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Original Paper

Generation of transgenic rice expressing heat shock protein genes under cool conditions

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Abstract The heat shock response of rice, including expression of heat shock transcription factors (*Hsf*s), was investigated to elucidate the molecular regulation of its high-temperature tolerance. In silico analysis revealed that the rice genome encodes more than 19 species of *Hsf* genes that can be organized into three classes, A, B, and C. Rice seedlings treated with high temperature, express three class A *Hsf* genes (*HsfA2a*, *HsfA2c*, and *HsfA2d*) and two class B *Hsf* genes (*HsfB2b* and *HsfB2c*) at significantly elevated levels. Transgenic rice plants overexpressing these three class A *Hsf* genes controlled by the rice *actin 1* promoter or the wheat cold response (*WCR*) promoter expressed the transgenes, but did not express heat shock response genes such as *small HSP*s, whose expression is controlled by *Hsf*s. Treatment with geldanamycin, an inhibitor of HSP90, elevated the expression of *HSP*s in the *WCR::Hsf* transformant. Therefore, transgenic rice co-expressing *WCR::Hsf*s and a dominant negative HSP90 mutant were generated in which the heat shock response could be induced under *WCR* promoter-activating conditions. Successful induction of the heat shock response under cool conditions in this co-expression line suggests that HSP90 controls the heat shock response via the activity of *Hsf* in rice cells.

Key words: Heat shock factor, heat shock response, HSP90, transgenic rice.

Rice is a staple food in East Asia and comprises a large part of the daily diet of half of the people on earth. Therefore, stable rice yields are very important. Towards this goal, crops have been selected for environmental stress tolerance, including tolerance to extreme temperature, desiccation or salt tolerance. In fact, the rice-growing region has been spreading northward in Asia due both to global warming and the enhancement of cold tolerance through selective breeding. It will continue to be very important to confer stress tolerance to rice due to the broadening of growing regions and to enhance rice yields. Plants have developed mechanisms for dealing with environmental stress because they cannot physically avoid suboptimal environments, such as those with extreme temperatures, aridity, flooding, or chemical toxicity such as that in high salinity regions, or move to more optimal environments. To date, numerous experiment have been conducted to elucidate these stress tolerance mechanisms, and some transgenic plants that are highly tolerant to stresses such as low temperature, drought, or salt have been developed (Gao et al. 2011; Li et al. 2011; Xu et al. 2011).

Heat shock transcription factors (*Hsf*s) play a central role in the heat shock response. In fact, there are several reports describing high temperature tolerance conferred by *Hsf*s (Lee et al. 1995; Liu et al. 2009; Lohmann et al. 2004; Mishra et al. 2002; Ogawa et al. 2007; Prändl et al. 1998; Yokotani et al. 2007). Higher plants contain coding sequences for many more *Hsf*s in their genomes than do animals or *Chlamydomonas*. Mammals have three *Hsf* genes, whereas *Chlamydomonas* has only two *Hsf* genes, and only one of these (*Hsf1*) acts in the thermoresistance response (Morimoto 1998; Schulz-Raffelt et al. 2007). Yeast and *Drosophila* have only a single *Hsf* each; however, *Arabidopsis*, rice, and tobacco have at least 21, 25 or 26, and 17 *Hsf* genes, respectively (Baniwal et al. 2004; Guo et al. 2008; Mishra et al. 2002; Mittal et al. 2009; von Koskull-Döring 2007). This high number of *Hsf* genes in higher plants suggests that plants have a complex and highly regulated system by which they cope with high temperatures and survive in a broader temperature range than animals or *Chlamydomonas*.

To assess the functions of individual *Hsf*s, several transgenic approaches have been undertaken. Transgenic *Arabidopsis* expressing *AtHsf1* fused to *GUS* under the control of the CaMV *35S* promoter constitutively expressed *HSP18* and *HSP70* at approximately 20% of the maximum heat-inducible levels, and the *AtHsf1*

Abbreviations: HSP, heat shock protein; HSF, heat shock transcription factor; WCR, wheat cold response; GDM, geldanamycin.
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gene conferred basic thermotolerance via induction of heat-shock responsive genes (Lee et al. 1995). In contrast, overexpression of *Hsf3*, but not overexpression of *Hsf4*, in *Arabidopsis* derepressed the expression of several small HSPs (sHSPs) and led to an elevated basal thermotolerance (Prändl et al. 1998). However, there have been no prior reports analyzing the effects of overexpressing *Hsf*s in transgenic rice.

Rice is well known to be relatively tolerant to high temperatures because it originated in a tropical region; however, rice seedlings can also exhibit greater cold tolerance following a transient exposure to high temperature (Sato et al. 2001). This observation suggests that the heat-shock inducible genes also function to help plants endure cold temperatures. Recently, overexpression of *Arabidopsis AtHsfA2* or rice *OsHsfA2e* conferred not only increased thermotolerance, but also increased salt-stress tolerance (Ogawa et al. 2007; Yokotani et al. 2007). Because the heat shock response confers tolerance not only to high temperature stress but also to other abiotic stresses such as low temperature or salt stress due to crosstalk among stress signaling pathways, elucidating the mechanism for high temperature response in rice will facilitate the development of crops tolerant to multiple abiotic stresses.

