Metabolome Analysis of Response to Oxidative Stress in Rice Suspension Cells Overexpressing Cell Death Suppressor Bax Inhibitor-1

Toshiki Ishikawa$^{1,2,3}$, Kentaro Takahara$^2$, Takayuki Hirabayashi$^2$, Hideo Matsumura$^4$, Shizuko Fujisawa$^4$, Ryohei Terauchi$^5$, Hirofumi Uchimiya$^{2,4}$ and Maki Kawai-Yamada$^{1,3,5,*}$

$^1$Graduate School of Science and Engineering, Saitama University, Saitama, Japan
$^2$Iwate Biotechnology Research Center, Kitakami, Iwate, Japan
$^3$Japan Science and Technology Agency (JST), Core Research for Evolutional Science and Technology (CREST), Kawaguchi, Japan
$^4$Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan
$^5$Institute for Environmental Science and Technology, Saitama University, Saitama, Japan

*Corresponding author: E-mail, mkawai@mail.saitama-u.ac.jp; Fax, +81-48-858-9269

(Received July 13, 2009; Accepted November 9, 2009)

Bax inhibitor-1 (BI-1) is a cell death suppression factor widely conserved in higher plants and animals. Overexpression of Arabidopsis BI-1 (AtBI-1) in plants confers tolerance to various cell death-inducible stresses. However, apart from the cell death-suppressing activity, little is known about the physiological roles of BI-1-overexpressing plants. In this study, we evaluated the effects of AtBI-1 overexpression on the rice metabolome in response to oxidative stress. AtBI-1-overexpressing rice cells in suspension displayed enhanced tolerance to menadione-induced oxidative stress compared with vector control cells, whereas AtBI-1 overexpression did not influence the increase of intracellular H$_2$O$_2$ concentration or inhibition of oxidative stress-sensitive aconitase activity. Capillary electrophoresis–mass spectrometry (CE-MS)-based metabolome analysis revealed dynamic metabolic changes in oxidatively stressed rice cells, e.g., depletion of the central metabolic pathway, imbalance of the redox state and energy charge, and accumulation of amino acids. Furthermore, comparative metabolome analysis demonstrated that AtBI-1 overexpression did not affect primary metabolism in rice cells under normal growth conditions but significantly altered metabolite composition within several distinct pathways under cell death-inducible oxidative stress. The AtBI-1-mediated metabolic alteration included recovery of the redox state and energy charge, which are known as important factors for metabolic defense against oxidative stress. These observations suggest that although AtBI-1 does not affect rice metabolism directly, its cell death suppression activity leads to enhanced capacity to acclimate oxidative stress.

**Keywords**: Bax inhibitor-1 • cell death • CE-MS • metabolome • oxidative stress • Rice (*Oryza sativa* L.).

**Abbreviations**: BI-1, Bax inhibitor-1; CE-MS, capillary electrophoresis–mass spectrometry; DMSO, dimethylsulfoxide; γEC, γ-glutamyl-L-cysteine; ER, endoplasmic reticulum; GSH, glutathione; HCA, hierarchical cluster analysis; MD, menadione; OAS, O-acetyl-L-serine; OPPP, oxidative pentose phosphate pathway; OX, AtBI-1 overexpressor; PCA, principal component analysis; ROS, reactive oxygen species; RT–PCR, reverse transcription–PCR; TCA, tricarboxylic acid; VC, vector control.

**Introduction**

Bax inhibitor-1 (BI-1) is an endoplasmic reticulum (ER)-localized membrane protein identified in mammals and plants as a suppressor of Bax-induced cell death (Xu et al. 1998, Kawai et al. 1999). Overexpression of Arabidopsis BI-1 (AtBI-1) results in enhanced tolerance to cell death induced by a wide range of stresses (Kawai-Yamada et al. 2001, Matsumura et al. 2003, Kawai-Yamada et al. 2004, Ihara-Ohori et al. 2007). Watanabe and Lam (2006, 2008) demonstrated the vital role of AtBI-1 in attenuation of ER stress-induced cell death. Although the exact molecular mechanism of BI-1-mediated stress tolerance has not been identified, recent studies demonstrated the molecular interactions of BI-1 with calmodulin and Cyt $b_6$ suggesting the involvement of BI-1 in the Ca$^{2+}$-mediated signal transduction pathway and lipid metabolism (Ihara-Ohori et al. 2007, Nagano et al. 2009). Relationships of BI-1 with calcium signaling/homeostasis and ER-localized cytochrome (Cyt P450) were also observed in mammalian cells (Kim et al. 2008, Kim et al. 2009). However, apart from enhanced cell death resistance, the physiological roles of BI-1-induced stress tolerance remain unclear in plants.

