a p-value of ≤ 0.1 only six genes showed up regulation from HNT, and five showed a down-regulation in transcript values. To find a pattern or a trend in the genes that were affected by HNT, a gene set enrichment analysis (GSEA) was performed using AgriGO GSEA analysis tool (Du et al., 2010). This analysis was performed using all the genes identified in a differential comparison with a p-value ≤0.5. This list contained 609 genes with a higher level of expression under HNT, and 576 genes that showed lower

expression from HNT. The GSEA of genes that were suppressed by HNT returned no gene ontology (GO) terms with significant enrichment. However, of the 609 genes that displayed up regulation by HNT returned 43 GO terms with significant representation. All of the GO terms were high-level terms such as translation (GO:0006412), gene expression (GO:0010467), and primary metabolic process (GO:0044238). The GSEA did not return any low level terms, which identify a more specific function than high level.

Filtering the differences of expression with a p-value of ≤ 0.5 really means there is a 50/50 chance the difference is even real, because of that we increased the stringencies for our temperature comparison within LaGrue to a p-value ≤ 0.1 (Table 8). Applying these cutoffs results in a list of six genes with an annotated function that are up-regulated by HNT, five genes with an annotated function that are down-regulated by HNT, and also three genes down-regulated by HNT with no associated function assigned. Genes up regulated were typical in that a few of them are involved in metabolism of some sort. A predicated glycosyl hydrolase gene (LOC_Os08g40680) was up-regulated three-fold under HNT treatment. This same gene showed the similar up regulation in Cypress. Two genes were three-fold down regulated by HNT are predicted to encode membrane bound proteins that may be involved in iron transport. One gene is predicted to be an YS1-like Metal-nicotianamine transporter (LOC_Os02g43370), and the other is described as encoding a metal transporter Nramp6 domain (LOC_Os07g15460).

Physiological differences between cultivars

Source to sink relationships are very important for carbon partitioning in plants. In rice plants, the flag leaf is the primary source for carbon ultimately deposited in the developing endosperm (Zhang et al., 2015). Photosynthetic rates measured in flag leaves of field grown

plants (Figure 11) were not significantly different between the cultivars in the early reproductive growth stage R2 (Cypress: 11.61 ± 0.43 $\mu molCO_2m^{-2}s^{-1}$, LaGrue: 10.53 ± 0.38 $\mu molCO_2m^{-2}s^{-1}$; Figure 10A). Plants are classified growth stage R2 when the collar has formed on the flag leaf, just before panicle exertion from the boot (Counce et al., 2000). Measurements were collected again when the plants had reached growth stage R6. During this stage a large proportion of grains are considered to be in the filling stage and the photosynthetic rates were significantly higher in LaGrue (Cypress: 15.22 ± 0.4 $\mu molCO_2m^{-2}s^{-1}$, LaGrue 17.98 ± 0.35 $\mu molCO_2m^{-2}s^{-1}$; Figure 10B). The photosynthetic rates remained significantly higher in LaGrue when measurements were collected during growth stage R8 (Cypress: 8.45 ± 0.42 $\mu molCO_2m^{-2}s^{-1}$, LaGrue: 11.74 ± 0.29 $\mu molCO_2m^{-2}s^{-1}$; Figure 10C). Rates were higher in both cultivars during growth stage R6 compared to rates during stages R2 and R8 (Figure 10).

Quantitative PCR Results

To further verify differences in gene expression levels identified in the microarray, seven genes that displayed high differential expression between cultivars were selected for quantitative PCR analysis. All of the genes had significant differential expression between the one-way cultivar comparison regardless of nighttime temperature treatment. Quantitative PCR was preformed on cDNA synthesized from RNA collected from a replicate experiment with the same treatment regime. Plants, LaGrue and Cypress were grown under the same conditions in the greenhouse and sets were moved into the growth chamber for nighttime temperature treatments. The qPCR expression analysis for fatty acid dehydrogenase (FAD; LOC_Os11g0134) showed significantly higher expression in Cypress versus LaGrue after both nighttime treatments.

Expression of a gene that encodes fatty acid dehydrogenase (FAD; LOC_Os11g0134) measured by qPCR showed higher levels of RNA in Cypress agreed with microarray results with significantly higher expression in Cypress after both temperature treatments. A gene, with a domain of unknown function (DUF09; LOC_Os09g1297) had very high expression in Cypress compared to LaGrue, twenty-fold higher at both temperatures in microarray analysis. However in qPCR results DUF09 was not expressed significantly higher in Cypress compared to LaGrue after exposure to HNT. Expression in Cypress at CNT was about thirty-times higher that LaGrue, which did agree with microarray results. Another difference observed was in expression levels for a hAT domain-containing gene (hAT; LOC_Os01g1892). Microarray analysis showed transcript accumulation for the hAT gene was significantly higher in Cypress under both nighttime temperature treatments. Interestingly expression was not significantly different between cultivars during either nighttime temperature treatment. Three genes were chosen that showed higher expression in LaGrue over Cypress regardless of temperature treatment. Transcripts for DUF05 (LOC_Os05g37210), had three-fold higher RNA levels in LaGrue at both temperature treatments according to whole transcriptome analysis. This DUF05 however qPCR results did not show significantly higher expression in LaGrue over Cypress following either temperature treatment. Two different storage proteins had significantly higher levels of expression depending on variety. Cultivar LaGrue showed four-fold higher transcript accumulation after both treatments for PROML24 (LOC_Os12g16880), which codes for a prolamin precursor protein. Cultivar Cypress showed greater transcript accumulation for storage protein glutelin (LOC_Os02g1682), which was twenty-fold higher at CNT and four-fold higher under HNT.

E. Discussion

Starch synthesis gene expression

Rice quality has been of concern to scientists and breeders for some time now. Previous studies investigating the chalky phenotype in rice endosperm have correlated the occurrence with differential expression in several key starch biosynthesis genes. Many of these studies have showed a decrease in amylose content (AC) and an increase in amylopectin chain length with a reduction in branching frequency (Cheng et al., 2005; Cooper et al., 2008; Counce et al., 2005; Jiang et al., 2003; Lin et al., 2010; Yamakawa et al., 2007; Yamakawa & Hakata, 2010). These studies also found that high temperature caused a repression of certain genes involved in the starch biosynthesis pathway. Repression of GBSSI and BEIII were reported in many of the studies (Cheng et al., 2005; Jiang et al., 2003; Yamakawa et al., 2007; Yamakawa & Hakata, 2010).

The repression of the many enzymes involved in amylose synthesis is usually accompanied with the up-regulation of alpha-amylase genes (Hakata et al., 2012; Yamakawa et al., 2007). However the data in our study did not indicate differences in the expression of these genes. As shown in Figure 2 there was no differential expression of any starch synthase genes granule bound or soluble starch synthase. Our results indicated no differential expression found between any of the branching enzymes (Figure 7) and no differences in any alpha-amylase genes (Figure 8). Many studies have shown that down-regulation of some of these genes can result in improper starch packaging and in turn chalky grain formation. However transcription and translation are very dynamic and regulation of gene expression is a complex system. To completely investigate the effect that expression of these genes has on end products, it is helpful to collect tissue from multiple time points. Taking that fact into account, a time course study

looking at gene expression responses to temperature in cv. Nipponbare at multiple time points during grain development suggested equal expression at the start of grain filling, followed by up-regulation of amylase genes AmyIa, AmyIIID, and AmyIIIE (Yamakawa et al., 2007). Another study by Hakata et al. (2012) saw the same expression pattern for alpha-amylase genes in plants exposed to HNT versus CNT. In that same report they found that RNAi suppression of alpha-amylase genes gave a reduction in the amount of chalky grains formed. The data in this study showed no differential expression of alpha-amylase genes as a result of temperature treatment (Figure 8). The lack of significant difference was surprising because a study looking at grain quality examined gene expression in LaGrue and Cypress without temperature treatments and found higher expression of a starch branching enzyme, and two isoforms of amylase enzymes in Cypress (Venu et al., 2011). Venu et al. (2011) went on to perform sq-PCR on a time course of tissue samples and saw the ADPase and GBSSI had higher expression earlier in seed development in Cypress versus LaGrue however the levels became even at thirteen days after anthesis. For the deep sequencing at a single time point, Venu et al (2011) collected endosperm tissue samples 6 days after anthesis, and in the middle of the dark period. For their time course experiment they collected 3, 6, 9 12, and 15 days after anthesis. There are quite a few reasons that could explain the difference between previous work and the current project. The first explanation for the difference is the number of days after seed set they collected tissue was different from the current study. Also the fact that they collected the tissue in the middle of the dark period plays a big role in the difference. Our tissue was collected during the light period, several hours after temperatures were returned to normal daylight levels. The microarray data for starch biosynthesis genes agrees with the results of our semi-quantitative PCR. SQ-PCR is not a very sensitive analysis, it works well to look at absence or presence of a gene but for absolute

quantification of expression levels it is more difficult to make an argument about differences in our results. That is why we turned to analysis via microarray, this method is far more accurate in measuring gene expression levels and in our analysis it verified there was a lack of differential expression, or transcript accumulation for starch synthase genes.

In addition to starch in rice endosperm, storage proteins make up about six to ten percent of dry matter of de-branned rice and are important for nutrition and quality (Lin et al., 2010). Expression data in this experiment found differences in rice storage proteins glutelin and prolamin. Statistical analysis revealed storage protein glutelin transcripts are up in Cypress sixteen-fold at CNT and four-fold at HNT as compared to LaGrue. The gene that encodes for storage protein Prolm27 is up in LaGrue four-fold higher than in Cypress at both temperatures. In a study looking at protein accumulation in response to HNT, an increased accumulation of storage proteins early in grain filling with a reduction of prolamin towards the end (Lin et al., 2010). To further investigate storage protein gene expression response to HNT in cultivars LaGrue and Cypress, real time PCR quantification was employed. The results for the qPCR experiment were different from the microarray results; however there could be a few reasons for this difference. In the qPCR results the significant differences at the HNT dropped off, meaning glutelin was only significantly expression higher in Cypress than LaGrue at CNT, whereas at HNT they were the same. Also prolamin in LaGrue was only significantly higher expressed in LaGrue and CNT, after HNT the expression was equal in both cultivars. Variation may arise from amplification of a non-target gene sequence. The Affymetrix array probes were designed from the cv. Nipponbare genome. Primers for specific genes were also designed using the cv. Nipponbare genome sequence differences between rice varieties might result in incongruences and ultimately false-positive or negative results via PCR. Also, the RNA was isolated from tissue

that was treated in a separate experiment and the tissue in the second experiment were collected as soon as the lights came on in the growth chamber, at 09:00. Whereas, tissue for the first experiment was collected two hours after the chambers had been at equal temperatures with the lights on. Cypress maybe able to recover from stress situations more rapidly that LaGrue. Many of the genes measures in qPCR agreed with microarray expression and were significantly higher in Cypress after the CNT temperature treatment. However these genes were repressed in Cypress immediately following HNT and RNA levels were not higher. Because the RNA was collected from tissue that was collected two hours after temperatures had reduced to ambient, Cypress could have had enough time return to homeostatic levels. Another explanation might be starch biosynthesis in the endosperm may have already slowed or seized two hours after the lights had come on. Studies in rice and barley have shown that storage starch accumulates mainly in the dark, and have shown expression and accumulation of ADPase and other starch biosynthesis proteins increases in the dark and decrease during the day (Yu et al., 2012).