To investigate the induction mechanism for the heat shock response in rice, we generated transgenic rice overexpressing *Hsf*s; however, these transformants did not induce the expression of *sHSP*s, genes that are typically up-regulated during the heat shock response. When the seedlings of *WCR::Hsf* transformants were treated with 50 μM geldanamycin (GDM), an inhibitor of HSP90, the expression levels of *sHSP*s were higher than those of the non-transformant. These results strongly suggested that induction of the heat shock response in rice is controlled by HSP90 activity (Yamada et al. 2007). Therefore, we generated transgenic rice overexpressing *Hsf*s in a dominant negative *HSP90* mutant. These transformants induced the expression of many *sHSP*s under cool conditions that could activate the *WCR* (Wheat cold response) promoter. These results suggest that the heat shock response in rice is controlled by the activity of HSP90 and that the response in rice cells is more complicated than in *Arabidopsis*, a plant that originated in a temperate region. This is the first report of inducing the heat shock response under cool conditions.

Materials and methods

Expression analysis of Hsfs and transgenes in rice seedlings by RT-PCR

The full-length cDNA database at the Knowledge-Based *Oryza* Molecular Biological Encyclopedia (http://cdna01.dna.affrc.go.jp/cDNA/) was searched for full-length cDNA sequences of rice *Hsf*s and primer sets for each rice *Hsfs* were designed using Primer Express software Version 3.0 (Applied Biosystems).

Sterilized rice seeds (*Oryza sativa*, cv. Oboroduki) were germinated and grown for 10 days on 1/2 MS (Murashige and Skoog Plant Salt Mixture, Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) solid medium containing 0.3% Gelrite (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The seedlings were transferred to an incubator at 42°C for 10 min, 60 min, 120 min, or 300 min. The treated seedlings were homogenized under liquid nitrogen and RNA was extracted with RNAiso Plus (TAKARA, Kyoto, Japan). DNA in the samples was digested by DNaseI (Promega, Madison, WI) and then extracted with phenol : chloroform (1 : 1). Equal amounts of purified RNA were reverse-transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA), and the products were used as templates for second-strand synthesis, followed by quantitative PCR analysis. The quantitative PCR was performed with a 7300 Real Time PCR system (Applied Biosystems) using the Power SYBR Green PCR Master Mix (Applied Biosystems). Gene-specific primer sequences for *ubiquitin*, the *sHSP*s, the *Hsf*s, and *HSP90* (Table S1) were designed using Primer Express software Version 3.0. The primer set used to amplify each of the *sHSP*s was designed in the highly conserved region of *HSP16.9* (AK121025, AK242299), *HSP17.4* (AK119717, AK073671, AK119239), and *HSP18.0* (AK119664). *Ubiquitin* mRNA was used as an internal control in all experiments. Expression values are the averages of three independent experiments $\pm$ *SD*.

Overexpression of Hsf genes and mutated HSP90 in rice transformants

Hsf genes (*HsfA2a*, AK069579; *HsfA2c*, AK072391; *HsfA2d*, AK066844) that are expressed in rice seedlings treated at high temperature were cloned and fused downstream of the rice *actin 1* or *WCR* promoters (AB676782). The promoter::*Hsf* cDNAs with an added *Nos* terminator sequence were inserted between both border sequences of pBIN19 in which the antibiotic-resistance gene had been replaced with a hygromycin-resistance gene under control of the CaMV *35S* promoter. The double *WCR::Hsf* transformant was developed by crossing the *WCR::HsfA2a* and *WCR::HsfA2c* transformants. A dominant negative mutant of *HSP90* (AK061896, a candidate for the *Arabidopsis HSP90.2* homologue in rice (Yamada et al. 2007) was created in which Asp^{80} was converted to Asn^{80} by PCR (Pnaretou et al. 1998), and was designated the *HSP90-D80N* gene. *HSP90-D80N* was fused downstream of the maize *ubiquitin* promoter, and the promoter::*HSP90-D80N* with an added *Nos* terminator were then inserted between both border sequences of the modified pBIN19. Transformation of *Agrobacterium tumefaciens* (strain EHA105) with the binary vector, inoculation of explants with the transformed *Agrobacterium*, and regeneration of transformed calli were conducted according to the method of Goto et al. (1999).

The *WCR::HsfA2a*/*WCR::HsfA2c* transformant was generated by crossing the *WCR::HsfA2a* transformant with the *WCR::HsfA2a* transformant. The *WCR::HsfA2a*/*WCR::*

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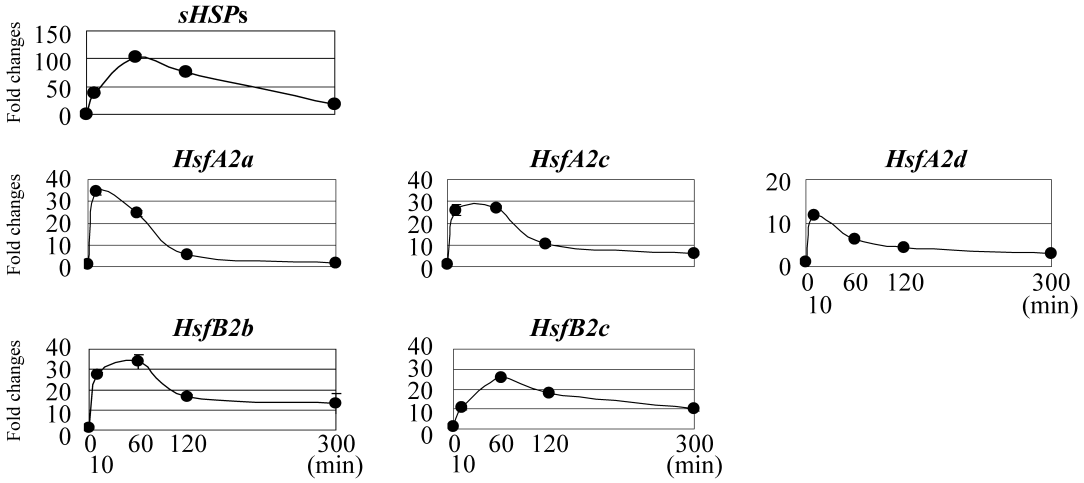

Figure 1. Expression levels of rice *Hsf*s under high temperature conditions. Total RNA was isolated from 10-day-old seedlings that were treated at 42°C for 10, 60, 120, or 300 min. The levels of each gene transcript were measured using quantitative RT-PCR. The values are normalized using *ubiquitin* mRNA as an internal control. The relative quantity of the untreated control was defined as 1. Values are averages of three independent experiments ± *SD*.

