Development of Rice Tolerant to Heat During Flowering

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Abstract

High temperature stress on crops results in significant yield reduction. It also affects the grain quality which results in economic loss due to low market price. Rice is also known to be sensitive to high temperature stress particularly at anthesis stage. Temperature exceeding 38°C results in significant decrees in spikelet fertility, anther dehiscense and pollen germination on stigma. An aus type variety *Nagina* 22 (N22), identified as heat tolerant variety, shows higher rate of spike fertility, anther dehiscense and pollen germination on stigma under high temperature stress (39°C). Whereas heat-sensitive variety *Moroberekan* shows a sever impairment on those. To identify the molecular mechanism, heat treated anther and spikelet proteome form N22 were analyzed by 2D gel electrophoresis. Deferentially expressed proteins were identified from the spots by sequencing using MALDI-TOF. Using BLAST search these protein sequences were identified which includes heat shock protein, cold shock protein, dirigent like protein, pollen allergens and extensin family protein. Genes coding for the identified proteins were cloned and sequenced from N22. However, no sequence polymorphisms were identified compared to *Nipponbare* alleles. Gene expression analyses by semi-quantitative and quantitative PCR using RNA from pollinated stigmas confirmed induction of two heat shock protein coding genes upon heat stress. These two genes were selected for validating their role in high temperature stress tolerance by over-expression in heat-sensitive variety background IR64. These genes were cloned into a binary vector pMDC32 under constitutive CaMV 35S promoter and immature rice embryos from IR64 were transformed mediated by *agrobacterium.* Plants were regenerated through tissue culture. Using southern analysis T_0 plants containing single copy of the transgene were selected. Both the T_1 lines over-expressing CaMV::17.4kDaHSP and CaMV::16.9kDaHSP shows fairly increased amount of transgene expression compared to the endogenous gene in IR64. Upon heat stress the transgenic plants showed an increased rate of spikelet fertility (maximum 80.98% and 86.52% in 17.4 kDa HSP and 16.9 kDa respectively) than the non-transgenic background IR64 (maximum 57.57%). Whereas the spikelets that were not exposed to heat stress in T_1 plants shows the spikelet fertility rate similar to that of non-transgenic IR64. Although the fertility found in the transgenic lines was not as good as *N22* (>76%), it can be concluded that the transgene had improved the tolerance to heat stress. Among the two heat shock proteins, the 16.9 kDa sHSP transformed lines show better performance upon heat stress.

Introduction

Population and world food demand

World population is increasing. At the beginning of 2014, the estimated world population was about 7.2 billion (world population datasheet 2014 by UN and PRB). Although between 2010 and 2014, the world's population grew at a rate of 1.2 per cent per annum, on its current trajectory, the world's population is expected to reach 8.1 billion in 2025 and 9.6 billion in 2050. By 2050, however, more than 80 per cent of the global increase will take place in Africa, with only 12 per cent in Asia (world population datasheet 2014 by UN). China and certain European countries are expected to maintain steady population.

Growing population will have increasing demand for food. According to International Fund for Agricultural Development (IFAD), food production will need to nearly double by 2050 in developing countries. Increasing population and rapid industrialization is consuming the cultivable land reducing the area under production. In addition to that, about 40 per cent of the world's arable land is degraded to some degree and will be further affected by climate change.

Food /rice production issue due to climate change

On a global average, 42% of the daily energy comes from cereal crops where rice alone contributes 19%, followed by wheat 18% (Emily 2014). The picture is different for developing countries or less developed countries where the major portion of the dietary energy comes from rice. Therefore, when it is the issue of fighting the hunger, rice has a crucial role play and makes it the most important crop.

Rice production has been tripled since 1960 (Emily 2014). This was possible by improved rice varieties, better management practice like irrigation, fertilizer, insecticide etc. given that the cultivable land is limited, the productivity has increased. The areas required for production of 1 ton of rice has decreased from 0.49 hectors in 1960s to only 0.24 hectors in 2000s(Emily 2014). So far China is the largest producer, followed by India, Bangladesh, Thailand and Vietnam. Again, these countries house some of the highest numbers of people of the world accounting almost half of the global population.

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The demand for rice as food source is also high. Figure 1.1 shows the rice production and consumption of different regions of the world.
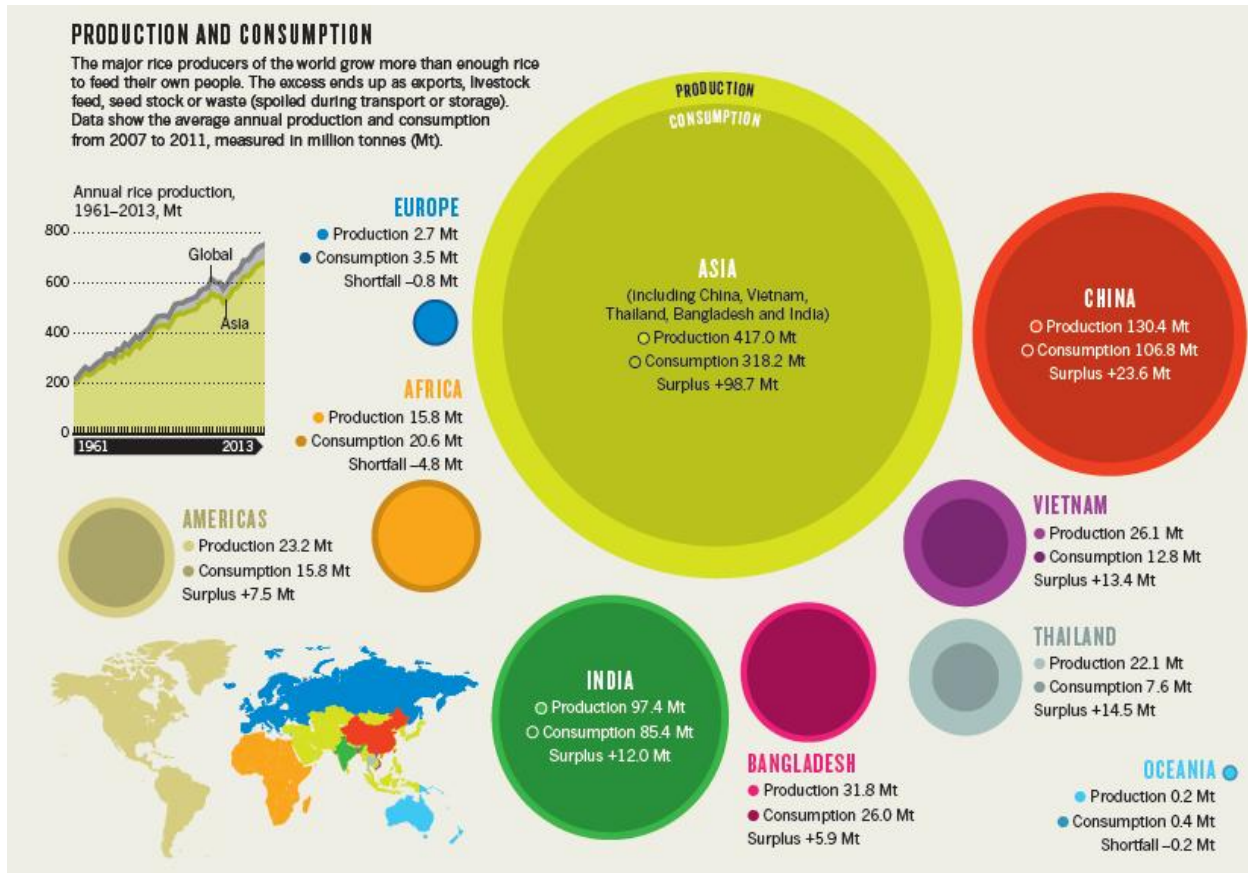
Figure 1.1: **Rice production and consumption.** China India and Bangladesh are the largest producer and consumer as well. Thailand and Vietnam are the largest exporters. 2007-2011 we have produced just enough to meet the demand (Emily 2014).

Currently we are producing just enough to meet the global requirement. But the challenge lies ahead with the increasing population and decreasing cultivable land. Added to this is the climate change scenario. Global warming and extreme weather pattern has affected the crop production. By 2050 the surface temperature is likely to increase 1.8 to 4.0 degree Celsius under different scenario (IPCC 2007). This is likely to be accompanied by rainfall and its pattern, CO_2 emission sea level raise etc, leading to a vulnerable condition for crop production. Taking these in account IFPRI projected (Figure 1.2) production of major crops by year 2050 where wheat and rice faces significant decrease in production.

Region	CSIRO No CF	NCAR No CF	CSIRO CF	NCAR CF
Maize, irrigated				
Developing countries	−2.0	−2.8	−1.4	−2.1
Developed countries	−1.2	−8.7	−1.2	−8.6
Maize, rainfed				
Developing countries	0.2	−2.9	2.6	−0.8
Developed countries	0.6	−5.7	9.5	2.5
Rice, irrigated				
Developing countries	−14.4	−18.5	2.4	−0.5
Developed countries	−3.5	−5.5	10.5	9.0
Rice, rainfed				
Developing countries	−1.3	−1.4	6.5	6.4
Developed countries	17.3	10.3	23.4	17.8
Wheat, irrigated				
Developing countries	−28.3	−34.3	−20.8	−27.2
Developed countries	−5.7	−4.9	−1.3	−0.1
Wheat, rainfed				
Developing countries	−1.4	−1.1	9.3	8.5
Developed countries	3.1	2.4	9.7	9.5

Note: For each crop and management system, this table reports the area weighted average change in yield for a crop grown with 2050 climate instead of 2000 climate. CF = with CO_2 fertilization; No CF = without CO_2 fertilization.

Figure 1.2: **Change in crop yield (%) from 2000 to 2050 induced by Climate-change**. Following the climate-change scenario by IPCC, rice and wheat production is likely to be decreased considerably (IFPRI 2009).

Affect of climate change

In general, climate change refers to long-term trends of weather, usually over the decades or centuries. Whereas, climate variability refers to individual events generally fluctuates in seasonal or annual basis. Magnitude of these events gradually translates into climate change. Both of them can affect crop production directly or indirectly. For instance, temperature increase or change in precipitation pattern can alter the optimal growth environment resulting yield loss. On the other hand, intense precipitation causing flood can damage infrastructure like roads, storage facility, irrigation system, causing indirect damage to crop production.

Temperature

Temperature is the most pronounced factor of climate change. IPCC projects 2-4 degree increase in global temperature. However, this is only global average. A lot of local variation is expected (figure 1.3). High latitude regions in northern hemisphere will experience the highest increase in mean temperature with lesser increase in tropical and sub-tropical regions. Land will experience higher increase then oceans. Part of China is may see higher increase in temperature then neighboring region (IPCC, 2007). In addition to this, extreme temperature events are also expected as observed by Zhai et al. in China (Zhai et. al 2003). They observed increasing trends of number of warm days as well as cold days over the last half of 20th century. This is expected to occur more frequently and widely in future.
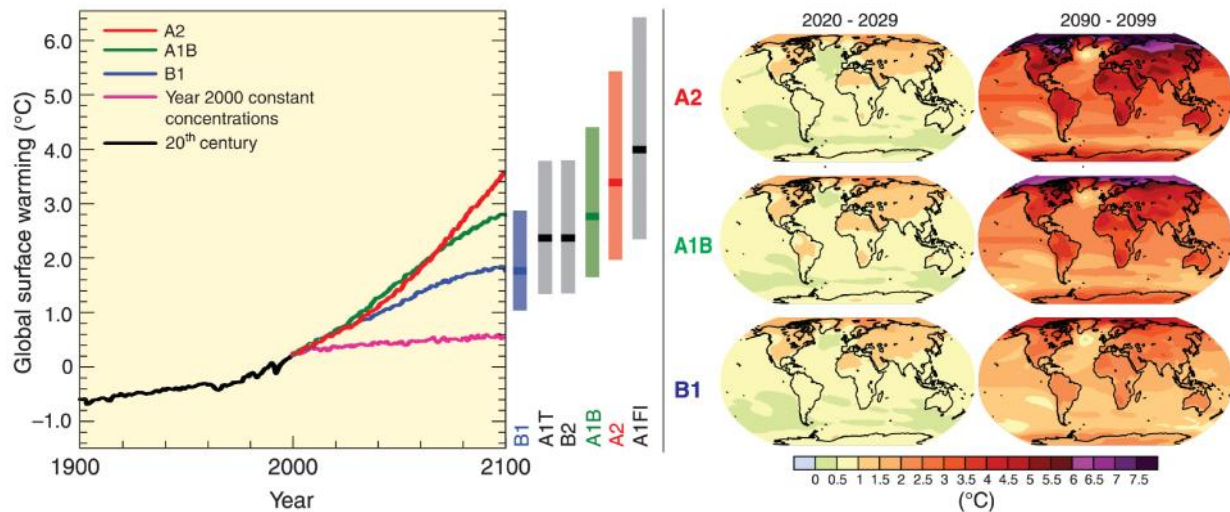
Figure 1.3: **Global surface warming.** Historical global temperature and predicted temperature on different scenario by end of 21st century. Left panel shows average increase in surface temperature over 20th century and predicted over 21st century. Right panel shows local increase in temperature.

Future climatic condition with increased temperature coinciding with key stage of development can be devastating (Wollenweber et al., 2003). The yields of many crops can be drastically reduced by temperatures above 32 °C during the flowering stage: for instance, rice grain sterility occurs in temperatures in the mid-30s (Porter and Gawith, 1999; Wheeler et al., 2000; Vara Prasad et al., 2003;

Jagadish et al. 2007). This is supported by experience in 2003 where European heat-wave resulted in yield losses of up to 36% in Italy for corn (Stott et al., 2003).

Drought and flood

Climate change also predicts change in precipitation pattern. In general, temperature increase will result in more active hydrological cycle. This can translate into more rainfall in places causing flood and also less rainfall causing drought (IPCC, 2007). Water requirement for different developmental stage is different. Historical practice of crop production is as such that the precipitation pattern will meet the requirement. Change in precipitation pattern will alter the optimal growth condition resulting in yield loss. Although, in modern practice, we can provide water through irrigation, but often irrigation through underground water causes salt deposition on top soil hence increasing soil salinity.

High temperature stress and rice

Vegetative growth of rice can continue at as high as 40°C day temperature. The reproductive stage is extremely sensitive to heat which includes booting stage and floret fertilization (Satake and Yoshida, 1978). Temperature induced floret sterility shows highest sensitivity. The temperature at which sterility occurs, vary among genotypes. In general, above 35°C during anthesis can result in upto 90% floret sterility (De Datta, 1981) which is translated to yield loss. On the other hand, high night temperature during grain development can result in poor grain quality hence reduced market value. High nighttime temperatures are related to decreased panicle mass and increased numbers of chalky kernels, increase in amylose content and decrease in the proportion of long chains of amylopectin (Krishnan et al. 2011).

Other factor affecting temperature stress

Relative humidity (RH) plays a vital role in temperature stress. Matsui et al. (1997) showed that fertility of spikelet at 37.5° C was highest at 45% RH followed by that at 60% RH and lowest at 80% RH. RH affects the anther dehiscence and pollen germination on stigma (Krishnan et al. 2011). Surface water and wind flow can significantly increase the transpiration rate which helps to reduce the canopy temperature under high-temperature stress.

Action to mitigate temperature stress

With the increasing demand of food, best exploitation of available resources is needed. As far as rice is concerned, we are already in highest state of productivity with the given plant architecture. Unless we can create dramatic change in capacity, as aimed in C4-rice, there would not be a significant increase in productivity. Therefore we need to focus more on improving rice varieties that can adopt with the climate change scenario, likely to be stress tolerant rice variety.

Review of literatures

Heat stress is often defined as the rise in temperature beyond a threshold level for a period of time sufficient to cause irreversible damage to plant growth and development. In general, a transient elevation in temperature, usually 10–15 ∘C above ambient, is considered heat shock or heat stress (Wahid, 2007). Heat tolerance is generally defined as the ability of the plant to grow and produce economic yield under high temperatures. According to a report of the Intergovernmental Panel on Climatic Change (IPCC), global mean temperature will rise 0.3 ∘C per decade (Jones et al., 1999) reaching to approximately 1 and 3 ∘C above the present value by years 2025 and 2100, respectively, and leading to global warming.

Immediately after exposure to high temperatures and perception of signals, changes occur both physiologically and molecular level. Altered gene expression and transcript accumulation leads to synthesis of stress related protein (Iba, 2002) among which heat-shock protein plays an important role (Feder and Hoffman, 1999). The small heats-shock proteins (sHSPs) are known to have chaperone-like functions (Schoffl et al., 1999) and confers tolerance by improving photosynthesis, assimilate partitioning, water and nutrient use efficiency, and membrane stability (Camejo et al., 2005; Ahn and Zimmerman, 2006; Momcilovic and Ristic, 2007). This translates into better performance of plant growth and development under heat stress. However, the capability varies among genotype and species leaving opportunities to improve by conventional breeding or genetic transformation.

Heat stress Threshold

A threshold temperature can be defined as the daily mean temperature where plants growth and development is ceased. However, plant behavior differs significantly with respect to other environmental factors and therefore is difficult to determine a

consistent threshold temperature (Miller et al., 2001). The staple cereal crops can tolerate only narrow temperature ranges, which if exceeded during the flowering phase can damage fertilization and seed production, resulting in reduced yield (Porter, 2005). For example, in tomato, germination, growth, flowering and fruit set is affected with temperature exceeding 35°C. For other plants the threshold may be higher or lower.

Mechanism of heat tolerance

The mechanism of heat tolerance is still enigmatic. Plant's immobility limits the range of their behavioral responses and therefore, for protection and adaptation they rely on physiological and cellular mechanism. Changing leaf orientation, transpirational cooling, or alteration of membrane lipid compositions are some phenological or morphological changes in short-term adaptation mechanism. In some plants early maturation was observed to minimize the yield loss (Adams et al., 2001), where as in wild rice, early morning flowering was observed to avoid high temperature exposure during anthesis (Ishimaru et al, 2010).

Plants temperature sensing mechanism is still not identified. But it initiates downstream signaling processes which activates a variety of stress-response mechanism to re-establish cell homeostasis and damage protection. A significant portion of this response includes heat shock proteins. Primary function of this group of proteins are to provide for new or distorted proteins to fold into shapes essential for their normal functions, shuttling proteins from one compartment to another and transporting old proteins to "garbage disposals" inside the cell. Among other proteins, peroxidase and super-oxide-dismutase are free-radical scavengers and involved in protection from reactive oxygen species (ROS) to minimize heat-induced damage. Inadequate responses at one or more steps in the signaling and gene activation processes might ultimately result in irreversible damages in cellular homeostasis and destruction of functional and structural proteins and membranes, leading to cell death (Vinocur and Altman, 2005; Bohnert et al., 2006). Hormones like abscisic acid, ethylene, salicylic acid comes into

action and different solutes and antioxidant starts accumulating which plays role in long-term adaptation.

Plant responses to heat stress

Morphological changes

Different development stage reacts differently to high temperature stress. Extreme heat known to delayed germination or loss of vigor, declines in shoot dry mass, relative growth rate and net assimilation rate in maize, pearl millet and sugarcane, though leaf expansion was minimally affected (Ashraf and Hafeez, 2004; Wahid, 2007). The response of rice to high temperatures differs according to the developmental stage. High temperature tolerance at one developmental stage may or may not necessarily lead to tolerance during other stages. An independent extreme heat episode during vegetative stages was shown to have no influence on reproductive stage (Porter and Semenov, 2005).

During vegetative stage, rice can tolerate relatively high temperatures (35/25 °C). Temperatures beyond this critical level could reduce plant height, tiller number and total dry weight (Yoshida et al., 1981), reduced photosynthesis (Oh-e et al., 2007). The loss of photosynthesis activity is explained by structural changes in the organization of thylakoids (Karim et al., 1997), more specifically due to loss of stacking of grana in the chloroplast or its ability to swell (Wahid et al., 2007). High temperature also increase the membrane kinetic energy which leads to increased fluidity of lipid layer resulting increased solute leakage and membrane instability (Wahid et al., 2007, Savchenko et al., 2002).

Reproductive stage is considered more vulnerable then vegetative stage. High temperature affects pollination and fertilization which results in reduced seed set. A reciprocal study with pollen from control plants on heat-stressed pistils and vice versa, showed that ale gametophyte and not the pistil is responsible for spikelet sterility during high temperature stress (Yoshida *et al.*, 1981). Physiological processes involved are anther dehiscence where spikelet opening triggers rapid pollen swelling leading to

pollen discharge through basal and apical pore of anther, germination of pollen on stigma which extends as pollen tube to reach embryo sac to complete fertilization. Typically it takes about 30 min for pollen tube to reach embryo sac after pollination (Cho, 1956). However, anther dehiscence is most critically affected by high temperature (Matsui *et al.*, 1997*a*, *b*, 2001). Basal and apical pore size pollination (Matsui and Kagata, 2003) as well as stigma length shows significant variation among genotypes (Matsui *et al.*, 1997*a*) and can contribute in avoiding high temperature exposure by rapid discharge of pollen through larger basal and apical pores and proximity of stigma surface by larger stigma. Reproductive processes are markedly affected by high temperatures in most plants, which ultimately affect fertilization and post-fertilization processes leading to reduced crop yield.

Anatomical changes

Although limited details are available, anatomical changes in some plants observed under high ambient temperatures are generally similar to those under drought stress. At the whole plant level, reduced cell size, closure of stomata and increased stomatal densities, and greater xylem vessels of both root and shoot was observed (A˜non et al., 2004). At the sub-cellular level, major modifications occur in chloroplasts, which affect photosynthesis. In response to high temperature stress structural organization of thylakoids changes (Karim et al., 1997) due to loss of grana stacking or its swelling (Wahid et al 2007). Even finer details studied in grape plants showed swollen stroma lamellae, clumped vacuole contents, disruption of cristae leading to empty mitochondria (Zhang et al., 2005). These changes often lead to formation of antenna-depleted photosystem-II (PSII) and significantly reduced photosynthetic and respiratory activities (Zhang et al., 2005). In general, high temperature considerably affects anatomical structures at the tissue and cellular levels as well as at the sub-cellular level. The cumulative effects of all these changes under high temperature stress may result in poor plant growth and productivity.

Phenological changes

Observation of changes in plant phenology in response to heat stress can reveal a better understanding of interactions between stress atmosphere and the plant. Vulnerability of species and cultivars to high temperatures may vary with the stage of plant development, but all vegetative and reproductive stages are affected by heat stress to some extent (Wahid et al. 2007). Rice plants when exposed to high temperatures during critical stages can avoid heat by maintaining their microclimate temperature below critical levels by efficient transpiration cooling. Moreover, the effect of high temperature is closely related to the ambient relative humidity and hence the level of transpiration cooling is determined by vapor pressure deficit than temperature (Jagadish et al. 2007). Weerakoon et al. (2008) using a combination of high temperatures (32-36 °C) and relative humidity (RH) (60% and 85%) RH recorded high spikelet sterility with simultaneous increase in temperature and RH. Hence it can be concluded that the reduction in spikelet temperature in relation to RH is avoidance while the performance of a variety at a given spikelet temperature to be true tolerance (R. Wassmann 2009). On the basis of the interaction between high temperature and relative humidity, rice cultivation regions in the tropics and sub-tropics can be classified into hot/dry or hot/humid regions. It can be assumed that rice cultivation in hot/dry regions where temperatures may exceed 40 °C (e.g., Pakistan, Iran, India) has been facilitated through unintentional selection for efficient transpiration cooling under sufficient supply of water. An exceptionally high temperature difference of 6.8 °C between crop canopy and ambient air temperature (34.5 °C) was recorded in Riverina region of New South Wales, Australia which was primarily due to extremely low humidity of 20%, resulting in strong transpiration cooling mainly driven by high wind velocity of 3.2-4.2ms^{-1} (Matsui et al., 2007).