Differences in gene expression between cultivars

Fatty acids make up the bulk of organelle membranes and play a role in membrane fluidity. Cypress had significantly higher expression in two genes that encode for fatty acid desaturase, which are involved in fatty acid metabolism. Suppression of the omega-3 fatty acid desaturase (FAD3) gene in tobacco plant chloroplast has been reported to increases high temperature tolerance, however the role FAD3 plays in temperature tolerance for plastids in non-photosynthetic tissue has not been examined (Murakami et al., 2000). A group generated transgenic rice lines that overexpressed the chloroplast localized omega-3 fatty acid desaturase (FAD7) of *Arabidopsis thaliana* driven by the maize ubiquitin promoter (Song et al., 2004).

Different research has shown plants exposed to low temperatures had a higher expression of FAD, correlation with higher levels of trienoic fatty (TA) acids in cell membranes (Wang et al., 2006). The increase in TA is thought to maintain membrane fluidity at low temperature allowing plant survival. Similar research has found a reduction in the expression of FAD in response to high temperature (Liu et al., 2006). However in the current study transcription levels of two FAD encoding genes was higher in Cypress compared to LaGrue at both nighttime temperature treatments. It is possible that the higher expression in Cypress causes amyloplast membranes to become more rigid even at HNT, which might allow for a more uniform filling of starch granules in the amyloplast.

Another gene family, glycerophosphoryl diester phosphodiesterase (GPDL) may be important in cell wall organization and different isoforms showed differential expression depending on in a cultivar by temperature treatment. One isoform of GDPL (LOC_Os02g37590) showed up-regulation in LaGrue after both temperature treatments (Table 6). This gene displayed thirty-fold higher expression at CNT, and seventeen-fold higher expression at the HNT. Another isoform GDPL (LOC_Os02g31030) also encoded on chromosome 2, was the only gene with significantly higher expression six-fold (p-value ≥ 0.5) in Cypress following HNT treatment. Also another putative isoform of a GPDL gene (LOC_Os02g31030) has three-fold higher expression in LaGrue versus Cypress and the CNT but not after HNT treatment (Table 8). The gene that encodes this GPDL isoform is the only gene that has significantly higher expression is Cypress versus Cypress after HNT treatment (Table 7). GDPL catalyzes the hydrolysis of glycerophosphoryl diester to a glycerol-3-phosphate and alcohol (Hayashi et al., 2008). Proteins containing the GPDL domain play important roles in cell wall organization in *Arabidopsis* root hairs and may be involved in membrane degradation and uptake of nutrients

(Hayashi et al., 2008). In *Arabidopsis* GPDL gene knock-outs displayed signs of decreased rigidity of the cell wall, indicating that GPDL and homologs may play important roles in cell wall organization (Hayashi et al., 2008).

The subset of genes expressed higher in LaGrue after HNT treatment combine with genes higher at both temperatures are enriched in genes that encode proteins involved in protein metabolism. Higher expression of protein metabolism related genes indicates LaGrue working harder to correct cellular damage caused by HNT. It is possible that LaGrue is going too far in fine-tuning starch chain elongation and branching, which could lead to non-optimal starch crystallization.

Varietal effects on physiological processes

To explore the idea that source sucrose may be the limiting factor for starch biosynthesis leading to chalk, daytime photosynthetic rates were measured via gas exchange and compared between cultivars. We attempted to take measurements on plants treated in the growth chambers, however readings were very inconsistent. Because of this we chose to take measurements from rice grown in the field in Stuttgart, AR. The average nighttime temperature (22:00-07:00) on July 25th and 26th was 21 ºC and the daytime average from (07:00-12:00) was 24 ºC. This date was before the initiation of grain filling, photosynthetic rates were low in both cultivars and there was no significant difference between the rates as seen in Figure 10. The next measurements were collected on August 15th & 16th, when average nighttime temperature was 17 ºC and daytime temperature average was 21 ºC. At this point of the growing season both cultivars had reached growth stage R6 and displayed an increase in photosynthetic rate. Plants at stage R6 have just begun filling and grain filling continues until R9. At R6 the photosynthetic rate in LaGrue was

significantly higher than Cypress. Photosynthetic rate was measured towards the end of grain filling on August 29th & 30th the plants measured were in plant growth stage R8. The average nighttime temperature for the last collection was 22 ºC and the average daytime temperature was 28 ºC. There is considerable coordination of source and sink activity in plants. Positive photosynthetic responses to sink demand have been determined with objective data in some cases. The cultivar LaGrue has more grains per panicle, allowing for a higher yield, but also more variable head rice yields than Cypress. A contributing factor of the increased head rice yield is likely related to a longer panicle length in LaGrue (Counce et al., 2015). The longer panicle allows for a greater number of grains per panicle, and therefore a longer R8 duration for LaGrue compared to Cypress therefore, there is a longer duration for photosynthate demand by LaGrue sinks, i.e. the filling grains.

The effect of sink stimulation on photosynthesis is the result of a complex set of feedback signals between the source and the sink. Source-sink coordination is both logical and verifiable but verifying the relationship requires extensive experimentation at both the whole plant level and at the gene expression level for individual source and sink tissue. Southern U.S. long-grain rice, for instance, differs greatly from high yielding Chinese *indica* rice such as Guichao2 for photosynthesis in the late reproductive growth stages such as R6, R7 and R8 (Black et al., 1995). Without further investigation, the relationship of photosynthesis and sink demand in LaGrue and Cypress is far from clear but the higher photosynthetic rate of LaGrue compared to Cypress is matched to the greater sink demand for LaGrue at R8. It is possible that nighttime temperatures have an impact on daytime photosynthesis, affecting LaGrue more significantly because it has more grains to fill per panicle.

This study investigated the differential effects HNT has on gene expression in rice cultivars with opposite grain quality responses and will be useful for rice breeding and reproduction. However, mechanistic connections between the expression of genes encoding starch biosynthetic enzymes and the resultant fine structure of strain granules in amyloplasts under HNT stress need further investigation.

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Table 1. List of genes expressed at a higher level in cv. Cypress compared to expression in cv. LaGrue regardless of temperature treatment. Eighteen genes that were identified have a predicted function associated, and sixteen genes are hypothetical or expressed proteins with no associated function. (Fold change (FC) ≥ 2.0, p-value ≤ 0.05)

Gene Symbol	Gene Description	18°C FC (log)	Adj. P-Value	30°C FC (log)	Adj. P-Value
LOC_Os09g12970	plant protein of unknown function domain containing protein, expressed	4.673	6.350E-06	4.883	3.94E-06
LOC_Os02g16820	glutelin, putative, expressed	4.134	5.350E-05	2.458	1.80E-03
LOC_Os11g01340	omega-3 fatty acid desaturase, chloroplast precursor, putative, expressed	3.405	8.98E-04	3.369	8.48E-04
LOC_Os01g18920	hAT dimerisation domain-containing protein, putative	2.769	8.98E-04	2.824	8.25E-04
LOC_Os01g18910	peroxidase precursor, putative	2.486	2.40E-03	2.883	8.42E-04
LOC_Os02g21630	SEC14 cytosolic factor family protein, putative, expressed	2.395	0.013	1.982	0.037
LOC_Os02g16480	nucleolar transcription factor 1, putative	2.277	1.77E-03	2.309	1.68E-03
LOC_Os07g43060	transcription elongation factor SPT4 homolog 1, putative, expressed	2.166	0.008	2.029	0.013
LOC_Os02g13140	xaa-Pro dipeptidase, putative, expressed	2.023	7.71E-04	2.184	4.63E-04
LOC_Os12g01370	fatty acid desaturase, putative, expressed	2.005	7.77E-04	1.897	9.68E-04
LOC_Os08g34830	hAT dimerisation domain-containing protein, putative	1.994	0.012	1.856	0.019
LOC_Os02g40784	WAX2, putative, expressed	1.650	0.016	1.380	0.042
LOC_Os11g36430	zinc finger, C3HC4 type domain containing protein, expressed	1.359	0.010	1.977	8.42E-04
LOC_Os07g22640	lipid binding protein, putative, expressed	1.220	0.044	1.601	0.013
LOC_Os02g04950	splicing factor 3B subunit 1, putative, expressed	1.170	0.021	1.412	0.009
LOC_Os04g05080	vacuolar ATP synthase subunit F, putative, expressed	1.151	0.028	1.415	0.012
LOC_Os03g55810	uncharacterized PE-PGRS family protein PE_PGRS46, putative, expressed	1.135	0.019	1.527	0.004
LOC_Os01g67520	VTC2, putative, expressed	1.131	0.024	1.101	0.029
LOC_Os09g21689	expressed protein	3.127	1.904E-04	3.179	1.37E-04
LOC_Os10g36260	expressed protein	2.936	0.010	2.420	0.029
LOC_Os09g15284	expressed protein	2.823	0.009	2.035	0.049
LOC_Os02g14800	expressed protein	2.746	7.820E-06	2.475	2.39E-05

Table 1. List of genes expressed at a higher level in cv. Cypress compared to expression in cv. LaGrue regardless of temperature treatment. Eighteen genes that were identified have a predicted function associated and sixteen genes are hypothetical or expressed proteins with no associated function. (Fold change (FC) ≥ 2.0, p-value ≤ 0.05) (cont.)