HsfA2c/*HSP90-D80N* transformant was generated by crossing the *WCR::HsfA2a*/*WCR::HsfA2c* transformant with the *HSP90-D80N* transformant.

Treatment with geldanamycin

Sterilized seeds of the *WCR::Hsf*s transformant were germinated and grown on 1/2 MS solid medium containing 0.3% Gelrite at 27°C. Ten-day-old seedlings were removed from the solid medium and treated with fresh 1/2 MS liquid medium containing 50 μM GDM (Tokyo Chemical Industry Co., Ltd., Japan) at 12°C overnight (ca. 16 h) followed by incubation at 27°C for 30 min. After this treatment, the expression level of transgenes and heat shock response genes were assessed according to the procedure as described above.

Microarray analysis

Microarray analysis was performed according to the manufacturer's instructions (Agilent Technologies, Palo Alto, CA). Total RNAs were prepared from leaves of wild type, *HSP90-D80N*, *WCR::HsfA2a*/*WCR::HsfA2c*, and *WCR::HsfA2a*/*WCR::HsfA2c*/*HSP90-D80N* transformants (the transformants carrying the *WCR* promoter were treated at 12°C overnight). The extracted RNAs were used to prepare reciprocally Cy3- or Cy5-labelled cRNA using the Quick Amp Labeling Kit (Agilent Technologies). Equal volumes of the two fluorescently labeled cRNAs were mixed and hybridized with the Agilent 44K Rice Oligo DNA Microarray RAP-DB (Agilent Technologies). The hybridized slides were scanned using a DNA microarray scanner (Agilent Technologies). Signal intensities were measured by feature extraction software (Agilent Technologies). The *HSP*s that are up-regulated more than 10-fold higher in the transformants than in the wild type are listed in Table S2–S5.

Results

Expression profiles of rice Hsfs

We searched for *Hsf* genes in the rice full-length cDNA database (Knowledge-Based *Oryza* Molecular Biological Encyclopedia) and found 19 *Hsf* genes that are expressed in rice. When the expression of these 19 *Hsf* genes was examined in high-temperature treated (42°C) rice seedlings, only five *Hsf* genes (three genes from *Hsf* class A and two genes from class B) were highly induced (5-fold higher expression than the control) by the high-temperature treatment (Figure 1). The expression levels of the three class A *Hsf*s (*HsfA2a*, *HsfA2c* and *HsfA2d*) increased significantly within 10 min; however, *HsfB2b* and *HsfB2c* expression levels increased continuously for up to 60 min after the high temperature treatment. The highest expression levels of four of the *Hsf*s (*HsfA2a*, *HsfA2c*, *HsfB2b*, and *HsfB2c*) were 25- to 35-fold higher than those of the non-treated control. Heat shock response genes such as *sHSP*s (*HSP16.9*, *HSP17.4* and *HSP18.0*) were also induced, followed by the expression of *Hsf* genes (Figure 1).

Overexpression of HsfA2a, HsfA2c, and HsfA2d genes

To further investigate the function and mechanism of the heat shock response, we generated transgenic plants that overexpress the *HsfA2a*, *HsfA2c*, and *HsfA2d* genes under the control of the rice *actin 1* promoter. The expression of each introduced gene was examined in 10-day-old seedlings of transformants in the T2 generation without heat shock treatment. The expression levels of *HsfA2c* and *HsfA2d* in each *act::Hsf* transformant were approximately 10-fold and 6.2-fold higher than those of non-transformants, respectively (Table 1). The expression

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level of *HsfA2a* in the *act::HsfA2a* transformant was only slightly higher (2.6-fold) than that of the non-transformants (Table 1). Further, the expression levels of *sHSP*s in the *act::Hsf* transformants were also analyzed by quantitative PCR. The heat shock response genes were never induced in the seedlings of the *act::Hsf* transformants without prior heat shock treatment (Table 1).

Induction of heat shock response genes was not observed in any of the *act::Hsf* transformants. Next, we generated transformants expressing the *HsfA2a*, *HsfA2c*, and *HsfA2d* genes under the control of a cold-inducible promoter originating from wheat (*WCR*, wheat cold response promoter). The *WCR* promoter is a stronger promoter than the rice *actin 1* promoter when plants are treated with cold temperature (below 16°C). In the *WCR::HsfA2c* transformants, the expression level of *HsfA2a* and *HsfA2c* were nearly 13-fold and 180-fold higher than that of the non-transformants, respectively, when seedlings of the transformant were treated at 12°C overnight (Table 1). In the *WCR::HsfA2a* and *WCR::HsfA2d* transformants, expression levels of *HsfA2a* and *HsfA2d* were nearly 10-fold and 20-fold higher than those of the non-transformants, respectively (Table 1). The expression levels of *HsfA2c* and *HsfA2d* in the transformants treated overnight at 12°C were higher than the expression levels in non-transformants treated at 42°C for 30–60 min (compare Figure 1 to Table 1).