Anther dehiscence is the most susceptible process during anthesis under high temperature (Matsui et al., 1999b). High temperature results in increased vapor pressure deficit, enhanced evaporation from the anthers and thereby depriving the crucial moisture needed for pollen grain swelling which is inevitable for anther

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dehiscence. Heat avoiding genotypes thrive well in hot and dry rice cultivation regions of the world while for hot and humid regions either heat escape or true tolerance is essential to maintain productivity. However, with predicted increased mean surface air temperature rather than just increased maximum temperature the rice plant could be exposed to increased day and night temperatures further indicating the importance of true heat tolerance.

Physiological responses

Water retentions

Plant water status is an important variable in any stress including temperature change (Mazorra et al., 2002). Plants tend to maintain stable tissue water status in any temperature when substantial moisture is present. Limited water severely impairs this ability (Machado and Paulsen, 2001). High temperature stress is frequently associated with reduced water availability in field conditions (Simoes-Araujo et al., 2003). Upon heat exposure, leaf water potential and its components were changed in sugarcane even though the soil water supply and relative humidity conditions were optimal (Wahid and Close, 2007). Similarly, in tomato heat stress perturbed the leaf water relations and root hydraulic conductivity (Morales et al., 2003).

Accumulation of compatible osmolytes

A general adaptive mechanism to any stress including salinity, water deficit and extreme temperature, plant tends to accumulate certain low molecular weight organic compounds (Hare et al., 1998; Sakamoto and Murata, 2002). These organic compounds are reffered to as compatible osmolytes, includes sugars and sugar alcohols (polyols), proline, tertiary and quaternary ammonium compounds, and tertiary sulphonium compounds (Sairam and Tyagi, 2004). Accumulation of such solutes may vary according to species and contribute to enhanced stress tolerance of plants.
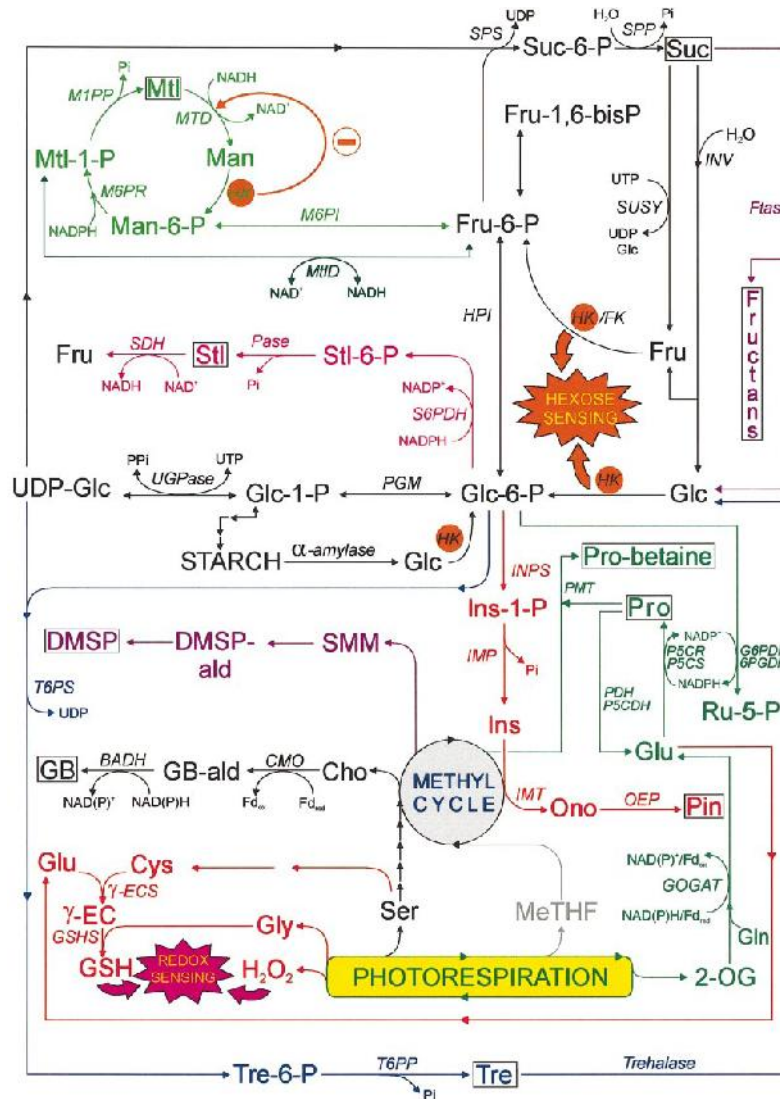
Figure 2.1: **Role of osmolyte in stress**. Hare et al. 1998

Accumulation of soluble sugars under heat stress has been reported in sugarcane (Wahid and Close, 2007) whereas fruit set in tomato plants failed due to the disruption of sugar metabolism and proline (Sato et al., 2006). Introduction of Proline or Glycine Betaine (GB) (Wahid and Close, 2007; Sakamoto and Murata, 2002; Quan et al., 2004) or hexose sensing (Hare et al., 1998) in transgenic plants engineered to produce solutes that are normally not synthesized, provides an important contributory factor to the stress-tolerant phenotypes. Among other osmolytes, cytosolic level of Ca plays an

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important role in high temperature stress which in turn leads to calmodulin-mediated activation of GABA synthesis (Taiz and Zeiger, 2006).

Photosynthesis

Photosynthesis is an important parameter for any stress as change of photosynthesis will effect in growth and development of plant. Heat stress is often accompanied by high light intensity leading to more light energy absorption then used in photosynthesis and results oxidative stress (*Anja Krieger-Liszkay 2004*). On the other hand, heat induced dissociation or loss of regeneration capacity results disruption in various component of photosynthesis such as ribulose-1,5-bisphosphate (RuBP) (Wise et al., 2004), D1 and/or the D2 proteins (De Las Rivas and Barber, 1997), oxygen evolving complex (OEC) (De Ronde et al., 2004; Salvucci and Crafts-Brandner, 2004b), manganese (Mn)-stabilizing 33-kDa protein at PSII (Yamane et al., 1998). This may be due to the properties of thylakoid membranes where PSII is located (Mcdonald and Paulsen, 1997). Heat stress is often accompanied by high light intensity. This often causes more light energy absorption then used in photosynthesis and results oxidative stress (*Anja Krieger-Liszkay 2004*). Recent investigations on the combined action of moderate light intensity and heat stress suggest that moderately high temperatures do not cause serious PSII damage but inhibit the repair of PSII. The latter largely involves de novo synthesis of proteins, particularly the D1 protein of the photosynthetic machinery that is damaged due to generation of reactive oxygen species (ROS), resulting in the reduction of carbon fixation and oxygen evolution, as well as disruption of the linear electron flow. The attack of ROS during moderate heat stress principally affects the repair system of PSII, but not directly the PSII reaction center (RC). Heat stress additionally induces cleavage and aggregation of RC proteins. On the other hand, membrane linked sensors seem to trigger the accumulation of compatible solutes like glycinebetaine in the neighborhood of PSII membranes. They also induce the expression of stress proteins that alleviate the ROS-mediated inhibition of repair of the stress damaged

photosynthetic machinery and are required for the acclimation process (Allakhverdiev et al. 2008).

Cell membrane thermostability

Heat stress accelerates the kinetic energy and movement of molecules across membranes thereby disturbing the stability of the molecules of biological membranes by either denaturation of proteins changing tertiary and quaternary structures or change in fatty acid composition (Savchenko et al., 2002). Such alterations enhance the permeability of membranes, increases loss of electrolytes, decreases cell membrane thermostability (CMT) which has long been used as an indirect measure of heat-stress tolerance in diverse plant species, including soybean (Martineau et al., 1979), potato and tomato (Chen et al., 1982), wheat (Blum et al., 2001), cotton (Ashraf et al., 1994), sorghum (Marcum, 1998), cowpea (Ismail and Hall, 1999) and barley (Wahid and Shabbir, 2005). However, electrolyte leakage is influenced by a variety of factors including plant/tissue age, sampling organ, developmental stage, growing season, degree of hardening and plant species.

Hormonal changes

Hormones play an important role in sensing and reacting adverse environment conditions. Cross-talk between hormones reflects the ability of the organism to adopt in that extreme condition which varies among species and genotype. Studying different mutants of Arabidopsis that effects different hormonal pathways Larkindale et al. 2005 postulated a nice framework about the role of different hormones in basal and acquired thermotolerance (Fig: 2.2).
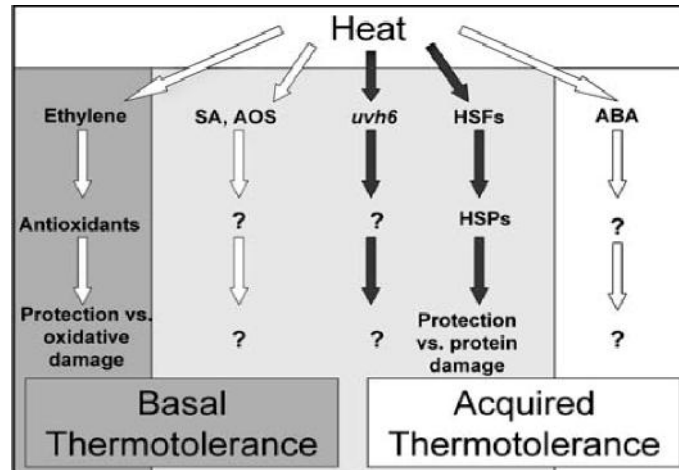
Figure 2.2: **Hormonal response upon heat stress** (Larkindale et al. 2005)

Abscisic acid (ABA) and ethylene (C_2H_4), as known before as stress hormones, are involved in the process of thermotolerance but in a different fashion. ABA signaling plays critical role for acquired thermotolerance than for basal thermotolerance, while the converse is true for ethylene signaling and antioxidant protection (Larkindale et al. 2005). The action of ABA in response to stress involves modification of gene expression a numerous genes (Xiong et al., 2002) including HSPs to confer thermotolerance (Pareek et al., 1998) and also may act synergistically with heat shock transcription factor 3 (HSF3) on sHSP promoter (Rojas et al., 1999). On the other hand, ethylene has been associated with oxidative stress responses as a signal to activate oxidative defenses during heat stress (Kato et al., 2000; Argandona et al., 2001; Bortier et al., 2001; Moeder et al., 2002; Manning et al., 2003). Oxidative damage is a major component in basal thermotolerance where ethylene plays role by means of antioxidant pathway to provide protection (Larkindale et al. 2005).

Salicylic acid (SA) has been suggested to be involved in heat-stress responses as an important component of signaling pathways in response to systemic acquired resistance (SAR) and the hypersensitive response (HR) (Kawano et al., 1998). A derivative of SA, sulphosalisylic acid (SSA) treatment shows increased activity of catalase, a key enzyme to remove H_2O_2 as wel as glutathione peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR) under heat stress (Shi et al.,

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2006) indicates a possible involvement of SA in Active oxygen species (AOS) pathway for protection. Gibberellins and cytokinins on high temperature tolerance are opposite to that of ABA as studied in barley (Vettakkorumakankav et al., 1999), creeping bentgrass (Liu and Huang, 2005) and wheat (Banowetz et al., 1999).

Molecular responses

Oxidative stress and antioxidants

In addition to tissue dehydration, heat stress induces oxidative stress produced mainly by reactivated oxygen species (ROS) including singlet oxygen ($:O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Liu and Huang, 2000). ROS modify membrane function by autocatalytic peroxidation of membrane lipids and pigments thus leading to the loss of membrane semi-permeability (Xu et al., 2006). Superoxide radical is regularly synthesized in the cellular organelles like chloroplast and mitochondrion and some quantities in microbodies. The scavenging action of superoxide dismutase (SOD) on O_2^- results in the production of H_2O_2, which is removed by APX or CAT. The OH^- can damage chlorophyll, protein, DNA, lipids and other important macromolecules, thus fatally affecting plant metabolism and limiting growth and yield (Sairam and Tyagi, 2004).

ROS are naturally produced in cells and hence plants have developed detoxification systems to counteract ROS, thereby protecting cells from oxidative damage (Sairam and Tyagi, 2004). Therefore, altering the capacity can affect some other processes. For example, overexpression of SOD in plants affect a number of physiological phenomena, which include the removal of H_2O_2, oxidation of toxic reductants, biosynthesis and degradation of lignin in cell walls, auxin catabolism, defensive responses to wounding, defense against pathogen or insect attack, and some respiratory processes (Scandalios, 1993). More specifically, expression and activation of APX is related to the appearance of physiological injuries caused in plants by thermal stress (Mazorra et al., 2002). On the other hand, decrease in antioxidant activity in

stressed tissues results in higher levels of ROS that may contribute to injury (Fadzillah et al., 1996).

Stress proteins

Expression of stress proteins is an important adaptation to cope with environmental stresses. A number of proteins are expressed upon exposure to heat. But majority of them belongs to a gene super family commonly known as heat shock proteins (HSPs) and are most abundant in any stress.

Heat shock proteins: Induction of HSPs seems to be a universal response to temperature stress in all organisms ranging from bacteria to human (Vierling, 1991) where its production increases upon abrupt or gradual increase in temperature (Nakamoto and Hiyama, 1999; Sch¨offl et al., 1999). HSPs are involved in various functions including cell cycle and developmental control (Hopf et al., 1992) and hence are expressed in small amount but the type of HSPs are restricted to certain stages of development, such as embryogenesis, germination, pollen development and fruit maturation (Prasinos et al., 2005). In higher plants, HSPs are usually induced under heat shock at any stage of development and major HSPs are highly homologous among distinct organisms (Vierling, 1991).

Other heat induced proteins: Besides HSPs, there are a number of other plant proteins, including ubiquitin (Sun and Callis, 1997), cytosolic Cu/Zn-SOD (Herouart and Inz´e, 1994) and Mn-POD (Brown et al., 1993), whose expressions are stimulated upon heat stress. For example, ubiquitin and conjugated-ubiquitin synthesis provides important mechanism of tolerance to high temperature in soybean (Ortiz and Cardemil, 2001). Heat shock induces Mn-peroxidase, which plays a vital role in minimizing oxidative damages (Iba, 2002). Late embryogenesis abundant (LEA) proteins can prevent aggregation and protect the citrate synthase from desiccating conditions like heat- and drought-stress (Goyal et al., 2005). Proteomic study in hexaploid wheat shows enhanced expressions of LEA during grain filling (Majoul et al., 2003). A number of osmotin like proteins induced by heat and nitrogen stresses, collectively called Pir proteins, were

found to be overexpressed in the yeast cells under heat stress conferring them resistance to tobacco osmotin (an antifungal) (Yun et al., 1997). Dehydrin proteins were found to be over-expressed in geranium (Arora et al., 1998) and sugarcane (Wahid and Close, 2007) leaves exposed to drought and heat stress. Dehydrin proteins are low molecular weight proteins, apperently related to protein degradation pathway and may play role to minimize the adverse effect of water and high temperature stress (Sch¨offl et al., 1999).

In essence, expression of stress proteins is an important adaptation toward heat-stress tolerance by plants. Of these, expression of low and high molecular weight HSPs, widely reported in a number of plant species, is the most important one. These proteins show organelle- and tissue-specific expression with deduced function like chaperones, folding and unfolding of cellular proteins and protection of functional sites from the adverse effects of high temperature. Among other stress proteins, expression of ubiquitin, Pir proteins, LEA and dehydrins has also been established under heat stress. A main function of these proteins appears to be protection of cellular and sub-cellular structures against oxidative damage and dehydrative forces.

Classification of HSP

HSP are characterized by the presence of carboxy-terminal domain named heat-shock domain. Its expression is observed widely, from bacteria to human and in wide range of cells. Their biosynthesis is extremely fast and intensive, especially in response to stress. HSP70 and HSP90 mRNAs can increase ten-fold, while low molecular weight (LMW) HSPs can increase as much as 200-fold (Feussner et al., 1997). The members of this family vary widely in molecular size, expression pattern, cellular localization and function. Immuno-localization studies have determined that HSPs normally associate with particular cellular structures, such as cell wall, chloroplasts, ribosomes and mitochondria (Nieto-Sotelo et al., 2002; Yang et al., 2006). In plants, based on their molecular size and functions, five principal classes of HSPs are suggested (Schlesinger,

1990; Schoffl et al., 1998; Kotak et al., 2007): (1) Hsp100, (2) Hsp90, (3) Hsp70, (4) Hsp60, and (5) small heat-shock.

HSP100: The function of this class of HSP is not very well studied. It appears that they are involved in reactivation of protein aggregates by resolubilizing them (Parsell and Lindquist, 1993) or direct the irreversibly damaged polypeptides (Bosl et al., 2006; Kim et al., 2007) for degradation via proteasome ubiquitin pathway. Members of this family provides housekeeping functions, however, they plays essential role in acclimation during high temperature and afterwards (Lee et al., 2006; Gurley, 2000).

HSP 90: Apart from the role being molecular chaperones which is attributed to all HSPs, HSP 90 is also reported to be involved in signal transduction. HSP90 can bind HSP70 in many chaperone complexes and has important role in signaling protein function and trafficking (Pratt and Toft, 2003). However, most studied role of HSP90 is regulation of the cellular signals by interacting with steroid receptors i.e. glucocorticoid receptor (GR) (Pratt et al., 2004). HSP90 interacts with and maintains these receptors in cytoplasm and guides the signal transduction process such as regulation of corresponding gene transcription. Cytoplasmic Hsp90 also reacts with resistance protein (R) from pathogen and acts as a signal receptor complex to provide pathogen resistance as observed in *A. thaliana* and two species of (*Nicotiana tabacum* and *Nicotiana benthamiana*) (Hubert et al., 2003; Liu et al., 2004). This mechanism resembles the regulating mechanism of steroid receptor in animals (Schulze-Lefert, 2004). Thao et al. (2007) have reported that Hsp90 was an essential component of innate-immune response and pathogenic resistance in rice. There are some indication that HSP90 negatively inhibited Heat Shock Factor (HSF) in the absence stress (i.e. heat) and thus acting as a feedback regulator of heat shock response (Yamada et al., 2007).

HSP70: HSP70 is present in all organism and function as chaperones for newly synthesized proteins. They prevent aggregation and provide proper folding during and after synthesis until it reaches the final destination (Sung et al., 2001; Su and Li, 2008). Cellular localization such as stomata (Schroda et al., 1999) or chloroplast (Jackson-Constan et al., 2001; Soll, 2002) suggest that HSP 70 also participates repairing

denatured protein or as a part of guidance complex import (translocon) that bound to protein precursor to be transferred through the membranes. A recent study shows the necessity of Hsp70 found in chloroplast of germinating seeds of *A. thaliana* to provide tolerance to heat (Su and Li, 2008). However, sHSPs plays an integral part of this function. It is suggested that sHSP is responsible for prevention of aggregation whereas HSP70 provides proper folding of the native protein (Lee and Vierling, 2000). Together they play crucial role in stress response. Furthermore, HSP70 and sHSPs primarily act as molecular chaperone and play a crucial role in protecting plant cell from the detrimental effects of heat stress (Rouch et al., 2004; Lee and Vierling, 2000). Hsp70 seems to be able to participate in disposal of damaged or defective proteins by interaction with E3 ubiquitin ligase which allows Hsp70 to pass proteins to the ubiquitination and proteolysis pathways (Lüders et. al., 2000).

HSP60: also named as chaperonins, most pronounced role of this class is assisting plastid such as Rubisco (Wang et al., 2004). Some studies also indicates their possible role in folding and aggregation of many proteins that were transported to organelles such as chloroplast and mitochondria (Lubben et al., 1989). These Hsps60 bind different types of proteins after their transcription and before folding to prevent their aggregation (Parsell and Lindquist, 1993). Functionally, plant chaperonins are limited and the general idea is that stromal chaperones (Hsp70 and Hsp60) are involved in attaining functional conformation of newly imported proteins to the chloroplast (Jackson-Constan et al., 2001).

Small heat-shock protein (sHSP): These proteins have a common alpha-crystallin domain containing 80–100 amino acid residues located in the C-terminal region (Seo et al., 2006). One of the characteristic functions of this class is the degradation of the proteins that have unsuitable folding (Ferguson et al., 1990). Their activity is independent from ATP (Miernyk, 1999). However, the sHSPs cannot refold non-native proteins, but they can bind to partially folded or denatured substrates proteins, preventing irreversible unfolding or wrong protein aggregation (Sun et al., 2002). Recent findings showed that the sHSPs binds to unfolded proteins and allows further refolding

by Hsp70/Hsp100 complexes (Mogk et al., 2003). It was noticed that there was a positive qualitative relation between the accumulation of sHSPs in different organelles and thermotolerance of heat shock (Downs et al., 1998; Downs and Heckathorn, 1998). There is also evidence of sHSPs playing important role in maintaining integrity especially under stress conditions (Nakamoto and Vigh, 2007). A recent review concluded that there were some indications that small heatshock proteins play an important role in membrane quality control and thereby potentially contribute to the maintenance of membrane.

In general, HSPs are expressed in large quantities during stress and have related functions as they ameliorate the problems of unsuitable folding and aggregation (Queitschet al., 2000).

Temperature sensing and signaling

Perception of stress and relay of the signal for turning on adaptive response mechanisms are key steps towards plant stress tolerance. Although the presence of a plant thermometer has not been established, it is suggested that changing membrane fluidity plays a central role in sensing and influencing gene expression both under high and low temperatures. This suggests that sensors are located in microdomains of membranes, which are capable of detecting physical phase transition, eventually leading to conformational changes and/or phosphorylation/dephosphorylation cycles due to changes in temperature (Plieth, 1999). In this regard, a model for temperature sensing and regulation of heat shock response integrates observed membrane alterations. Changes in the ratio of saturated to unsaturated fatty acid on the set point of temperature for the heat shock response (HSR) alters activities of HSFs.

Rigidification of thylakoid membranes appears to invoke altered expression profiles of heat shock genes, suggesting that the temperature sensing mechanism may be located on the thylakoid membrane (Horvath et al., 1998). The prospect of the thylakoid membrane acting as a heat sensor is physiologically crucial, because it is

susceptible to temperature upshift, owing to its highly unsaturated character, and the presence of photosystems, which are fragile to temperature changes (Sung et al., 2003). Various signaling ions and molecules are involved in temperature sensing and signaling. As a signaling response to temperature stress, cytosolic Ca^{2+} sharply rises (Larkindale and Knight, 2002), which seems to be linked to the acquisition of tolerance possibly by transducing high temperature-induced signals to MAPK. MAPK cascades are important parts of signal transduction pathways in plants and thought to function ubiquitously in many responses to external signals (Kaur and Gupta, 2005). A heat-shock activated MAPK (HAMK) has been identified, the activation of which was triggered by apparent opposite changes in membrane fluidity coupled with cytoskeletal remodeling (Sangwan and Dhindsa, 2002). Ca^{2+} influx and the action of Ca-dependent protein kinases (CDPK) have been closely correlated with the expression of HSPs (Sangwan and Dhindsa, 2002). However, another study suggested that Ca^{2+} is not required for production of HSPs in plants, despite the fact that heat stress induces uptake of Ca^{2+} and induction of some calmodulin (CaM) related genes (Gong et al., 1997). As a mediator of Ca^{2+} signal, CaM is activated by binding Ca^{2+}, inducing a cascade of regulatory events and regulation of many HSP genes (Liu et al., 2003). Several studies have shown that Ca^{2+} is involved in the regulation of plant responses to various environmental stresses, including high temperature. Increasing cytosolic Ca^{2+} content under heat stress may alleviate heat injury, such as increased activity of antioxidants (Gong et al., 1997), turgor maintenance in the guard cells (Webb et al., 1996) and enable plant cells to better survive. However, excessive Ca^{2+} released into the cytosol and sustained high cytosolic Ca^{2+} concentration might be cytotoxic (Wang and Li, 1999).