Reactive oxygen species (ROS) play a key role in responses to biotic and abiotic stresses. Although ROS themselves are sensor molecules for induction of a series of defense responses, increased levels of ROS cause damage to various cellular components, such as enzyme inhibition, protein degradation,
DNA and RNA damage, and membrane lipid peroxidation, which ultimately culminate in cell death. To avoid oxidative damage, a steady-state ROS level is strictly maintained by detoxification of excess ROS via scavenging enzymes and antioxidants. The ascorbate–glutathione (GSH) cycle exists in most cellular compartments and is considered to play a crucial role in regulating the cellular ROS level (Mittler 2002). Redox states of ascorbate and GSH pools are closely related to regulation of cellular ROS levels and stress tolerance (Sanmartin et al. 2003, Ding et al. 2009). The re-reducing system of the oxidized forms of ascorbate and GSH (GSSG) requires NADPH as a reducing power, which is derived from the oxidative pentose phosphate pathway (OPPPP) in yeast and animals, which is also supported in plants by observations of significant accumulation of OPPP intermediates under oxidative stress (Baxter et al. 2007, Lehman et al. 2008). Metabolic alterations under oxidative stress are not limited to ROS-scavenging systems but extend to the entire primary metabolism. Oxidative stress induced by menadione (MD) causes dynamic alteration in primary metabolism in Arabidopsis cultured cells and root, such as carbon distribution in the central pathway and large sectors of amino acid metabolism (Baxter et al. 2007, Lehman et al. 2008). Takahashi et al. (2008) reported that in rice suspension cells, blast fungus-derived elicitor, which induces the oxidative burst and cell death, affected primary metabolism including the tricarboxylic acid (TCA) cycle, amino acid metabolism, and redox and energy metabolism. Moreover, broad changes in primary metabolism were also observed in various abiotic stresses, such as high and/or low temperatures (Kaplan et al. 2004), salt stress (Kim et al. 2007) and sulfur deficiency (Nikiforova et al. 2005). These metabolomic studies indicate that primary metabolism is closely involved in plant metabolic responses to stresses, i.e. early damage and the subsequent metabolic acclimation.

The aim of the present study was to evaluate the effects of AtBI-1-mediated stress tolerance on metabolic stress responses. For this purpose, primary metabolite compositions of rice suspension cultures under cell death-inducible oxidative stress were profiled using capillary electrophoresis–mass spectrometry (CE-MS). Comparative metabolome analysis revealed that AtBI-1-overexpressing rice cells exhibit enhanced capacity for metabolic acclimation to oxidative stress represented by recovery and rebalance of oxidative damage in primary metabolism.

**Results**

**Tolerance of AtBI-1-overexpressing rice suspension cells to menadione-induced cell death**

To evaluate how stress-induced metabolic responses are affected by cell death suppression mediated by AtBI-1 overexpression, we employed a rice suspension culture that has been reported to be a model in which constitutive overexpression of AtBI-1 leads to suppression of cell death induced by a pathogen elicitor and salicylic acid (Matsumura et al. 2003). Suspension cells were induced from seeds of two independent AtBI-1-overexpressing rice lines (OX1 and OX2) and a vector control plant (VC), followed by evaluation of cell death induced by oxidative stress. MD was used to induce oxidative stress (Yoshinaga et al. 2006). MD is a redox-active quinone known to generate ROS in mitochondria (Thor et al. 1982). MD induced reproducible cell death in 4-day-old rice cells in a dose-dependent manner (Fig. 1A–C). In OX cells 24 h after treatment, cell death was suppressed by 40–50% at 400 µM and by 20–25% at 600 µM, compared with VC cells. Thus, an MD concentration of 400 µM was used in the following experiments as the appropriate condition to obtain the cell death-suppressing phenotype of OX cells. Under this condition, cell growth was severely attenuated in VC cells, whereas OX cells exhibited recovery of growth inhibition at 48 h following cell death suppression (Fig. 1D).

Reverse transcription–PCR (RT–PCR) analysis revealed that endogenous OsBI-1 expression was significantly up-regulated in MD-treated cells (Fig. 2). This suggests that OsBI-1 is involved in the response to MD-induced oxidative stress but may be incapable of suppressing cell death under this condition. Overexpression of heterogeneous AtBI-1 enhanced the capacity of oxidative stress tolerance without affecting the tendency of MD-induced up-regulation of OsBI-1 expression. Notably, the expression level of AtBI-1 seemed to be higher in OX2 than in OX1 (Fig. 2). Quantitative real-time PCR analysis confirmed that the transcripts of AtBI-1 in OX2 cells were 1.6-fold higher than those in OX1 cells. This indicates that the cell death inhibition activity of the OX lines correlated with the expression level of AtBI-1.

**Oxidative damage in AtBI-1-overexpressing rice cells**

To test whether the enhanced tolerance to oxidative stress involves alteration of MD-induced ROS generation or ROS-eliminating activity, H2O2 concentration and aconitase activity were determined in MD-treated cells. MD treatment increased the intracellular H2O2 concentration to a 4-fold peak level at 2 h, and this subsequently gradually decreased but sustained high concentrations throughout 24 h (Fig. 3A). Differences in H2O2 contents between VC and OX cells were not significant. Aconitase is one of the most sensitive enzymes to direct inactivation by ROS (Verniquet et al. 1991). In all cell lines, aconitase activity decreased 2 h after treatment and the low levels were persistently observed throughout 24 h (Fig. 3B). These results suggest that MD-induced accumulation of ROS is similar in VC and OX cells, resulting in a similar degree of oxidative stress.