We examined whether the heat shock response genes were induced in *WCR::Hsf* transformants that had been treated overnight at 12°C and then moved to 27°C for 30 min in order to maximize translation of *Hsf* genes and transcription of *sHSP*s. Under these conditions, the expression of *sHSP*s was barely induced, even when individual *Hsf* genes were highly expressed (Table 1).

We generated a double *WCR::Hsf* transformant by crossing *WCR::HsfA2a* with *WCR::HsfA2c* transformants since high temperature treatment of the wild type induced higher levels of *HsfA2a* and *HsfA2c* genes than that of the *HsfA2d* gene (Figure 1). When these transformants were treated overnight at 12°C (Table 2), the *HsfA2a* and *HsfA2c* genes were induced in each transformant. We examined whether the heat shock response was induced in the *WCR::HsfA2a*/*WCR::HsfA2c* double transformants treated under the same conditions and found that the heat shock response genes were slightly induced (Table 2). However, the induction level observed under cooler conditions was weaker than when the seedlings were treated at 42°C (Figure 1).

Geldanamycin treatment

Geldanamycin (GDM) is an antibiotic that induces the heat shock response in the absence of a heat shock treatment. GDM acts as an inhibitor of HSP90 that may inhibit the interaction between HSP90 and HSF (Yamada et al. 2007). To investigate the interaction between HSP90 and the introduced HSFs, we treated the *WCR::Hsf* transformants with GDM.

We assessed the expression level of *sHSP*s in 10-day-old seedlings of *WCR::Hsf* transformants that were incubated in 1/2 MS medium containing 50 μM GDM with the cold treatment (12°C overnight) to activate the *WCR* promoter. The transcript levels of the *HsfA2a*, *HsfA2c* and *HsfA2d* genes in the GDM treated *WCR::HsfA2a*, *WCR::HsfA2c* and *WCR::HsfA2d* transformant were 20.52±7.6-, 33.33±3.79- and 13.71±

Table 1. Relative transcript abundance of *Hsf* genes and *sHSP*s in the *act::Hsf*s and *WCR::Hsf*s transformants.

	HsfA2a	*HsfA2c*	*HsfA2d*	*sHSPs*
act::HsfA2a	2.61±0.57	0.89±0.16	0.85±0.20	1.39±0.35
act::HsfA2c	1.51±0.30	10.07±0.30	1.03±0.08	1.76±0.10
act::HsfA2d	2.87±1.34	1.86±1.35	6.19±3.38	1.82±0.98
WCR::HsfA2a	9.78±7.45	1.14±0.78	0.71±0.35	0.78±0.06
WCR::HsfA2c	12.91±7.71	183.8±67.7	0.96±0.35	2.20±0.54
WCR::HsfA2d	3.42±0.63	1.58±0.54	21.93±3.22	0.97±0.26

Transcript abundance was normalized to the expression level of *ubiquitin* and compared with transcript abundance in the non-transformant from same treatment that was defined as 1. Relative transcript abundance of *HsfA2a*, *HsfA2c* and *HsfA2d* in 10 days old seedlings of the *WCR::Hsf* transformants were measured after an overnight treatment at 12°C. The relative transcript abundance of *sHSP*s was measured after an overnight treatment at 12°C and then 27°C for 30 min. Values are averages of three independent experiments±*SD*.

Table 2. Relative transcript abundance of *Hsf* genes, *HSP90* and *sHSP*s in the *WCR::HsfA2a*/*WCR::HsfA2c and WCR::HsfA2a*/*WCR::HsfA2c*/*HSP90-D80N* transformants.

	HsfA2a	*HsfA2c*	*HSP90* (*including HSP90-D80N*)	*sHSPs*
WCR::HsfA2a/*WCR::HsfA2c*	5.64±3.60	9.56±1.59	0.80±0.27	8.50±1.44
WCR::HsfA2a/*WCR::HsfA2c*/*HSP90-D80N line 1*	21.55±10.53	21.76±3.49	2.93±1.27	77.20±5.94
WCR::HsfA2a/*WCR::HsfA2c*/*HSP90-D80N line 9*	9.36±1.45	7.43±2.54	1.94±0.54	20.61±1.56

Transcript abundance was normalized to the expression level of *ubiquitin* and compared with transcript abundance in the non-transformant from same treatment that was defined as 1. Relative transcript abundance of *HsfA2a*, *HsfA2c* and *HSP90-D80N* (*including HSP90-D80N*) in 10 days old seedlings of the triple transformants was measured after an overnight treatment at 12°C. Values are averages of three independent experiments±*SD*.

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3.93-fold higher than the same treatment of wild type, respectively.

In the GDM-treated *WCR::HsfA2a*, *WCR::HsfA2c*, and *WCR::HsfA2d* transformants incubated overnight at 12°C, the expression of *sHSP*s was induced to 8-, 9-, and 5-fold higher levels, respectively, than that of the non-transformant (Figure 2). These results suggest that *HsfA2a* and *HsfA2c* are important for induction of *sHSP* expression and that HSP90 is also a factor responsible for induction of *sHSP*s.