Specific groups of potential signaling molecules like SA, ABA, $CaCl_2$, H_2O_2, and ACC may induce tolerance of plants to heat stress by reducing oxidative damage (Larkindale and Huang, 2004). Being molecules of somewhat novel interest in the last fewyears,H_2O_2 and NO have emerged to be central players in the world of plant cell signaling under stressful situations (Dat et al., 2000). A protein phosphorylation cascade has been shown to be activated by H_2O_2 is a MAPK cascade. Methyl-SA has a major

signaling role in the gene activation under heat stress up to 1.8 nmol g−1 dry mass of tissue, beyond which it becomes lethal to cell metabolism (Llusia et al., 2005).

In short, sensing of high temperature and induction of signaling cascades are important adaptive steps in coping with adversaries of heat stress. Although numerous molecules including ROS, hormones and ethylene have been identified for the perception of heat stress cues, role of Ca^{2+} is exclusive.

Regulation of heat shock proteins are attributed to Heat shock factors (HSF). They are best known as inducible transcriptional regulators of genes encoding molecular chaperones and other stress proteins. Members of the HSF family are also important for normal development and lifespan-enhancing pathways. However, its involvement in various other target reveals a complex network of regulation which is maintained by layers of post-translational modifications of HSFs that integrate the metabolic state of the cell with stress biology (Åkerfelt et al., 2010).

A series of new studies have demonstrated that certain RNA sequences can directly sense ambient temperature or any of a variety of small molecule metabolites. Remarkably, these sensors allow associated mRNAs to regulate their own transcription or translation accordingly, without the need for regulatory proteins (Lai 2003). A temperature influenced secondary structure element referred as ROSE (repression of heat shock gene expression) was shown to be the regulator of rizobial heat shock response (Nocker et. al., 2001). On the other hand, H2A.Z was described as a master regulator of the entire temperature transcriptome. H2A.Z-containing nucleosomes represent the major node of regulation of the temperature transcriptome in plants. H2A.Z nucleosomes wrap DNA more tightly, which influences the ability of RNA polymerase (Pol) II to transcribe genes in response to temperature, suggesting a mechanism by which the transcriptome can be thermally regulated (Kumar et al., 2010).

Chapter 1: *Understanding the mechanism of high temperature tolerance*

Materials and Methods

Greenhouse

Plants were grown in a temperature controlled greenhouse facility in International Rice Research Institute (IRRI), Philippines. Growth condition was maintained at 29/21°C day/night temperature and relative humidity (RH) at 75%. Ambient temperature and RH were measured using thermocouples (Chessell 392, USA). Natural day light was used. Plants were grown in pots and were placed on a bench.

Crop husbandry

Two rice varieties were used. Moroberekan (IRGC 12048), which is a *japonica* type rice variety, is drought tolerant but highly susceptible to high temperature during anthesis. Nagina 22 (IRGC 19379), commonly known as N22, is an *Aus* type rice variety, is highly tolerant to high temperature. Pregerminated seeds were sown into trays containing natural clay loam soil. Seeds were germinated on seedling tray. 15 days old seedlings were transplanted to pots containing 6.0kg of same clay loam soil. 7.5g $(NH_4)_2SO_4$, 1.5g KCL and 1.5g SSP was applied into each pots before transplanting. Additional 2.5 g of $(NH_4)_2SO_4$ was applied into each pots ~30 days after transplanting.

Growth chamber and treatment

On the first day of anthesis, plants were transferred into growth chamber (Thermoline, Australia) at 8am. By 9am the temperature of the chamber was set to increase from 29°C to 39°C and maintained at 39°C until 3pm (Figure 3.1.1). Relative humidity (RH) of 75% was maintained throughout the treatment. Immediately after the heat treatment, plants were transferred to control condition at 29/21 °C day/night temperature. Plants were returned to the growth cabinet under the same conditions on the following morning at 8am for a total exposure of 3 days of high temperature treatment. A thermocouple (Chessell 392, USA) was placed above the canopy inside the growth chamber to measure

the air temperature. Ambient air was cycled from outside into the chamber which was warmed with the help of a heater to maintain the temperature. Photon flux density inside the chamber was maintained at 640 µmol m^{-2}S^{-1}. CO_2 or any other gas was not measured.
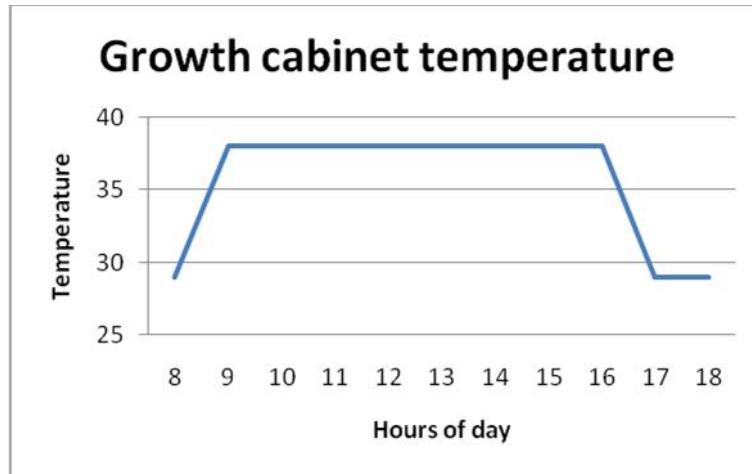
Figure 3.1.1: **Temperature curve of the growth chamber during the plants under high temperature stress.**

Water-deficit stress was imposed by withholding watering of the plants and the stoppers fitted at the bottom of the pots were removed to drain out any excess water. Three replicates from each group of 10 plants were chosen to assess the water-deficit stress by measuring relative water content (RWC). RWC of the plants were measured from the flag leaf using the formula

$$RWC (\%) = [(FW-DW)/(TW-DW)] \times 100$$

Where FW= Initial fresh weight of the leaf, DW= dry weight and TW= turgid weight (Liu et al. 2006). Water-deficit-stressed plants were exposed to high temperature stress as mentioned above starting on the day of anthesis when the RWC was expected to reach 65-70%.
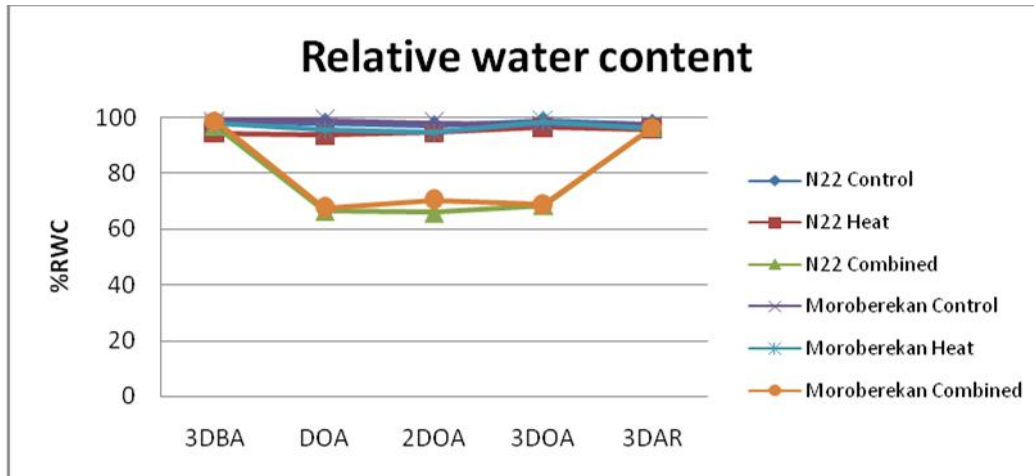
Figure 3.1.2: **Relative water content of plants during the stress**. Flag leaf was collected from N22 and Moroberekan plants under control condition, heat stress and combined heat and water-deficit stress at 3 day before anthesis (3DBA), on the day of anthesis (DOA), 2nd day of anthesis (2DOA) 3rd day of anthesis (3DOA) and 3 days after re-watering the water-deficit plants and relative water content was measured.

Soil and leaf temperature:

Soil and leaf temperatures were recorded using Infrared (IR) thermometer. An infrared thermometer infers temperature from a portion of the thermal radiation sometimes called blackbody radiation emitted by the object being measured. They are sometimes called laser thermometers if a laser is used to help aim the thermometer, or non-contact thermometers or temperature guns, to describe the device's ability to measure temperature from a distance. By knowing the amount of infrared energy emitted by the object and its emissivity, the object's temperature can be determined. For soil, temperature was recorded by holding the thermometer perpendicular to the pot soil. Leaf temperature was recorded from the middle part of the flag leaf blade of main tiller. Laser pointer of the thermometer was used as a guide to adjust the area of coverage of the thermometer. To avoid errors, all measurements were recorded form roughly equal distance at 11am throughout the treatment.

Figure 3.1.3: **Infrared (IR) thermometer**. A non-contact type thermometer gun. It can measure object temperature by calculating the amount of infrared radiation emission.

Sample collection for spikelet fertility, anther dehiscence and pollen germination:

To measure spikelet fertility, spikelet opening between 9am and 3pm during the high temperature treatment were marked using acrylic paint of 3 different colors for three days of treatment. They were allowed to mature and marked spikelets were scored for fertility as filled or unfilled grains.

Just after closing of the spikelet, images of the anther hanging from the closed spikelet was taken using a digital camera. From the image anthers were identified and scored as dehisced and not dehisced.

10 spikelet were collected at 30, 60 and 240 min after opening from all treatment and control plants and were immediately transferred into fixative containing 1:3 glacial acetic acid: absolute ethanol in a glass vial for microscopic observation of pollen germination.

Microscopic observations:

The dissection of the spikelet was done using a stereomicroscope (Olympus SZX7, Olympus corp., Japan). Number of pollen on stigma and number of pollen germinated on stigma were observed using Axioplane 2 microscope (Carl Zeiss, Germany) at ×100 magnification and images were taken using the DP70 digital camera attached to it

.

Results

Leaf temperature and cooling effect:

Like animals, plants can also cool themselves. This was observed in this experiment. In control condition, where green house temperature was set at 29°C during the day, leaf temperatures were observed to be 26.85 (±0.3), 27.47 (±0.5), 26.77 (±0.8), 26.91 (±0.4), 28.18 (±0.9) on the 3rd day before anthesis (3DBA), on the day of anthesis (DOA), 2nd day of anthesis (2DOA), 3rd day of anthesis (3DOA) and 3 days after re-watering (3DAR) respectively. But during high temperature stress, when chamber temperature was 39°C, leaf temperature of N22 plants were observed from a minimum of 31.9°C to maximum 34.8°C for the 3 days of treatment. Before and after the heat stress, plants were kept in a green house and showed similar leaf temperature pattern to that of plants under control conditions (average 25.68±0.5 and 26.15±0.5 before and after respectively). But in case of combined treatment, where plants were in an additional water stress, leaf temperatures were observed to be very high (average 39.38±0.3, 37.36±0.7, 37.55±0.5 for the three days of treatment) which were close to the chamber temperature (39°C) and cooling effect was not observed. Heat sensitive moroberekan also shows similar temperature pattern (Figure 3.1.4).

Soil temperature:

Unlike the live plant parts, soil temperature tends to change according to the condition applied. In control condition, where chamber temperature was set to 29°C, the observed average temperatures in the pot soil were between 26.51±0.35°C and 30.56±1.58°C for N22 and 29.14±0.95°C and 31.54±0.63°C for Moroberekan. But in case of high temperature stress and combined stress, when the chamber temperature was set to 39°C, elevated soil temperature was observed. For high temperature stress, pot soil temperatures were between (32.25±0.65°C and 34.94±0.89°C) for N22 and (31.35±1.26°C and 32.20±0.87°C) for Moroberekan. In the combined stress treatment, the soil temperature was observed to be between (31.39±0.81°C and 33.5±1.08°C) for N22 and (31.32±0.93°C and 32.65±0.69°C) for Moroberekan. Figure 3.1.4 shows the average soil temperature of different pots across treatment.
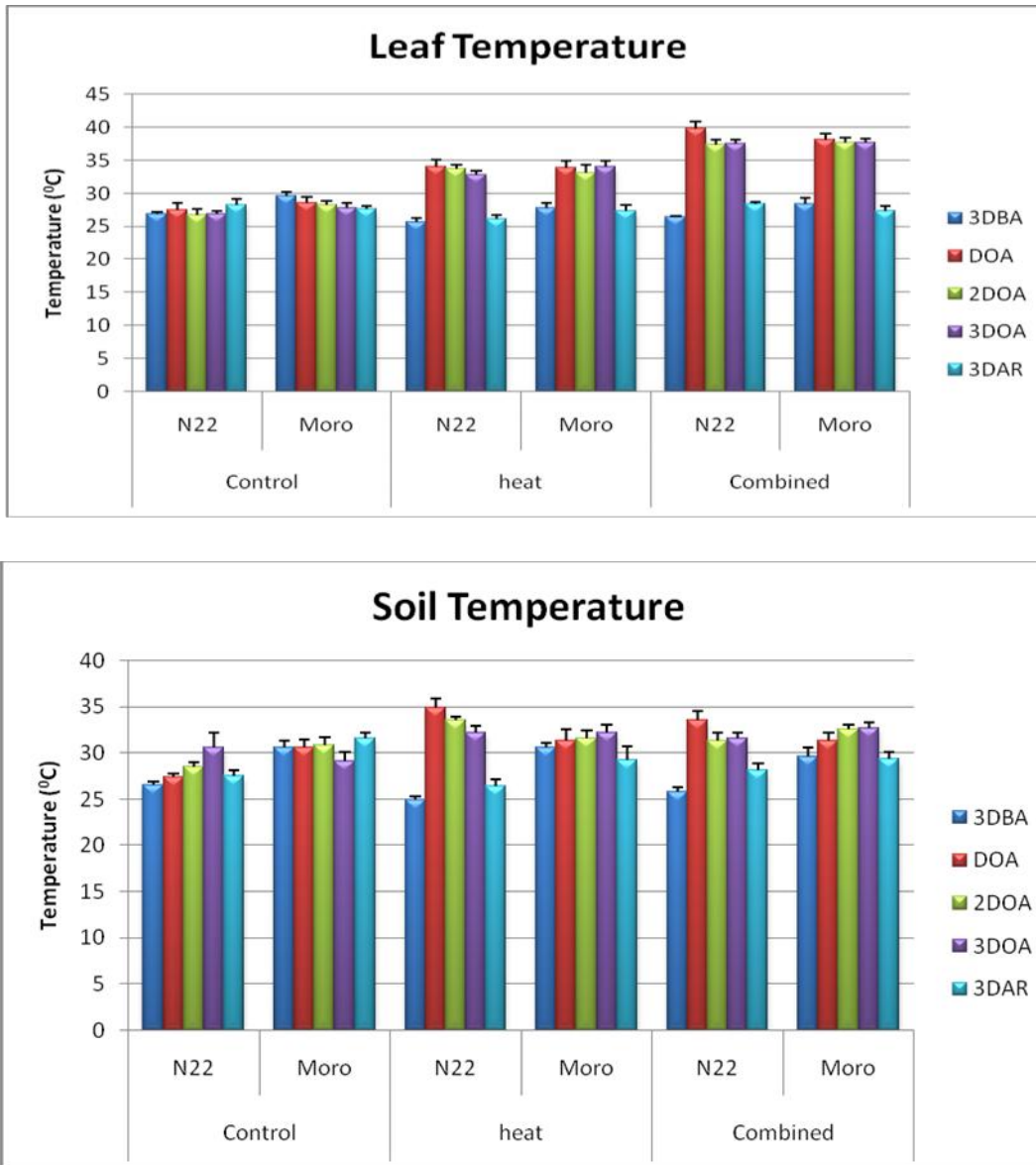
Figure 3.1.4: **Leaf and soil temperature in response to different stress treatment.** Infra Red (IR) thermometer readings were recorded from middle part of the flag leaf of main tiller at 3 days before anthesis (3DBA), on the day of anthesis (DOA), 2nd day after anthesis (2DOA), 3rd day after anthesis and 3 days after re-watering (3DAR) for the water stressed plants. Control condition plants (C) were grown in glasshouse at temperature 29°C/21°C. For high temperature stress (H), plants were transferred to growth chamber (39°C) at the day of anthesis. For combined stress (DH), water stress was applied so that at the day of anthesis the leaf water content reaches 70-75% and then were transferred to growth chamber (39°C) for high temperature stress. For soil temperature, IR thermometer readings from pot soil in these conditions were recorded.

Effect on spikelet fertility:

Heat sensitive Moroberekan shows high level of spikelet fertility (81.10±4.8, 87.02±7.7, 88.41±7.1 when subjected to three days under control conditions. But the spikelet fertility is dramatically reduced upon heat alone as well as combined treatment (0, 0, 2.6 and 5.09, 0, 1 for three days of treatment) (Figure 1.5). On the other hand, heat tolerant variety N22 shows spikelet fertility of 96.41±3.5, 98.18±2.7, and 96.22±2.9 under control conditions. Upon heat stress, the spikelet fertility was reduced to 31.48±6.3, 20.85±3.7and 14.39±1.6. And spikelet fertility of 16.23±7.1, 18.56±9.5 and 12.68±7.33 was observed under the combined treatment (Figure 3.1.5).
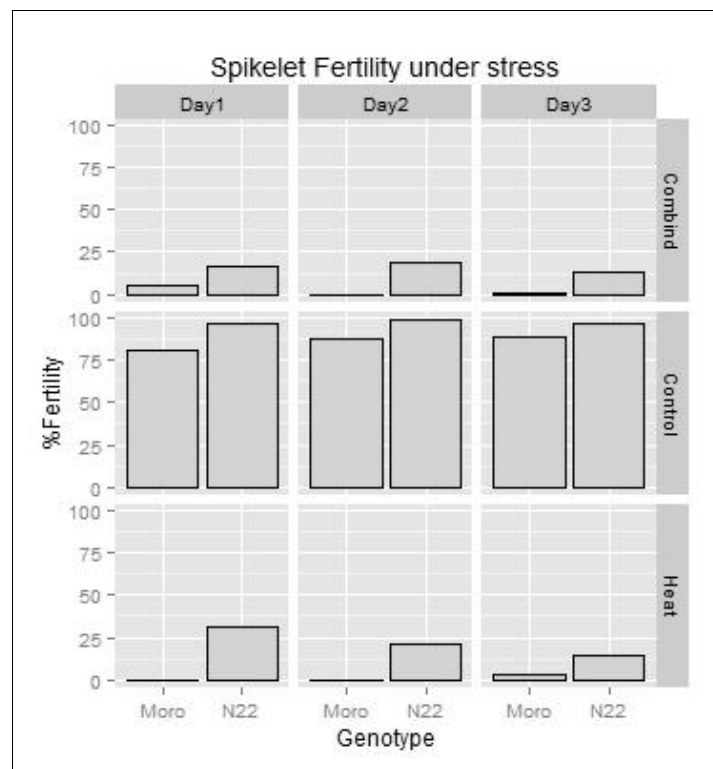
Figure 3.1.5: **Spikelet fertility under different stress treatment.** Plants were placed under heat stress (39°C) and combined heat and water stress starting from the day of anthesis till third day of anthesis. Spikelet opened during stress period were marked and allowed to mature. Filled and unfilled grains were counted and spikelet fertility was calculated.

***Effect on anther dehiscence*:**

On the day of anthesis, rice spikelet opens, anthers are pushed out of the spikelet (lemma and palea) and then anther discharges the pollen grain to reach the stigma for fertilization. This discharge of the pollen grain is termed as anther dehiscence and plays a vital role in the fertilization process. In control condition, Moroberekan shows complete dehiscence (99.28% and 99.16% on first and third day of observation) that is almost all the anthers empty its pollen content so that they can reach the stigma for fertilization (Figure 3.1.6). But upon heat stress, the process is severely affected. The anther dehiscence rate is decreased to 16.62% on first day of high temperature treatment and 14.75% on the third day (Figure 3.1.6). In combined high temperature and water deficit stress, the rate is further reduced to 2.61% and 8.65% on first day and third day respectively (Figure 3.1.6). On the other hand, high temperature tolerant variety N22 shows 92.23% and 86.20% anther dehiscence on first and third day of high temperature treatment where in control condition it was measured 99.64% and 97.15% respectively (Figure 3.1.6). In combined stress treatment it was reduced to 41.28% and 12.90% (Figure 3.1.6) which is a dramatic reduction compared to high temperature stress alone, but compared to sensitive Moroberekan, N22 shows a much higher rate of dehiscence.
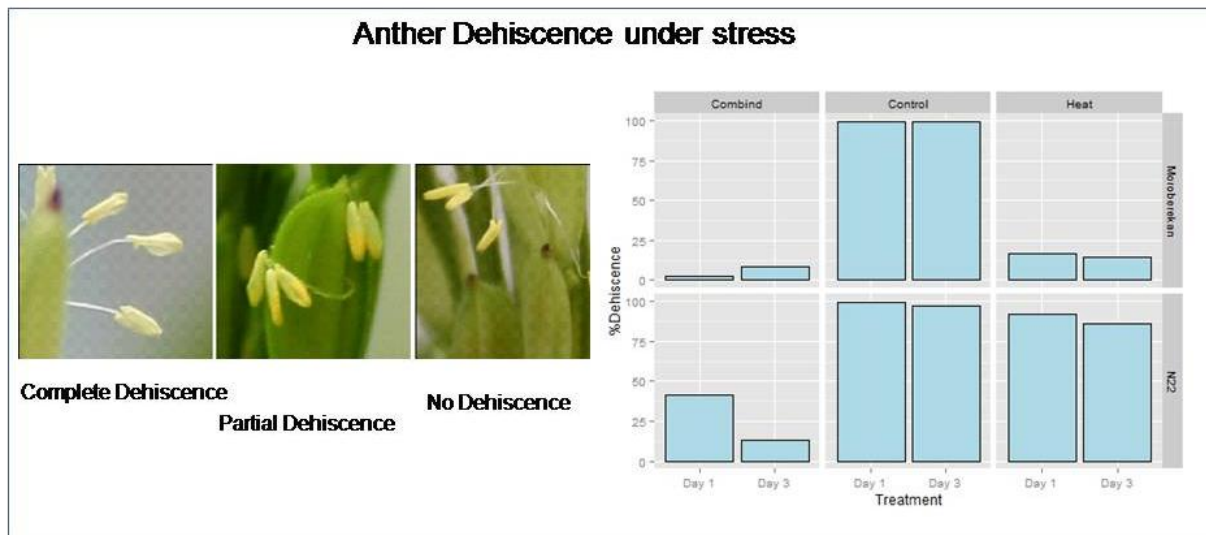
Figure 3.1.6: **Effect of anther dehiscence of under stress treatment.** Anther photographs of different plants under treatment were taken after anthesis. Complete/partially dehisced and not dehisced anthers were identified and counted.