Gene Symbol	Gene Description	18°C FC (log)	Adj. P-Value	30°C FC (log)	Adj. P-Value
LOC_Os07g20164	expressed protein	2.668	7.71E-04	1.879	0.008
LOC_Os02g22590	expressed protein	2.314	7.71E-04	1.928	2.04E-03
LOC_Os07g01890	expressed protein	2.100	6.25E-04	1.940	8.25E-04
LOC_Os06g40010	expressed protein	2.040	0.008	1.662	0.025
LOC_Os11g17280	conserved hypothetical protein	1.671	0.045	1.664	0.049
LOC_Os02g16490	expressed protein	1.640	0.006	1.766	0.004
LOC_Os02g05199	expressed protein	1.435	0.004	1.190	0.012
LOC_Os08g19105	expressed protein	1.372	0.048	2.320	3.19E-03
LOC_Os09g31462	hypothetical protein	1.335	0.021	1.689	0.007
LOC_Os02g23930	expressed protein	1.316	3.32E-03	1.156	0.008
LOC_Os04g05750	hypothetical protein	1.184	0.015	1.066	0.028
LOC_Os02g17840	hypothetical protein	1.024	0.029	1.119	0.021

Table 2. List of genes expressed at a higher level in cv. Cypress compared to cv. LaGrue treated with high nighttime temperature (30ºC). Thirteen of the identified genes have a predicted function associated and seven are hypothetical or expressed proteins with no associated function. (Fold change (FC) ≥ 2.0, p-value ≤ 0.05)

Gene Symbol	Gene Description	30°C FC (log)	adj.P.val
LOC_Os11g37200	transmembrane BAX inhibitor motif-containing protein, putative, expressed	1.800	3.74E-02
LOC_Os02g31030	glycerophosphoryl diester phosphodiesterase family protein, putative, expressed	1.637	2.89E-02
LOC_Os04g32970	OTU-like cysteine protease family protein, putative, expressed	1.566	3.89E-03
LOC_Os09g37452	OsSAUR50 - Auxin-responsive SAUR gene family member	1.424	4.15E-02
LOC_Os02g52910	zinc finger protein, putative, expressed	1.354	1.89E-02
LOC_Os08g29530	double-stranded RNA binding motif containing protein, expressed	1.300	4.28E-02
LOC_Os07g38260	insulin-degrading enzyme, putative, expressed	1.278	2.51E-02
LOC_Os03g32314	allene oxide cyclase 4, chloroplast precursor, putative, expressed	1.250	2.05E-02
LOC_Os02g16040	ubiquitin-conjugating enzyme, putative, expressed	1.248	1.74E-02
LOC_Os11g37640	ADP-ribosylation factor, putative, expressed	1.214	4.68E-02
LOC_Os07g40290	OsGH3.8 - Probable indole-3-acetic acid-amido synthetase, expressed	1.214	2.89E-02
LOC_Os05g14040	G-patch domain containing protein	1.044	4.12E-02
LOC_Os02g13850	leucine-rich repeat-containing protein kinase family protein, putative expressed	1.038	4.92E-02
LOC_Os11g27120	hypothetical protein	2.328	1.37E-02
LOC_Os06g25090	conserved hypothetical protein	1.775	1.57E-02
LOC_Os04g06520	expressed protein	1.400	3.89E-02
LOC_Os02g28860	expressed protein	1.340	2.55E-02
LOC_Os01g24849	hypothetical protein	1.203	1.24E-02
LOC_Os05g08044	expressed protein	1.070	2.43E-02
LOC_Os07g45194	expressed protein	1.005	4.92E-02

Table 3. List of genes expressed at a higher level in cv. Cypress compared to cv. LaGrue when treated with the control nighttime temperature of 18º C. Three of the genes listed have a predicted function and eleven are hypothetical expressed proteins. (Fold change (FC) ≥ 2.0, p-value ≤ 0.05)

Gene Symbol	Gene Description	18°C FC (log)	adj.P.val
LOC_Os01g65850	CHD3-type chromatin-remodeling factor PICKLE, putative	2.196	2.10E-02
LOC_Os08g20440	OsMADS69 - MADS-box family gene with M-alpha type-box, expressed	1.324	2.20E-02
LOC_Os11g36450	OsFBO15 - F-box and other domain containing protein, expressed	1.176	5.37E-03
LOC_Os08g36610	expressed protein	2.171	3.82E-02
LOC_Os02g21359	hypothetical protein	2.008	2.83E-02
LOC_Os02g22580	conserved hypothetical protein	1.471	2.67E-02
LOC_Os02g24109	hypothetical protein	1.465	1.41E-02
LOC_Os04g09350	expressed protein	1.462	3.47E-02
LOC_Os07g44700	expressed protein	1.406	3.06E-02
LOC_Os05g05290	expressed protein	1.341	1.61E-02
LOC_Os02g26790	expressed protein	1.253	1.44E-02
LOC_Os01g32520	hypothetical protein	1.228	1.77E-02
LOC_Os04g08800	expressed protein	1.170	3.82E-02
LOC_Os05g45560	hypothetical protein	1.006	2.67E-02

Table 4. List of genes expressed at a higher level in cv. LaGrue compared to expression in cv. Cypress regardless of temperature treatment. Fifteen of these predicted genes have a putative function associated, and sixteen are hypothetical or expressed proteins with no associated function. (Fold change (FC) ≥ 2.0, p-value ≤ 0.05)

Gene Symbol	Gene Description	18°C FC (log)	Adj P-value	30°C FC (log)	Adj P-value
LOC_Os02g37590	glycerophosphoryl diester phosphodiesterase family protein, putative, expressed	4.90	7.50E-08	4.13	4.87E-07
LOC_Os09g38010	no apical meristem protein, putative, expressed	4.33	1.06E-03	2.67	2.08E-02
LOC_Os09g16000	NBS-LRR disease resistance protein, putative	3.11	7.17E-05	3.37	3.77E-05
LOC_Os12g02880	E2F-related protein, putative, expressed	2.82	2.02E-03	2.31	8.19E-03
LOC_Os02g40280	piwi domain containing protein, putative, expressed	2.58	8.77E-04	1.13	3.40E-04
LOC_Os12g16880	PROLM27 - Prolamin precursor, expressed	2.07	2.10E-02	2.30	1.37E-02
LOC_Os07g46780	tyrosine-specific transport protein, putative, expressed	2.04	7.71E-04	2.11	4.63E-04
LOC_Os08g23590	ankyrin repeat domain containing protein	1.90	2.62E-03	1.68	6.97E-03
LOC_Os05g37210	eukaryotic protein of unknown function DUF914 domain containing protein	1.87	2.67E-02	2.82	2.75E-03
LOC_Os01g65920	F-box/LRR-repeat protein 2, putative, expressed	1.77	1.88E-02	1.50	4.57E-02
LOC_Os08g31228	ribosomal protein L27, putative, expressed	1.58	2.83E-02	2.06	8.18E-03
LOC_Os01g02200	armadillo/beta-catenin repeat family protein, putative, expressed	1.54	5.79E-03	1.57	6.65E-03
LOC_Os07g31460	peptide-Nasparagine amidase, putative, expressed	1.48	1.44E-02	1.20	4.18E-02
LOC_Os07g30960	monooxygenase, putative, expressed	1.28	4.47E-02	1.29	4.81E-02
LOC_Os03g32590	transcription initiation factor, putative, expressed	1.16	1.02E-02	1.13	1.34E-02
LOC_Os02g20676	expressed protein	3.38	1.40E-03	3.29	1.68E-03
LOC_Os09g15980	expressed protein	1.91	1.62E-03	2.34	4.63E-04
LOC_Os07g29224	expressed protein	1.71	9.51E-03	1.39	2.87E-02
LOC_Os02g05240	hypothetical protein	1.67	5.57E-03	1.39	1.75E-02
LOC_Os05g37220	conserved hypothetical protein	1.49	4.44E-02	1.82	1.88E-02
LOC_Os02g22060	expressed protein	1.47	4.02E-02	1.47	4.34E-02
LOC_Os10g13880	hypothetical protein	1.44	4.60E-03	1.20	1.40E-02

Table 4. List of genes expressed at a higher level in cv. LaGrue compared to expression in cv. Cypress regardless of temperature treatment. Fifteen of these predicted genes have a putative function associated, and sixteen are hypothetical or expressed proteins with no associated function. (Fold change (FC) ≥ 2.0, p-value ≤ 0.05) (cont.)

Gene Symbol	Gene Description	18°C FC (log)	Adj P-value	30°C FC (log)	Adj P-value
LOC_Os10g13880	hypothetical protein	1.44	4.60E-03	1.20	1.40E-02
LOC_Os02g27110	expressed protein	1.40	1.02E-02	1.30	1.57E-02
LOC_Os09g29270	expressed protein	1.36	2.89E-02	1.62	1.34E-02
LOC_Os02g55670	expressed protein	1.35	1.44E-02	1.46	1.06E-02
LOC_Os11g47370	expressed protein	1.34	5.48E-03	1.92	4.63E-04
LOC_Os10g13694	expressed protein	1.28	1.52E-02	1.64	4.29E-03
LOC_Os01g69904	expressed protein	1.20	4.07E-02	1.24	3.94E-02
LOC_Os11g45809	expressed protein	1.18	3.12E-04	1.35	1.25E-04
LOC_Os02g55710	hypothetical protein	1.16	1.17E-02	1.13	1.40E-02

Table 5. List of genes expressed at a higher level in cv. LaGrue compared to expression in cv. Cypress when treated with high nighttime temperature. Nine of the genes listed have a predicted function and thirteen have no associated function but are hypothetical expressed proteins. (Fold change (FC) ≥ 2.0, p-value ≤ 0.05)

Gene Symbol	Gene Description	30°C FC (log)	Adj P-value
LOC_Os12g01160	dehydrogenase, putative	1.73	8.13E-03
LOC_Os12g38770	nucleotide pyrophosphatase/phosphodiesterase, putative, expressed	1.62	6.65E-03
LOC_Os07g43510	40S ribosomal protein S9, putative, expressed	1.33	6.11E-03
LOC_Os09g30446	transporter, monovalent cation:proton antiporter-2 family, putative, expressed	1.29	2.89E-02
LOC_Os02g06200	phytosulfokine receptor precursor, putative, expressed	1.19	1.74E-02
LOC_Os12g33220	F-box/LRR-repeat protein 14, putative	1.09	2.08E-02
LOC_Os06g11330	OsMADS55 - MADS-box family gene with MIKCc type-box, expressed	1.07	2.92E-02
LOC_Os01g70010	ribosomal protein L7Ae, putative, expressed	1.02	4.92E-02
LOC_Os07g23200	F-actin-capping protein subunit alpha, putative, expressed	1.02	2.98E-02
LOC_Os03g62388	expressed protein	1.88	3.82E-02
LOC_Os07g23390	conserved hypothetical protein	1.56	4.18E-02
LOC_Os12g01880	expressed protein	1.51	1.24E-02
LOC_Os04g12920	hypothetical protein	1.43	4.92E-02
LOC_Os10g34370	expressed protein	1.40	4.27E-02
LOC_Os02g55770	expressed protein	1.39	3.69E-02
LOC_Os02g32650	expressed protein	1.31	1.57E-02
LOC_Os03g48950	expressed protein	1.30	1.37E-02
LOC_Os07g45195	expressed protein	1.16	4.13E-02
LOC_Os07g45439	expressed protein	1.13	3.82E-02
LOC_Os02g19220	expressed protein	1.10	2.69E-02
LOC_Os02g02970	expressed protein	1.10	1.57E-02
LOC_Os08g05340	expressed protein	1.04	2.19E-02

