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Proteomic Response of Rice Floral Organs and Lemma-palea to High Temperature Stress

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ABSTRACT

In rice, a brief period of 6 h of high temperature at flowering had a significant effect on reproductive processes. High temperature stress had a significant effect on the number of spikelets opening per day and spikelet fertility ($P<0.001$). High temperature stress inhibited normal anther dehiscence and pollination, thereby reducing spikelet fertility. 2D-PAGE analysis of control and high temperature-stressed floral organs of rice spikelets, *i.e.*, anthers (male), pistil (female reproductive organ), and lemma-palea, revealed that anthers had more proteins with significant changes in expression, followed by the lemma-palea. The pistil, on the other hand, had no significant changes in protein expression. This study provides evidence at the molecular level that the anther is the most responsive and sensitive floral organ to high temperatures, facilitating tissue-specific physiological and molecular studies to induce high temperature tolerance in rice varieties.

Keywords: anther, high temperature stress, lemma-palea, pistil, proteomics, rice

INTRODUCTION

Rice, the major staple food crop of the world, is mainly grown under tropical and sub-tropical environments where abiotic stresses such as drought, salinity, and, more recently, high temperature are becoming major factors that reduce yields. Global climate models predict an increase of 2.0 to 4.5°C in mean air temperature by the end of this century (International Panel on Climate Change 2007), with increased fluctuations around the mean. Accordingly, rice breeders will have to consciously select material capable of adapting to the gradually increasing mean temperatures while selecting for high grain yield. However, the major concern with high temperature in future climates will be the extreme fluctuations resulting in high temperature stress coinciding with critical developmental stages. Flowering is the most sensitive stage to high temperature stress, and temperatures >35°C even for an hour can lead to spikelet sterility (Yoshida *et al.* 1981; Jagadish *et al.* 2007). During the past two to three decades, emphasis has been given to understanding the physiological, genetic, and molecular basis of drought, salinity, and submergence tolerance in rice (Flowers *et al.* 2000; Bernier *et al.* 2007; Neeraja *et al.* 2007). In contrast, studies on high temperature in rice are relatively new (Prasad *et al.* 2006; Jagadish *et al.* 2010; Kobayasi *et al.* 2010) and this subject warrants further studies to develop rice varieties that can withstand extreme high-temperature fluctuations coinciding with the critical stages.

High temperature during the vegetative stage can cause damage to the leaf photosynthetic apparatus, thereby reducing carbon dioxide assimilation rates and membrane stability. However, these adverse effects can be reversed during the cooler hours of the evening and with the removal of stress. But high temperature coinciding with reproductive organ development, pollination, and fertilization results in irreversible change, leading to increased pollen and spikelet sterility. High temperature-induced sterility in rice is caused by floral abnormalities, such as stamen hypoplasia and pistil hyperplasia (Takeoka *et al.* 1991), poor anther dehiscence (caused by the tight closure of the locules), low pollen production, and a reduced number of germinating pollen grains on the stigma (Matsui *et al.* 2001; Matsui and Omasa 2002; Prasad *et al.* 2006; Jagadish *et al.* 2010). With the anther (male reproductive organ) receiving most emphasis, little information is available on the effect of high temperature on the female reproductive organ (pistil) and morphological structures such as the lemma-palea, which protects the anther and pistil from adverse climatic conditions. Yoshida *et al.* (1981), using reciprocal crosses, indicated that the female gametophyte had greater high temperature tolerance but no systematic studies have been carried out to understand the response of the pistil or the lemma-palea to high temperature stress. Hence, our study was undertaken to assess the effect of high temperature on the physiological functioning of the rice plant at anthesis and the proteomic responses of the floral organs, *i.e.*, anthers, pistil and lemma-palea.

MATERIALS AND METHODS

Plant material

Rice seeds (*Oryza sativa* cultivar 'IR 64') were pre-treated at 50°C for three days to break dormancy. Seeds were direct sown in seeding trays and 14 day-old-seedlings were transplanted into pots containing six kilograms of clay loam soil. Adequate basal fertilizer [2.0 g $(NH_4)_2SO_4$, 1.0 g KCL and 1.0 g SSP] was added before transplanting and an additional 2.5 g of urea $[(NH_2)_2CO]$ was added 30 days after transplanting. Plants were grown in a temperature-controlled glasshouse maintained at 29/21°C day/night temperature and relative humidity (RH) of 75% under natural sunlight conditions at the International Rice Research Institute (IRRI), Philippines. Plants were placed with 30-cm spacing on a bench to avoid shading effects. Ambient air temperature and RH were measured using thermocouples (Chessell 392, USA). The plants were maintained free of pest or disease problems.