Co-expression of Hsf genes and HSP90-D80N

The results of GDM treatment strongly suggested that the heat shock response in rice is controlled by the activity of HSP90. Therefore, we generated transgenic rice overexpressing the dominant negative mutant of *HSF90* (*HSP90-D80N*). Some of the HSF90-D80N transformants in the T0 generation had dwarf phenotypes and were not selected for further investigation. None of the selected lines in the T2 generation had dwarf phenotypes or alterations in leaf number or seed fertility problems when plants were grown in the glasshouse. The expression level of *HSP90* in the *HSP90-D80N* transformant was 2.79±1.17-fold higher than that of wild type. The *HSP90-D80N* transformant was crossed with the *WCR::Hsf* transformant to generate the *WCR::HsfA2a/WCR::HsfA2c/HSP90-D80N* transformant, which was expected to induce the expression of *sHSP*s after exposure to cool (12°C) temperatures. The expression levels of *HSP90* in the transformants and wild type were not elevated by the cool temperature treatment.

Microarray analysis was used to assess the expression of *sHSP*s in the *HSP90-D80N*, *WCR::HsfA2a/WCR::HsfA2c*, and *WCR::HsfA2a/WCR::HsfA2c/HSP90-D80N* transformants. We found that overexpression of *HSP90-D80N* alone did not induce the heat shock response in the transformant (Figure 3), as only one up-regulated gene (heat shock protein DnaJ family protein) with >10-fold elevated expression was detected (Table S2). On the other hand, the expression of seven *HSPs* was induced 10-fold over that of wild type in the cool-temperature treated *WCR::HsfA2a/WCR::HsfA2c* transformant (Figure 3, Table S3).

When gene expression was compared between cool temperature-treated wild type and the *WCR::HsfA2a/WCR::HsfA2c/HSP90-D80N* transformants, many *HSP* genes, including high molecular-weight *HSP*s, were induced in the transformants (Figure 3, Table S4, S5). The expression levels of *HSP*s in the *WCR::HsfA2a/WCR::HsfA2c/HSP90-D80N* transformants were higher and the number of species of *HSP*s whose expression was induced was greater than in the *WCR::HsfA2a/WCR::HsfA2c* transformant. These results clearly suggested that the heat shock response could be induced by cool conditions in rice cells in this system (Figure 2, Table S4, S5) and that *Hsf*s under the control of HSP90 are necessary for the expression of the heat shock response in rice cells.

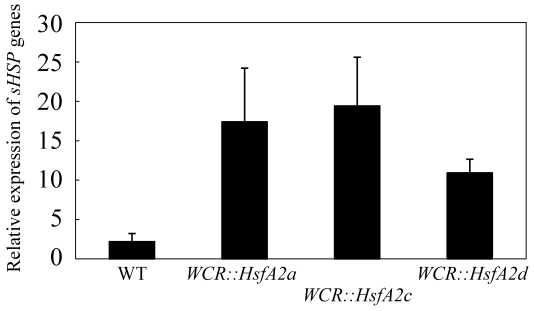

Figure 2. Expression levels of *sHSP*s in GDM-treated *WCR::Hsf*s transformants. The 10-day-old seedlings were incubated with 50 μM GDM according to the procedure outlined in Materials and methods. The values were normalized to the expression level of *ubiquitin* mRNA as a standard. The value for the non-transformant (WT) without GDM treatment was defined as 1. Values are averages of three independent plants±*SD*.

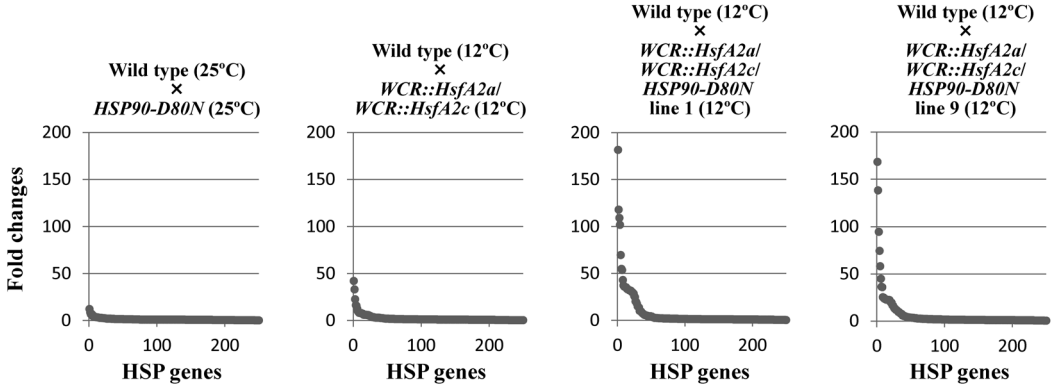

Figure 3. Expression levels of all *HSP*s on rice DNA oligo microarrays in normal (25°C) and cool temperature (12°C)-treated transformants. The methods used for microarrays are described in Materials and methods. The value for the *HSP*s of wild type under same temperature condition was defined as 1. The horizontal axis indicates the spots of *HSP*s on the array in descending order of fold change, and the combination of microarray experiments is described above the chart.

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Discussion

We detected at least 19 *Hsf*s in the rice full-length cDNA database and found that these *Hsf*s could be divided into three classes, based on whether the HR-A/B region contained an insertion of either 21 (class A), 0 (class B), or 7 (class C) amino acid residues between the A and B regions (Nover et al. 2001). In general, the rice genome encodes at least 26 *Hsf*s, including one variant that lacks a DNA-binding domain, a nuclear localization signal, a nuclear export signal, and an AHA-type activation domain (Guo et al. 2008). Twenty-two of these 26 *Hsf*s were induced throughout the rice plant by heat shock treatment. When 10-day-old seedlings were heat shocked, at least 16 *Hsf*s were up-regulated by >2-fold (Mittal et al. 2009). In our study, the expression levels of only five *Hsf*s (*HsfA2a*, *HsfA2c*, *HsfA2d*, *HsfB2b*, and *HsfB2c*) increased more than 5-fold in response to high temperature, and some other *Hsf*s such as *HsfA2b*, *HsfA2e*, *HsfA4b*, *HsfA4d*, *HsfA7*, *HsfA9*, *HsfB4b*, and *HsfC2a*, were slightly up-regulated from 2- to 5-fold in the seedlings treated after high temperature treatment (data not shown). Based on their high level of induction in response to high temperature, these five *Hsf*s (Figure 1) seem to be chiefly responsible for the expression of the heat shock response genes in rice seedlings.