Table 6. List of genes expressed at a higher level in cv. LaGrue compared to expression in cv. Cypress at control nighttime temperature. Thirty-four genes listed have function associated and twelve are hypothetical expressed proteins. (Fold change (FC) ≥ 2.0, p-value ≤ 0.05)

Gene Symbol	Gene Description	18°C FC (log)	Adj P-value
LOC_Os04g54240	wound induced protein, putative, expressed	1.88	1.44E-02
LOC_Os08g37380	glucose-6-phosphate isomerase, putative, expressed	1.77	5.37E-03
LOC_Os03g49440	phosphatase, putative, expressed	1.74	2.89E-02
LOC_Os02g31030	glycerophosphoryl diester phosphodiesterase family protein, putative, expressed	1.69	2.32E-02
LOC_Os10g02750	Ser/Thr protein phosphatase family protein, putative, expressed	1.65	3.24E-03
LOC_Os02g16620	reticulon domain containing protein, putative, expressed	1.63	5.57E-03
LOC_Os02g57160	ELMO/CED-12 family protein, putative, expressed	1.58	5.79E-03
LOC_Os07g47510	stress-related protein, putative, expressed	1.57	6.45E-03
LOC_Os03g45210	2-aminoethanethiol dioxygenase, putative, expressed	1.57	2.96E-03
LOC_Os10g36650	actin, putative, expressed	1.54	4.47E-02
LOC_Os04g58570	C2 domain containing protein, putative, expressed	1.41	5.76E-03
LOC_Os08g31219	ribosomal protein L27, putative, expressed	1.40	1.44E-02
LOC_Os01g55310	haloacid dehalogenase-like hydrolase domain-containing protein 1A, putative, expressed	1.39	2.85E-02
LOC_Os01g16450	peroxidase precursor, putative, expressed	1.38	3.47E-02
LOC_Os06g17390	auxin-independent growth promoter protein, putative, expressed	1.34	4.77E-02
LOC_Os03g48920	DUF1517 domain containing protein, putative, expressed	1.31	1.50E-02
LOC_Os05g18730	generative cell specific-1, putative, expressed	1.27	3.59E-02
LOC_Os06g01850	ferredoxin--NADP reductase, chloroplast precursor, putative, expressed	1.22	2.52E-02
LOC_Os05g32544	glycosyltransferase, putative, expressed	1.20	8.64E-03
LOC_Os12g18860	OsPOP23 - Putative Prolyl Oligopeptidase homologue, expressed	1.20	3.30E-02
LOC_Os01g51290	protein kinase family protein, putative, expressed	1.19	2.83E-02
LOC_Os07g15460	metal transporter Nramp6, putative, expressed	1.18	3.30E-02
LOC_Os01g70780	WD40-like domain containing protein, putative, expressed	1.18	1.40E-03
LOC_Os04g56730	repressor of RNA polymerase III transcription MAF1, putative, expressed	1.17	2.04E-02
LOC_Os11g28340	ER lumen protein retaining receptor, putative, expressed	1.13	3.17E-02

Table 6. List of genes expressed at a higher level in cv. LaGrue compared to expression in cv. Cypress at control nighttime temperature. Thirty-four genes listed have function associated and twelve are hypothetical expressed proteins. (Fold change (FC) ≥ 2.0, p-value ≤ 0.05) (Cont.)

Gene Symbol	Gene Description	18°C FC (log)	Adj P-value
LOC_Os01g54670	coiled-coil domain-containing protein 25, putative, expressed	1.12	2.83E-02
LOC_Os06g28124	glycosyltransferase, putative, expressed	1.12	3.25E-02
LOC_Os12g14699	protein kinase domain containing protein, expressed	1.05	1.93E-02
LOC_Os04g46390	chaperone protein dnaJ, putative, expressed	1.04	3.30E-02
LOC_Os04g50204	protein transport protein-related, putative, expressed	1.04	1.90E-02
LOC_Os07g46410	bifunctional thioredoxin reductase/thioredoxin, putative, expressed	1.04	4.69E-02
LOC_Os02g06480	SCO1 protein homolog, mitochondrial precursor, putative, expressed	1.03	1.50E-02
LOC_Os07g43360	MYST-like histone acetyltransferase 1, putative, expressed	1.03	2.08E-02
LOC_Os01g66580	RNA polymerase III RPC4 domain containing protein, expressed	1.01	4.23E-03
LOC_Os11g47453	expressed protein	1.79	1.50E-02
LOC_Os07g31000	expressed protein	1.78	7.85E-03
LOC_Os02g37150	expressed protein	1.74	1.12E-02
LOC_Os10g34270	expressed protein	1.47	2.61E-02
LOC_Os10g22630	expressed protein	1.45	4.87E-02
LOC_Os08g42940	hypothetical protein	1.33	2.38E-02
LOC_Os01g46720	expressed protein	1.32	1.18E-02
LOC_Os02g43490	hypothetical protein	1.25	4.77E-02
LOC_Os02g14720	expressed protein	1.17	3.39E-02
LOC_Os01g66150	expressed protein	1.12	2.24E-02
LOC_Os01g39840	expressed protein	1.04	4.61E-02
LOC_Os12g02150	expressed protein	1.01	2.28E-02

Table 7. Lists genes that were identified by comparing the effect of temperature on gene expression within the cv. Cypress. Genes with a negative fold change are expressed higher in Cypress with the high nighttime temperature, whereas genes listed with a positive FC are up regulated at control nighttime temperature versus the high temperature. Five of the six genes up in cv. Cypress at 30ºC have a function associated, one gene is a hypothetical protein. One gene up regulated in cv. Cypress at 18ºC is identified as a retrotransposon, and the other two are expressed with no function associated. (Fold change (FC) ≥ 1.5, p-value ≤ 0.5)

Gene Symbol	Gene Description	logFC	adj.P.val
LOC_Os02g31030	glycerophosphoryl diester phosphodiesterase family protein, putative, expressed	-2.64	0.04
LOC_Os02g17292	retrotransposon protein, putative, unclassified, expressed	-1.54	0.08
LOC_Os08g40680	glycosyl hydrolase, putative, expressed	-1.49	0.08
LOC_Os01g24849	hypothetical protein	-1.11	0.23
LOC_Os03g19500	ubiquitin-conjugating enzyme, putative, expressed	-0.82	0.34
LOC_Os08g06010	transporter, major facilitator family, putative, expressed	-0.80	0.29
LOC_Os08g39810	retrotransposon protein, putative, unclassified, expressed	0.73	0.09
LOC_Os05g05290	expressed protein	1.23	0.34
LOC_Os02g26790	expressed protein	1.24	0.23

Table 8. Genes identified in this list display down regulated in LaGrue treated with high nighttime temperature compared to LaGrue treated with a low nighttime temperature. Six with higher expression from high nighttime temperature and five with lower expression from HNT exposure. (Fold change (FC) ≤ 1.5, p-value ≤ 0.1)

Gene Symbol	Gene Description	logFC	adj.P.val
LOC_Os06g36740	pollen-specific protein SF21, putative, expressed	-1.28	9.76E-02
LOC_Os08g40680	glycosyl hydrolase, putative, expressed	-1.19	9.76E-02
LOC_Os05g01444	polygalacturonase inhibitor precursor, putative, expressed	-0.95	3.91E-02
LOC_Os12g41930	SRP40, C-terminal domain containing protein, expressed	-0.79	9.76E-02
LOC_Os08g16480	ATPase, AFG1 family domain containing protein, expressed	-0.76	9.76E-02
LOC_Os05g43130	brix domain-containing protein 1, putative, expressed	-0.68	9.76E-02
LOC_Os07g15460	metal transporter Nramp6, putative, expressed	1.69	3.91E-02
LOC_Os02g43370	YS1-like Metal-nicotianamine transporter (blastp)	1.64	3.91E-02
LOC_Os10g02750	Ser/Thr protein phosphatase family protein, putative, expressed	1.53	3.91E-02
LOC_Os01g55270	SGS domain containing protein, expressed	0.95	4.55E-02
LOC_Os08g43334	HSF-type DNA-binding domain containing protein, expressed	0.90	3.91E-02
LOC_Os02g38084	hypothetical protein	3.15	3.91E-02
LOC_Os03g49740	hypothetical protein	1.03	9.76E-02
LOC_Os02g57600	expressed protein	0.88	9.76E-02

Table 9. Gene-specific primers for use in qPCR analysis of transcripts that encode enzymes involved in starch biosynthesis pathway in rice endosperm.

Gene Description	Direction	Sequence	Length	Predicated Product
Plant Protein domain of unknown function	F	5'-CGAGCCCAAACACAACTACC-3'	20	
	R	5'-GCAGTGTCACGCAGTTCCTA-3'	20	101
Glutelin, putative, expressed	F	5'-GAGAACGAGGCCTCAGAGTG-3'	20	
	R	5'-GAACAGAACCGCCACAAAGT-3'	20	100
ω-3 fatty acid desaturase	F	5'-ACTTCACCTGTTTGGCGTTC-3'	20	
	R	5'-AGTCAGTGCCGTTCAAGCTG-3'	20	110
hAT dimerisation domain containing protein	F	'5-CTCGTAAATGGCAAGGTGGT-3'	20	
	R	'5-TTCCCGGTTCAAAGAAAATG-3'	20	132
Similar to solute carrier family 35, member F1	F	'5-GGGACTTGCATGACTGGACT-3'	20	
	R	'5-CCACAATGATAAGGGCATCC-3'	20	109
PROLM27 - Prolamin precursor, expressed	F	5'-ACGGTCGGTGGTATCTGGTA-3'	20	
	R	5'-TGTATCGCCTCGATGTTTCA-3'	20	107
No apical meristem putative protein	F	5'-CAGCATCGACCTCGACATAA-3'	20	
	R	5'-GCCCAAAGAAGTACCACTCG-3'	20	110
Ubiquitin 5*	F	5'-ACCACTTCGACCGCCACTACT-3'	21	
	R	5'-ACGCCTAAGCCTGCTGGTT-3'	19	69

*Primers came from (Jain et al., 2006).

Table 10. Gene specific primers for preliminary study to investigate transcripts for enzymes involved in the starch biosynthesis pathway.