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High-temperature treatment

Plants on the first day of anthesis were transferred into growth chambers (Thermoline, Australia) and exposed to 6 h of high temperature. Plants were transferred at 0800 with temperatures gradually increasing from 29 to 38°C (SD=0.15) by 0900 and being maintained at 38°C until 1500. The RH was maintained around 75% (SD=3.0).

Physiological observations and tissue sampling

At the end of the high temperature-stress period, flag leaves were collected from both control and stressed plants and relative water content (RWC) measured as described by Liu *et al*. (2006). Spikelets opening during the 6 h of high temperature stress and in control plants were marked with acrylic paint for identification and left till maturity under control conditions (Jagadish *et al.* 2007, 2008). Spikelet fertility was estimated following Prasad *et al.* (2006) and Mohammed and Tarpley (2009) by separating filled and unfilled grains. The ratio of filled grains to the total number of reproductive sites (spikelets) was estimated and expressed as a percentage. The number of filled grains included both completely and partially filled grains.

Protein extraction and 2D-PAGE analysis

At the end of high temperature-stress treatment, spikelets expected to open on the next day with both reproductive organs fully matured (located just below the spikelets opening on the day of the treatment) were collected from both control and high temperature-stressed plants and stored at -80°C till further use. Three biological replicates of anthers, pistil, and lemma-palea from the stored spikelets were dissected meticulously and used for protein extraction by trichloroacetic acid (TCA) precipitation as suggested by Jagadish *et al.* (2010). 2D-PAGE (2-dimensional polyacrylamide gel electrophoresis) separation of proteins and staining was carried out with minor modifications according to Jagadish *et al.* (2010). The protein spots in analytical gels were visualized by staining with silver nitrate according to Blum *et al.* (1987) and scanned using a GS-800 densitometer (Bio-Rad). Image visualization, spot detection, and protein quantification were carried out using Melanie 3 software (GeneBio, Geneva, Switzerland). The % volume was determined based on the area occupied and the intensity of the protein spot using the optimized parameters. Comparison between treatments across genotypes was done by calculating the abundance ratio of spots (% volume of spot in treated samples/% volume of spot in control samples) (Yan *et al.* 2005; Jagadish *et al.* 2010).

Statistical analyses

The effect of high temperature on all physiological parameters recorded including spikelet fertility were analyzed as completely randomized design using Genstat version 11 (Rothamsted Experimental Station, Harpenden, UK). Three replicate values for % volume of protein spot under control and stress conditions in anthers, pistil and lemma-palea were tested separately for significance using the same statistical package as above and expressed in abundance ratio (% volume of the protein under stress over control).

Table 1 Effect of high temperature stress on flag-leaf water content, peduncle length, spikelet opening, and spikelet fertility in rice. Values in parentheses are ±SD.

Trait	Control	Heat
Flag-leaf RWC (%)	97.95 (±1.01)	92.37 (±1.79)
Peduncle length (cm)	31.1 (±0.98)	29 (±1.91)
Total number of spikelets opened	48 (±8.7)	22.2 (±14.1)
Filled grains	45.6 (±7.07)	4 (±3.1)
Unfilled grains	3.1 (±2.3)	18.4 (±12.4)
Spikelet fertility (%)	93.6 (±2.7)	21.7 (±13.2)

RESULTS AND DISCUSSION

Physiological processes and spikelet fertility

A short 6-h high-temperature stress had a small, though significant effect on the relative water content of flag-leaf tissues of rice plants ($P<0.05$; **Table 1**). At this RWC only moderate impacts on stomatal opening, gas exchange, photosynthesis, and transpiration would be expected (Crafts-Brandner and Law 2000; Stone 2001).