HsfA2a, *HsfA2c*, and *HsfA2d* responded to high temperature rapidly, within 10 min. Similar results were reported by Liu et al. (*HsfA2c* (Liu et al. 2005), *HsfA2d* (Liu et al. 2010)), and the expression of *HsfA2c* reached a maximum level within 5 min after high temperature treatment. For class B *Hsf*s, *HsfB2b* and *HsfB2c*, the level of expression continued to increase until 60 min after the high temperature treatment. These results suggest that *HsfA2a*, *HsfA2c*, and *HsfA2d* play an important role in the initial reaction to the high temperature response, and that *HsfB2b* and *HsfB2c* play a role in prolonging or intensifying the response to the treatment.

In tomato, *HsfA1a* (*LeHsfA1a*) acts as the "master regulator" of the heat stress response (Mishra et al. 2002), whereas, in *Arabidopsis*, at least two or more *HsfA1s* play the master regulator role despite similarities in the composition of the *Hsf* families of tomato and *Arabidopsis* (Liu et al. 2011; Nishizawa-Yokoi et al. 2011; Yoshida et al. 2011). To date, the master regulator of the heat stress response in rice has not been identified. The difficulty in identifying a master regulator may suggest that the heat stress responses in rice are more complex than that of tomato, in which the heat shock response is regulated by one gene (*LeHsfA1a*). Our results are good agreement with the suggestion that the expression of *sHSP*s were slightly induced in the double transformants containing *WCR::HsfA2a* and *WCR::HsfA2c* at the low temperature, although *sHSP*s were not induced in the single transformants containing the *WCR::HsfA2x* gene under identical conditions (Table 2). These results also indicated that the mechanism for induction of the heat shock response in rice is similar to that in *Arabidopsis*, but the species of *HsfA* is different. To elucidate this complex mechanism in rice, we generate transformants that simultaneously overexpressed multiple *Hsf*s.

The function of *Hsf*s as transcriptional activators is based on the structure of the proteins' four domains. Three domains (DNA-binding, oligomerization, and localization domains) are present in all three classes (A, B, and C) of *Hsf*s, but the activation domain only exists in class A *Hsf*s (Kotak et al. 2004; Nover et al. 2001). The *HsfA1* and *HsfA2* families are responsible for the heat shock response (Busch et al. 2005; Mishra et al. 2002; Nishizawa et al. 2006; Nishizawa-Yokoi et al. 2011; Ogawa et al. 2007; Schramm et al. 2006). Notably, rice has an *HsfA2* group consisting of five genes (Baniwal et al. 2004; von Koskull-Döring et al. 2007). Some of the *HsfA2* group genes reacted to heat shock treatment within 10 min (Figure 1), but *HsfA1a* did not (data not shown). These results suggest that the activation of the heat shock response is regulated by the A2-type of *Hsf*s in rice cells. If the heat shock response is regulated by the A2-type of *Hsf*s, then *HsfA1a* may be associated with another stage such as amplification or maintenance of the heat shock response. When *OsHsfA2e* was overexpressed in *Arabidopsis*, the expression of heat shock response genes (*HSP*s, *GolS1*, and others) was induced in the transformants in the absence of heat stress, and the transformants had an enhanced tolerance to high temperature (Yokotani et al. 2007). In contrast, when *OsHSF7* (similar to *HsfA2d*) was overexpressed in *Arabidopsis*, the transformants did not induce the expression of most heat shock response genes under normal conditions, except for a few HSPs (Liu et al. 2009). These results suggested that *OsHsfA2e* may be one of the master regulators like *LeHsfA1a* and that *OsHsfA2e* is necessary for activation of the heat shock response in *Arabidopsis*, unlike *OsHSF7*. In our experiments, transgenic rice overexpressing *HsfA2e* under the control of the rice *actin 1* and *WCR* promoters did not induce heat shock response genes under normal and cool temperature conditions, respectively (data not shown). Furthermore, we developed transgenic rice overexpressing A2-type *Hsf*s (*HsfA2a*, *HsfA2c* and *HsfA2d*) that were inducible with high temperature treatment (Figure 1). Four (*HsfA2a*, *HsfA2c*, *HsfA2d* and *HsfA2e*) out of five class A *Hsf*s could not induce a heat shock response, unlike in *Arabidopsis* (Nishizawa et al. 2006; Ogawa et al. 2007; Prändl et al. 1998; Yokotani et al. 2007) and tomato (Mishra et al. 2002), even when these transcription factor genes were overexpressed in rice cells. These results suggest at least two possibilities: (i) the transcripts of introduced *Hsf*s were not translated due to an unknown mechanism in rice cells or (ii) the levels

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of transcripts produced by the transgene were too low to induce the heat shock response. However, the transcript levels of *HsfA2c* and *HsfA2d* in each transformant under control of the *WCR* promoter treated at 4°C were higher than the expression level in non-transformants treated at 42°C (Figure 1, Table I). Although translation product accumulation was not examined, the level of overexpression and efficient translation of *HsfA2* were very important for inducing the heat shock response (Ogawa et al. 2007). Therefore, we propose that rice cells may inhibit the translation of *Hsf* genes under normal temperatures.

