Cross-tolerance to Thermal Stresses and its Application to the Development of Cold Tolerant Rice

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Abstract
Having originated in tropical regions, rice is more sensitive to cold stress than other grain crops, such as rye, wheat, and barley, which originated in temperate regions. Despite the limited knowledge about the exact mechanisms of cold tolerance, breeders have generated new rice cultivars that can grow in Hokkaido, Japan's northern limit of rice cultivation, for more than a hundred years. However, greater cold tolerance in rice would enable more stable production and elevate productivity. Because cold tolerance is a complex quantitative trait, it is difficult to pyramid the genes that could improve plant tolerance to this abiotic stress. Therefore, it is important to develop effective strategies for improving cold tolerance in rice. In this review, the use of a 'cross-tolerance' strategy is proposed, and the current status of such strategy introduced.

Introduction
The expansion of areas for rice production is important for world food security in the face of significant population growth, particularly in Asia and Africa. Hokkaido, in northern Japan, represents the northern limit for the economic production of rice. Rice breeders in Hokkaido must contend with cool summers and relatively short durations of sunshine during rice heading. However, rice growers in the area still face such problems as ensuring the cropping season and avoiding chilling damage.

There are two major critical periods for the cultivation of rice in cold regions. These include the stage from germination through seedling growth and the booting stage. Low temperatures adversely affect germination and early growth, as rice seedlings are sensitive to cold. Therefore, farmers grow rice seedlings in greenhouse nurseries to ensure germination and adequate early development of the seedlings. Later, cold stress at the booting stage inhibits pollen development, which leads to reduced fertility and yield. Cold tolerance at the booting stage is a more important issue as it directly affects farmers’ incomes. Over the past few decades, conventional breeding has generated rice cultivars with cold tolerance at the booting stage, thereby gradually improving cold tolerance. The adverse effects of cold stress have thus been avoided at early stages by a special cultivation technique and at the booting stage by the effective use of genetic resources.

Global warming can certainly increase the incidence of extreme weather patterns and events, such as hot and cool summers or localized torrential rains. Even now, plant cold tolerance is one of the most important agricultural problems to be addressed in the Hokkaido region. Due to their immobility, plants have evolved several molecular mechanisms to withstand abiotic stresses in the environment. Therefore, the mechanisms of tolerance to abiotic stresses due to the changeable environment should be investigated in detail, with the resulting information being used to create more stable and higher-yielding crops.

We have been conducting experiments designed to control the cold tolerance of rice at two critical periods: from germination through the seedling stage, and at the booting stage. The results of these experiments suggest that cold tolerance at both stages might be enhanced by the overexpression of reactive oxygen species (ROS)-scavenging enzymes and activation of the heat shock response under cold conditions. In this review, we discuss the relationship between cold tolerance, ROS, and the heat shock response,
and introduce strategies to mitigate cold stress by exploiting these pathways.

Response to abiotic stress

1. Response to cold

When plants that originated in tropical regions are exposed to low temperatures below 10-12°C, they can experience chilling damage due to physiological dysfunction (Lyons 1973). Low temperatures in Australia reduce the average annual rice yield by 5-10%, and unpredictable cold snaps can result in yield losses of 20-40% (Oliver et al. 2005). In Japan, similar yield losses are caused by unpredictable cold snaps that occur roughly every three to four years.

As plants are sessile, they have evolved morphological and physiological responses to abiotic stress involving genes in stress-responsive pathways in order to survive or increase their range of distribution. Although unique sensors for low temperature have yet to be identified, plant cells may sense cold stress via changes in the fluidity of cellular membranes (Yadav 2010, Miura & Furumoto 2013). These changes in the consistency of cell membranes can result in increased cytosolic calcium levels (Knight et al. 1991). These signals can then induce such cold-stress responsive genes as OsICE1/2 and activate the DREB-CRT/DRE pathway (Zhu et al. 2007, Nakamura et al. 2011).

Many genes responsive to cold temperatures including chilling (0-15°C) and freezing (below 0°C) conditions have been identified in the last two decades. The functions of cold-responsive genes and QTL in the cold tolerance of rice (Table 1) have been reviewed and summarized by da Cruz et al. (2013) and Zhang et al. (2014). The expression and function of genes involved in stress response are controlled by stress-inducible transcription factors (TFs) such as DREB. Because DREB is one of the most important TFs under cold-stress conditions, the DREB pathway is a powerful tool for conferring cold tolerance to crops (Ito et al. 2006). Other TFs such as NAC and MYB can also influence cold tolerance, as can CDPK and MAPK, which are involved in signal transduction. These results suggest that the enhancement of cold tolerance requires harmonization of the expression and function of several genes, including genes that affect TFs and the genes controlled by TFs.