Observations on anthers under control and high-temperature conditions revealed that anther dehiscence was found to be significantly affected during high-temperature stress (**Fig. 1**). Under control conditions, the basal and apical pore of anthers opened, allowing normal pollen shedding on the stigma to ensure successful pollination and fertilization (**Fig. 1A**). All the anthers under control conditions appeared white and transparent, indicating no retention of pollen grains within 1 h of anthesis (**Fig. 1B**). However, high temperature significantly affected the basal and apical pore opening, resulting in retention of pollen grains within the anthers even after successful anthesis (spikelet opening), with the appearance of fully or partially yellow anthers (**Fig. 1C**). Jagadish *et al.* (2010) reported that, in susceptible rice cultivar 'Moroberekan', high temperature stress causes significantly high expression of dirigent proteins, mainly responsible for lignin biosynthesis, to strengthen/repair damaged cell walls acting as physical barriers. This may have led to increased lignification, preventing anther dehiscence. In N22, a high temperature-tolerant rice cultivar, the same protein was absent, allowing pollination. Similar effects of high temperature leading to abnormal anther dehiscence have been documented (Matsui and Omasa 2002; Jagadish *et al.* 2010).

Liu *et al.* (2006), exposed 'IR64' anthers to drought stress followed by re-watering, also documented the failure of the basal pore to open. They also observed that most of the pollen failed to exit through the open apical pores because of stress-induced adherence of pollen to anthers,

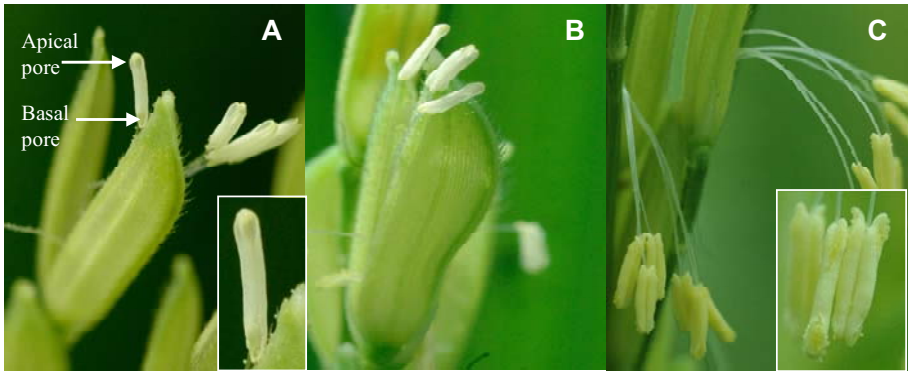

Fig. 1 Anther dehiscence behavior under normal and high-temperature conditions in rice cultivar 'IR64'. (**A**) Opening of basal and apical pores under control conditions; (**B**) white and transparent anthers showing complete pollen dispersal under control temperature; (**C**) failure of pore opening under high-temperature conditions (inset showing increased pollen stickiness and poor pollen dispersal even after normal anther dehiscence).

Table 2 Differentially expressed proteins (% volume of spot) within different floral organs, *i.e.*, anthers (A), pistil (P) and lemma-palea (LP), under control and high-temperature stress and the mean abundance ratio (ratio of the % volume of the protein under high temperature over control). Variation in protein expression level is given as ±SE (n=3). Protein spots significantly up-regulated are in bold while the down-regulated ones are in normal font and spots not indicated with asterisk were not significant. *, **, *** indicate significance at 5%, 1%, and 0.1%, respectively.