Effect of heat stress on the germination of pollen on stigma:

The next step after anther dehiscence in the fertilization process is pollen germination, where the pollen grains germinate pollen tubes and the male nuclei reach the ovary. To observe the effect of temperature on pollen germination, spikelets were collected at 30 min, 60 min and 240 min after the flower opened. Germination was observed under microscope where a germination tube at least the size of the pollen grain was scored as germinated. In control condition Moroberekan shows very high rate of germination (62.18%-95.91%) (Figure 3.1.7). But in both the treatment conditions no pollen germination was observed (except 4.16% on 240 min sample of first day of high temperature stress treatment). On the other hand, in N22 control samples, germination rate ranges from 89.83% to 99.70% (Figure 3.1.7). Upon high temperature stress treatment, the rate was reduced drastically on the first day (43.14%-54.16%) but recovered on second and third day of treatment (57.52%-96.04%) (Figure 3.1.7).
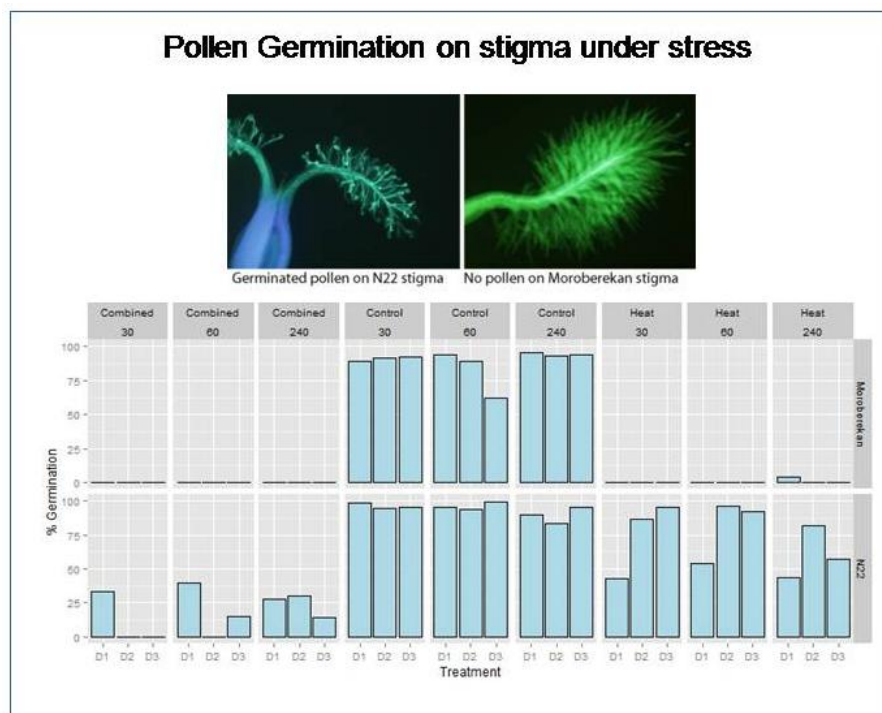
Figure 3.1.7: **Pollen germination on stigma under stress.** Plants were allowed to flower under control (29°C), high temperature stress (39°C) and combined water-deficit and high temperature condition. Spikelets were collected in fixative solution at 30, 60 and 240 min interval after the spikelet opens. Spikelets were dissected and observed under microscope with aniline blue staining. Pollen tube growing larger than the pollen size was scored as germinated.

***Effect of peduncle elongation and panicle entrapment under stress*:**

The length from the first node to the base of the panicle is termed as peduncle length. Usually it is trapped inside flag-leaf collar. In control condition N22 has peduncle length of 338 mm. Upon heat stress it was observed 364 mm and in combined stress 231.66 mm. Whereas Moroberekan shows peduncle length of 376.25 mm, 332.50 mm and 319 mm in control condition, high temperature stress and combined stress respectively (Figure 3.1.8).

Usually the whole panicle is exerted for flowering and fertilization. But some genotypes, like N22, have a natural tendency to leave a part of the panicle inside the collar (trapped). But upon stress this phenomenon appears to be changed. As shown in this experiment, N22 in control condition, 27 mm of panicle was trapped out of 255 mm of total length (~10%). But on high temperature stress and combined stress, trapped lengths were 3.5 mm and 101.11 mm respectively from the total lengths 257.50 mm and 242.22 mm (1.35% and 41.74% respectively). Moroberekan shows no entrapment in control and high temperature stress (panicle lengths were 274.37 mm and 239.50 mm respectively), where as in combined stress 24 mm length was trapped of 263 mm (9.12%). Figure 3.1.8 shows total length of N22 and Moroberekan panicle with relative trapped parts.
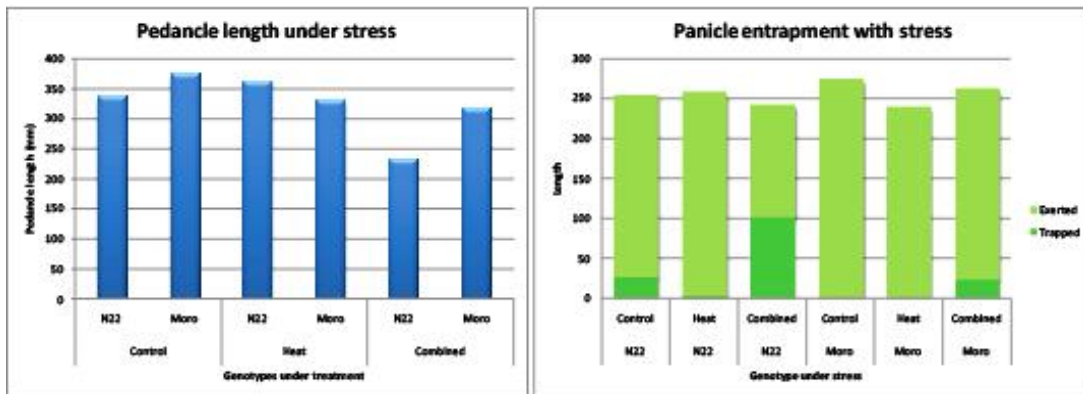
Figure 3.1.8**: Effect of peduncle length under stress.** N22 and Moroberekan plants were grown under control condition (29°C), heat stressed (39°C) and combined heat and water limiting condition and allowed to mature. Upon harvest leaf collar was opened and peduncle length, total length of the panicle as well as the length of trapped and exerted parted were recorded.

In summary, it has been observed that plants tend to adopt themselves in heat stress to avoid heat stress. Transpirational cooling can reduce leaf temperature significantly compared to ambient (chamber) temperature. Similar trends was observed in panicle entrapment where upon heat stress, plants tend to fully exert the panicle. In both these mechanisms, water is important factor. In combined stress heat and water-deficit stress reduced transpirational cooling was observed as water is limited. However, it showed no difference in tolerance of N22 compared to sensitive Moroberekan, hence it is more likely to be a general adoptive response to heat stress. On the other hand, anther dehiscence and pollen germination is affected upon heat stress and has a significant difference in tolerance over sensitive variety. This is translated in spikelet fertility, the key phenotype to characterize heat stress damage. Therefore, it can be concluded that upon heat stress the fertilization process is significantly affected but tolerant variety N22 is less affected and hence shows the tolerance.

Chapter 2: *Small Heat Shock proteins in rice*

N22 shows tolerance to heat stress during flowering stage. In previous chapter we examined spikelet fertility, anther dehiscence, pollen germination on stigma compared to a heat sensitive variety Moroberekan, to determine the mechanism of tolerance. However, these tolerances are often driven by one or more genes that influence the plant to react differently upon stress. To identify the proteins involved in high-temperature stress tolerance, the protein expression pattern of combined stressed (heat and water-deficit) spikelet was analyzed by 2D-PAGE approach (Jagadish et al 2011). 11 spots were identified to have greater than or equal to two fold expressions. 8 out of these 11 genes encoding these mentioned proteins were used in this study as candidate genes for further analysis. Fruktokinase, one of the 11 genes, is used in another study and hence was not included in this study. Two other genes were not used as sufficient information was not available.

Materials and Methods

Sequencing of candidate genes from N22

To amplify the genomic sequence of the candidate genes, primers were designed from the flanking regions of the coding sequence using the *nipponbare* sequence information from Gramene database (www.gramene.org). Nagina 22 (N22) genomic DNA was extracted and used as template for PCR amplification of the candidate genes. PCR reactions were carried out with 1× PCR buffer, 0.66mM dNTP mix, 10µM of each primers and 1 unit of taq polymerase (SBS Genetech, Beijing). The PCR cycle settings were 94°C for 2 min, followed by 35 cycles of 94°C for 30s, primer specific annealing (See Appendix A.1), 72°C for 1 min and a final extension at 72°C for 10 min. PCR amplicons were checked by 0.8% agarose gel electrophoresis for the correct size. Amplicons were then cloned into pCR8/GW/TOPO cloning vector (Invitrogen) as per manufacturer's instruction and were sent for sequencing (Macrogen, Korea).

Sequence Alignment

Full length DNA sequence for the candidate genes from *nipponbare* were extracted from gramene using Biomart tool (http://www.gramene.org/biomart/martview/). Respective forward and

reverse sequences for N22 were extracted from the tracer file provided by Macrogen. They were aligned using MAFFT sequence alignment (http://mafft.cbrc.jp/alignment/server/) online version. Aligned sequences were compared for any changes from the *nipponbare* sequence.

Reverse transcriptase PCR (RT-PCR) for gene expression under heat

Plants were grown in greenhouse under normal conditions as mentioned before. High temperature, drought, combined heat-and-drought stress were applied subsequently. Spikelets were collected 1 and 4 hours after applying heat as well as drought frozen in liquid nitrogen and stored in a -80°C freezer. Control spikelets were also collected the same way. RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instruction. RNA samples were treated with RNAse free DNAse (Promega) to remove any genomic DNA contamination. cDNA synthesis were carried out at 55°C for 1 hour in 20µl reaction containing 500ng DNAse treated total RNA, 2.5 mM oligo(dT), 0.5 mM dNTP mix, 0.01 M dithiothreitol, 1× first strand synthesis buffer and 200 units of superscript II reverse transcriptase (Invitrogen).PCR was carried out in 20 µl reaction containing 0.5 µl cDNA, candidate (17.4kDa sHSP or 16.9kDa sHSP) gene specific primer (0.2 mM each, sequence provided in appendix A.2), 0.5 mM dNTP mix, 1X PCR buffer and 1 unit of Taq DNA polymerase (SBS genetech, Beijing). The PCR cycle settings were 95°c for 2 min, followed by 30 cycles of 95°C for 30s, 55°C for 30s and 72°C for 1 min and a final extension of 72°C for 10 min. The glyceraldehydes-3-phosphate (GAPDH) gene was used as control of successful amplification as well as in determining the absence of genomic DNA. RT product were separated by agarose electrophoresis and stained with SYBR safe (Invitrogen).

Expression from rice genome 51K array

The Affymetrix 51K (also known as 57K Rice Genome GeneChip[TM] as it has 57,381 probe sets in reference to the 51,279 transcripts used to construct the chip) was develop to study the change in gene expression profile due to mutagenesis, transgene insertion , parasite and bacterial blight infection in rice (Qiu et al 2008, Batista et al 2008, Swarbrick et al 2008, Yang et al 2007). Since then it has been widely used to study gene expression profiling in different conditions. Data from many of these studies are available online. The Online based Genevestigator plant biology tool (https://www.genevestigator.com/gv/plant.jsp) was used to extract gene specific expression. The software equilibrates the expression based on the experimental control and compares with other

control genes used in different experiments from the database, to enable the data comparison across experiments.   Expression for the small Heat Shock Proteins (sHSP) (LOC_Os01g04370 and LOC_Os03g15960) were extracted and plotted according to different tissues (anatomical parts) in rice throughout its lifecycle using the Genevestigator anatomy tool.

Protein domain search

The protein sequences were used for domain search by blast search against NCBI conserved domain database (CDD), expasy prosite database and Pfam database. NCBI conserved domain search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) protein sequences were blasted against CDD v3.10 which consists of 44354 Position-Specific Scoring Matrix (PSSM). Other parameters were used as default. Expasy ScanProsite tool (http://prosite.expasy.org/scanprosite/) was used with database release 20.95. Protein sequences were submitted with default parameter to scan against prosite collection of motifs. Sequence search option was used to blast against Pfam database 27.0. Pfam database is a large collection of protein families, each represented by multiple sequence alignments and hidden Markov models (HMMs).

Phylogenetic analysis of sHSP in rice

Protein domain search revealed that the sHSP contains HSP20 domain which constitutes major part of the protein. According to PFam database classification HSP20 family is identified as PF00011. This was used as filter in gramene biomart tool to extract the HSP20 containing proteins in rice. Extracted protein sequences were aligned in MEGA software v5 using clustalW algorithm. Phylogenetic tree was built by UPGMA method from the aligned sequences (Tamura et. al. 2011).

Results

Proteins from rice spikelets (CV N22) under heat stress and water deficit condition were extracted and were subjected to 2D-PAGE analysis by Jagadish et. al. (2011). Spots of differentially expressed proteins at significant level were analyzed by mass spectroscopy. Peptide mass fingerprint database was used for search in TIGR (www.tigr.org/tdb/e2k1/osa1/index.shtml) and similarities to annotated protein found are listed below (table 3.2.1). These proteins were considered as candidate proteins in this study.

Table 3.2.1: **Differentially expressed proteins in spikelet under heat stress and water deficit condition**

Accession number	Chromosome	Location(Mbp)	Gene Annotation
LOC_Os01g04370	1	1.95	hsp20_Alpha_Crystallin_Family_16.9
LOC_Os02g55649	2	34.08	Unknown protein
LOC_Os03g01650	3	0.40	Expansin_Precursor
LOC_Os03g15960	3	8.80	hsp20_Alpha_Crystallin_Family_17.4
LOC_Os04g25160	4	14.53	Pollen specific protein _Two
LOC_Os06g36240	6	21.22	Pollen specific protein _Three
LOC_Os06g44470	6	26.86	Pollen specific protein_One
LOC_Os08g12160	8	7.16	Expressed_Protein_Unknown

Sequencing of candidate genes

All the 8 candidate genes were amplified from tolerant variety N22 and were compared with published *nipponbare* genome. Since the sHSP family genes are very closely related and contains high degree of similarity in sequence, it was difficult find a suitable primer to amplify the full length of mRNA. Hence only the coding region (including intron) was considered for the alignment. The sequence alignment shows no difference in the coding region for the candidate genes (alignment provided in appendix A.3) indicating they express the same protein as *nipponbare* genome. However they might be differentially regulated in order to show tolerance to heat stress and therefore, expression of these genes under heat stress were analyzed.

Expression of the candidate genes under heat stress

Semi-quantitative RT-PCR was performed to evaluate the expression of the candidate genes under stress in a heat tolerant variety (N22) and a heat sensitive variety (Morobarekan). Since flowering stage is most sensitive under heat stress, spikelets were used to check expression. Both the heat shock genes show a significant difference in expression induced by heat (Figure 3.2.1). Pollen specific proteins, beta-expansin and two unknown genes did not show any change-pattern due to stress. Since two heat-shock proteins showed enhanced expression in response to heat stress, they were selected for evaluation of their performance when over-expressed in transgenic plants.
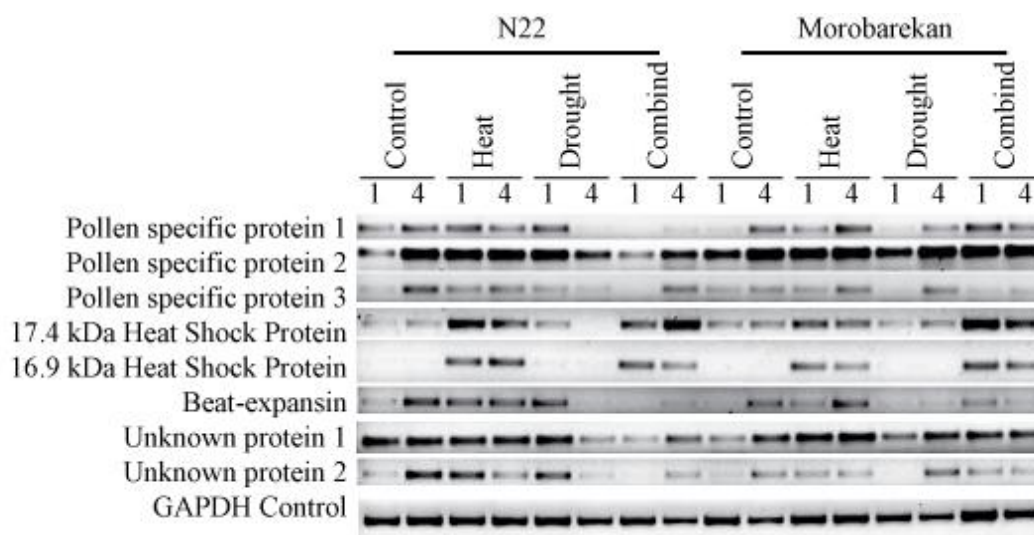
Figure 3.2.1: **Semi-quantitative RT-PCR of candidate genes under stress.** Stress was applied to plants at flowering stage and spikelets were collected at 1 hr and 4 hrs after opening of the spikelet. RNA was extracted and RT-PCR was performed using gene specific primers. GAPDH was used as endogenous gene expression control.

Expression from rice genome 51K array

51,279 transcripts were used in affimetrix to analyze the expression of rice genes in different organ/tissue and different environment/conditions. This data was analyzed using GENEVESTIGATOR tool to identify the expression of two sHSP genes. Figure 3.2.2 and 3.2.3 represents the expression of 17.4 kDa sHSP and 16.9 kDa sHSP respectively in different tissue.
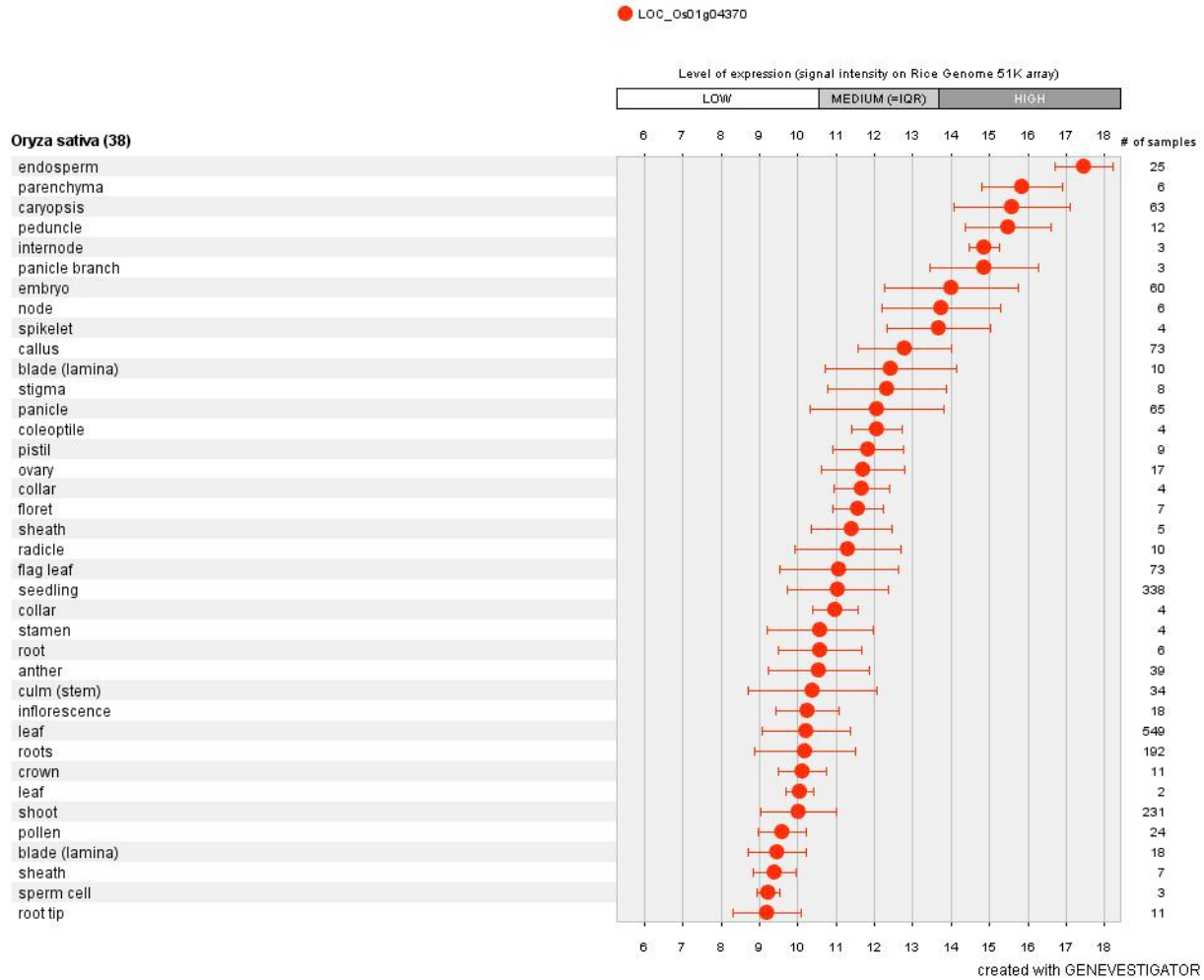
Figure 3.2.2: **Expression of 17.4 kDa small Heat Shock protein (LOC_Os01g04370) in different tissue.** Signal intensity which is proportional to level expression, was extracted for the gene LOC_Os01g04370 from rice genome 51K array experiment using basic version of GENEVESTIGATOR software (https://www.genevestigator.com) and plotted against different tissue. Figure shows the average level of expression of LOC_Os01g04370 (17.4 kDa HSP) in different tissues. The numbers on the right represent the number of samples available for the average of specific tissue type.