2. Thermal stress and ROS generation

Abiotic stresses such as heat, cold, drought, salinity, and ozone generally accelerate the generation of ROS such as hydrogen peroxide, the hydroxyl radical, and superoxide (Mittler 2002, Apel & Hirt 2004, Suzuki & Mittler 2006, Miller et al. 2008). Although cold stress signaling in plant cells can be controlled by ROS accumulation as a second messenger, excessive levels of ROS lead to cell death (Sharma et al. 2012). Therefore, when plant cells are exposed to any abiotic stress condition, the expression of reactive oxygen-scavenging enzymes is usually elevated. Thus, different stress conditions can induce the same ROS response, which suggests that the homeostasis of redox balance in stressed cells is very important.

When rice seedlings were pre-treated under heat shock conditions (42°C), the seedlings became more tolerant to cold. Although the mechanism is not completely understood, overexpression of the ascorbate peroxidase (APX) gene, which is induced by high temperature, can confer cold tolerance (Sato et al. 2001). APX is a typical ROS-scavenging enzyme that catalyzes the conversion of H₂O₂ to its detoxified form H₂O. Further, overexpression of DHAR (dehydroascorbate reductase), which is indirectly related to the detoxification of ROS, can also confer cold tolerance to rice (Yasuda et al. unpublished data). These results suggest that enhancing ROS-scavenging ability could be an effective strategy for enhancing plant tolerance to cold stress.

Heat stress also induces the production of excess amounts of ROS in plant cells; therefore, the activities of many ROS-scavenging enzymes in several plant species are elevated under heat stress conditions (Kumar et al. 2012, Lin et al. 2010). Miller and Mittler (2006) found that ROS induces the expression of genes encoding heat shock factors (HSFs) and Davletova et al. (2005) described the central role of HSFs in sensing H₂O₂ stress. These reports indicated that HSFs play a pivotal role in mitigating excess ROS.

HSFs are known to be transcription factors that induce the expression of HSPs, and play a central role in the heat shock response. HSFs are encoded by plant, animal, and bacterium genomes, but plant HSFs are more diverse than those in other kingdoms. For instance, HSFs in plants are encoded by multi-gene families and the Arabidopsis, rice, and tobacco genomes each encode at least 21, 25, and 17 Hsf genes, respectively (Baniwal et al. 2004, Guo et al. 2008, Mishra et al. 2002, Mittal et al. 2009, von Koskull-Döring et al. 2007). Conversely, mammals, Chlamidomonas, yeast, and Drosophila have only three genes, two genes, one gene, and one Hsf gene, respectively (Morimoto 1998, Schulz-Raffelt et al. 2007). Taken together, this suggests that plants have a complex and highly regulated system for heat shock response.

In general, HSFs are classified into three classes (A, B, and C) based on domain structure. The activation function in transcriptional activators only has the A-type of HSF, because the activation domain only exists in class A HSFs (Kotak et al. 2004, Nover et al. 2001). Members of the Hsf gene family have various functions; for example, HSFA1 in tomato and Arabidopsis acts as a master regulator of the heat stress response (Mishra et al. 2002, Liu et al. 2011), HSFA2 in rice and Arabidopsis confers tolerance to
heat shock, high light, oxidative, salt, and anoxia stresses (Chang et al. 2007, Ogawa et al. 2007, Yokotani et al. 2008, Banti et al. 2010), HSFA3 in *Arabidopsis* influences drought stress tolerance (Sakuma et al. 2006, Yoshida et al. 2008), and HSFA4 in wheat and *Arabidopsis* confers tolerance to cadmium (Shim et al. 2009) and salt (Perez-Salamo et al. 2014). Thus, HSFs have been implicated in tolerance to a wide range of abiotic stresses. Expression of HSFA3 in rice is also induced by low temperature (Nakamura et al. 2011), but there is no evidence that HSFs confer cold stress tolerance.