**Metabolome analysis of rice suspension cells**

Next, metabolome analysis was performed to evaluate the effects of AtBI-1-mediated stress tolerance on rice metabolism. Using CE-MS and photometric assays, we targeted 84 metabolites mainly in primary metabolism, and 73 were quantitatively detected in rice suspension cells with or without MD treatment. All the targeted metabolites are listed in Supplementary...
Table S1, including their abbreviations and other information. The metabolome data were analyzed statistically for the following: (i) effects of AtBI-1 overexpression on constitutive metabolite composition; (ii) characterization of the metabolic responses to MD-induced oxidative stress in rice cells (without AtBI-1 expression); and (iii) metabolic effects of AtBI-1-mediated cell death suppression in response to oxidative stress.

Effects of AtBI-1 overexpression on the metabolite profile in non-stressed rice cells. First, we analyzed the metabolite composition in 4-day-old VC and OX cells under normal growth conditions. The composition of all metabolites was similar in VC and OX cells, and there were no metabolites whose abundance in both types of OX lines was significantly different from that in VC cells (Supplementary Table S2). In addition, cell line-dependent separation could not be achieved by principal component analysis (PCA) based on the metabolite profiles (Fig. 4). These results indicate that constitutive overexpression of AtBI-1 produced no or very little effect on primary metabolism in rice cultured cells.

Characterization of MD-induced metabolome alteration in rice suspension cells. We then investigated the metabolic responses to cell death-inducible oxidative stress in rice cells. To characterize the effects of oxidative stress on the metabolome, the metabolite profiles were analyzed in VC cells at 2, 4, 8, and 24 h after treatment with 400 µM MD (as set by cell death analysis, Fig. 1). All metabolite data are listed in Supplementary Table S3 as abundance in MD-treated cells relative to dimethylsulfoxide (DMSO)-treated controls. In DMSO-treated VC cells, the metabolite levels were stable throughout the period of the experiment, whereas most analytes were significantly altered and temporally fluctuated in MD-treated cells. Table 1 lists the metabolites that were significantly different between DMSO-treated controls and MD-treated cells (>2-fold alteration, with P < 0.05). In general, the number of increased
metabolites peaked at 4–8 h, whereas the numbers of those that decreased increased with time. To classify the metabolites according to their patterns of alteration, the data set was subjected to hierarchical cluster analysis (HCA). Fig. 5 shows a dendrogram created by HCA with a heat map colored according to the relative abundance of each metabolite (red and blue indicate increased and decreased content, respectively). HCA generated six clusters (indicated on the dendrogram in Fig. 5). Clusters 1 and 2 included increased metabolites, while clusters 3–5 included decreased metabolites in MD-treated cells compared with DMSO-treated controls. Metabolites in cluster 6 showed a borderline and insignificant decrease. These clustering patterns provided an outline of oxidative stress-induced metabolic alteration in primary metabolism. In the targeted intermediates of glycolysis [pyruvate, 3-phosphoglyceric acid (3PGA), dihydroxyacetone phosphate (DHAP), fructose-6-phosphate (F6P), glucose-1-phosphate (G1P), G6P, G3P, phosphoenolpyruvate (PEP)] and the TCA cycle [2-oxoglutarate (2OG), aconitate, citrate, fumarate, isocitrate, malate, succinate], most sugar phosphates and organic acids, were depleted in MD-treated cells. On the other hand, the intermediates of the OPPP [6-phosphogluconate (6PG), ribose 5-phosphate (R5P), ribulose 5-phosphate (Ru5P)] were significantly increased by MD treatment. These results suggest a shift in carbon flow from the central pathway to the OPPP in response to MD-induced oxidative stress. This is probably due to increased demand for NADPH as reducing power, since oxidative stress causes significant decreases in reductants, such as GSH and ascorbate as well as NAD(P)H. The GSH biosynthetic pathway was also up-regulated in MD-treated cells: the intermediates O-acetyl-L-serine (OAS), cysteine and γ-glutamyl-L-cysteine (γEC) were included in cluster 2. Oxidative stress also altered energy metabolism. ATP and other high-energy phosphoric compounds were classified in cluster 5, which decreased gradually with time after MD treatment. This seems to result from an imbalance between generation and consumption of ATP; replacing oxidatively damaged components requires enormous amounts of ATP (Møller et al. 2007). On the other hand, the ATP-generating activity in MD-treated cells decreased due to obstruction of glycolysis and the TCA cycle.

### Table 1: Significantly altered metabolites in MD-treated rice cultures

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Increased metabolites</th>
<th>Decreased metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R &lt; 2</td>
<td>R &gt; 2</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>8</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>24</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Differences between groups were tested by the Student’s t-test (P < 0.05).