17.4 kDa HSP shows highest level of expression in endosperm, whereas 16.9 kDa HSP shows highest expression in sperm cells. Both of them are expressed in a wide variety of cells. It may be because they are involved in chaperone activity and are therefore essential for normal development process.
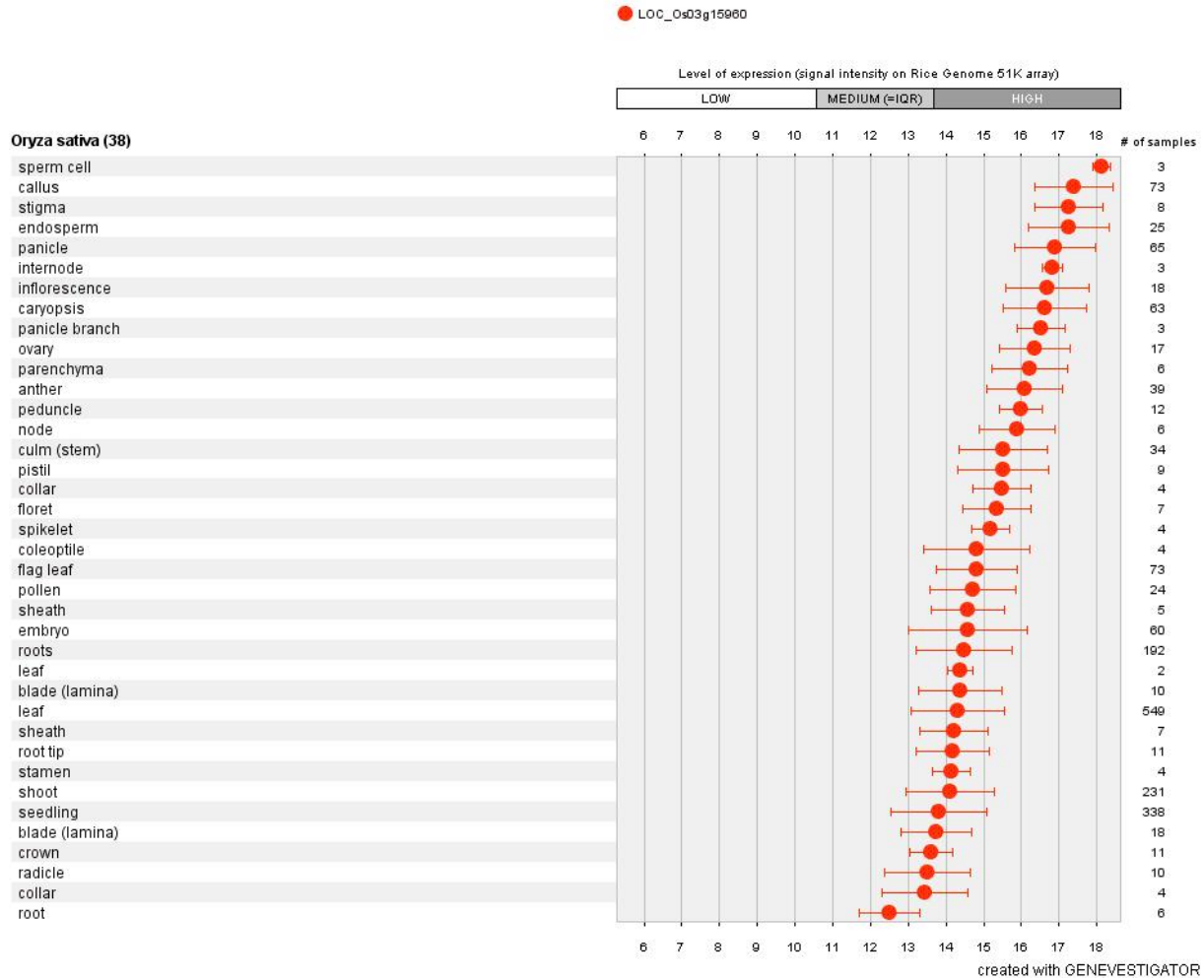
Figure 3.2.3: **Expression of 16.9 kDa small Heat Shock protein (LOC_Os03g15960) in different tissue.** Signal intensity which is proportional to level expression, was extracted for the gene LOC_Os01g04370 from rice genome 51K array experiment using basic version of GENEVESTIGATOR software (https://www.genevestigator.com) and plotted against different tissue. Figure shows the average level of expression of LOC_Os01g04370 in different tissue. The numbers on the right represent the number of samples available for the average of specific tissue type.

Protein domain search

NCBI Conserved domain Search

Three protein sequences, LOC_OS01g04370 (16.9 kDa sHSP) and two splice variant for LOC_Os03g15960 (17.4 kDa sHSP), show similar match pattern upon NCBI conserved domain search. All three sequences show specific hit with ACD_ScHsp26_like [cd06472], Alpha crystallin domain (ACD) found in Saccharomyces cerevisiae (Sc) small heat shock protein (Marchler-Bauer A et al. 2011). The conserved domain database describes alpha crystallin domain (ACD) found in Saccharomyces cerevisiae (Sc) small heat shock protein (Hsp)26 and similar proteins. sHsps are molecular chaperones that suppress protein aggregation and protect against cell stress, and are generally active as large oligomers consisting of multiple subunits. ScHsp26 is temperature-regulated; it switches from an inactive to a chaperone-active form upon elevation in temperature (Marchler-Bauer A et al. 2011). It associates into large 24-mer storage forms which upon heat shock disassociate into dimers. These dimers initiate the interaction with non-native substrate proteins and re-assemble into large globular assemblies having one monomer of substrate bound per dimer. Figure 3.2.4 shows the graphical alignment view of the conserved domain with the query protein sequence and Figure 2.5 shows the sequence alignment of the Cdd:cd06472 conserved domain with the protein query sequence (Marchler-Bauer A et al. 2011).
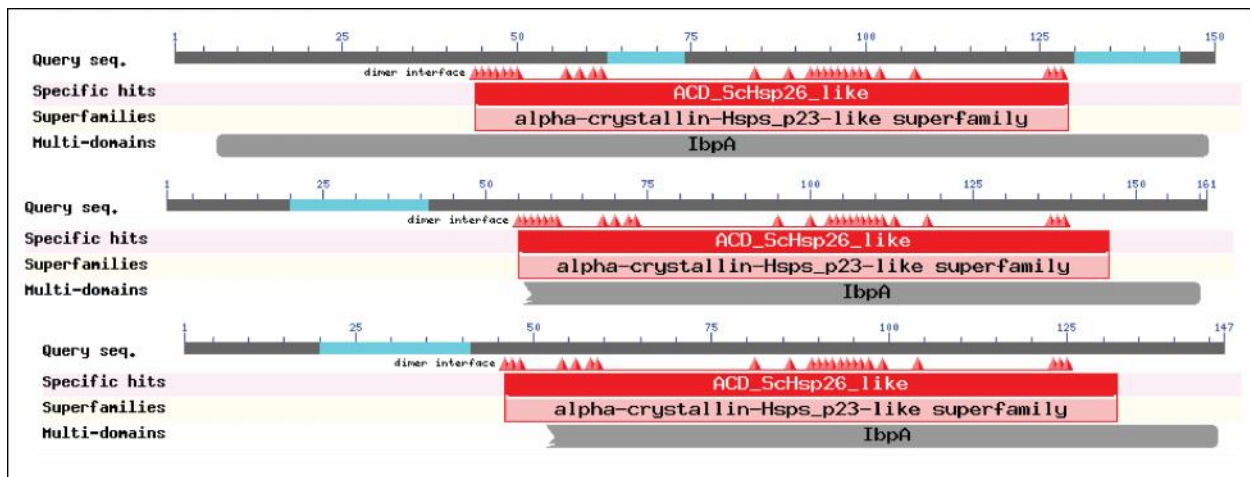
Figure 3.2.4: **Graphical representation of NCBI conserved domain on query sequence.** Query sequence (LOC_Os01g04370 at top, LOC_Os03g15960.1 at middle and LOC_Os03g15960.2 at bottom) were matched against NCBI conserved domain database (CDD). All three proteins have specific hit with ACD_ScHsp26_like domain which belongs to alpha-crystalin-HSPS_p23_like superfamily.

This group also contains the Arabidopsis thaliana (Ath) Hsp15.7, a peroxisomal matrix protein which can complement the morphological phenotype of S. cerevisiae mutants deficient in Hsps26. AthHsp15.7 is minimally expressed under normal conditions and is strongly induced by heat and oxidative stress. Also belonging to this group is wheat HSP16.9 which differs in quaternary structure from the shell-type particles of ScHsp26; it assembles as a dodecameric double disc, with each disc organized as a trimer of dimers (Marchler-Bauer A et al. 2011).

Figure 3.2.5: **Sequence alignment of conserved domain with query sequence**. Full length proteins were used to search conserved domain against NCBI CDD. All three sequence shows match with Cdd:cd06472 with length 92. Respective bit score and E-value are shown in the figure. In alignment, letters in red indicates exact match, blue indicates mismatch, "-" indicates gap.

Prosite Scan

All three protein sequences were analyzed with ExPasy Prosite scan. All three showed hit with the PROSITE entry PS01031 which describes a 90 residues heat shock hsp20 proteins family profile. According to the PS01031 entry description this is a protein family with an average 20 Kd molecular weight and seem to act as chaperones that can protect other proteins against heat-induced denaturation and aggregation. Hsp20 proteins seem to form large hetero-oligomeric aggregates. This family is composed of members from vertebrate, drosophila, *Caenorhabditis elegans*, Fungus and a variety of prokaryotic organisms (Sigrist et al 2012).
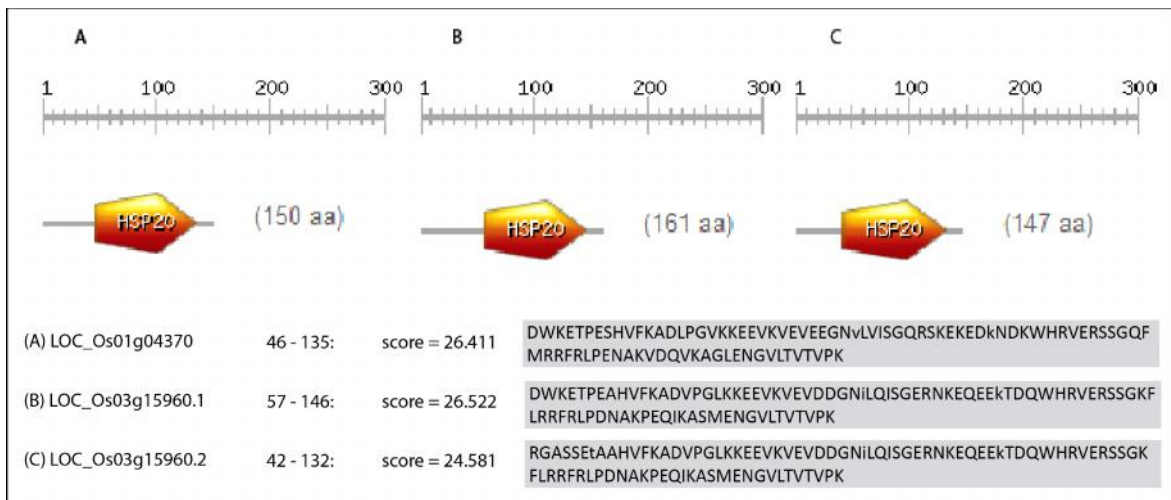
Figure 3.2.6: **Prosite scan of candidate genes.** A, B and C indicates three candidate genes LOC_Os01g04370, LOC_Os03g15960.1 and LOC_Os03g15960.2 respectively. Upper panel shows graphical presentation of the proteins with HSP20 domain positions. Lower panel shows the sequence of the protein (position shown) that matches the conserved domain sequence in database. Upper case in the sequence represents match positions and lower case insert positions relative to the matching profile.

PFam scan

Pfam version 27.0 was produced at the European Bioinformatics Institute using a sequence database called Pfamseq. LOC_OS01g04370 (16.9 kDa sHSP) and two splice variant for LOC_Os03g15960 (17.4 kDa sHSP) were analyzed using web based Pfam BLAST tool. Both the full length protein sequence matches significantly with HSP20/Alpha crystalline family with E-value 1.3e-33 and 1.8e-34 where as the truncated splice variant of 17.4 kDa sHSP shows a partial match with the same entry with E-value 9.3e-33. Like NCBI and Prosite scan database HSP20/Alpha crystalline family with Pfam entry PF00011 describes similar characteristics for the family (Finn et al. 2014). The family is found in both prokaryotic and eukaryotic organisms and is responsive to heat-shock and other environmental stress. Structurally, this family is characterized by the presence of a conserved C-terminal domain of about 100 residues as observed in this study. The three sequences in study matches with HMM motif (length 102) as 49-149, 57-160 and 47-146th residues which are at the C-terminal end of the protein. Figure 3.2.7 shows alignment region with HMM, bit score, E-value and sequence alignment with HMM with the two candidate proteins and the splice variant.

A) LOC_Os01g04370

Family	Description	Entry Type	Envelope		Alignment		HMM		HMM length	Bit Score	E-value
			Start	End	Start	End	From	To			
HSP20	HSP20/alpha crystalline family	Family	46	149	46	149	1	102	102	114.8	1.3e-33

B) LOC_Os03g15960.1

Family	Description	Entry Type	Envelope		Alignment		HMM		HMM length	Bit Score	E-value
			Start	End	Start	End	From	To			
HSP20	HSP20/alpha crystalline family	Family	57	160	57	160	1	102	102	117.6	1.8e-34

C) LOC_Os03g15960.2

Family	Description	Entry Type	Envelope		Alignment		HMM		HMM length	Bit Score	E-value
			Start	End	Start	End	From	To			
HSP20	HSP20/alpha crystalline family	Family	44	146	47	146	5	102	102	112.1	9.3e-33

Figure 3.2.7: **Pfam scan of candidate genes.** The protein sequences of the 16.9 kDa sHSP and 17..4 kDa sHSP along with its splice variant were analyzed with Pfam database. BLAST tool reveals HMM profile of the family that matches the protein sequence. LOC_Os01g04370 and LOC_Os03g15960.1 matches the complete profile (1-102) where as the splice variant LOC_Os03g15960.2 matches a partial profile (5-102) with HSP20 (PF00011).

Phylogenetic analysis of small heat shock proteins

For the profile scan with different database it is evident that both the proteins contains heat shock domain which described in PFam database as PF00011. Using PF00011 domain as filter in gramene database reveals that rice contains 37 genes that codes for proteins containing such a similar heat shock protein domain. Phylogenetic analysis of these proteins shows that these proteins display wide diversity (Figure 3.2.8). The two target proteins LOC_Os01g04370 and LOC_Os03g15960 are very closely related and are placed under a small node of 7 protein coding genes. Interestingly the proteins in this node are separated by two branches where proteins in each branch are consecutive genes in the genome, like LOC_Os01g04360, LOC_Os01g04370 and LOC_Os01g04380 in one branch and LOC_Os03g15960, LOC_Os03g16020, LOC_Os03g16030 and LOC_Os03g16040 in the second branch. It may be hypothesized that they may have evolved by gene duplication with acquired changes but may be involved in similar functions.
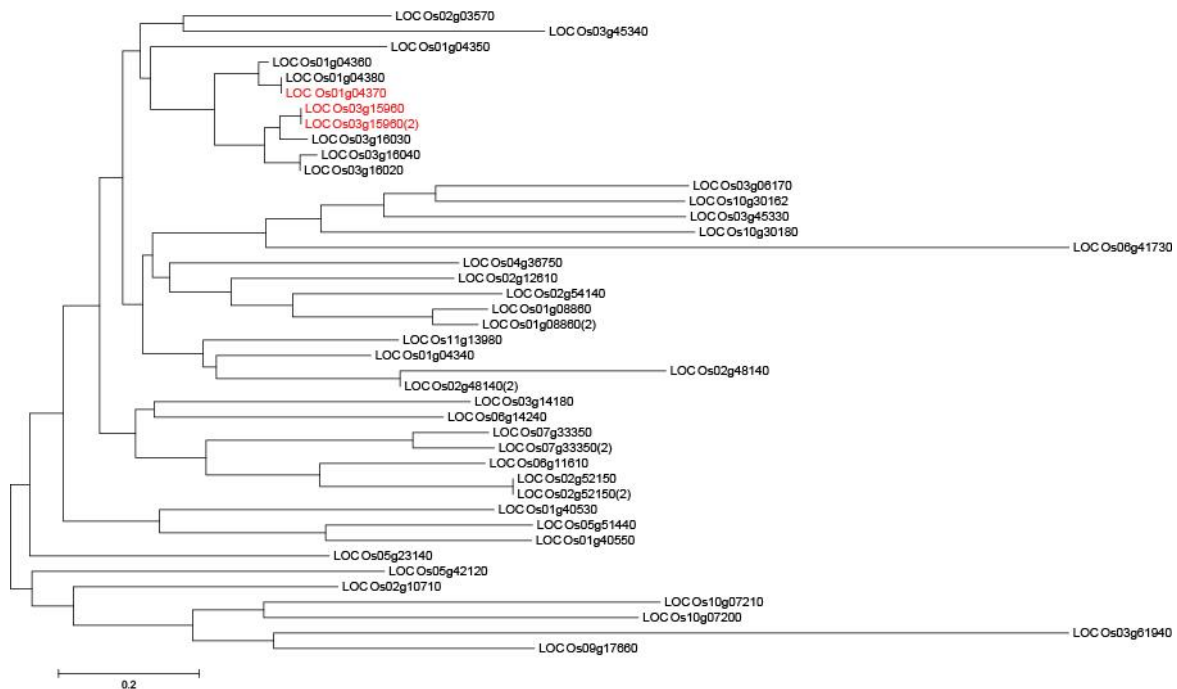
Figure 3.2.8: **Phylogenetic analysis of small heat shock proteins in rice.** All 37 small heat shock proteins in rice containing PF00011 domain were extracted from gramene database (http://www.gramene.org/) using the Biomart tool. The sequences were aligned with clustalW alignment tool provided in the software MEGA. The alignment was then used to build phylogenetic tree. Gene ID red font shows the candidate genes. LOC_Os03g15960 (2) is the splice variant of the gene LOC_Os03g15960 as indicated for others.

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Candidate genes for heat stress were selected following study by Jagdish (Jagadish et al 2011) where several proteins showed differential expression in response to heat such as sHSP, pollen specific protein, expansin precursor. DNA sequence amplified from N22 genome for these genes showed no difference compared to Nipponbare genome. Whereas semi-quantitative RT-PCR showed heat-induced expression only in two of the sHSPs. Moreover, pollen specific proteins mentioned above were recognized as pollen allergen in public annotations i.e gramene.org. Producing transgenic lines with these gene(s) could raise bio-safety concerns. Therefore, two small heat-shock proteins were selected for farther analysis.

Protein sequence for the two heat shock protein named 17.4kDa sHSP and 16.9kDa sHSP, were analyzed for conserved protein domain. All three database analyzed, suggested that both these protein contains Alpha Crystaline Domain (ACD). However, rice contains 37 sHSPs that contains the ACD domain. Phylogenetic analysis shows that these two proteins are very closely related among the 37. In general heatshock proteins are involved in stress response. But it might be possible that specific subset is involved in specific type of stress i.e heat.

Chapter 3: *Cloning and transformation of heat shock proteins*

Materials and methods

Amplification of the candidate genes

The 17.4kDa sHSP and 16.9 kDa sHSP were amplified by PCR amplification, using specific primer pairs (See appendix A1), and N22 genomic DNA as template. Amplification reaction was carried on G-storm thermal cycler with 1x reaction buffer, 0.33 mM dNTP mix, 0.33 mM of each primer and 2 units of Taq polymerase for a total 15μl reaction. Amplified PCR products were separated by agarose gel electrophoresis (1%) and observed for correct size amplification under UV light.

Cloning into pCR8 GW TOPO cloning vector

The target sequence for both sHSP were amplified by PCR reaction (confirmed by agarose gel electrophoresis) and were cloned into pCR8GW TOPO® cloning vector (invitrogen) as per manufacturer's instruction. Cloned mixture were transformed into One Shot® chemically competent *E. coli* (Invitrogen) as per manufacturer's instruction and were grown overnight at 37°C on a LB agar plate containing 100 μg/ml spectinomycin. Successfully transformed bacteria developed colonies. 6 individual colonies were selected randomly and plasmids were isolated by plasmid isolation kit (5-Prime). Plasmids were sent for sequencing (Macrogen, Korea) to confirm the correct cloned sequences and their orientation.
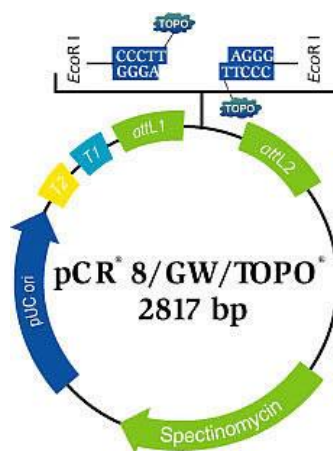
Figure 3.3.1: **pCR8 GW TOPO cloning vector.** The gene of interest was transferred to pCR8 vector in between attL1 and attL2 mediated by Topoisomerase attached to the cloning site. attL1 and attL2 facilitates transfer of gene of interest to another vector i.e. destination vector through recombination reaction.

Recombination to pMDC32 destination vector

pMDC32 binary destination vector (Curtis and Grossniklaus, 2003) contains 35S promoter and NOS terminator sequence flanking the recombination site. The individual entry clones in pCR8GW TOPO containing sHSP 16.9 and sHSP 17.4, the CDS were sub-cloned into two pMDC32 destination vector through LR recombinase reaction (Invitrogen) producing 35S::sHSP16.9 and 35S::sHSP17.4. Correct sequences were confirmed by sequencing.
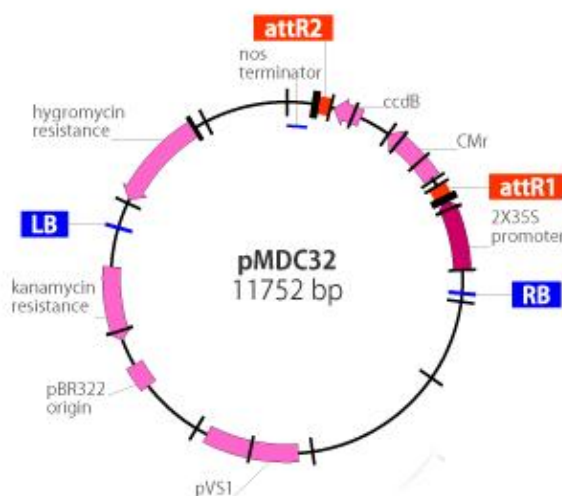
Figure 3.3.2: **pMDC32 binary destination vector.** Gene of interest from entry vector was cloned into multiple cloning site in between attL1 and attL2 through recombination reaction replacing ccdB-CMr. The vector contains kanamycin resistance for bacterial selection and hygromycin resistance for plant selection.

Generation of 35S::sHSP17.4 and 35S::sHSP16.9 IR64 transgenic lines

Transformation of the construct into *indica*-type IR64 variety was mediated by *Agrobacterium tumefaciens* strain LBA4404 according to a published protocol (Hiei et al., 2006) with modifications (Slamet-Loedin et al., IRRI, unpublished). 300 immature rice embryos (IR64) were infected using each construct. After co-cultivation with *Agrobacterium* containing the binary vector, embryos were transferred to selection media containing hygromycin. The binary vector contains hygromycin resistance gene which facilitates the growth of successfully transformed embryos. At this stage, Claforan (cefotaxime) and Carbenicillin were used to suppress *Agrobacterium* growth.Presence of the transgene in the plantlets was tested in the T_0 generation by genomic PCR for the presence of hygromycin phosphotransferase gene (HPT; A.5). PCR was carried out in a total volume of 20 µl. Reaction conditions:

100ng genomic DNA, primers (0.2µM each of forward and reverse primer), 1X PCR buffer, 0.5 mM dNTP mix and 1.5 U Taq DNA polymerase (5-prime). PCR cycle setting: 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C 1 min and a final extension at 72°C for 10 min.