3. Cross-tolerance to multiple stresses

An interesting phenotype known as cross-tolerance, in which exposure to one type of stress condition confers tolerance to other types of stress, is a phenomenon commonly observed in plants (Bartoli et al. 2013). Table 1 lists the useful genes that can confer stress tolerance to multiple stresses in rice, when the expression and function of the genes are activated. Most of these genes encode TFs or enzymes involved in the synthesis of compatible solutes. Because TFs can simultaneously induce many downstream genes that act to acquire abiotic stress tolerance, using TF has an advantage compared to using a single functional

### Table 1. List of genes which coincidently enhanced multiple stress tolerance in rice

<table>
<thead>
<tr>
<th>Gene</th>
<th>Origin of gene</th>
<th>Function</th>
<th>Tolerance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlSAP</td>
<td><em>Aeluropus littoralis</em></td>
<td>Transcription control</td>
<td>cold, drought, salt</td>
<td>Saad et al. 2012</td>
</tr>
<tr>
<td>bZIP23</td>
<td>rice</td>
<td>Transcription control</td>
<td>drought, salt</td>
<td>Xiang et al. 2008</td>
</tr>
<tr>
<td>CBF</td>
<td>maize</td>
<td>Transcription control</td>
<td>cold, drought, salt</td>
<td>Xu et al. 2011</td>
</tr>
<tr>
<td>COIN</td>
<td>rice</td>
<td>Transcription control</td>
<td>cold, drought, salt</td>
<td>Liu et al. 2007</td>
</tr>
<tr>
<td>DREB1s</td>
<td>rice</td>
<td>Transcription control</td>
<td>cold, drought, salt</td>
<td>Ito et al. 2006</td>
</tr>
<tr>
<td>DREB1F</td>
<td>rice</td>
<td>Transcription control</td>
<td>cold, drought, salt</td>
<td>Wang et al. 2008</td>
</tr>
<tr>
<td>MYB2</td>
<td>rice</td>
<td>Transcription control</td>
<td>cold, drought, salt</td>
<td>Yang et al. 2012</td>
</tr>
<tr>
<td>MYB48-1</td>
<td>rice</td>
<td></td>
<td>drought, salt</td>
<td>Xiong et al. 2014</td>
</tr>
<tr>
<td>NAC5</td>
<td>rice</td>
<td>Transcription control</td>
<td>cold, drought, salt</td>
<td>Song et al. 2011</td>
</tr>
<tr>
<td>SNAC2</td>
<td>rice</td>
<td>Transcription control</td>
<td>cold, drought, salt</td>
<td>Hu et al. 2008</td>
</tr>
<tr>
<td>WRKY11</td>
<td>rice</td>
<td>Transcription control</td>
<td>drought, heat</td>
<td>Wu et al. 2009</td>
</tr>
<tr>
<td>ZFPs</td>
<td>rice</td>
<td>Transcription control</td>
<td>cold, drought, salt</td>
<td>Huang et al. 2009, 2012, Xu et al. 2008</td>
</tr>
<tr>
<td>ADC</td>
<td><em>Datura stramonium</em> oat</td>
<td>Synthesis of polyamine</td>
<td>drought, salt</td>
<td>Capell et al. 2004, Roy &amp; Wu 2001</td>
</tr>
<tr>
<td>codA</td>
<td><em>Arthrobacter globiformis</em></td>
<td>Synthesis of glycinebetaine</td>
<td>cold, salt</td>
<td>Sakamoto et al. 1998</td>
</tr>
<tr>
<td>P5CS</td>
<td>moth</td>
<td>Synthesis of proline</td>
<td>drought, salt</td>
<td>Zhu et al. 1998</td>
</tr>
<tr>
<td>TPSP</td>
<td><em>Escherichia coli</em> rice</td>
<td>Synthesis of trehalose</td>
<td>cold, drought, salt</td>
<td>Garg et al. 2002</td>
</tr>
<tr>
<td>TPS1</td>
<td>rice</td>
<td></td>
<td>cold, drought, salt</td>
<td>Li et al. 2011</td>
</tr>
<tr>
<td>HVA1</td>
<td>barley</td>
<td>LEA protein</td>
<td>drought, salt</td>
<td>Xu et al. 1996</td>
</tr>
<tr>
<td>LEA4</td>
<td>rice</td>
<td>LEA protein</td>
<td>drought, salt, heavy metal</td>
<td>Hu et al. 2016</td>
</tr>
<tr>
<td>HSP17.0</td>
<td>rice</td>
<td>Molecular chaperon</td>
<td>drought, salt</td>
<td>Zou et al. 2011</td>
</tr>
<tr>
<td>HSP23.7</td>
<td>rice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP18.6</td>
<td>rice</td>
<td>Molecular chaperon</td>
<td>cold, drought, salt, heat</td>
<td>Wang et al. 2015</td>
</tr>
<tr>
<td>sHSP17.7</td>
<td>rice</td>
<td>Molecular chaperon</td>
<td>heat, UV-B, drought</td>
<td>Murakami et al. 2004, Sato &amp; Yokoya 2008</td>
</tr>
<tr>
<td>CDPK7</td>
<td>rice</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-dependent protein kinase</td>
<td>cold, drought, salt</td>
<td>Saijo et al. 2000</td>
</tr>
<tr>
<td>MAPK5</td>
<td>rice</td>
<td>Mitogen-activated protein kinase</td>
<td>cold, drought, salt</td>
<td>Xiong &amp; Yang 2003</td>
</tr>
<tr>
<td>PYL5</td>
<td>rice</td>
<td>Hormone receptor</td>
<td>drought, salt</td>
<td>Kim et al. 2014</td>
</tr>
<tr>
<td>TaSTRG</td>
<td>wheat</td>
<td>Salt-induced unknown</td>
<td>drought, salt</td>
<td>Zhou et al. 2009</td>
</tr>
</tbody>
</table>
gene. However, the transformants with several introduced TF genes might represent such off-target effects as dwarfism and retarded growth, when expression of the TF was enhanced under normal conditions (Ito et al. 2006, Wu et al. 2009, Xu et al. 2011).