*R*, relative metabolite abundance (MD-treated/DMSO-treated control).
Fig. 5 Hierarchical cluster analysis (HCA) of metabolite alteration under oxidative stress. A dendrogram obtained by HCA and a heat map showing relative abundance of metabolites (MD-treated/DMSO-treated, log2 scale) in VC cells with a color scale (n = 4). The numbers on each cluster are referred to in the text. See Supplementary Table S1 for abbreviations of metabolites.
In contrast to the depression of carbon metabolism in the central pathway, marked accumulation of most amino acids derived from PEP (phenylalanine, tryptophan and tyrosine), pyruvate (alanine, isoleucine and valine) and oxaloacetate (asparagine, leucine, lysine and threonine) was noted in MD-treated cells.

Effects of AtBI-1 overexpression on metabolic response to oxidative stress. The above results confirmed that cell death-inducible oxidative stress leads to dynamic alteration in primary metabolism in rice cells. We then investigated whether AtBI-1-mediated suppression of cell death affects oxidative metabolic alteration. For this purpose, metabolites in OX cells were analyzed in a manner similar to that in VC cells. Similar to non-treated cells as shown above, there were no significant changes in the lines in DMSO-treated cells throughout the experiment (data not shown). To focus on the differences in MD-induced metabolic alterations between cell lines, all metabolite data of MD-treated cells were expressed relative to those of DMSO-treated controls (Supplementary Table S3) before statistical analysis.

To outline differences in metabolite alteration patterns between VC and OX cells, the data set including relative metabolite abundance in the three lines vs. four different time points (2, 4, 8 and 24 h) were subjected to PCA. The obtained principal components 1 and 2 (PC1 and PC2) accounted for 24.1 and 21.9% of total variance within the data set, respectively. The two components showed clear segregation according to sample types, whereas subsequent minor PCs did not. Thus, the scatter plots of PC1 vs. PC2 were chosen and are shown in Fig. 6. In the PC1 vs. PC2 plot, OXs and VC were clearly segregated at only 24 h. Other time points were separated from each other, but within them the cell lines were not differentiated, unlike samples treated for 24 h. These results indicate that metabolic alterations in MD-treated cells showed a similar trend between OXs and VC in the early response (2–8 h), whereas the late response at 24 h included differences in metabolite profiles between the cell lines. Table 2 shows the numbers of metabolites with significant differences in relative metabolite abundance between VC and each OX (the difference was set to >1.5-fold with \( P < 0.05 \)). During 2–8 h, the differences in metabolite abundance were much less than those at 24 h. In addition, there were only a few metabolites in each of the OX cells that were significantly different compared with the VC. On the other hand, much larger numbers of metabolites showed significant differences at 24 h compared with the VC. Moreover, most of them were common in both OX lines. Based on these observations, we further focused on the differences in metabolite profiles at 24 h between VC and OX cells, and selected metabolites with significant differences between both OXs and VC. Significant differences were observed in 27 metabolites that are listed in Table 3 according to the order of HCA cluster numbers (Fig. 5). Clusters 1 and 2 included increased metabolites in VC cells (Fig. 5) and, in these clusters, several amino acids (asparagine, OAS, tryptophan and \( \gamma \)EC) increased further in OX cells, whereas levels of nucleotide monophosphates (AMP, GMP and UMP) decreased compared with VC cells. On the other hand, metabolites in the other clusters, which decreased in VC cells, increased significantly in both OX lines.

Mapping of the metabolites on their pathways clearly illustrated that AtBI-1-mediated metabolic alterations were seen within typical metabolic pathways, i.e. glycolysis, GSH biosynthesis, amino acids of the glutamate and aspartate families, and reduced pyridine nucleotides and high energy phosphoric nucleotides (Fig. 7). These metabolic alterations affected the total metabolite pools of amino acids, protein and sugar phosphates. In addition, increases in reductants and energy metabolites resulted in marked recovery of the redox balance and adenylate energy charge in OX cells at 24 h.

**Table 2** Differences in numbers of metabolites with altered abundance between OX and VC cells

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>High (( P &lt; 0.05, R &gt; 1.5 ))</th>
<th>Low (( P &lt; 0.05, R &lt; 0.66 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OX1</td>
<td>OX2</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>24</td>
<td>23</td>
<td>28</td>
</tr>
</tbody>
</table>

Differences are >1.5-fold change (R) with statistical significance (\( P \)).