To investigate further the induction machinery of the heat shock response in rice cells, the *WCR::Hsf* transformants were treated with GDM, an antibiotic that induces the heat shock response in *Arabidopsis* without heat shock treatment (Nishizawa-Yokoi et al. 2011; Yamada et al. 2007). After GDM treatment, the expression level of heat shock response genes (*sHSP*s) was enhanced in all rice transformants, even if the expression level was lower than when the seedlings were treated with high temperature (Figure 2). In addition, the expression level of HSP70 was also enhanced several-fold relative to that of the non-transformants upon the same treatment (data not shown). These results indicate that the inhibition of HSP90 activity may require the onset of a heat shock response. In this report, we found that the GDM-induced expression of heat shock response genes was enhanced by the pre-expression of *HsfA2* genes, especially *HsfA2a* and *HsfA2c*. This result suggests that the transgenic product of *HsfA2* was sequestered by endogenous HSP90; however, the transcript level of *HSP90* was not enhanced in the *WCR::Hsf* transformants without GDM treatment (data not shown). These findings suggest the existence of other mechanism(s) in rice cells that inhibit the activity of *Hsf*s in all transformants.

To elucidate the relationship between the activity of HSP90 and the initiation of the heat shock response in rice cells, we are generating another rice transformant that expresses the dominant negative form of HSP90 (*HSP90-D80N*), and are further analyzing the relationship between the activity of HSP90 and the heat shock response. When expressing *HSP90-D80N* under control of the maize *ubiquitin* promoter, only one *HSP* (a DnaJ family protein) was induced in the transformant (Figure 3, Table S2). However, many other genes in the transformant were strongly up- or down-regulated compared to the wild type (data not shown). These results suggest that the overexpression of the mutant form of HSP90 affects the expression of many genes due to dysfunction of the chaperone activity of HSP90.

The *WCR::HsfA2a*/*WCR::HsfA2c* transformant was able to induce a small number of HSPs (seven spots on the array with greater than 10-fold elevated expression) under cool conditions (Table 2, S3). These results suggest that simultaneous expression of some of the class A *Hsf*s would induce the heat shock response in the transformants. However, the induction rate in multiple *Hsf* transformants was very low compared to that of the wild type treated at 42°C (Figure 1).

The transformants overexpressing *HSP90-D80N*, *HsfA2a*, and *HsfA2c* induced the heat shock response under cool conditions (Figure 3, Table 2, S4, S5). In particular, the transformant designated as line 1 expressed heat shock protein genes to a significant degree (Table 2, S4). When the results of microarray analysis for line 1 were compared to those for line 9 transformants, the expression levels of HSPs were found to be elevated with increasing levels of transgene expression in line 1 (Table 2, S4, S5). These results indicate that if the transgenes (class A *Hsf*s and *HSP90-D80N*) could be expressed at higher levels, the heat shock response could be more strongly induced. Furthermore, when the heat shock response of line 9 was compared to that of the *WCR::HsfA2a*/*WCR::HsfA2c* transformant, we confirmed that the expression level of the *sHSP*s was controlled by the expression of *HSP90-D80N*. These results strongly suggested that the activity of HSP90 was very important for the induction of the heat shock response in rice cells. In future studies, we will also assess the cold tolerance of the *WCR::HsfA2a*/*WCR::HsfA2c*/*HSP90-D80N* transformants.

To our knowledge, this is the first report of a rice transformant that can induce the heat shock response under cool conditions. These results provide a method to manipulate environmental stress tolerance in crops at the molecular level.

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Table S1. Gene specific primers for quantitative PCR used in this study.

Gene name	Forward primer	Reverse primer
Ubiquitin	5′-AACCAGCTGAGGCCCAAGA-3′	5′-ACGATTGATTTAACCAGTCCATGA-3′
sHSPs	5′-GCTGAAGAAGGAGGAGGTCAAG-3′	5′-CCGCTGATCTGCAGGATGTT-3′
HsfA2a	5′-CCTCCCGCGCTACTTCAA-3′	5′-TTGAGCTGGCGGACGAA-3′
HsfA2c	5′-ATGTGAAAGCCATGGAAGATAGG-3′	5′-CCCCATCATCTGGACCTGTT-3′
HsfA2d	5′-CAGCCAGGGCGATCAACT-3′	5′-TTCAGCAAATGGCCTTGGA-3′
HsfB2b	5′-CGCGCCCGTATCAGACA-3′	5′-TGGAGACGCCGAAGAGCTT-3′
HsfB2c	5′-CACGCGAAAACTGCCTTTCT-3′	5′-GCGAGGACCGACCGTAGTAG-3′
HSP90	5'-GGAACATCTGGCTGTCAAGCA-3'	5'-AACAGGATGGCCTTGAATTCA-3'

Table S2. Up-regulated HSPs (more than 10-fold higher) in the *HSP90-D80N* transformant

	Accession	HSP gene	Fold change
1	AK071515	Heat shock protein DnaJ family protein.	12.13

Table S3. Up-regulated HSPs (more than 10-fold higher) in the *WCR::A2c / WCR::A2a* transformant treated at 12ºC for overnight.