Southern blot analysis for single copy determination

The copy number of transgene in selected plants was determined by southern blot analysis. Genomic DNA were digested with EcoRV, separated by agarose gel electrophoresis and blotted to nylon membrane. The membrane bound with digested DNA was hybridized with DIG-labeled HPT probe and was exposed to X-ray films. Digestion with EcoRV produces a single cut of the transgene. A second cut side on the 3' end would appear on the genomic region of host plant. Position of this cut-site would vary depending on the site of integration of the transgene. Therefore, it would produce only one band upon southern blot if there is only one copy integrated to the genome. Integration at multiple sites will produce a variable length of fragment after digestion which can be detected by the probe (Figure 3.3.3). On the other hand, multiple copy integration in tandem array, will produce multiple digestion site inside the integrated region. Upon digestion with EcoRV will produce a fragment of same size as insert except the last fragment, where the 3' end digestion site will be at the genomic region and therefore, will produce a different length of fragment (Figure 3.3.3). Upon southern blot analysis, this will produce 2 bands. Plants with independent transformation event and carrying a single copy gene were selected for phenotyping.
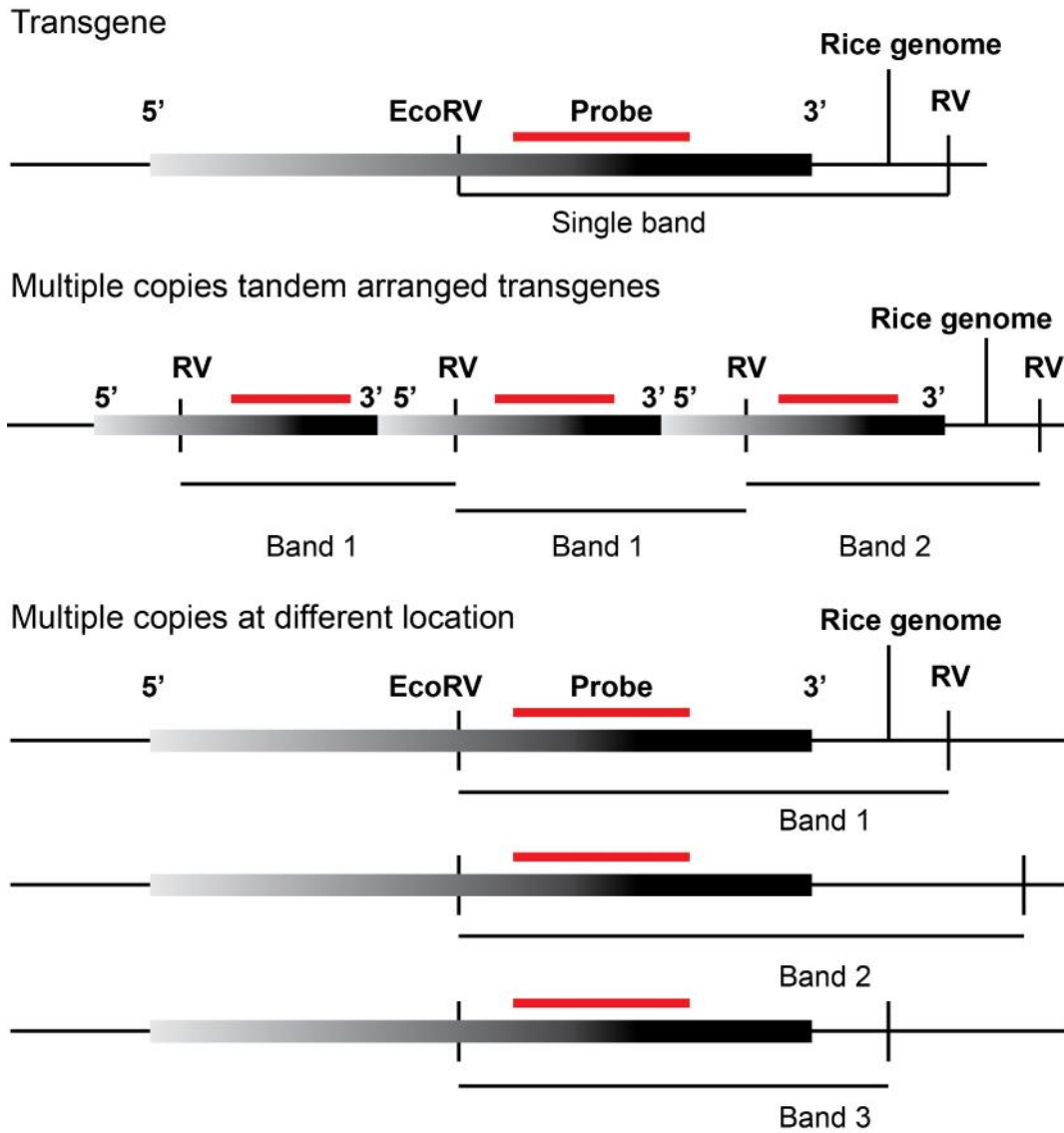
Figure 3.3.3: **Southern blot analysis to identify transgene with a single copy insert.** Genomic DNA was digested with EcoRV. After blot the membrane was hybridized with DIG-labeled hpt probe and chemi-luminescence was captured with X-ray films. EcoRV digestion produces a single cut inside the transgene insert. A second digestion site on 3' end is in the genomic region which produces a variable position depending on the site of integration. Therefore a single copy insert produces a single band upon digestion with EcoRV which was detected by DIG-labled probe after blot. More than one band indicates multiple copy insert either in tandem array or at multiple sites.

Results

Both 17.4 kDa sHSP and 16.9 kDa sHSP gene were cloned into pMDC32 destination vector. Figure 3.3.5 shows the insert region of destination vector after recombination reaction. Successfully recombined plasmid were confirmed by sequencing and were transferred to *Agrobacterium* LBA4404 strain and was used to infect rice (variety IR64) immature embryo.
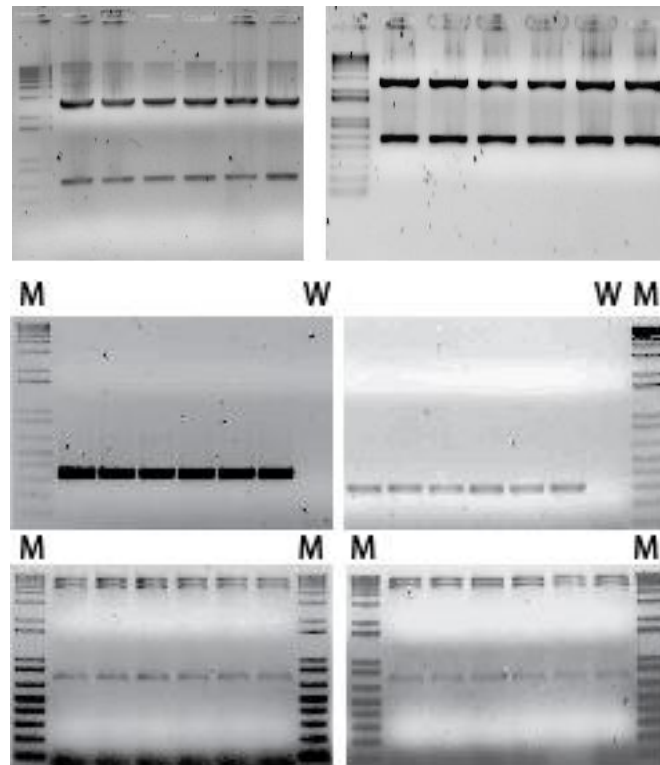
Figure 3.3.4: **Confirmation of clones.** Successful clones into pCR8 GW TOPO (top) and pMDC32 (middle and bottom) vector were confirmed by restriction digestion with EcoRI (top and bottom) and PCR using gene specific primer (middle). Digestion with EcoRI releases the insert which match the corresponding gene insert sizes of 17.4 kDa sHSP (left) and 16.9kDa sHSP (right).
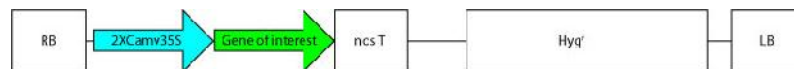
Figure 3.3.5: **The transfer region of pMDC32 destination vector**. The gene of interest was transferred to pMDC32 binary vector in between CaMV35S promoter and nosT terminator in a sequence specific manner mediated by LR recombinase. The transfer region (LB-RB) also contains hygromycin resistance gene as a selectable marker.

Successfully growing embryos were transferred to regeneration media to grow shoot and root in rooting media and then transferred to soil pots after hardening in Yoshida solution. Leaf sample were collected from individual plantlets, DNA was extracted to check the presence of the transgene by PCR using hpt primers. For 17.4kDa HSP construct 98 plantlets were regenerated of which 46 showed the presence of transgene through PCR. For the 16.9 kDa HSP construct transformation event 35 out of 78 plantlets were found positive. Figure 3.3.7 shows the agarose gel electrophoresis of PCR amplification with hpt primer for plantlets regenerated from 17.4 kDa and 16.9 kDa HSP construct transformation events.
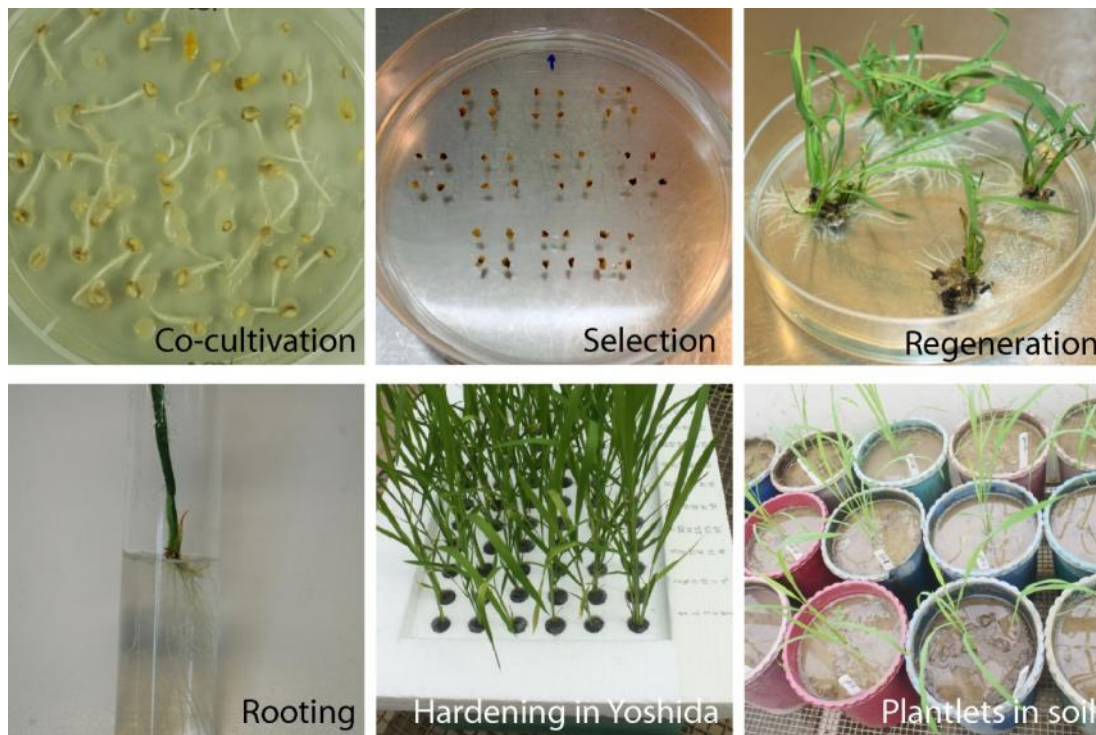
Figure 3.3.6: **Developing of transgenic lines using sHSP candidate genes**. Agrobacterium culture containing binary vector with the gene of interest were co-cultivated with rice immature embryos. Selection media containing hygromycin facilitates the growth of transgenic embryos only. Successfully growing embryos were transferred to proper media to regenerate shoot and root. Plantlets were transferred to Yoshida solution for hardening and then were transferred to pots containing soil.
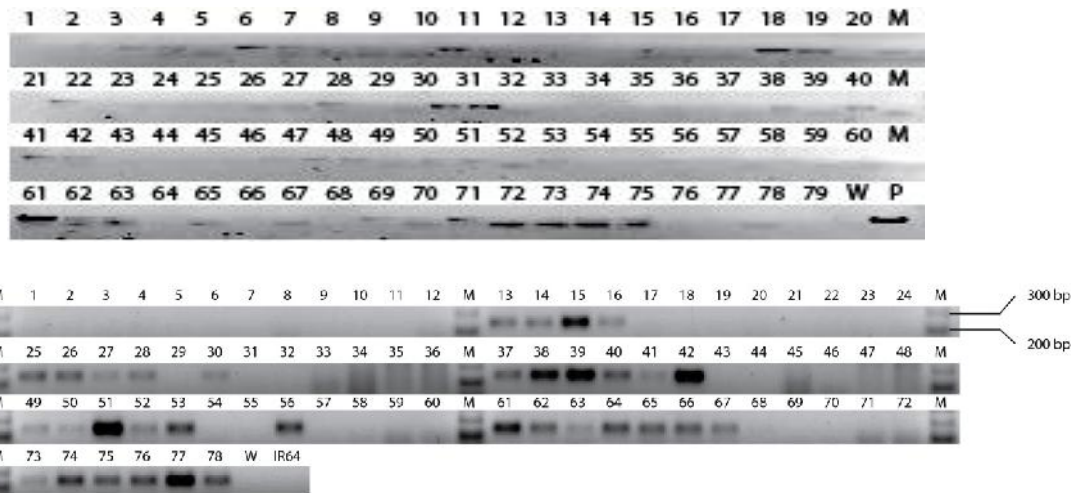
Figure 3.3.7: **PCR analysis of transgenic lines for the presence of transgene**. The hpt primer pair was used for PCR amplification. M: Molecular weight marker, [numbers]: lines for screening, W: water control, IR64: IR64 non-transgenic control.

DNA from these selected plantlets was analyzed by Southern blot analysis for single copy insert. The insert for both genes have the same sequence as their respective endogenous genes. Therefore, to identify the transgene, hpt gene sequence present in the transgenic region, was used as probe. Genomic DNA digested EcoRV which has a recognition sequence upstream of the insert. A single copy transformation event will produce one band only after blotting. Whereas multiple copy insert produces more than one band. Transgenic lines producing a single band were selected. 18 lines from 17.4 kDa HSP and 10 lines from 16.9 kDa HSP were found to have single copy insert.
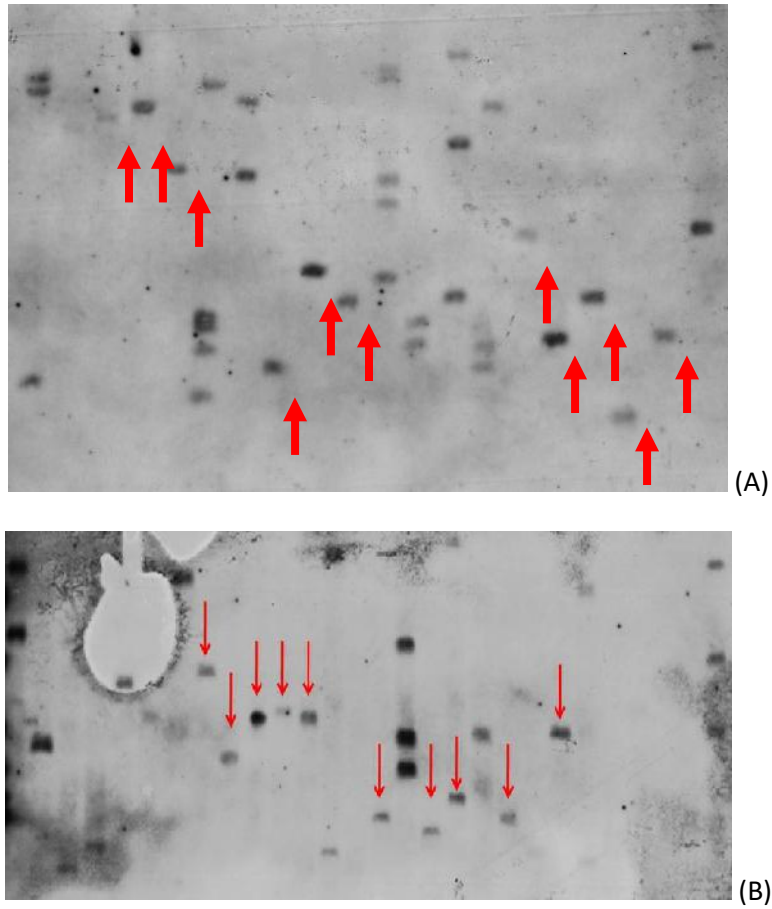
Figure 3.3.8: **Southern blot analysis to identify transgene with a single copy insert.** Genomic DNA was digested with EcoRV. After blot the membrane was hybridized with DIG-labeled hpt probe and chemi-luminescence was captured with X-ray films. A single band in a lane (red arrow) indicates single copy insert lines. Southern blot image of (A) 17.4 kDa HSP (B) 16.9 kDa HSP transformed transgenic lines.
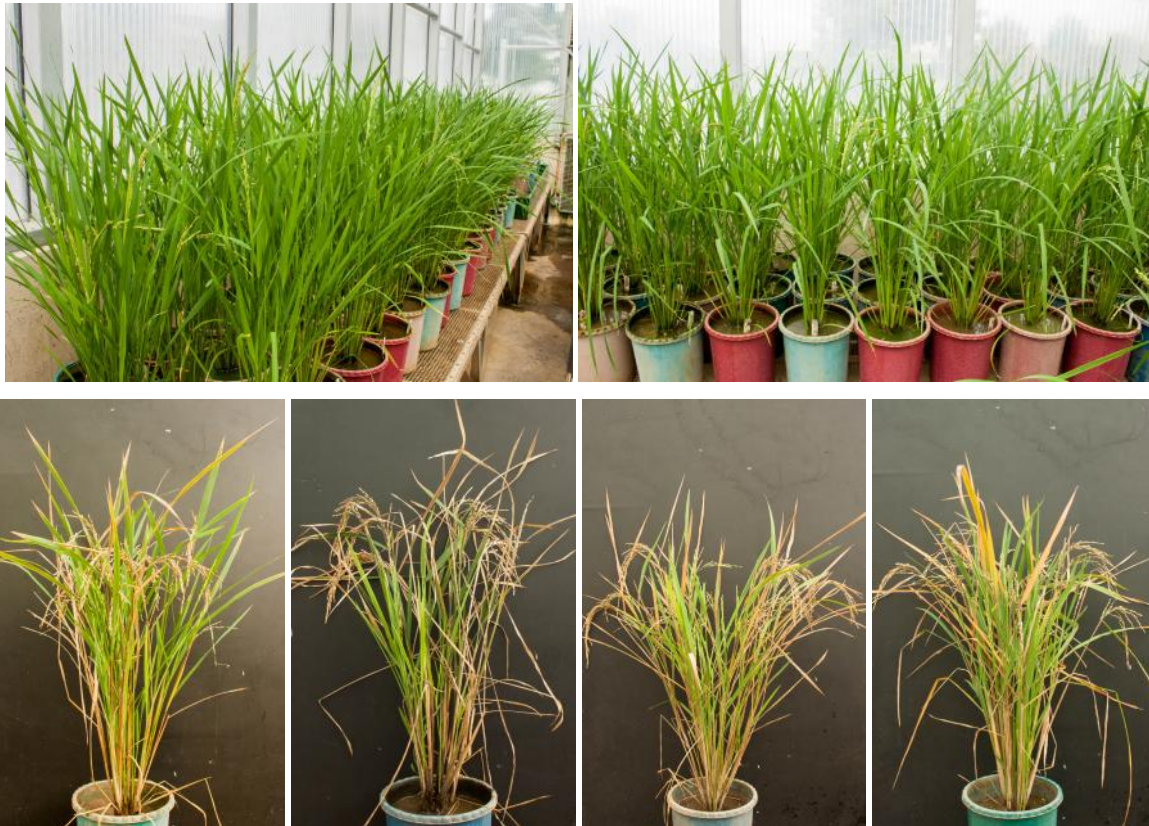
Figure 3.3.9: **Some selected transgenic lines with confirmed single copy gene insert present.** IR64 variety was transformed by 17.4 kDa sHSP and 16.9 kDa sHSP construct using immature embryo transformation method (Hiei et al., 2006). Regenerated plants were analyzed for single copy transgene. Selected plants were allowed to mature and seed were harvested for heat tolerance performance at T_1. Top panel shows plants after regeneration, bottom panel shows some plants at seed maturation.

Chapter 4: *Phenotyping of transgenic lines*

Materials and methods

Phenotyping of transgenic lines in T_1

Plants were grown in a temperature controlled CL4 facility in International Rice Research Institute (IRRI), Philippines. CL4 facility is a confined growth area where air/water is filtered before circulation/drain-out to prevent any contaminant to leak out. Access to this facility is locked double door and any materials going in or coming out are strictly monitored. Growth condition was maintained at 29/21°C day/night. Plants were grown in pots and were placed on a bench. Pre-germinated seeds were shown into Styrofoam trays fitted with nets and floated on Yoshida solution. 15 days old seedlings were transplanted to pots containing 6.0kg of clay loam soil. 7.5g $(NH_4)_2SO_4$, 1.5g KCL and 1.5g SSP was applied into each pots before transplanting. Additional 2.5 g of $(NH_4)_2SO_4$ was applied into each pots ~30 days after transplanting.

Since the plants in study were transgenic, to avoid contamination with other plants in indoor growth chamber facility at IRRI, two growth chambers (Thermoline, Australia) were isolated from the rest by building a wall with plastic and wood (Figure 3.4.1). The air inlet and outlet for the growth chambers were fitted with a filter to avoid pollen leak out. All plant parts were cleaned and properly disposed before and after the experiment. All activities were monitored by the Institutional Biosafety Committee (IBC). Plants were transferred into growth chamber at 8am. By 9am the temperature of the chamber was gradually increased from 29°C to 39°C and maintained at 39°C until 3pm. Relative humidity (RH) of 75% was maintained throughout the treatment. Temperature and humidity were recorded by sensors attached in growth chamber.

Figure 3.4.1: **Isolating the growth chambers for transgenic screening.** A plastic and wooden structure was built to prevent the pollen and other plant parts to escape and contaminate the environment. A door fitted with lock was installed to restrict entry.

Expression of transgene in T_1 plants

T1 plants were grown in CL4 facilities at the International Rice Research Institute (IRRI) as mentioned before. Leaves were collected and RNA was isolated using Trizol (invitrogen) as per manufacturer's instruction. RNA samples were treated with RNAse free DNAse (Promega) to remove any genomic DNA contamination. cDNA synthesis were carried out at 55°C for 1 hour in 20µl reaction containing 500ng DNAse treated total RNA, 2.5 mM oligo(dT), 0.5 mM dNTP mix, 0.01 M dithiothreitol, 1× first strand synthesis buffer and 200 unites of superscript II reverse transcriptase (Invitrogen).PCR was carried out in 20 µl reaction containing 0.5 µl cDNA, gene specific primer (0.2 mM each, sequence provided in appendix A.2), 0.5 mM dNTP mix, 1X PCR buffer and 1 unit of Taq DNA polymerase (SBS genetech, Beijing). The PCR cycle settings were 95°C for 2 min, followed by 30 cycles of 95°C for 30s, 55°C for 30s and 72°C for 1 min and a final extension of 72°C for 10 min. the glyceraldehydes-3-phosphate (GAPDH) gene was used as control of successful amplification as well as in determining the absence of genomic DNA. RT product were separated by agarose electrophoresis and stained with SYBR safe (Invitrogen).