The expression of most TFs listed in Table 1 is enhanced by stress conditions (Sakuma et al. 2002, Agarwal et al. 2007, Saad et al. 2010, Takasaki et al. 2010). TFs sometimes respond to the presence of ROS such as H$_2$O$_2$; via protein kinases, through Ca$^{2+}$ as a second messenger, a mechanism common to plant responses to several abiotic stresses (Wang et al. 2003). These results suggest that intracellular ROS could induce molecular cascades that lead to abiotic stress tolerance. This phenomenon could thus form the basis of a strategy for generating abiotic-stress-tolerant crops.

Utilization of HSFs for enhancing cold stress tolerance

Taken together, the results above raise the question of why the heat shock response could confer cold tolerance to rice, for several reasons. First, some heat-shock inducible genes in rice and Brassica napus such as low-molecular-mass HSP (Wang et al. 2015), high-molecular-mass HSPs (Krishna et al. 1995, Pareek et al. 1995), and ascorbate peroxidase (Sato et al. 2001) are upregulated under cold stress. Secondly, heat shock factors function as H$_2$O$_2$ sensors (Miller and Mittler 2006) and heat shock responsive genes have been implicated in ROS equilibrium (Davletova et al. 2005, Kumar et al. 2012).

To activate the heat shock response under cool conditions, we used a cold-responsive wheat promoter (WCR promoter) to control the expression of HsfA2 in rice (Yasuda et al. 2013). However, the heat shock response was not clearly activated in the transformants, even though they overexpressed the transgenes under cool conditions. Therefore, an attempt was made for the co-expression in rice of HsfA2, together with the dominant negative form of HSP90 that controls the HsfA2 function. HSP90 is a negative regulator of HSF, and inhibition of HSP90 is required for the induction of heat-inducible genes (Yamada & Nishimura 2007). When the rice transformants co-expressed both the gene encoding HsfA2 and the gene encoding the dominant negative form of HSP90, the heat shock response was activated in rice under cool conditions (Yasuda et al. 2013). These results suggest that the heat shock response is strongly regulated in plant cells (Fig. 1). Although this experiment is ongoing, so far these rice transformants have improved cold stress tolerance during the seedling stage.

**Fig. 1. A model for activation of rice heat shock response (HSR).**
Left: induction of HSR under heat shock condition in wild type cells; Right: induction of HSR under cool condition in the transformants described by Yasuda et al. (2013).
Future perspectives

Due to the rapid advance of climate change caused by global warming, crops tolerant to abiotic stresses should be generated as quickly as possible. Climate change not only involves warming but is also predicted to induce drought or cold locally, which would have significant effects on crop yields. Table 1 shows that one gene conferred multiple forms of stress tolerance to rice; whereas most of the genes confer tolerance to cold, drought, and salt. However, the mechanisms of this tolerance have yet to be completely elucidated, and most of the genes listed are involved in the synthesis of osmolytes or are chaperones, except for the transcription factor. These results suggest that these three stresses induce similar cellular damage in response to osmotic and/or oxidative stress (Wang et al., 2003), and activate similar signal pathways for maintaining homeostasis of the cellular environment under stress (Zhu et al., 2002). If it were possible to completely control the activity of these genes, we would be able to grow complete crops highly tolerant to multiple severe stress conditions, including cold. It is necessary to develop new crops with greater tolerance to multiple abiotic stress and higher or at least stable yield under stress, in order to feed the world’s growing population. Thus, cross-tolerance should be a key word for the next generation of crop breeding.

References

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van Berkel, J. et al. (1994) Transcripts accumulating during