Primer Name		Primer Sequences	Predicted Size
GBSSI	F	5'-AACGTGGCTGCTCCTTGAA-3'	218
	R	5'-TTGGCAATAAGCCACACACA-3'	
SSIIa	F	5'-GGCCAAGTACCAATGGTGAA-3'	272
	R	5'-GCATGATGCATCTGAAACAAAGC-3'	
BEIIb	F	5'-ATGCTAGAGTTTGACCGC-3'	261
	R	5'-AGTGTGATGGAT CCT GCC-3'	
Amy3e	F	5'-GAGCACGCTGGACATCCTCA-3'	309
	R	5'-GCTCGTACACATCTCGCAGCA-3'	
UGP	F	5'-TCCTGGCCCGGTTTAAGTCA-3'	258
	R	5'-TGCCGAATGCACACGACAAT-3'	
cyPPDKB	F	5'-GCTCCGGCTCAATGTGCTCGT-3'	165
	R	5'-CTCCGTCGACACCGTGAAC-3'	
16kD Pro	F	5'-TTGCCAGGCTATTTGCACCA-3'	285
	R	5'-CGAACAGCCAAAGACTATTCCAAA-3'	
Glb-like	F	5'-CGAGAACGGCGAGAAGTGGT-3'	214
	R	5'-GCCCTTGCTGAAGCTCGACA-3'	
RAcII	F	5'-CTTCAACACCCCTGCTATG-3'	310
	R	5'-TCCATCAGGCTCGTAG-3'	

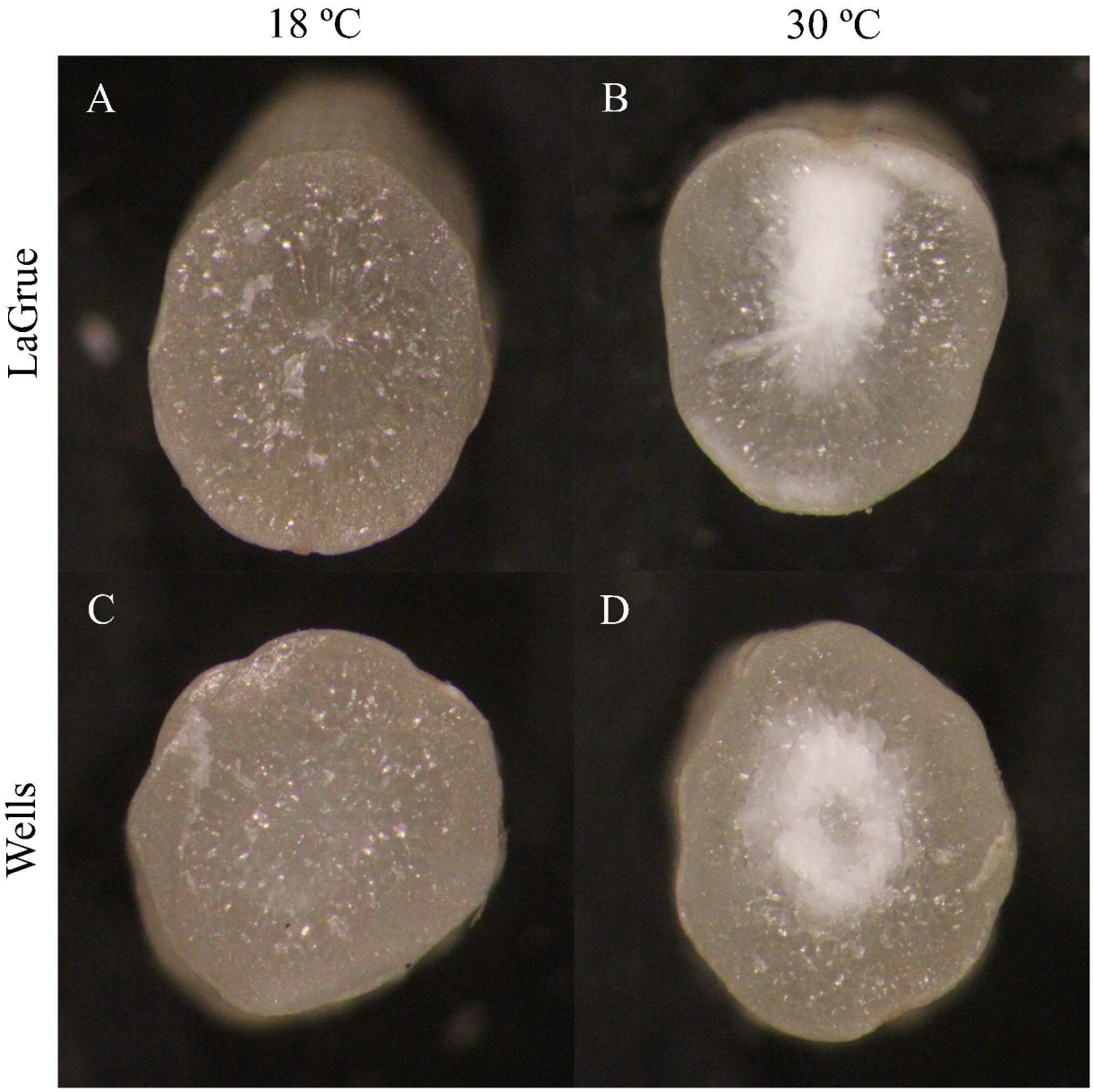

Figure 1. Nighttime temperatures affect starch packing in the central area of rice endosperm. These grains were bisected in the middle of the grain using a razor blade. The grains in panels A and B are from LaGrue. The grain in A was ripened under the control nighttime temperature 18 ºC. The grain depicted in B was treated with HNT during starch deposition. The grains in C and D are from Wells, C was ripened under control nighttime temperatures, and D is a depiction of a grain ripened under HNT.

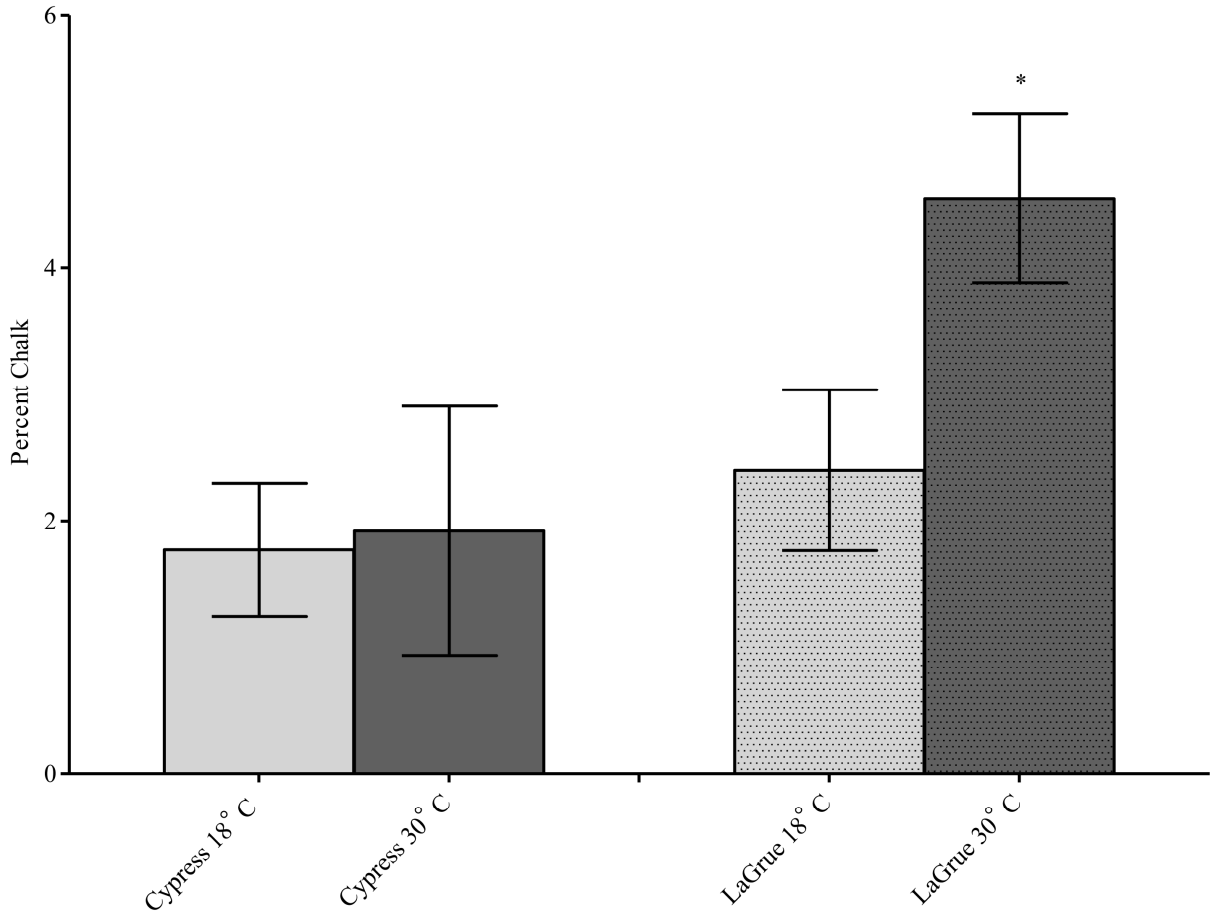

Figure 2. Percentage of chalky area in mature grain from plants treated with nighttime temperatures of either 18 ºC or 30 ºC growth chambers from plant growth stage R4 to maturity. Chalk levels were determined by digital scanning of brown rice followed by analysis with WinSeedle Pro 2005™ software. Asterisk above the column indicates a significant statistical difference between measurements (Student's *t*-test, $p>0.05$, $n=3$).