	% Volume of spot		Mean
	Control	Heat	abundance ratio
LP1	0.618 (±0.014)	0.378 (±0.086)	0.612
LP2	0.225 (±0.016)	0.098 (±0.020)	0.434*
LP3	0.109 (±0.007)	0.245 (±0.012)	**2.246****
LP4	0.228 (±0.101)	0.162 (±0.033)	0.710
LP5	0.093 (±0.029)	0.119 (±0.007)	1.279
LP6	0.203 (±0.024)	0.111 (±0.023)	0.548
P1	0.233 (±0.033)	0.183 (±0.047)	0.789
P2	0.169 (±0.056)	0.169 (±0.046)	0.996
P3	0.150 (±0.009)	0.274 (±0.054)	1.829
P4	0.110 (±0.006)	0.091 (±0.009)	0.828
P5	0.418 (±0.106)	0.306 (±0.033)	0.731
P6	0.181 (±0.104)	0.319 (±0.109)	1.759
P7	0.223 (±0.100)	0.195 (±0.050)	0.872
P8	0.210 (±0.058)	0.140 (±0.020)	0.665
P9	0.491 (±0.085)	0.468 (±0.024)	0.952
P10	0.107 (±0.043)	0.103 (±0.034)	0.960
P11	0.146 (±0.024)	0.113 (±0.018)	0.771
P12	0.164 (±0.015)	0.179 (±0.007)	1.089
P13	0.217 (±0.013)	0.256 (±0.015)	1.181
P14	0.075 (±0.001)	0.106 (±0.012)	1.410
P15	0.098 (±0.013)	0.113 (±0.027)	1.153
P16	0.205 (±0.046)	0.117 (±0.020)	0.573
P17	0.208 (±0.085)	0.135 (±0.057)	0.646
P18	0.179 (±0.042)	0.191 (±0.005)	1.066
A1	0.140 (±0.022)	0.195 (±0.015)	1.396
A2	0.252 (±0.022)	0.221 (±0.009)	0.878
A3	0.279 (±0.010)	0.339 (±0.008)	**1.218****
A4	0.347 (±0.023)	0.269 (±0.005)	0.776*
A5	0.230 (±0.023)	0.372 (±0.046)	**1.616***
A6	0.109 (±0.014)	0.023 (±0.004)	0.214**
A7	0.151 (±0.033)	0.001 (±0.000)	0.006*
A8	0.155 (±0.052)	0.271 (±0.024)	1.743
A9	0.176 (±0.014)	0.115 (±0.015)	0.654*
A10	0.138 (±0.012)	0.102 (±0.002)	0.741*
A11	0.097 (±0.016)	0.198 (±0.023)	**2.043***
A12	0.148 (±0.012)	0.091 (±0.011)	0.617*
A13	0.090 (±0.000)	0.168 (±0.017)	**1.864****
A14	0.288 (±0.010)	0.251 (±0.013)	0.870
A15	0.300 (±0.059)	0.244 (±0.014)	0.813
A16	0.007 (±0.003)	0.075 (±0.015)	**10.789****
A17	0.130 (±0.019)	0.208 (±0.015)	**1.606***
A18	0.098 (±0.018)	0.148 (±0.009)	1.510
A19	0.088 (±0.004)	0.042 (±0.003)	0.476***

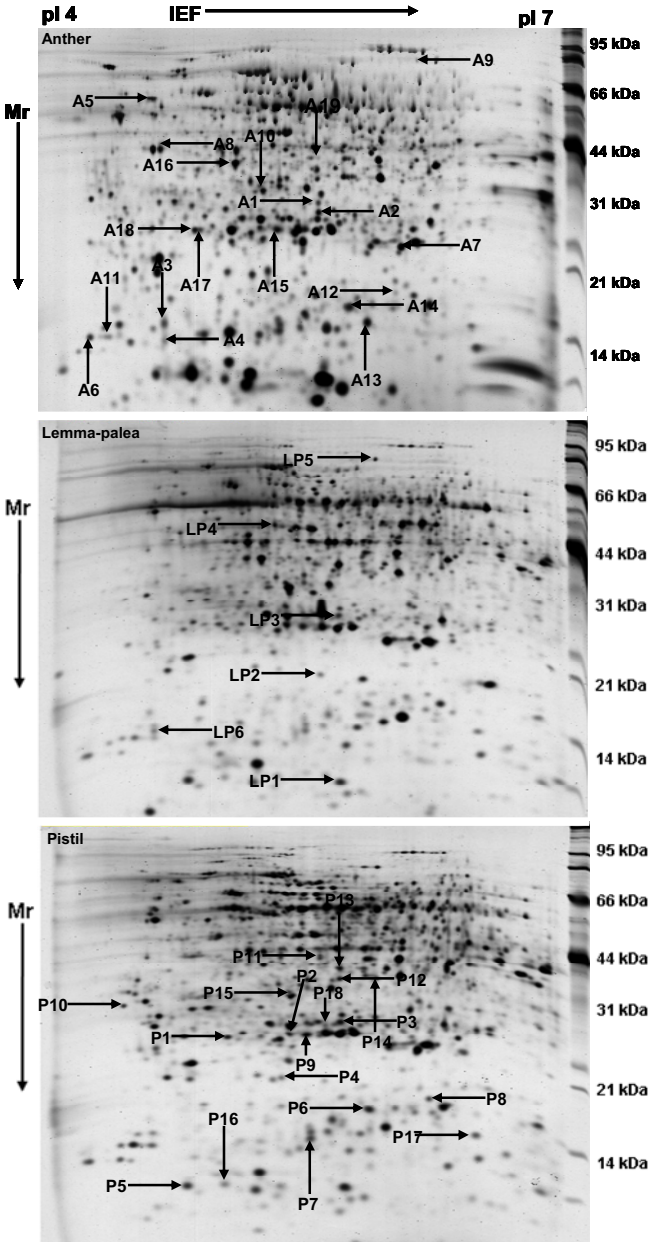

Fig. 2 Representative 2D gel electrophoresis of protein spots differentially expressed in anthers, lemma and palea, and pistil. Differentially changing protein spots are highlighted using arrows. First-dimensional focusing (IEF) was done by using 17-cm IPG strips with a pH 4-7 loaded with 100 µg of total spikelet protein. In the second-dimension SDS-PAGE, 12% gels were used. Experimental molecular weight was fixed by internal markers identified earlier with the sample and indicated by Mr.