**Fig. 6** Principal component analysis (PCA) of temporal metabolite alteration in control and AtBI-1-overexpressing rice cells under oxidative stress. PCA was performed using four replicate data of relative metabolite abundance in VC, OX1 and OX2 cells at 2, 4, 8 and 24 h after MD treatment, and the generated PC1 and PC2 were plotted.
Table 3 Metabolites altered in OX cells at 24 h after MD treatment

<table>
<thead>
<tr>
<th>HCA*</th>
<th>Fold change (mean ± SEM)</th>
<th>Significance*</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VC</td>
<td>OX1</td>
<td>OX2</td>
</tr>
<tr>
<td>Leu</td>
<td>1</td>
<td>2.89±0.07</td>
<td>2.06±0.22</td>
</tr>
<tr>
<td>Asn</td>
<td>2</td>
<td>1.26±0.12</td>
<td>1.76±0.10</td>
</tr>
<tr>
<td>OAS</td>
<td>2</td>
<td>0.98±0.13</td>
<td>2.01±0.10</td>
</tr>
<tr>
<td>Trp</td>
<td>2</td>
<td>1.02±0.20</td>
<td>1.77±0.16</td>
</tr>
<tr>
<td>γEC</td>
<td>2</td>
<td>3.38±0.23</td>
<td>10.8±0.51</td>
</tr>
<tr>
<td>AMP</td>
<td>2</td>
<td>3.63±0.32</td>
<td>2.12±0.18</td>
</tr>
<tr>
<td>GMP</td>
<td>2</td>
<td>1.93±0.28</td>
<td>1.14±0.19</td>
</tr>
<tr>
<td>UMP</td>
<td>2</td>
<td>1.75±0.12</td>
<td>1.15±0.09</td>
</tr>
<tr>
<td>Asp</td>
<td>3</td>
<td>0.64±0.06</td>
<td>1.16±0.08</td>
</tr>
<tr>
<td>Glu</td>
<td>3</td>
<td>0.40±0.04</td>
<td>1.05±0.14</td>
</tr>
<tr>
<td>Met</td>
<td>3</td>
<td>0.58±0.06</td>
<td>1.00±0.09</td>
</tr>
<tr>
<td>GSH</td>
<td>3</td>
<td>0.05±0.01</td>
<td>0.90±0.11</td>
</tr>
<tr>
<td>G3P</td>
<td>3</td>
<td>0.33±0.05</td>
<td>0.60±0.08</td>
</tr>
<tr>
<td>G6P</td>
<td>3</td>
<td>0.32±0.04</td>
<td>0.85±0.10</td>
</tr>
<tr>
<td>F6P</td>
<td>3</td>
<td>0.37±0.04</td>
<td>0.92±0.04</td>
</tr>
<tr>
<td>GTP</td>
<td>3</td>
<td>0.46±0.02</td>
<td>1.00±0.06</td>
</tr>
<tr>
<td>Pro</td>
<td>4</td>
<td>0.43±0.04</td>
<td>0.84±0.05</td>
</tr>
<tr>
<td>Gln</td>
<td>4</td>
<td>0.64±0.10</td>
<td>1.79±0.09</td>
</tr>
<tr>
<td>Citrulline</td>
<td>4</td>
<td>0.85±0.05</td>
<td>1.16±0.06</td>
</tr>
<tr>
<td>CTP</td>
<td>4</td>
<td>0.54±0.06</td>
<td>1.25±0.09</td>
</tr>
<tr>
<td>NADH</td>
<td>5</td>
<td>0.28±0.04</td>
<td>0.65±0.07</td>
</tr>
<tr>
<td>NADPH</td>
<td>5</td>
<td>0.05±0.01</td>
<td>0.33±0.05</td>
</tr>
<tr>
<td>ATP</td>
<td>5</td>
<td>0.36±0.03</td>
<td>0.67±0.03</td>
</tr>
<tr>
<td>GTP</td>
<td>5</td>
<td>0.41±0.06</td>
<td>0.83±0.02</td>
</tr>
<tr>
<td>UTP</td>
<td>5</td>
<td>0.25±0.03</td>
<td>0.81±0.08</td>
</tr>
<tr>
<td>HomoSer</td>
<td>6</td>
<td>1.15±0.09</td>
<td>1.62±0.12</td>
</tr>
<tr>
<td>GSSG</td>
<td>6</td>
<td>0.81±0.15</td>
<td>2.76±0.24</td>
</tr>
</tbody>
</table>

*HCA cluster numbers (Fig. 5).
**P < 0.05, ***P < 0.01, ****P < 0.001.
*Ratio of fold change value to VC.

Discussion

Overexpression of AtBI-1 in rice leads to enhanced tolerance of cell death induced by oxidative stress without alteration in ROS generation and detoxification

In a previous study, overexpression of AtBI-1 in rice cultured cells resulted in enhanced tolerance to elicitor- or salicylic acid-induced cell death (Matsumura et al. 2003). In the present study, it was confirmed that AtBI-1-overexpressing rice cells were tolerant to MD-mediated oxidative stress. As evaluated by H₂O₂ contents and aconitase activity, AtBI-1 overexpression did not affect the generation and detoxification of ROS. This observation is consistent with previous studies in Arabidopsis, in which ROS generation accompanied by cell death induction was independent of altered AtBI-1 expression levels (Kawai-Yamada et al. 2004, Watanabe and Lam 2005). Thus, it seems likely that BI-1-mediated suppression of ROS-induced cell death is independent of generation and action of ROS.