Results

Expression of transgene

The transgene is under CaMV35s promoter and therefore, is ubiquitously expressed. Expression from the transgene should increase the intensity of expression compared to non-transgenic background IR64 as observed in this study. Figure 3.4.2 shows the expression of different transgenic lines for 17.4 kDa HSP and 16.9 kDa HSP compared to non transgenic IR64 and gene source N22. GAPDH was used as endogenous gene expression control and shows that all samples in this study has equal level of gene expression.
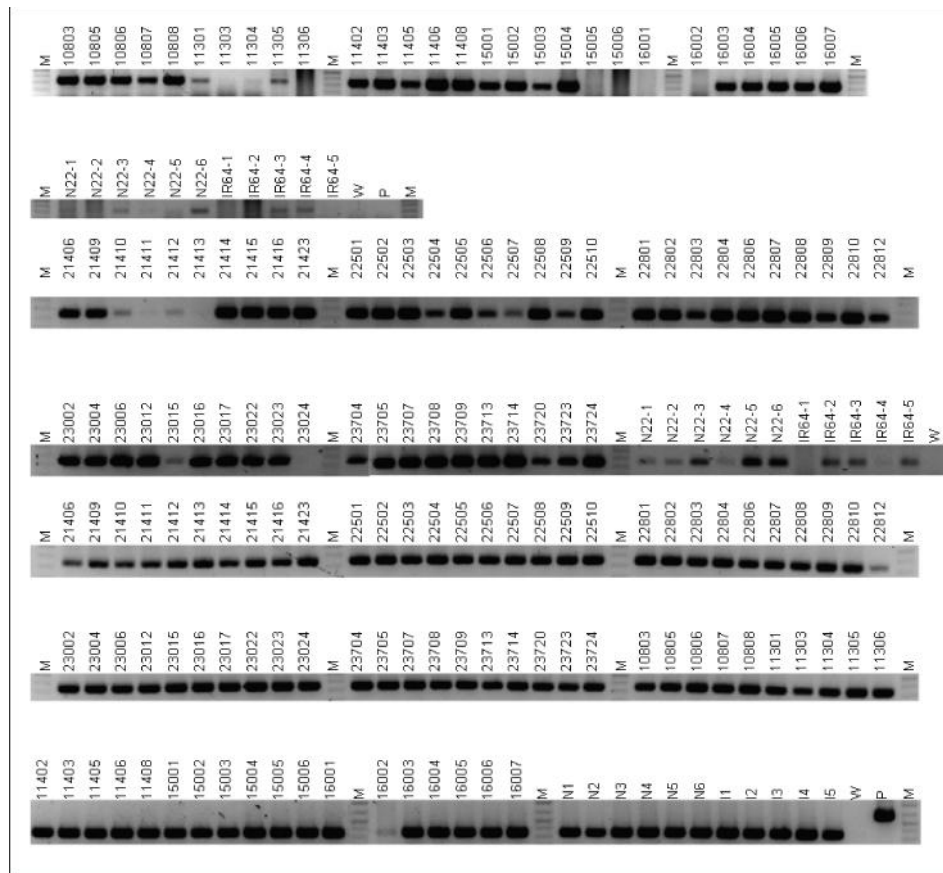
Figure 3.4.2: **Expression of transgene in different transgenic lines.** RNA was extracted for leaf sample, cDNA was synthesized from DNAse treated RNA and was used as template for PCR using gene specific primers. N22 and IR64 was used as non transgenic control and shows low level of expression from endogenous gene were as transgenic lines for 17.4kDa HSP (A) and 16.9kDa HSP (B) shows higher level of expression. GAPDH expression was used as endogenous gene expression level (C). M: molecular marker, W: water control, P: genomic DNA.

Phenotyping of transgenic lines

T_0 plants from tissue culture were confirmed by southern blot analysis for successful integration of single copy transgene. T_1 seeds from those selected T_0 plants were germinated and grown in pots until flowering stage at controlled CL4 facility. Since each T_1 plants are not identical, the expression and phenotype would be different. Therefore, it is not practical to compare a subset in control and others in stress condition as they are not comparable. For this reason, primary tiller of individual plants were allowed to flower in control condition and treated as control for that individual plant. On flowering of secondary tillers, plants were transferred to indoor growth chambers for high temperature stress at 38°C for 5 days. Spikelet that opened during the stress treatment was marked with water based colors. Tillers that flowered before and after the heat stress were marked and harvested separately from those flowered during the stress. Spikelet fertility was calculated. Table 3.4.1 summarizes the heat tolerance performance of different transgenic lines of 17.4 kDa HSP and 16.9 kDa HSP. 17.4 kDa HSP and 16.9 kDa transgenic plants shows maximum fertility of 80.98% and 86.52% under heat stress where as non-transgenic IR64 (variety where the transgene was introduced) shows maximum 57.57% spikelet fertility. Figure 3.4.3 graphically represents the spikelet fertility of the transgenic plants under heat stress.

Table 3.4.1: Spikelet fertility of different transgenic lines from heat treated spikelet and before and after heat treatment.

	Line no	%Fertility		
		Heat stress	Before heat stress	After heat stress
17.4 kDa sHSP over-expression lines	10803	29.88	80.65	71.19
	10805	72.69	-	77.83
	10806	65.36	-	77.98
	11301	80.98	75.22	83.26
	11304	18.04	-	-
	11403	15.80	57.58	49.63
	16001	27.07	-	70.36
	16004	76.02	-	65.49
	16006	79.77	-	66.31
16.9 kDa sHSP over-expression lines	21406	75.96	90.38	83.29
	21411	29.85	87.1	81.76
	21412	75.04	-	75.3
	21413	9.02	60.54	86.43
	21416	77.52	-	77.15
	22501	5.14	-	64.48
	22509	69.22	58.49	63.25
	22510	82.68	79.31	79.64
	22801	81.96	-	88.24

	22802	25.90	-	75.56
	22803	36.26	77.17	43.63
	22804	5.39	78.95	66.93
	22808	82.13	90.91	85.61
	22809	80.28	92.06	85.73
	23006	25.13	-	33.19
	23016	70.35	-	71.96
	23017	27.23	31.03	37.75
	23022	18.59	43.56	31.99
	23023	29.52	-	32.56
	23024	17.45	34.69	33.53
	23704	21.80	-	58.05
	23705	56.18	78.72	68.49
	23708	83.35	96.06	85.69
	23709	69.64	90.74	82.15
	23713	80.38	-	72.73
	23714	71.60	-	63.08
	23720	9.02	68.42	71.31
	23723	71.90	-	67.26
	23724	86.52	-	81.15
Heat sensitive check (IR64)	IR64-1	13.91	-	69.27
	IR64-2	52.80	45.65	82.35
	IR64-3	47.24	97.65	75.79
	IR64-4	57.57	-	85.79
	IR64-5	57.57	98.06	87.31
Heat tolerant check (N22)	N22-1	95.71	-	61.97
	N22-3	76.22	-	85.47
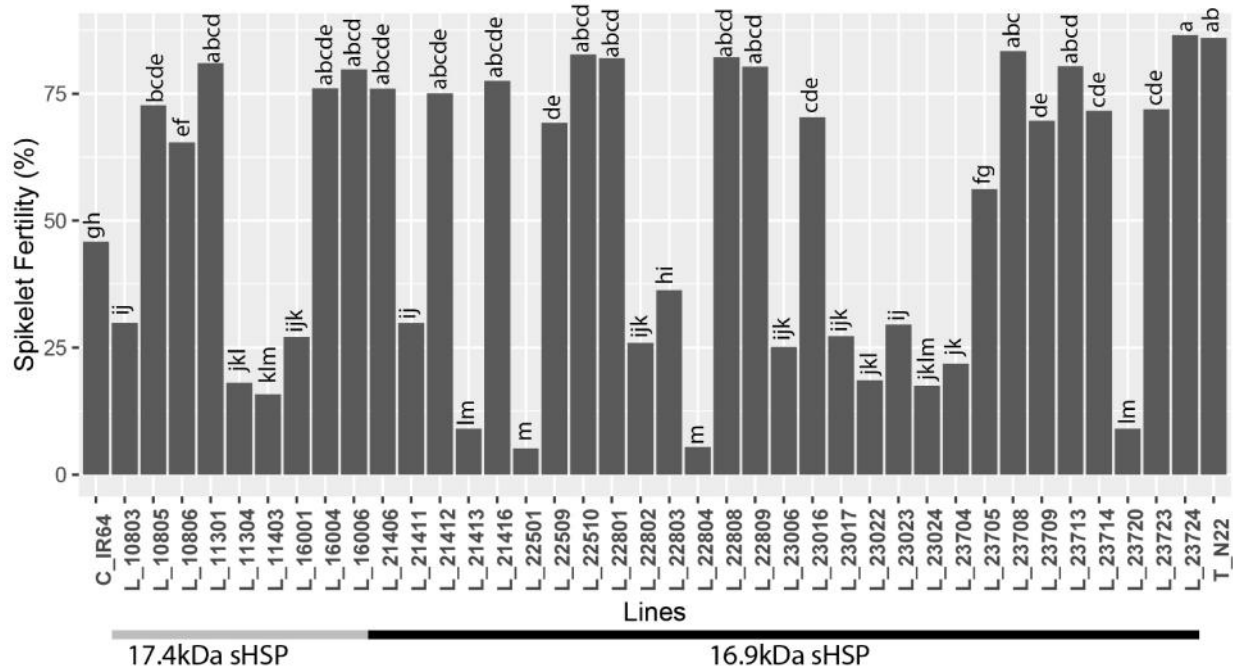
Figure 3.4.3: **Spikelet fertility of the transgenic lines under heat stress to evaluate the heat tolerance performance compared to sensitive IR64 (transgenic background) and tolerant N22.** T_1 transgenic lines were grown in controlled condition (29°/21° C). Heat stress was applied at 39°C for 6 hrs during the flowering stage. Spikelet opened during stress treatment was marked with water based color. On maturation seeds were harvested and spikelet fertility was calculated. Each bar in the graph represents average spikelet fertility recorded from 3 different tillers exposed to heat stress. Different letters in each bar indicates significant differences ($P < 0.05$, ANOVA and Duncan test).

The aim of this study was to evaluate the effect of over-expression of 17.4 kDa HSP and 16.9 kDa HSP under high temperature stress. Transgenic T_1 plants ware evaluated under stress. Although the effect varies, there is a overall increase in tolerance was observed compared to non-transgenic IR64. However, this effect was observed in T_1 where single plants per transformation events were exposed to heat stress only. Therefore, further evaluation of progenies are required to confirm the effect. On the other hand, effect of individual sHSP over-expression may be increased by combining them in one plant.

Discussion

Understanding the mechanism of high temperature tolerance

The overall goal of this study was to identify the mechanism involved in high temperature tolerance. Leaf temperature and cooling effect study suggest that there is a significant amount of cooling at the canopy level. Where the ambient temperature was 39°C, the leaf temperature was observed to be below 35°C during heat stress. Transpiration in well-watered condition may possibly help plants to reduce the temperature to protect from heat-induced damage which was not observed in the combined heat and water deficit condition where the leaf temperature was recorded approximately equal to the ambient temperature of 39°C. This also suggests that air moisture can also affect the heat induced damage as low moisture supports higher rate of transpiration in well-watered condition. However, no difference was observed between tolerant N22 and sensitive moroberekan in leaf temperature patterns indicating this might be a general defense mechanism to high temperature rather than tolerance specific. Based on temperature and humidity, rice cultivation regions were described as hot/dry or hot/humid where similar effect was observed (Matsui et al., 2007). In these hot/dry regions (e.g., Pakistan, Iran, India) low humidity supports transpirational cooling which was important for survival under heat.

Spikelet fertility is an important determinant of heat induced damage since flowering stage shows the most sensitivity and is translated to yield. Heat-sensitive variety *moroberekan* shows spikelet fertility of close to zero upon heat stress. Tolerant *N22* has a reduced fertility compared to controlled condition yet maintains it at ~20% after heat stress. This can be co-related with anther dehiscence where *moroberekan* has a rate below 20%, while *N22* maintains a rate above 80%. Greater the anther dehiscence rate, the better chance for pollen to reach the stigma for fertilization. Matsui et al. (199b) described anther dehiscence as the most susceptible process during anthesis under high temperature. Similar observations were made for pollen germination rate in *N22* where significant amounts of pollen reaching the stigma were germinated. For *moroberekan* the germination rate was near zero.

Peduncle length and panicle entrapment can also influence heat stress damage. It appears that upon heat stress, plants tend to exert the panicle more than it is in normal condition where as in combined stress the opposite was observed.

The phenomena of cooling through transpiration, variation in peduncle length and panicle entrapment do not show significant difference across sensitive and tolerant genotype suggesting these may be a general defense mechanism that plants apply upon heat stress. On the other hand, there was a significant difference in spikelet fertility, anther dehiscence and pollen germination on stigma across sensitive and tolerant genotypes suggesting that this stage in the fertilization process is the most critical to that could cause a difference in tolerance. A plant that can protect these processes from heat damage will be able to show increased tolerance.

Small Heat Shock proteins in rice

Differentially expressed proteins were studied from spikelet under combined heat and water-deficit condition (Jagadish et al. 2011). Differentially expressed proteins in 2D-gel electrophoresis were analyzed by mass spectroscopy and were matched against nipponbare proteins. From this a total of 8 candidate genes were selected for this study. Sequencing of these candidate genes amplified from N22 genome showed that there was no difference in their coding region compared to the *Nipponbare* genome. This indicates that the proteins perform the same functions in both sensitive and tolerant varieties but transcriptional, translational or post-translational regulation in the tolerant variety may make the difference. Study of regulation pathway of a protein requires extensive experiments which were not performed in this study. However semi-quantitative RT-PCR study under heat stress spikelet from *N22* and *moroberekan* indicated that the two heat shock proteins show a significant amount of induction upon heat stress. The other candidate genes did not show a differential significant pattern upon stress. Bioinformatics analysis of these two heat shock proteins shows that both contain an hsp20 subunit. These proteins are evolutionarily conserved. They are expressed in a variety of stresses including heat. They generally remain as large oligomers and upon stress they are dissociated to dimers to bind to their substrate. This group of protein is generally small, consisting approximately of 160 amino acid residues of which about 100 residues at the C-terminal end, is the characteristic hsp20 domain. The N-terminal region is variable among different proteins. Rice (nipponbare genome) has 37 sHSP. Phylogeny study shows that the two candidate sHSP in this study are very closely related. Interestingly there are several other members that are found in tandem with these two proteins in chromosome 1 and 3 where they are located. It is possibly originated by gene duplication and has acquired the changes but may be they are involved in very similar functions.

Expression of heat shock proteins

sHSP is a large group of protein and are expressed in wide variety of tissue as found in 51k rice microarray study (chapter 3.2, figure 3.2.2, 3.2.3). In general they are induced by a variety of stress and are involved in chaperonic activity and therefore may play an important role in development processes. However their exact mechanism of function in heat stress is yet to be discovered. Since flowering in rice is the most sensitive stage to heat stress, spikelets were analyzed for the candidate sHSP genes by semi-quantitative RT-PCR. Results reveal that in normal condition they are expressed in small quantities but their expression are induced upon heat stress. Since sHSP can play a protective role by keeping the denatured proteins in soluble form, these were presumed to be suitable candidates for further study. In line with this rationale, each of these two proteins were transformed into a heat sensitive rice cultivar IR64 and the effect of this transformation studied.

Phenotyping of transgenic lines

Both the CaMV::17.4kDaHSP and CaMV::16.9kDaHSP transformed lines shows fairly increased amount of transgene expression in different T_1 plants compared to the endogenous gene in IR64. Upon heat stress the transgenic plants showed an increased rate of spikelet fertility (maximum 80.98% and 86.52% in 17.4 kDa HSP and 16.9 kDa respectively) than the non-transgenic background IR64 (maximum 57.57%). Whereas the spikelets that were not exposed to heat stress in T_1 plants shows the spikelet fertility rate similar to that of non-transgenic IR64. Although the fertility found in the transgenic lines was not as good as *N22* (>76%), it can be concluded that the transgene had improved the tolerance to heat stress. Among the two heat shock proteins, the 16.9 kDa sHSP transformed lines show better performance upon heat stress.

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Appendix

A.1 Primers for amplification of candidate gene form Nagina 22 (N22) genome

LOC_Os01g04370 (hsp20_Alpha_Crystallin_Family_16.9)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	GCTTCTTCCGTTCCATTTCAG	21	61.0	48	613 bp
Reverse	CAGACCATACTTGCACGACC	20	59.0	55	

LOC_Os02g55649 (Unknown protein)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	CTCAAATCCACACCAGTTACC	21	58.0	48	841 bp
Reverse	AGGACTAGGAGTAGATGCAC	20	57.0	50	

LOC_Os03g01650 (Expansin_Precursor)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	TATTGCAGCAATGGCATCCT	20	62.0	45	837 bp
Reverse	GGTTTCTTCCTTGCTTAAAATG	22	57.0	36	

LOC_Os03g15960 (hsp20_Alpha_Crystallin_Family_17.4)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	CTTCCAAAAATCCGCTTCCA	20	62.0	45.0	697 bp
Reverse	CGACACACGACTGTTCAAGA	20	58.0	50.0	

LOC_Os04g25160 (Pollen specific protein _Two)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	CGATCCATCCCTCTACCAAC	20	59.0	55.0	445 bp
Reverse	CACAAGAGTATGCATGCCTG	20	58.0	50.0	

LOC_Os06g36240 (Pollen specific protein _Three)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	CGACACCGATCGAGAGAGA	19	57.0	58	748 bp
Reverse	CATCTTGAGCTTCGGAGAGG	20	61.0	55	

LOC_Os06g44470 (Pollen specific protein_One)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	CATCGATCCATCCCTCTATTAT	22	57.0	41	506 bp
Reverse	TCTCATGCGACTCAACAAAAA	21	61.0	38	

LOC_Os08g12160 (Expressed_Protein_Unknown)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	GGCGGAGAGATAAATAATATACAG	24	57.0	38	1053 bp
Reverse	ATCAATGTGTGGGAAGCAAGTG	22	58.0	45	

A.2 Primers for amplification of candidate gene for expression analysis using Reverse Transcriptase PCR (RT-PCR) in N22 and transgenic lines.

LOC_Os01g04370 (hsp20_Alpha_Crystallin_Family_16.9)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	AACGTGTTCGACCCATTCTC	20	60.0	50	266 bp
Reverse	TGCCACTTGTCGTTCTTGTC	20	59.9	50	

LOC_Os02g55649 (Unknown protein)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	AGTACGAGCCACCGAAGAGA	20	60.0	55	200 bp
Reverse	TACACCCCTCATTGCACAAA	20	60.0	45	

LOC_Os03g01650 (Expansin_Precursor)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	CTCCCTTCTACTCGCCTGTG	20	60.0	60	297 bp
Reverse	GGCTTGGAGCACTTGATCTC	20	60.0	55	

LOC_Os03g15960 (hsp20_Alpha_Crystallin_Family_17.4)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	CAGCATCTTCCCGTCCTTC	19	60.8	57.9	352 bp
Reverse	CTTCTTGGCCTCCTCCTTG	19	59.9	57.9	

LOC_Os04g25160 (Pollen specific protein _Two)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	CAAGGTTGCTGAGGGCTCTA	20	60.5	55.0	216 bp
Reverse	ACATCATCGACGACACGGTA	20	60.0	50	

LOC_Os06g36240 (Pollen specific protein _Three)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	ACCAACGTCTCCAAAAGCAT	20	59.6	45	252 bp
Reverse	CTTGAGGCCGTTGTTGCT	18	60.0	55.6	

LOC_Os06g44470 (Pollen specific protein_One)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	TGGCCTCCTTATCCTCCTTC	20	60.5	55	224 bp
Reverse	GCTTTTGATCGTCCAGGTGT	20	60.1	50	

LOC_Os08g12160 (Expressed_Protein_Unknown)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	GTGGTGTTGATGGTCGTCAG	20	60.0	55	283 bp
Reverse	AGAGGTTGGTGCTCAGGATG	20	60.3	55	

A.3 Sequencing of candidate genes form *N22* genome and alignment with *Nipponbare*

Genomic sequence of the candidate genes were amplified using primers designed using the *nipponbare* sequence information from Gramene database (www.gramene.org). Nagina 22 (N22) genomic DNA was extracted and used as template for PCR amplification of the candidate genes. Amplicons were then cloned into pCR8/GW/TOPO cloning vector (Invitrogen) and were sent for sequencing (Macrogen, Korea). For each gene 6 clones were sequenced using vector specific M13 primer pair. Consensus sequence for 12 reads (6 clones×2 primers) were then aligned with corresponding nipponbare sequence (shown below). [*]- represents match N22 sequence to nipponbare sequence. all other genes match perfectly with nipponbare sequence except for LOC_Os01g04370 (hsp20_Alpha_Crystallin_Family_16.9) where is has a A-> C transversions in coding region (CDS). But it does not change the translated protein (alignment shown).

```
LOC_Os04g25160: Pollen specific protein _Two

Nipponbare      --------------------------------------------------------cgatcc
N22             tgcttttttataatgccaactttgtacaaaaaagcaggctccgaattcgcccttcgatcc
                                                                        ******
Nipponbare      atccctctaccaaccaagaaacaagaaacattacaccaaaaaactagctagctagccaat
N22             atccctctaccaaccaagaaacaagaaacattacaccaaaaaactagctagctagccaat
                ************************************************************
Nipponbare      ggcctccatgtcctccttccgacttgccgtggcggcggctgcactattggtcattggctc
N22             ggcctccatgtcctccttccgacttgccgtggcggcggctgcactattggtcattggctc
                ************************************************************
Nipponbare      atgtgccacagagctcaccttcaaggttgctgagggctctagcgccactagtctggagct
N22             atgtgccacagagctcaccttcaaggttgctgagggctctagcgccactagtctggagct
                ************************************************************
Nipponbare      cgtcaccaacgtcgccatctctgaggtggaggtcaaggagaagggcggcaaggactgggt
N22             cgtcaccaacgtcgccatctctgaggtggaggtcaaggagaagggcggcaaggactgggt
                ************************************************************
Nipponbare      ggggctcaaggagtcaggctctaacacctggacactcaaaagtgaagcaccactcaaggg
N22             ggggctcaaggagtcgggctctaacacctggacactcaaaagtgaagcaccactcaaggg
                ************************************************************

Nipponbare      ccccttctccgttcgcttccttgtcaagaacggtggctaccgtgtcgtcgatgatgtcat
N22             cccccttctccgttcgcttccttgtcaagaacggtggctaccgtgttgtcgatgatgtcat
                ************************************************************
Nipponbare      ccctgaaagctttactgccggctctgaatataagagtggcatccaactctaagtaacagg
N22             ccctgaaagctttactgccggctctgaatataagagtggcatccaactctaagtaacagg
                ************************************************************
Nipponbare      catgcatactcttgtgt--------------------------------------------
N22             catgcatactcttgtgtagggcgaattccgacccagctttcttgtacaaagttggcatta
                ****************
```