termed "pollen stickiness," and attributed this adhesion to an increase in glycoprotein content in the pollen. In some of the high temperature-stressed anthers, a similar phenomenon was observed (**Fig. 1C**, see inset), indicating that successful anther dehiscence is not necessarily correlated with pollen count on the stigma. Presently, information related to such changes and the effect of an asynchronous pollination and stigma receptivity are the missing links in understanding the effect of high temperature at anthesis in rice genotypes (Jagadish *et al.* 2010).

Elevated temperature did not show any significant inhibitory effect on peduncle length ($P>0.05$; **Table 1**) but it had a significant effect on the number of opened spikelets and spikelet fertility ($P<0.05$). The number of spikelets that opened on the day the high temperature stress was imposed declined by up to 50%, which in turn could extend the flowering period. Similar responses have been documented previously (Jagadish *et al.* 2007), indicating a probable escape strategy to overcome the high-temperature periods. Hence, flowering pattern should be considered while phenotyping for high temperature tolerance at flowering stage. In addition, the 6-h high-temperature stress significantly reduced spikelet fertility by 77% compared with the control ($P<0.001$) (**Table 1**). Reduced spikelet fertility due to improper anther dehiscence and failure of pollen tube growth has already been reported in rice (Jagadish *et al.* 2010), wheat (*Triticum aestivum*; Saini *et al.* 1983; Ferris *et al.* 1998), and maize (*Zea mays*; Stone 2001).

Proteomic response of floral organs and lemma-palea to high temperature stress

For successful seed set under high-temperature conditions, pollen must be viabile, stigmas receptive, and pollen tubes growing normally to reach and fertilize the ovules. With a

view to assess the impact of high-temperature stress on different floral organs of rice spikelets, protein profiling was done in control and high-temperature-stressed floral organs of rice, the anther, pistil, and lemma-palea, by the 2D-PAGE approach.

Proteomic analysis of the lemma and palea detected about 300 protein spots that gave reproducible silver staining patterns over a pH range of 4–7, with molecular weight ranging from 10 to 100 kDa (**Fig. 2**). Six proteins showed differential expression in lemma-palea during high-temperature stress, out of which two proteins were found to change significantly with one up-regulated and the other down-regulated (**Table 2**). Profiling proteins expressed in the female reproductive organs (ovary, style, and stigma) detected around 400 spots showing a reproducible spotting pattern (**Fig. 2**). Measuring the abundance of spot intensity of differentially expressed proteins between control and high-temperature-stressed ovary revealed 18 spots with differential expression but none of the spots varied significantly in expression in response to high temperature stress (**Table 2**). Physiologically, Yoshida *et al.* (1981), using cross pollination between high temperature-stressed pollen and stigma with pollen from control plants, recorded no effect of high temperature stress (38°C) on the functioning of the pistil. Similarly, wheat exposed to high-temperature stress capable of causing male sterility had no damaging influence on the functions of female reproductive organs, suggesting that the female gametophyte had greater tolerance to high temperatures (Saini and Aspinall 1982).

Proteomic analysis of high-temperature responsiveness in rice anthers revealed the differential expression of 19 protein spots, of which six were significantly up-regulated and seven down-regulated (**Table 2**). This clearly supports the observations made in the previous studies that the main cause of floret sterility induced by high-temperature stress at flowering in rice is the abnormal functioning of the anther and pollen (Matsui *et al.* 2001).

CONCLUSIONS

Results of this study indicated that high-temperature stress had a significant effect on the reproductive processes and floral tissue protein expression in rice. Among the floral organs and lemma-palea, the anther was shown to be more vulnerable to high-temperature injury than the female reproductive organs. Hence, this study provides evidence at the molecular level that the female reproductive organ in rice has a much higher critical level of high temperature-stress tolerance than the anthers. Future anatomical, physiological, and molecular studies involving anthers, pistils, and pollen-pistil interaction under high-temperature stress will lead to the identification of candidate genes or metabolic pathways involved in high temperature tolerance.

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