Oxidative stress induces marked alteration in primary metabolism in rice cells

CE-MS is a powerful tool for metabolome analysis, in which high resolution and quantitative performance are obtained without any derivatization procedures of analytes. We employed...
two extraction methods for cationic/anionic compounds and nucleotides, since several nucleotides required acidic conditions for stability in extracts from rice cells (data not shown). In addition, pyridine nucleotides were separately determined by photometric assays due to their instability in the two extraction methods (particularly NADH and NADPH). Seventy-three metabolites were quantitatively determined in rice cells (Supplementary Table S1). Prior to comparative analysis for evaluation of the metabolic effects of AtBI-1 overexpression under stress conditions, metabolome analysis was performed using VC cells treated with MD for general characterization of oxidative stress-induced metabolic alteration in rice cells. The metabolome data were subjected to HCA to classify metabolites, which showed dynamic alteration in primary metabolism in rice suspension cells under oxidative stress. In the central metabolic pathway, carbon flow shifted from glycolysis to the OPPP, as observed by substantial decreases in the abundances of G6P and downstream glycolytic intermediates, coupled with a marked increase in OPPP metabolites. This is consistent with previous metabolome analyses on treatment of Arabidopsis root and cultured cells with MD or of rice suspension culture with a pathogen elicitor, which induced oxidative burst in rice cells (Baxter et al. 2007, Lehman et al. 2008, Takahashi et al. 2008). Such a metabolic shift in carbon flow seems to be due to an increased requirement for NADPH as a reducing power against oxidative stress. MD-induced oxidative stress caused a significant decrease in major reductants ascorbate and GSH, as well as NAD(P)H, leading to an imbalance in the cellular redox system. Most metabolites in the TCA cycle were also decreased under oxidative stress, which is due to not only a decrease in the available carbon source from glycolysis but also disturbance of enzymes. A number of mitochondrial proteins

![Fig. 7 Metabolic map of AtBI-1-mediated metabolite alteration at 24 h after MD treatment. Metabolites and indices of redox/energy state, in which both OX cells showed statistical significance compared with VC cells (Table 3), are shown on their metabolic pathways. Red, higher in OXs; blue, lower in OXs; gray, not significant; open, not determined.](image-url)
are known to be inactivated or degraded by ROS. Isolated mitochondrial aconitase is directly inactivated by \( \text{H}_2\text{O}_2 \) exposure (Verniquet et al. 1991). Sweetlove et al. (2002) reported oxidative stress-induced degradation of a large number of mitochondrial proteins involved in the TCA cycle and respiratory chain, including lipoic acid-containing subunits of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase. Notably, pyruvate and succinate increased significantly but transiently at 2 h, whereas other metabolites involved in the TCA cycle decreased. Zhang et al. (1990) reported that bovine heart-derived mitochondrial respiratory chain enzymes, including succinate dehydrogenase, were inactivated with various sensitivities according to ROS molecules. Thus, the transient increases of pyruvate and succinate may indicate different sensitivities of rice mitochondrial enzymes to ROS generated by MD.

Obstruction of the TCA cycle results in depletion of NADH and ATP, which were found to decrease in MD-treated cells. Persistent depletion of ATP is probably important in oxidative stress accompanying cell death since ATP depletion was reported to be associated with programmed cell death in oxidative stress and flower senescence (Tiwari et al. 2002, Azad et al. 2008). In addition, ATP is also required at a large excess molar ratio to remove and/or repair oxidative damage in cellular compartments, such as protein, lipid and DNA (Møller et al. 2007). Thus, a persistently low level of ATP should lead to accumulation of oxidative damage in the cells.

Most amino acids accumulated in the stressed rice cells despite depletion of their precursors in the central metabolic pathways (e.g. 3PGA, PEP, pyruvate, 2OG). The increases of the amino acids and the decreases of their precursor organic acids fluctuated synergistically, with a peak at 4–8 h, indicating that the depletion of the glycolysis and TCA cycle intermediates partially resulted from increased amino acid metabolism.

This result is inconsistent with the observation in MD-treated Arabidopsis (Baxter et al. 2007, Lehman et al. 2008), in which several amino acids were found to decrease in parallel with decreased levels of their precursors. Fahnenstich et al. (2008) reported more complex changes of amino acid compositions in glycolate oxidase-overexpressing Arabidopsis, in which amino acids derived from TCA cycle acids increased, whereas those from glycolysis intermediates decreased. On the other hand, increases in the amino acid pool were also reported in various plants under abiotic stresses, e.g. in cowpea cells and Arabidopsis plants under temperature stress (Fougère et al. 1991, Kaplan et al. 2004), in alfalfa roots under salt stress (Mayer et al. 1990) and in sweet potato cells under osmotic stress (Wang et al. 1999). Recently, Takahashi et al. (2008) reported specific accumulation of γ-aminobutyric acid in rice suspension cells treated with blast fungus-derived elicitor. These findings suggest that amino acid metabolism has various roles in stress responses, although its physiological functions and regulatory mechanisms under stress are almost unknown, with few exceptions, e.g. proline biosynthesis as an osmoprotectant.