	Accession	HSP gene	Fold change
1	CI066180	Heat shock protein 70.	42.20
2	AK063700	22.7 kDa class IV heat shock protein precursor.	33.05
3	X60820	16.9 kDa class I heat shock protein.	22.71
4	AK063751	Heat shock protein 81-1 (Heat shock protein 83).	16.23
5	AK063618	Heat shock protein 26.	15.68
6	AK063698	Heat shock protein 81-1 (Heat shock protein 83).	12.73
7	AB020973	Heat shock protein 26.	10.07

Table S4. Up-regulated HSPs (more than 10-fold higher) in the *WCR::A2c / WCR::A2a / HSP90-D80N* transformant (line 1) treated at 12ºC for overnight.

	Accession	HSP gene	Fold change
1	AK063751	Heat shock protein 81-1 (Heat shock protein 83).	181.65
2	AK063700	22.7 kDa class IV heat shock protein precursor.	118.06
3	AK063618	Heat shock protein 26.	109.10
4	CI066180	Heat shock protein 70.	101.89
5	AB020973	Heat shock protein 26.	69.59
6	AK120048	Heat shock protein 26.	54.88
7	AK120045	Heat shock protein 26.	53.53
8	AK063698	Heat shock protein 81-1 (Heat shock protein 83).	42.86
9	AK119243	Low molecular mass heat shock protein Oshsp17.3.	37.24
10	AK101700	Heat shock factor RHSF2.	36.01
11	M80186	Low molecular mass heat shock protein Oshsp17.3.	35.84
12	D12635	Low molecular mass heat shock protein Oshsp17.3.	35.17
13	AK119717	Low molecular mass heat shock protein Oshsp17.3.	35.05
14	AK119239	Heat shock protein Hsp20 domain containing protein.	33.80
15	AK119664	Heat shock protein Hsp20 domain containing protein.	33.03
16	AK105317	Heat shock protein Hsp20 domain containing protein.	32.73
17	AK073671	Heat shock protein Hsp20 domain containing protein.	32.48
18	AK119675	Heat shock protein Hsp20 domain containing protein.	31.95
19	AK119616	Heat shock protein Hsp20 domain containing protein.	31.95
20	AF467729	Heat shock protein Hsp20 domain containing protein.	31.52
21	X60820	16.9 kDa class I heat shock protein.	30.40
22	AB110191	Heat shock protein Hsp20 domain containing protein.	30.06
23	AK104129	Heat shock protein Hsp20 domain containing protein.	29.44
24	AK105433	101 kDa heat shock protein.	28.53
25	AF332981	101 kDa heat shock protein.	25.99
26	AK100903	101 kDa heat shock protein.	24.61
27	AK121414	101 kDa heat shock protein.	20.06
28	AK063629	Heat shock protein 81-1 (Heat shock protein 83).	19.44
29	AK105370	Heat shock protein 81-1 (Heat shock protein 83).	17.75
30	X75616	Low molecular mass heat shock protein Oshsp18.0.	14.84
31	AK069547	LMW heat shock protein.	14.51
32	AK064389	Low molecular weight heat shock protein precursor.	13.69
33	AK105409	Heat shock factor protein 3 (HSF 3)	10.17
34	CI053053	16.9 kDa class I heat shock protein.	10.05

Table S5. Up-regulated HSPs (more than 10-fold higher) in the *WCR::A2c / WCR::A2a / HSP90-D80N* transformant (line 9) treated at 12ºC for overnight.

	Accession	HSP gene	Fold change
1	AK063751	Heat shock protein 81-1 (Heat shock protein 83).	168.21
2	AK063698	Heat shock protein 81-1 (Heat shock protein 83).	138.15
3	AK063700	22.7 kDa class IV heat shock protein precursor.	94.34
4	AK063618	Heat shock protein 26.	74.34
5	CI066180	Heat shock protein 70.	58.02
6	AB020973	Heat shock protein 26.	44.80
7	AK120045	Heat shock protein 26.	36.17
8	AK120048	Heat shock protein 26.	35.86
9	AK101700	Heat shock factor RHSF2.	25.22
10	M80186	Low molecular mass heat shock protein Oshsp17.3.	24.72
11	AK119243	Low molecular mass heat shock protein Oshsp17.3.	23.75
12	AK119717	Low molecular mass heat shock protein Oshsp17.3.	23.53
13	AK073671	Heat shock protein Hsp20 domain containing protein.	22.87
14	AF467729	Heat shock protein Hsp20 domain containing protein.	22.56
15	AK119675	Heat shock protein Hsp20 domain containing protein.	22.48
16	AK119664	Heat shock protein Hsp20 domain containing protein.	22.38
17	AK119616	Heat shock protein Hsp20 domain containing protein.	22.32
18	D12635	Low molecular mass heat shock protein Oshsp17.3.	22.30
19	AK119239	Heat shock protein Hsp20 domain containing protein.	22.04
20	AB110191	Heat shock protein Hsp20 domain containing protein.	20.27
21	AK105317	Heat shock protein Hsp20 domain containing protein.	19.48
22	AK104129	Heat shock protein Hsp20 domain containing protein.	19.32
23	AK105370	Heat shock protein 81-1 (Heat shock protein 83).	16.56
24	AK063629	Heat shock protein 81-1 (Heat shock protein 83).	16.32
25	AK064389	Low molecular weight heat shock protein precursor.	14.71
26	X60820	16.9 kDa class I heat shock protein.	13.41
27	AK106545	Heat shock factor protein 3 (HSF 3)	12.92
28	AK105464	Low molecular weight heat shock protein precursor.	11.94
29	AK105409	Heat shock factor protein 3 (HSF 3)	11.64
30	AK121414	101 kDa heat shock protein.	11.25
31	AK105433	101 kDa heat shock protein.	10.42