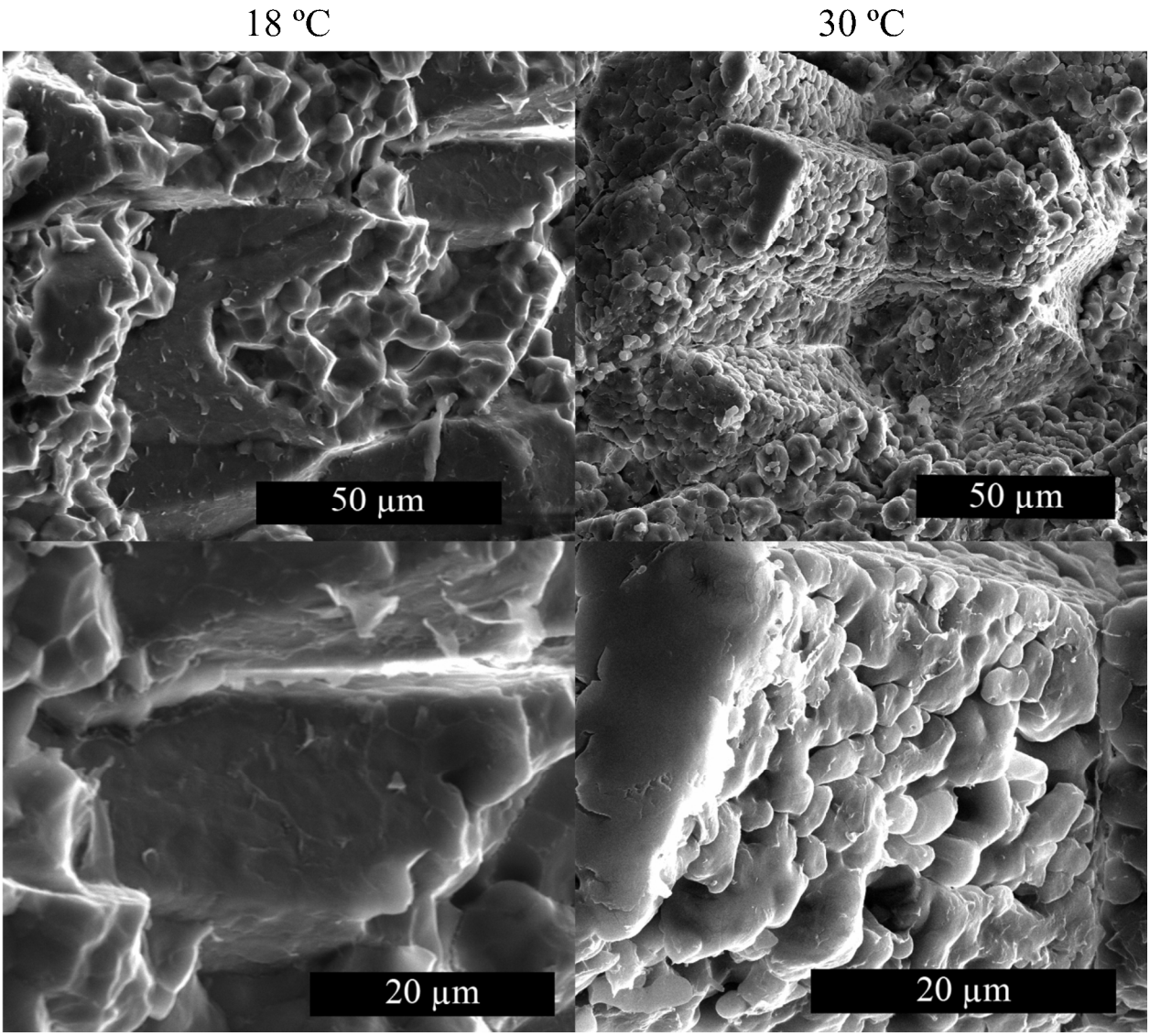

Figure 3. Scanning electron micrographs of the central area of transversely cut grains (LaGrue). The top two photos show starch granule packing in a non-chalky grain of LaGrue that was grown 18 ºC as indicated in the figure. The polygonal starch granules fit tightly together. The bottom photos show the starch granule packing in a chalky grain of LaGrue grown at a nighttime temperature of 30 ºC.

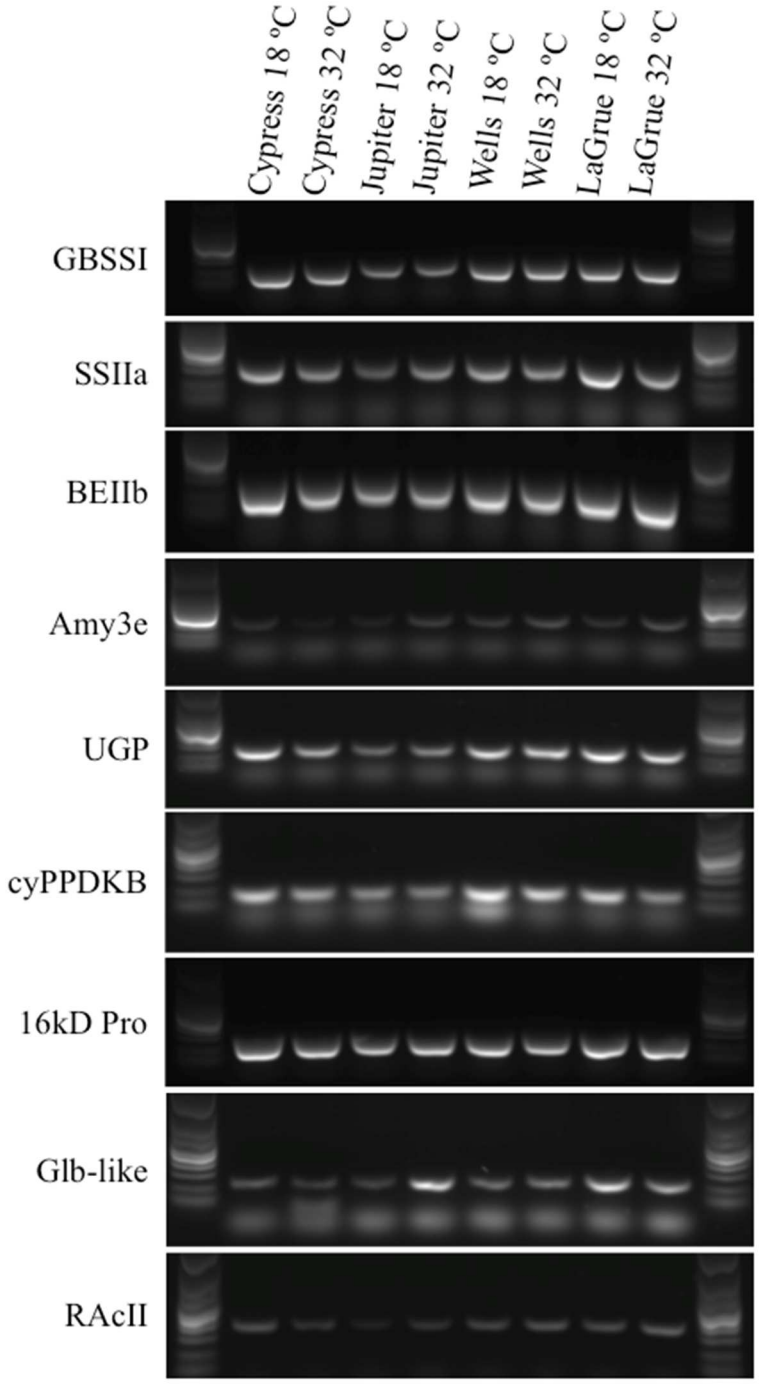

Figure 4. RT-PCR analysis of genes encoding granule bound starch synthase I (GBSSI; LOC_Os06g04200), soluble starch synthase IIa (SSIIa; LOC_Os06g12450), branching enzyme IIb (BEIIb, LOC_Os02g32660), alpha-amylase 3e (Amy3e; LOC_Os08g36900), UTP-glucose-1-phosphate uridylyltransferase (UGP; LOC_Os09g38030), pyruvate phosphate dikinase (cyPPDKB; LOC_Os05g33570), PROLM24-prolamin precursor (16kD Pro; LOC_Os06g31070), cupin domain containing protein (Glb-like, LOC_Os03g46100), Control: Actin (RAcII; LOC_Os03g50885). Labels at the top indicate rice variety and nighttime temperatures of each treatment.

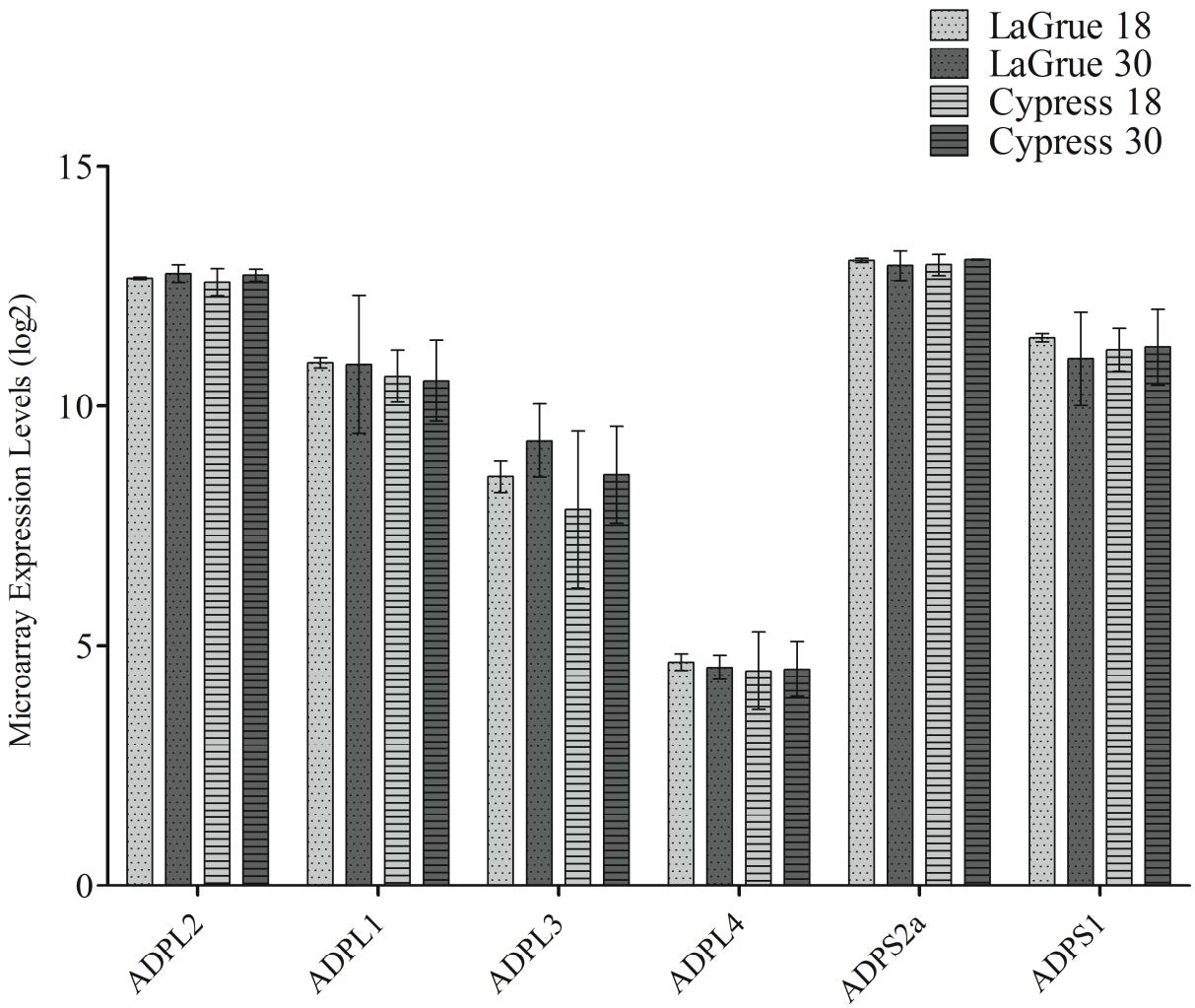

Figure 5. Expression patterns for all the subunits of glucose-1-phosphate adenylyltransferase. The ADPase enzyme is a heterotetrameric complex with four isoforms for the large subunit and two isoforms of the small subunit. Expression levels are converted from perfect match probe values collected from the Affymetrix Microarray chip (Error bars indicate S.E.M.).