LOC_Os06g36240: Pollen specific protein _Three

```
Nipponbare    --------------------cgacaccgatcgagagagagagatcgcaccgatcgacgt
N22           agctgggtcgaattcgcccttcgacaccgatcgagagagagagatcgcaccgatcgacgt
                                  ******************************************

Nipponbare    acggtacgagacgaccatggcgcgtcctcgcttcgccaccacggcgccattgttggcgct
N22           acggtacgagacgaccatggcgcgtcctcgcttcgccaccacggcgccattgttggcgct
              ************************************************************

Nipponbare    cgccgtcctcgccgccgtggtgtccgtcgccgtcgccaccgctcccgccggcaaggaccc
N22           cgccgtcctcgccgccgtggtgtccgtcgccgtcgccaccgctcccgccggcaaggaccc
              ************************************************************

Nipponbare    cggcggcttcgtcgtcaccgggcgcgtctactgcgacccctgccgcgccggcttcgagac
N22           cggcggcttcgtcgtcaccgggcgcgtctactgcgacccctgccgcgccggcttcgagac
              ************************************************************


Nipponbare    caacgtctccaaaagcatcccaggtgcgtagagatcgaatcgaatcgaagaagcaataag
N22           caacgtctccaaaagcatcccaggtgcgtagagatcgaatcgaatcgaagaagcaataag
              ************************************************************

Nipponbare    aaggcaggtgcaggcgaggtgactgaggaaatgcgtgcgtgcgtgcgtaggggcgacggt
N22           aaggcaggtgcaggcgaggtgactgaggaaatgcgtgcgtgcgtgcgtaggggcgacggt
              ************************************************************


Nipponbare    gtcggtggagtgcaggcactacggggcggggagggagagcctgaaggcggaggcgacgac
N22           gtcggtggagtgcaggcactacggggcggggagggagagcctgaaggcggaggcgacgac
              ************************************************************

Nipponbare    ggacgagaaggggtggtacaaggtggagatcgaccaggaccaccaggaggagatctgcga
N22           ggacgagaaggggtggtacaaggtggagatcgaccaggaccaccaggaggagatctgcga
              ************************************************************

Nipponbare    ggtggtgctggacaagagcagcgacccggcgtgctccgagacggagaagacccgcgaccg
N22           ggtggtgctggacaagagcagcgacccggcgtgctccgagacggagaagacccgcgaccg
              ************************************************************

Nipponbare    gtcccgcgtcccgctcaccagcaacaacggcctcaagcagaacggcatccgctacgccaa
N22           gtcccgcgtcccgctcaccagcaacaacggcctcaagcagaacggcatccgctacgccaa
              ************************************************************


Nipponbare    ccccatcgccttcttccgcaaggagcccctcgccgactgcggctccatcctccagaagta
N22           ccccatcgccttcttccgcaaggagcccctcgccgactgcggctccatcctccagaagta
              ************************************************************

Nipponbare    cgacctcaaggacgcccccgagaccccatgatcatcatcatatatatgtttctccttagc
N22           cgacctcaaggacgcccccgagaccccatgatcatcatcatatatatgtttctccttagc
              ************************************************************

Nipponbare    agtgcatcaaacgttccttcaaatttgagcctctccgaagctcaagatg-----------
N22           agtgcatcaaacgttccttcaaatttgagcctctccgaagctcaagatgaagggcgaatt
              *************************************************
```

LOC_Os03g15960: hsp20_Alpha_Crystallin_Family_17.4

```
Nipponbare    cttccaaaaatccgcttccaattcgcgaaactacactagtcgtaagcgccaaatccaacc
N22           cttccaaaaatccgcttccaattcgcgaaactacactagtcgtaagcgccaaatccaacc
              ************************************************************


Nipponbare    gacgatgtcgctgatccgccgcagcaacgtgttcgaccccttctccctcgacctctggga
N22           gacgatgtcgctgatccgccgcagcaacgtgttcgaccccttctccctcgacctctggga
              ************************************************************


Nipponbare    cccccttcgacggcttcccccttcggctccggcggcagcagcagcggcagcatcttcccgtc
N22           cccccttcgacggcttcccccttcggctccggcggcagcagcagcggcagcatcttcccgtc
              ************************************************************


Nipponbare    cttcccgcgcggcgcctcctccgagaccgcggccttcgccggcgcgcggatcgactggaa
N22           cttcccgcgcggcgcctcctccgagaccgcggccttcgccggcgcgcggatcgactggaa
              ************************************************************
```

```
Nipponbare    ggagacgcccgaggcgcacgtgttcaaggcggacgtgccggggctgaagaaggaggaggt
N22           ggagacgcccgaggcgcacgtgttcaaggcggacgtgccggggctgaagaaggaggaggt
              ************************************************************

Nipponbare    caaggtggaggtggacgacggcaacatcctgcagatcagcggcgagcgcaacaaggagca
N22           caaggtggaggtggacgacggcaacatcctgcagatcagcggcgagcgcaacaaggagca
              ************************************************************

Nipponbare    ggaggagaagacggaccagtggcaccgcgtggagcgcagcagcggcaagttcctccgcag
N22           ggaggagaagacggaccagtggcaccgcgtggagcgcagcagcggcaagttcctccgcag
              ************************************************************

Nipponbare    gttccgcctccccgacaacgccaagccggagcagatcaaggcgtccatggagaacggcgt
N22           gttccgcctccccgacaacgccaagccggagcagatcaaggcgtccatggagaacggcgt
              ************************************************************

Nipponbare    gctcaccgtcacggttcccaaggaggaggccaagaagcccgacgtcaagtccatccagat
N22           gctcaccgtcacggttcccaaggaggaggccaagaagcccgacgtcaagtccatccagat
              *********************************************** **************

Nipponbare    ctccggctaggcatcgccggcgtgccgcgtgcgcgacggagaggagcacggcggggtttt
N22           ctccggctaggcatcgccggcgtgccgcgtgcgcgacggagaggagcacggcggggtttt
              ************************************************************

Nipponbare    cgctttggcctggtttgtctgtcgtgaaggagcaaataaaatcgggtccggttgagtcca
N22           cgctttggcctggtttgtctgtcgtgaaggagcaaataaaatcgggtccggttgagtcca
              ************************************************************

Nipponbare    gtgtgtgtccgtgtctgtcttgaacagtcgtgtgtcg-----------------------
N22           gtgtgtgtccgtgtctgtcttgaacagtcgtgtgtcgaaggcgaattcgacccagcttta
              *************************************
```

LOC_Os01g04370: hsp20_Alpha_Crystallin_Family_16.9

```
Nip-Gen    gcttcttccgttccatttcagtagcccacaacttcgcatcagaaagcgaaagatagagca
N22-Gen    gcttcttccgttccatttcagtagcccacaacttcgcatcagaaagcgaaagttagagca
Nip-CDS    ------------------------------------------------------------
N22-CDS    ------------------------------------------------------------


Nip-Gen    accatgtcgctggtgaggcgcagcaacgtgttcgacccattctccctcgacctctgggac
N22-Gen    accatgtcgctggtgaggcgcagcaacgtgttcgacccattctccctcgacctctgggac
Nip-CDS    ---atgtcgctggtgaggcgcagcaacgtgttcgacccattctccctcgacctctgggac
N22-CDS    ---atgtcgctggtgaggcgcagcaacgtgttcgacccattctccctcgacctctgggac
              ***********************************************************

Nip-Gen    cccttcgacagcgtgttccgctccgtcgtcccggccacctccgacaacgacaccgccgcc
N22-Gen    cccttcgacagcgtgttccgctccgtcgtcccggccacctccgacaacgacaccgccgcc
Nip-CDS    cccttcgacagcgtgttccgctccgtcgtcccggccacctccgacaacgacaccgccgcc
N22-CDS    cccttcgacagcgtgttccgctccgtcgtcccggccacctccgacaacgacaccgccgcc
              ************************************************************

Nip-Gen    ttcgccaacgcccgcatcgactggaaggagacgccggagtcgcacgtcttcaaggccgac
N22-Gen    ttcgccaacgcccgcatcgactggaaggagacgccggagtcgcacgtcttcaaggccgac
Nip-CDS    ttcgccaacgcccgcatcgactggaaggagacgccggagtcgcacgtcttcaaggccgac
N22-CDS    ttcgccaacgcccgcatcgactggaaggagacgccggagtcgcacgtcttcaaggccgac
              ************************************************************
```

87

```
Nip-Gen     ctccccggcgtcaagaaggaggaggtgaaggtggaggtggaggaaggcaacgtgctggtg
N22-Gen     ctccccggcgtcaagaaggaggaggtgaaggtggaggtggaggaaggcaacgtgctggtg
Nip-CDS     ctccccggcgtcaagaaggaggaggtgaaggtggaggtggaggaaggcaacgtgctggtg
N22-CDS     ctccccggcgtcaagaaggaggaggtgaaggtggaggtggaggaaggcaacgtgctggtg
            ************************************************************

Nip-Gen     atcagcggccagcgcagcaaggagaaggaggacaagaacgacaagtggcaccgcgtggag
N22-Gen     atcagcggccagcgcagcaaggagaaggaggacaagaacgacaagtggcaccgcgtggag
Nip-CDS     atcagcggccagcgcagcaaggagaaggaggacaagaacgacaagtggcaccgcgtggag
N22-CDS     atcagcggccagcgcagcaaggagaaggaggacaagaacgacaagtggcaccgcgtggag
            ************************************************************

Nip-Gen     cgcagcagcgggcagttcatgcggcggttc**a**ggctgccggagaacgccaaggtggaccag
N22-Gen     cgcagcagcgggcagttcatgcggcggttc**c**ggctgccggagaacgccaaggtggaccag
Nip-CDS     cgcagcagcgggcagttcatgcggcggttc**a**ggctgccggagaacgccaaggtggaccag
N22-CDS     cgcagcagcgggcagttcatgcggcggttc**c**ggctgccggagaacgccaaggtggaccag
            *****************************  *****************************

Nip-Gen     gtgaaggccggactggagaacggcgtgctcactgtcaccgtgcccaaggccgaggtcaag
N22-Gen     gtgaaggccggactggagaacggcgtgctcactgtcaccgtgcccaaggccgaggtcaag
Nip-CDS     gtgaaggccggactggagaacggcgtgctcactgtcaccgtgcccaaggccgaggtcaag
N22-CDS     gtgaaggccggactggagaacggcgtgctcactgtcaccgtgcccaaggccgaggtcaag
            ************************************************************

Nip-Gen     aagcctgaggtgaaggccattgagatctccggttaagctcctgaagatgtgatcggtgag
N22-Gen     aagcctgaggtgaaggccattgagatctccggttaagctcctgaagatgtgatcggtgag
Nip-CDS     aagcctgaggtgaaggccattgagatctccggttaa------------------------
N22-CDS     aagcctgaggtgaaggccattgagatctccggttaa------------------------
            ************************************

Nip-Gen     ggaagaagtcatgtttggtgtcagtaattcagtatttcagtgtgtgtttgtttggtcgtg
N22-Gen     ggaagaagtcatgtttggtgtcagtaattcagtatttcagtgtgtgtttgtttggtcgtg
Nip-CDS     ------------------------------------------------------------
N22-CDS     ------------------------------------------------------------


Nip-Gen     caagtatggtctg
N22-Gen     caagtatggtctg
Nip-CDS     -------------
N22-CDS     -------------
```

Alignment of translated protein.

```
Nip-Prot    MSLVRRSNVFDPFSLDLWDPFDSVFRSVVPATSDNDTAAFANARIDWKETPESHVFKADL
N22-Prot    MSLVRRSNVFDPFSLDLWDPFDSVFRSVVPATSDNDTAAFANARIDWKETPESHVFKADL
            ***********************************************************

Nip-Prot    PGVKKEEVKVEVEEGNVLVISGQRSKEKEDKNDKWHRVERSSGQFMRRFRLPENAKVDQV
N22-Prot    PGVKKEEVKVEVEEGNVLVISGQRSKEKEDKNDKWHRVERSSGQFMRRFRLPENAKVDQV
            ***********************************************************

Nip-Prot    KAGLENGVLTVTVPKAEVKKPEVKAIEISG
N22-Prot    KAGLENGVLTVTVPKAEVKKPEVKAIEISG
            *****************************
```

88

LOC_Os03g01650: Expansin_Precursor

```
Nipponbare    ------------------------------------------------------------atg
N22           caatgctttttttataatgccaactttgtacaaaaaagcaggctccgaattcgcccttatg
                                                                          ***


Nipponbare    gcatcctcctcccttctactcgcctgtgttgtggtggcggctatggtgtccgccgtctcc
N22           gcatcctcctcccttctactcgcctgtgttgtggtggcggctatggtgtccgccgtctcc
              ************************************************************


Nipponbare    tgcgggccacccaaggtgccaccgggccccaacatcacgacaagctacggcgacaagtgg
N22           tgcgggccacccaaggtgccaccgggccccaacatcacgacaagctacggcgacaagtgg
              ************************************************************


Nipponbare    ctggaagccaaggccacctggtatggtgcgcccaagggtgctggccccaaggacaacggc
N22           ctggaagccaaggccacctggtatggtgcgcccaagggtgctggccccaaggacaacggc
              ************************************************************


Nipponbare    ggcgcctgcgggtacaaggatgtcgacaaggctcccttcctcggcatgaactcctgcggc
N22           ggcgcctgcgggtacaaggatgtcgacaaggctcccttcctcggcatgaactcctgcggc
              ************************************************************


Nipponbare    aacgaccccatcttcaaggacggcaagggctgcggctcatgcttcgagatcaagtgctcc
N22           aacgaccccatcttcaaggacggcaagggctgcagctcatgcttcgagatcaagtgctcc
              ************************************************************


Nipponbare    aagccggaggcctgctccgacaagcccgcccttatccacgtcaccgacatgaacgacgag
N22           aagccggaggcctgctccgacaagcccgcccttatccacgtcaccgacatgaacgacgag
              ************************************************************


Nipponbare    cccatcgctgcctaccactttgacctctccggccttgccttcggcgccatggctaaggat
N22           cccatcgctgcctaccactttgacctctccggccttgccttcggcgccatggctaaggat
              ************************************************************


Nipponbare    ggcaaggacgaagagctccgtaaggccggcatcatcgacacgcagttccgccgcgtcaag
N22           ggcaaggacgaagagctccgtaaggccggcatcatcgacacgcagttccgccgcgtcaag
              ************************************************************


Nipponbare    tgcaagtatcctgccgacaccaagatcaccttccacatcgagaaggcctccaaccccaac
N22           tgcaagtatcctgccgacaccaagatcaccttccacatcgagaaggcctccaaccccgac
              ************************************************************


Nipponbare    taccttgcgctgctagtcaagtacgtcgctggtgatggtgacgtcgtggaggtggaaatc
N22           taccttgcgctgctagtcaagtacgtcgctggtgatggtgacgtcgtggaggtggaaatc
              ************************************************************


Nipponbare    aaggagaagggctccgaggagtggaaggcgctcaaggagtcatggggtgccatttggagg
N22           aaggagaagggctccgaggagtggaaggcgctcaaggagtcatggggtgccatttggagg
              ************************************************************


Nipponbare    atagacaccccccaagccgctcaagggccccttctccgtccgcgtcaccaccgagggtggc
N22           atagacaccccccaagccgctcaagggccccttctccgtccgcgtcaccaccgagggtggc
              ************************************************************


Nipponbare    gagaagatcatcgccgaggacgccatccctgatggctggaaggccgacagcgtgtacaag
N22           gagaagatcatcgccgaggacgccatccctgatggctggaaggccgacagcgtgtacaag
              ************************************************************


Nipponbare    tccaacgtccaggccaagtgagcattttaagcaaggaagaaaccagcgtacgta------
N22           tccaacgtccaggccaagtgagcattttaagcaaggaagaaaccagcgtacgtaaagggc
              ******************************************************
```

LOC_Os02g55649: Unknown protein

```
Nipponbare     -----ctcaaatccacaccagttaccctcaatagaaacggtgctaatggcccagaacaag
N22            ctatcctcaaatcccnaccagttaccctcaatagaaacggtgctaatggcccagaacaag
               ************************************************************

Nipponbare     accatggctctgctccttgccacccttgtggcggtggttgcggtagtacgagccaccgaa
N22            accatggctctgctccttgccacccttgtggcggtggttgcggtagtacgagccaccgaa
               ************************************************************

Nipponbare     gagaaggatatagaagaagctgtgtgctcagagcattgcaacgacgaggaaaaagaaggc
N22            gagaaggatatagaagaagctgtgtgctcagagcattgcaacgacgaggaaaaagaaggc
               ************************************************************

Nipponbare     accatcgaccacaagcactgtgtagacatctgcatcctcacgaacagggaactttttggg
N22            accatcgaccacaagcactgtgtagacatctgcatcctcacgaacagggaactttttggg
               ************************************************************

Nipponbare     gccttggagagaggaatgaagccctcgatggagcaattcagcgctttgtgcaatgagggg
N22            gccttggagagaggaatgaagccctcgatggagcaattcagcgctttgtgcaatgagggg
               ************************************************************

Nipponbare     tgtagcaaagaatttaaggaggatcctgccaccaacaagaagtgcgtagacagttgcatc
N22            tgtagcaaagaatttaaggaggatcctgccaccaacaagaagtgcgtagacagttgcatc
               ************************************************************

Nipponbare     gtcgatgctaaggaactcaatggacacttagcgaaaggtggcgcttctagtgttcccgca
N22            gtcgatgctaaggaactcaatggacacttagcgaaaggtggcgcttctagtgttcccgca
               ************************************************************

Nipponbare     cgtgcatgattgtgtcgagctgcacatctgttccttcatccatgatcgagggacgatacc
N22            cgtgcatgattgtgtcgagctgcacatctgttccttcatccatgatcgagggacgatacc
               ************************************************************

Nipponbare     cattaatggttcccacccagcggtcggccttgtattacattgtcctgttgttccttttgc
N22            cattaatggttcccacccagcggtcggccttgtattacattgtcctgttgttccttttgc
               ************************************************************

Nipponbare     ctcttcaattgtttcattatttggtgttcataacttctatgtgatcgatctggtctgttg
N22            ctcttcaattgtttcattatttggtgttcataacttctatgtgatcgatctggtctgttg
               ************************************************************

Nipponbare     ttctacccaatattgttactaaaagcatttgttatctttaattgattaaaatagttccag
N22            ttctacccaatattgttactaaaagcatttgttatctttaattgattaaaatagttccag
               ************************************************************

Nipponbare     ttgtgctttgtcttttaacattcattaatatacggtctcatattccactttgagtacgtg
N22            ttgtgctttgtcttttaacattcattaatatacggtctcatattccactttgagtacgtg
               ************************************************************

Nipponbare     caatgttagtacatagatatagttcatgcttccttccgcgcgtccggtgcgtgccctgtg
N22            caatgttagtacatagatatagttcatgcttccttccgcgcgtccggtgcgtgccctgtg
               ************************************************************

Nipponbare     atcatgctctcttggaaaacgcaacaactagggcaccacgatacacgtgcatctactcct
N22            atcatgctctcttggaaaacgcaacaactagggcaccacgatacacgtgcatctactcct
               ************************************************************

Nipponbare     agtcct----------------
N22            agtcctagccttcaattaacaa
               ******
```

LOC_Os08g12160: Expressed_Protein_Unknown

```
Nipponbare      ------------------------ggcggagagataaataatatacagaaatgagcagg
N22             aaaagcaggctccgaattcgcccttggcggagagataaataatatacagaaatgagcagg
                                        ********************************

Nipponbare      tcgagctcgagcatggccaccgtcctcgtggtgttgatggtcgtcagcgccggcggctta
N22             tcgagctcgagcatggccaccgtcctcgtggtgttgatggtcgtcagcgccggcggctta
                ************************************************************

Nipponbare      tccccaccatgtgcggccgctgcgaaggaggagaagccagtggtggtgctgccaccagcg
N22             tccccaccatgtgcggccgctgcgaaggaggagaagccagtggtggtgctgccaccagcg
                ************************************************************

Nipponbare      gcggcgccaggggaggcgccctccgcggacgcggcagcgttcgtccgctcctgctgcgac
N22             gcggcgccaggggaggcgccctccgcggacgcggcagcgttcgtccgctcctgctgcgac
                ************************************************************

Nipponbare      accgccctgcaggccgaccgcgacggctccagcttctgctactaccatctcctcccctac
N22             accgccctgcaggccgaccgcgacggctccagcttctgctactaccatctcctcccctac
                ************************************************************

Nipponbare      gctgccttcttcgagggcaaccaggtgaaggtcgccgaggtggccgccaccatcctgagc
N22             gctgccttcttcgagggcaaccaggtgaaggtcgccgaggtggccgccaccatcctgagc
                ************************************************************

Nipponbare      accaacctctgggtctacgtcgaccaattgcgcaaggtccagggagggggccggcaagggg
N22             accaacctctgggtctacgtcgaccaattgcgcaaggtccagggagggggccggcaagggg
                ************************************************************

Nipponbare      gacccgaacctgaacgcctgcgtcgatgacttcagcgtagccgccggcgagaacatcacc
N22             gacccgaacctgaacgcctgcgtcgatgacttcagcgtagccgccggcgagaacatcacc
                ************************************************************

Nipponbare      agggaggcgctgcagagcctcggccgcctcgccgccgccggaaatggcaagcgcagcaag
N22             agggaggcgctgcagagcctcggccgcctcgccgccgccggaaatggcaagcgcagcaag
                ************************************************************

Nipponbare      gaggatctggagaatgcgcaaaagtggatcaaaggggtggagaaaccctacaacggtggg
N22             gaggatctggagaatgcgcaaaagtggatcaaaggggtggagaaaccctacaacggtggg
                ************************************************************

Nipponbare      atcgggaaagcatccggttgcgagataggttacctgttcacttatagcgatgatctcccc
N22             atcgggaaagcatccggttgcgagataggttacctgttcacttatagcgatgatctcccc
                ************************************************************

Nipponbare      gcccagaaaacccttggatatactttcgacacagctagctccctcatcaatcacataaag
N22             gcccagaaaacccttggatatactttcgacacagctagctccctcatcaatcacataaag
                ************************************************************

Nipponbare      ctttaataatctagctatatatatagccttccatgcatgctctctagctacctactaagt
N22             ctttaataatctagctatatatatagccttccatgcatgctctctagctacctactaagt
                ************************************************************

Nipponbare      ggaagtacattatttttatttattatacttgttgttaattcccatgatcgattagtggtgt
N22             ggaagtacattatttttatttattatacttgttgttaattcccatgatcgattagtggtgt
                ************************************************************

Nipponbare      tgtccacgattttttggtaacattgttttttctcaattttctatttcatatattcccaactc
N22             tgtccacgattttttggtaacattgttttttctcaattttctatttcatatattcccaactc
                ************************************************************
```

```
Nipponbare    tcaattctacattatatatcattttttcattcaacttcatacatacaattcctcagttct
N22           tcaattctacattatatatcattttttcattcaacttcatacatacaattcctcagttct
              ************************************************************

Nipponbare    ttcttatatataggccttacgctaaattttttttttggatacacaccaccgtcgattatat
N22           ttcttatatataggccttacgctaaattttttttttggatacacaccaccgtcgattatat
              ************************************************************

Nipponbare    cgtgcatacttttgtatttcaattaattaattgtgacacttgcttcccacacattgat--
N22           cgtgcatacttttgtatttcaattaattaattgtgacacttgcttcccacacattgataa
              **********************************************************
```

A.4 Primers for amplification of candidate genes for cloning and transformation into rice (IR64)

LOC_Os01g04370 (hsp20_Alpha_Crystallin_Family_16.9)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	AACGTGTTCGACCCATTCTC	20	60.0	50	266 bp
Reverse	TGCCACTTGTCGTTCTTGTC	20	59.9	50	

LOC_Os03g15960 (hsp20_Alpha_Crystallin_Family_17.4)

Primer	Sequence	Length	Tm	%GC	Product Size
Forward	CAGCATCTTCCCGTCCTTC	19	60.8	57.9	352 bp
Reverse	CTTCTTGGCCTCCTCCTTG	19	59.9	57.9	

A.5 Primers for hygromycin phosphotransferase gene for selection of positive transformation events

Primer Sequence	Length	Tm	%GC
GATGTTGGCGACCTCGTATT	20	56.5	50
GCGAAGAATCTCGTGCTTTC	20	56.1	50