**Metabolic shift accompanied by AtBI-1-mediated suppression of cell death may contribute to enhanced stress tolerance in AtBI-1-overexpressing rice cells**

PCAs indicated that AtBI-1 overexpression did not have significant effects on constitutive metabolite composition and early metabolic responses (2–8 h after treatment) to oxidative stress (Figs. 4, 6). On the other hand, clear differences between OX and control cells were identified following 24 h exposure to stress. The differences were, in general, regarded as recovery of metabolite composition depleted during the early response (Table 3). In addition, OX-specific metabolite changes were observed in several pathways, e.g. glycolysis, key amino acids glutamate and aspartate and their family amino acids (glutamine, proline, arginine and citrulline in the glutamate family, and asparagine, methionine and homoserine in the aspartate family), and components of redox and energy metabolism (Fig. 7). In particular, the GSH level showed a marked recovery compared with the control cells. This increase seems to be via de novo synthesis rather than a re-reducing system, since significant accumulation of its precursors OAS and γEC as well as the oxidized form GSSG was also noted, resulting in amplification of the total glutathione pool size. In addition, the increases in glycolytic sugar phosphates (G6P, F6P, G1P and G3P) would contribute to the generation of NADH and NADPH as the carbon source of the TCA cycle and the OPPP, respectively. However, the recovery rate of NAD(P)H was less than that of GSH, due to the abnormal metabolite composition in the OPPP and the TCA cycle (slightly recovered but the stressed states still continued, see Supplementary Table S3) and persistent oxidative inactivation of enzymes such as aconitase (Fig. 3).

The ATP level and adenylate energy charge also showed recovery in OX cells. This could be due to NADH recovery. Depleted ATP levels are associated with programmed cell death in oxidative stress and senescence (Tiwari et al. 2002, Azad et al. 2008). The observation of this study supports these results, and it is suggested that the recovery of energy metabolites also contributes to acclimation to oxidative stress via energy-consuming reparation of damaged cellular components.

Glutamate and aspartate are important amino acids as branching points for the synthesis of several amino acids and as donors of the amino group. These amino acids significantly decreased in MD-treated cells despite increases in most other amino acids, even in derivatives of glutamate and aspartate. This indicates that the balance of amino acid metabolism was also affected by oxidative stress, and its rebalance was facilitated in the OX cells.

Yoshinaga et al. (2005a, 2005b) observed oxidative stress-induced membrane disruption of mitochondria and chloroplasts as well as plasma membrane in Arabidopsis leaf cells. Leaky disruption of these organelle membranes results in unusual compartmentalization of metabolites and enzymes, which might partially contribute to metabolic changes under oxidative stress. In AtBI-1-overexpressing rice cells, attenuated...
plasma membrane disruption induced by MD-mediated oxidative stress was observed as a lower uptake of Evans blue, suggesting increased membrane stability against oxidative stress. Although it is difficult to evaluate the metabolic effects of disruption of such organelles based on our metabolome data at present, this point would be one of the important tasks to figure out the molecular mechanism of BI-1-mediated stress tolerance.

In conclusion, CE-MS-based metabolic profiling identified dynamic metabolic changes in oxidative-stressed rice cells. In addition, we found the metabolic phenotype of AtBI-1-overexpressing rice cells at 24 h after MD treatment, and focused on several typical metabolic pathways involved in rebalancing oxidatively damaged sectors, such as carbon flow, amino acid metabolism, redox states and energy charge. The results indicate that tolerance to severe oxidative stress in AtBI-1-overexpressing rice cells, represented by attenuation of cell death and growth inhibition, is due to enhanced capacity of metabolic acclimation. On the other hand, overexpression of AtBI-1 had no effect on metabolite profiles under no-stress conditions, suggesting that the association of BI-1 with factor(s) involved in metabolic acclimation is limited to stress conditions. Alternatively, the enhanced acclimation capacity of BI-1 overexpression might be secondary effects of cell death suppression. Further studies of the metabolic phenotype of Arabidopsis and other plants should be addressed in future studies. In addition, intermediates linking BI-1 function to cell death suppression and the stress acclimation pathway remain to be identified. Cyt b5 and calmodulin are potential interactors of BI-1 (Ihara-Ohori et al. 2007; Kawai-Yamada et al. 2009; Nagano et al. 2009). The next challenge is to clarify how BI-1-interacting calcium signaling and lipid metabolism lead to stress tolerance.

Materials and Methods

Plant materials and suspension culture
AtBI-1-overexpressing rice plants (Oryza sativa cv. Sasanishiki) were generated as described previously (Matsumura et al. 2003). As an empty vector control, transgenics harboring a T-DNA lesion of pcAMBIA were used. Calli were induced from seeds of independent rice lines on plates of modified N6 medium (Kuchitsu et al. 1993) containing 2 µg ml⁻¹ 2,4-D at 27°C under darkness. The cell suspension was cultured with 30 ml of liquid medium in a 100 ml flask under the same conditions with rotation at 130 r.p.m. The culture was transferred every week to a new medium to be diluted 5-fold.