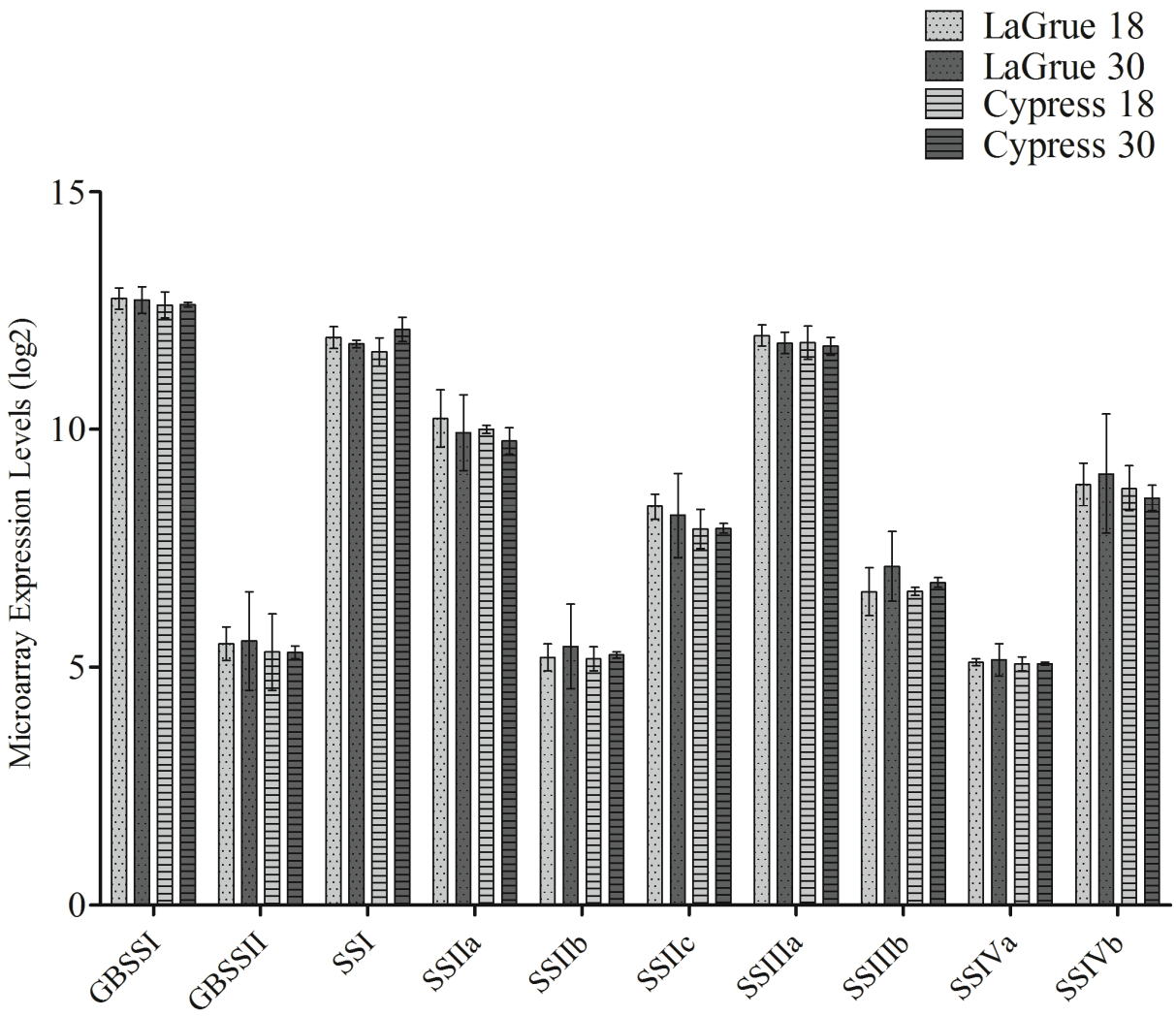

Figure 6. Expression levels for all the isoforms of starch synthase enzymes. The rice genome encodes two isoforms of granule bound starch synthase and eight known isoforms of soluble starch synthase. Expression levels are converted from perfect match probe values collected from the Affymetrix Microarray chip (Error bars indicate S.E.M.).

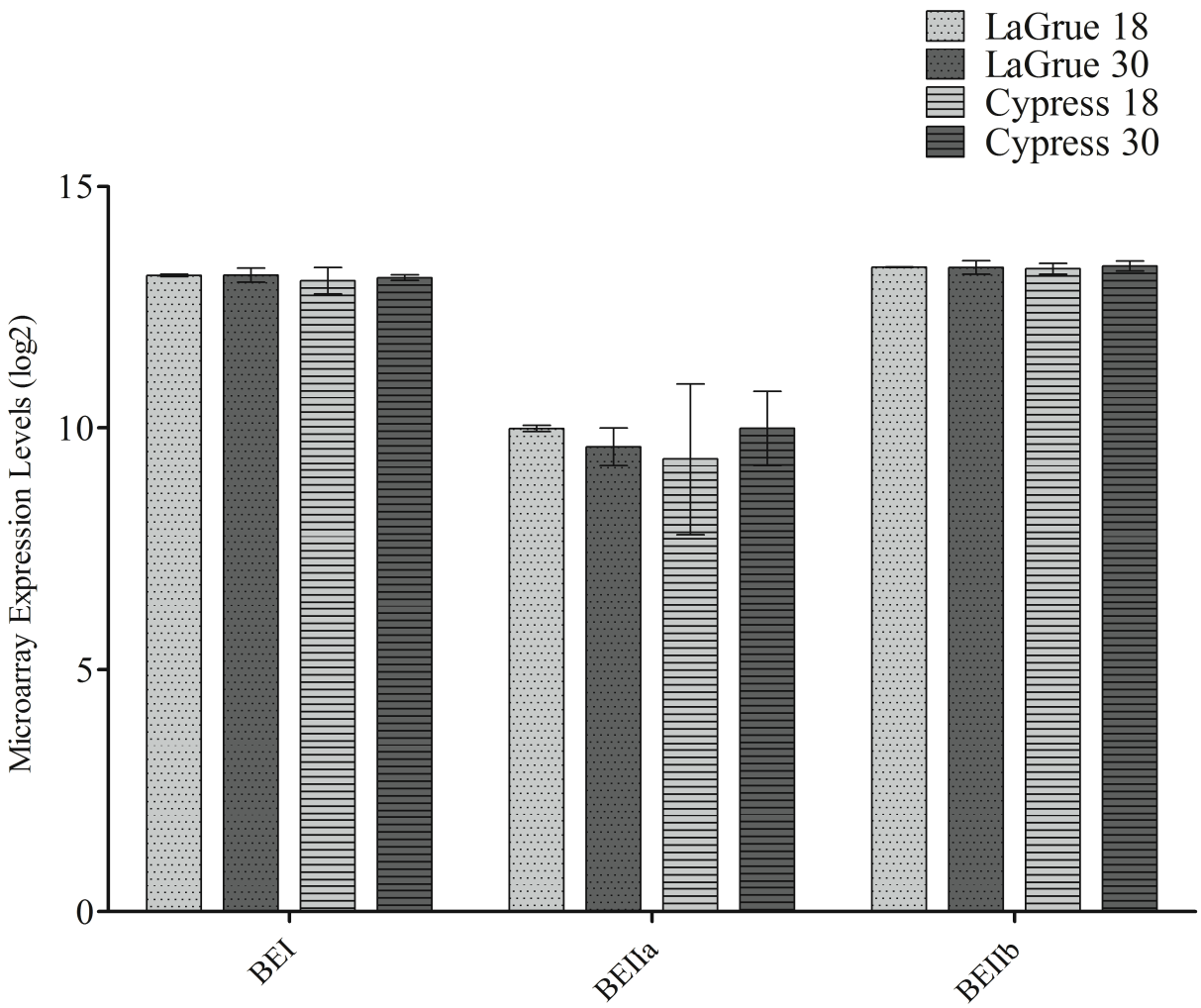

Figure 7. Expression levels for all the isoforms of starch branching enzymes. The rice genome encodes three isoforms of starch branching enzyme. Expression levels are converted from perfect match probe values collected from the Affymetrix Microarray chip (Error bars indicate S.E.M.).