Chemical treatment
MD was prepared at 500-fold concentration in DMSO. The solution was added to 4-day-old culture and mixed vigorously. DMSO was added to control cells. Cells were harvested at the indicated time points by filtration, washed four times with Milli Q water and used for Evans blue assay. The remaining cells were frozen immediately in liquid nitrogen and stored at −80°C.

RNA extraction and RT–PCR
Total RNA was prepared using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the instructions provided by the manufacturer. The PCR primers used are listed in Supplementary Table S5.

Evaluation of oxidative damage
Cell death was evaluated by Evans blue uptake (Hayashi et al. 2005). Fresh cells (30 mg) were incubated with 0.05% (w/v) Evans blue for 10 min. Cells were washed three times with distilled water. Dye was eluted by incubation in 1% (w/v) SDS in 50% (v/v) methanol for 2 h at 50°C and the A₄₉₀ was measured. Cell death (%) was represented as absorbance relative to that obtained from completely dead cells, which were prepared by twice repeating freeze–thaw.

The intracellular H₂O₂ concentration was determined by peroxidase-photometric assay according to the method described by Okuda et al. (1991). To take into account differences in background and inhibitory effects of the peroxidase reaction, each sample was pre-treated with catalase (for blank assay) or mixed with a known amount of H₂O₂ (for a standard curve considering the inhibitory effects).

Aconitase activity was measured as conversion of isocitrate to cis-aconitate. Frozen cells were ground in liquid N₂ and extracted with 10 vols. of 100 mM Tris–Cl (pH 7.5) containing 1 mM EDTA and 1 mM dithiothreitol. Cell debris was removed by centrifugation (15,000 x g for 5 min) and the supernatant was used for enzyme assay. The reaction was performed in 100 mM Tris–Cl (pH 7.5) at 25°C and started by adding 20 mM isocitrate. The increase in A₅₄₀ was monitored for 1 min. Aconitase activity was expressed as nmol gFW⁻¹ min⁻¹ using an extinction coefficient for cis-aconitate of 3.6 mM⁻¹ cm⁻¹ at A₅₄₀.

Metabolite measurement
Metabolites were separately determined by four methods as indicated in Supplementary Table S1: cationic compounds (method A), anionic compounds (method B) and nucleotides (method C) by CE-MS, and pyridine nucleotides by photometric assay (method D). Cationic and anionic metabolites were extracted simultaneously and purified according to Ohkama-Ohtsu (2008) using MES and methionine sulfone as internal standards. In brief, ground frozen tissue was extracted with 20 vols. of methanol containing 8 µM internal standards and cell debris was removed by centrifugation. The supernatant (500 µl) was mixed with 500 µl of chloroform and 200 µl of water. The mixture was subjected to a centrifugal concentrator to allow evaporation of methanol from the upper phase. The resultant water phase was passed through a 3 kDa cut-off filter (Millipore, Billerica, MA, USA) and further evaporated to dryness. The residue was dissolved in water and used for
CE-MS analyses. Cationic compounds were determined as described previously (Takahashi et al. 2006). Anionic metabolites were analyzed by pressure-assisted CE-MS (Harada et al. 2006, Harada et al. 2008) using 50 mM ammonium acetate (pH 9.0) as the electrolyte.

Nucleotides were separately extracted due to degradation in the procedure for cationic and anionic compounds. The appropriate extraction procedure for nucleotides was determined by checking the stabilities of authentic standards spiked into extracts from rice cells (data not shown). Instabilities of nucleotide phosphates were probably due to enzymatic degradation. The addition of formic acid to the extraction solvent and removal of proteins by ultrafiltration prior to evaporation markedly improved their stabilities. In brief, fine powder of ground sample was extracted with 20 vols. of methanol containing 5% (v/v) formic acid and 8 µM 10-camphorsulfonic acid as an internal standard. The suspension was added to an equal volume of chloroform and 0.4 vols. of water, and mixed thoroughly. After centrifugation, the upper water/methanol phase was filtered with a 3 kDa cut-off filter. The filtrate was dried using a centrifugal concentrator at 40°C and dissolved in water. Nucleotides were measured by CE-MS using a fused silica capillary pre-coated with phosphate according to the method described by Soga et al. (2007). All CE-MS data were processed using the R program with XCMS package (Smith et al. 2006).

Pyridine nucleotides were measured by enzyme-coupled spectrophotometric assay according to Queval and Noctor (2007). Protein concentration was determined by Bradford assay (Bradford 1976).

Statistical analysis

PCA and HCA were performed using the algorithms embedded in the R software package. Creation of the heat map and Student’s t-test were performed using Microsoft Excel (Microsoft Corporation, Seattle, WA, USA).

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the Ministry of Agriculture, Forestry and Fishery, Japan; the Japan Science and Technology Agency (JST) Core Research for Evolutional Science and Technology (CREST) project.

References