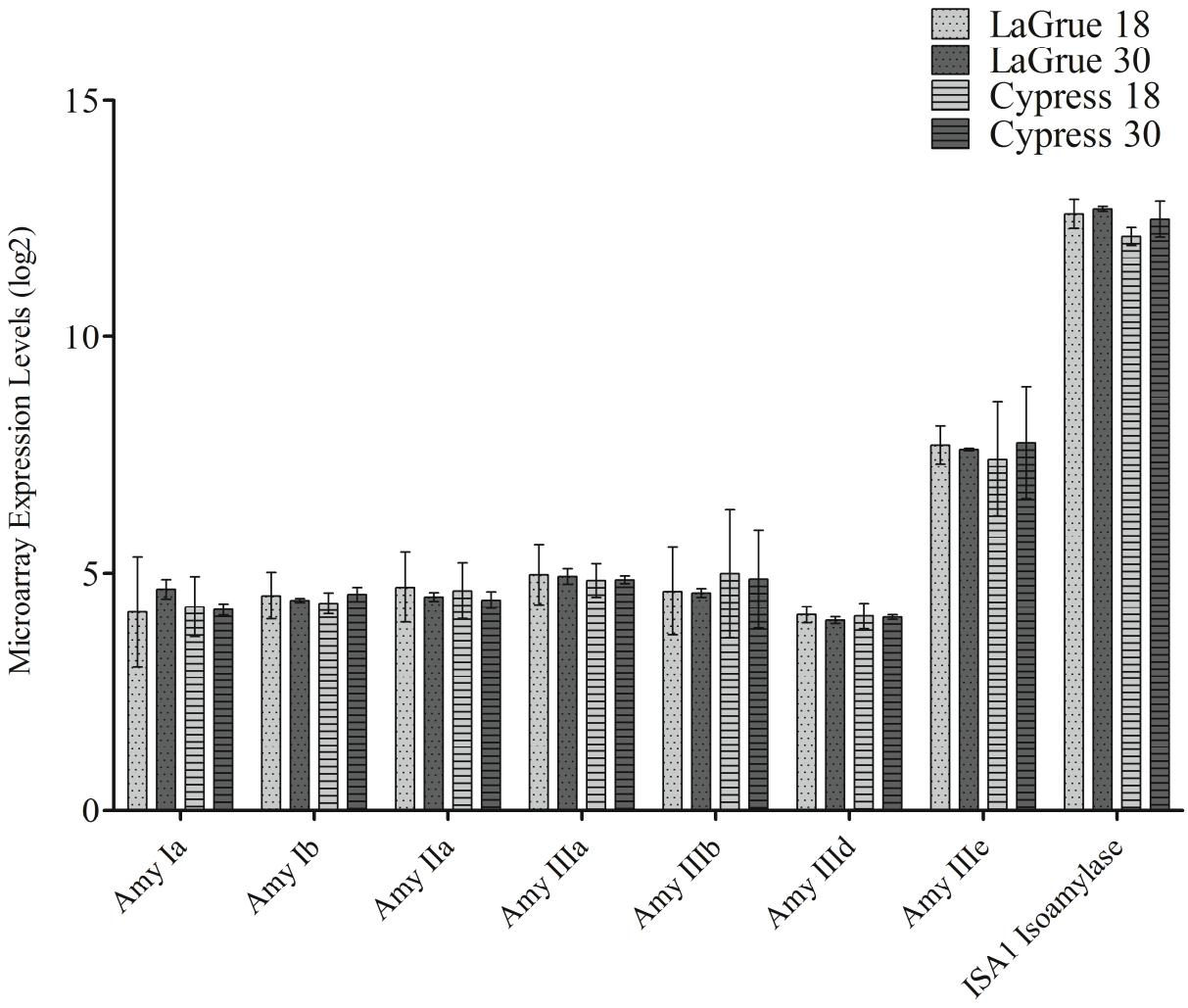

Figure 8. Expression levels for all the isoforms of alpha-amylase enzymes. The rice genome encodes seven known isoforms of alpha-amylase and one known isoforms of isoamylase. Expression levels are converted from perfect match probe values collected from the Affymetrix Microarray chip (Error bars indicate S.E.M.).

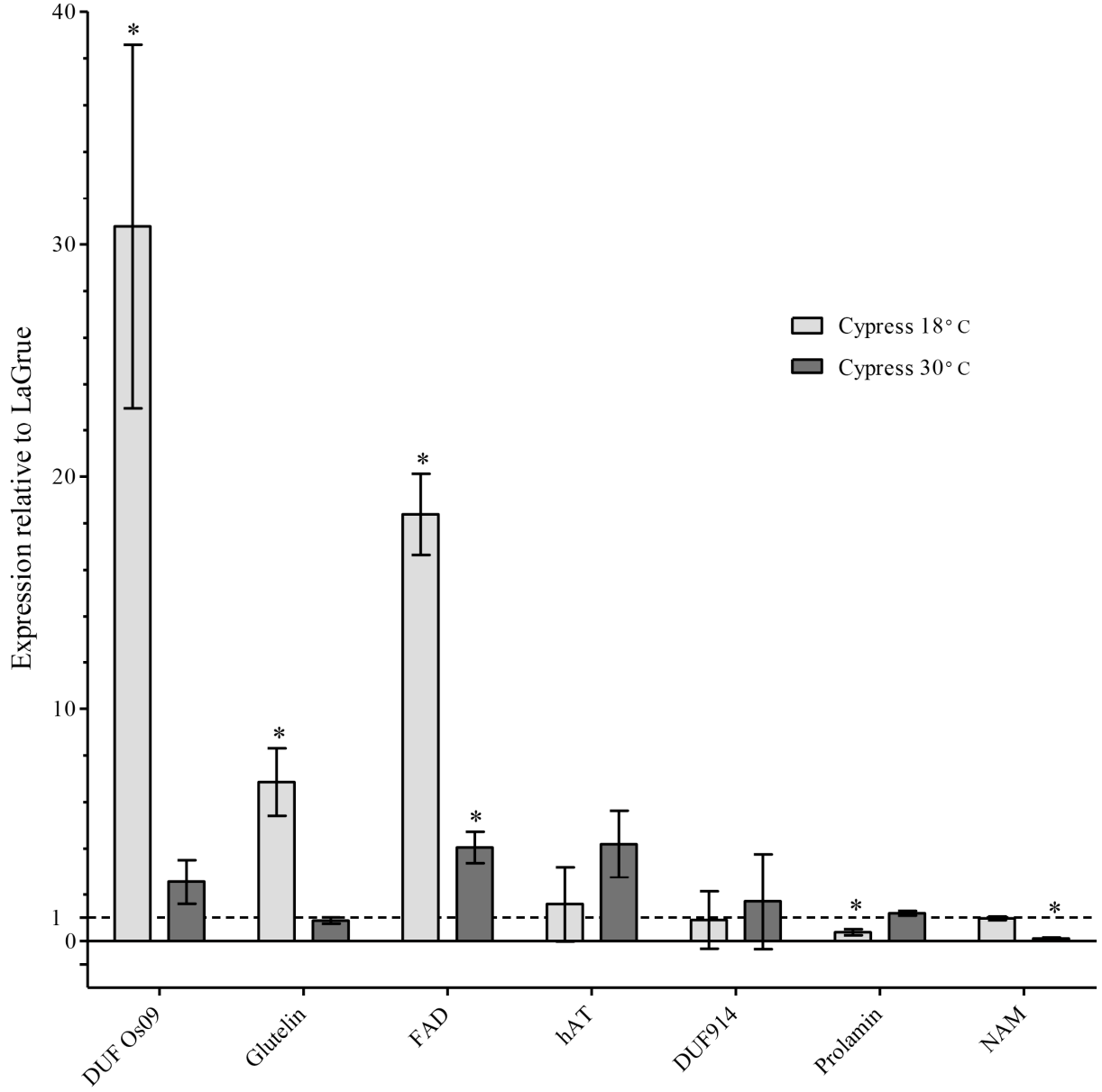

Figure 9. Relative gene expression of Cypress endosperm tissue subjected to low (light gray bars) and high (dark gray bars) nighttime temperature when compared to LaGrue treated with the same nighttime temperature. An asterisk above a column indicates a significant difference P value ≤0.05 (n=3). Error bars indicate SD. Abbreviations: DUF Os09: protein domain of unknown function (LOC_Os09g12970); Glutelin (LOC_Os02g16820); FAD: omega-3 fatty acid desaturase (LOC_Os11g01340); hAT: hAT dimerization domain-containing protein (LOC_Os01g18920); DUF914: similar to solute carrier family 35, member F1 (LOC_Os05g37210); Prolamin: PROLM27, prolamin precursor (LOC_Os12g16880); NAM: no apical meristem protein (LOC_Os09g38010).

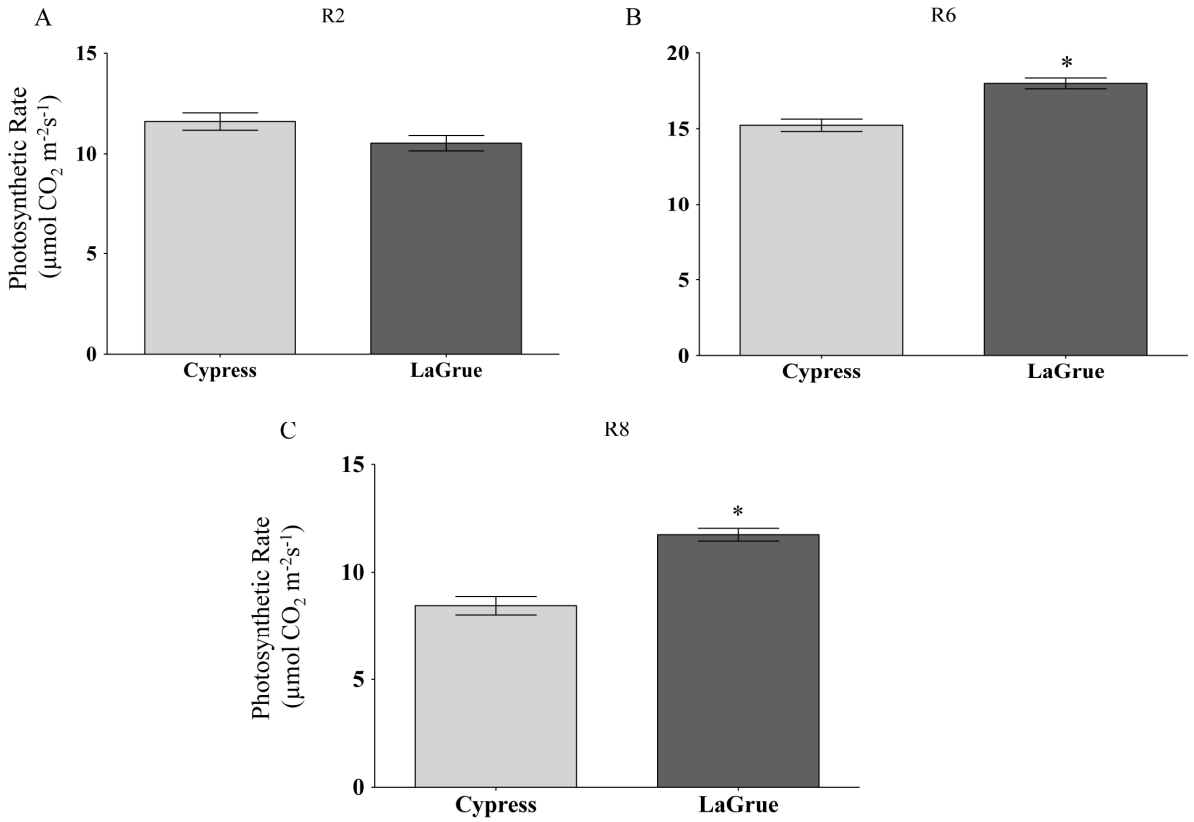

Figure 10. Net photosynthetic rate in Cypress (light gray) and LaGrue (dark gray). A. Photosynthetic rate of plants in R2 growth stage, no significant difference was found (Cypress: 11.61 ± 0.4320 µmol $CO_2m^{-2}s^{-1}$), LaGrue: 10.53 ± 0.3872 µmol $CO_2m^{-2}s^{-1}$). B. Photosynthetic rate of plants during R6 growth stage (Cypress: 15.22 ± 04034 µmol $CO_2m^{-2}s^{-1}$, LaGrue 17.98 ± 0.3562 µmol $CO_2m^{-2}s^{-1}$). Asterisk above the bar indicates P value ≤0.05 (n = 20). C. Photosynthetic rate of plants in the R8 stage (Cypress: 15.22 ± 04034 µmol $CO_2m^{-2}s^{-1}$, LaGrue 17.98 ± 0.3562 µmol $CO_2m^{-2}s^{-1}$). Asterisk above the bar indicates P value ≤0.05 (n = 20). Error bars indicate SE.

Figure 11. Research plots used to collect the photosynthesis measurements. A and B. Cypress plot in Stuttgart, AR. Plants in this plot are mainly in growth stage R8. C and D. LaGrue plot in Stuttgart, AR plants are also in growth stage R8.