Effect of early cold stress on the maturation of rice anthers

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Male reproductive development in rice (Oryza sativa Linnaeus) is very sensitive to various forms of environmental stresses including low temperature. Here, we present our findings on the proteomic analysis of the later developmental consequences of low temperature treatment on rice anthers. Anther proteins at the trinucleate stage, with or without cold treatment for four days at 12°C at the young microspore stage, were extracted, separated by two-dimensional gel electrophoresis (2-DE) and compared. More than 3000 rice anther proteins of cold-sensitive cultivar Doongara plants at the trinucleate stage were resolved on 2-DE gels over a pH range of 4–7 and detected by silver-staining. Seventy protein spots were differentially displayed after four days of cold treatment at the young microspore stage. Of these, 12 protein spots were newly-induced, 47 were up-regulated, and 11 were down-regulated by cold treatment at the early microspore stage. We identified 18 by matrix-assisted laser desorption/ionization mass spectrometry time of flight (MALDI-TOF) analysis. Of the identified proteins, seven were observed as breakdown (cleavage) products by a combination of 2-DE and MALDI-TOF analysis, thus demonstrating for the first time that cold temperature stress at the young microspore stage enhances and induces partial degradation of proteins in the rice anthers at the trinucleate stage.

Keywords: Cold-induced male sterility / Peptide mass fingerprint / Pollen development / Rice (Oryza sativa L.) / Two-dimensional gel electrophoresis

1 Introduction

Rice (Oryza sativa Linnaeus) is vulnerable to cold weather. In particular, the reproductive stage of rice is the most susceptible to cool weather damage [1]. The main cause of this damage is pollen sterility resulting from low temperature damage at the early stage of microspore development [2, 3]. The most sensitive stage to the various forms of environmental stresses, including cold damage, is just after meiosis, that is, the tetrad to early microspore phase or the young microspore stage [2]. In spite of much recent research on the subject, the underlying mechanism that cause cold-induced male sterility (CIMS) are poorly understood. In plants, several tissues or whole organs undergo cell death as part of their normal development, for example, in xylogenesis, anther dehiscence, and senescence, or in response to pathogens and environmental stresses. There are some excellent reviews on programmed cell death (PCD) in plants [4–8]. As part of the normal developmental sequence associated with pollen formation and release, several anther tissues undergo cell death in a precisely coordinated and temporal progression [9, 10]. The tapetal cells lyse and release lipid components that coat the pollen exine [11]. The stomium cells die and shear to allow release of the pollen from the anther loculus. The endothecium and epidermal cell layers undergo cell death, and special cell wall structures in the endothecium cells provide the mechanism whereby the loculus springs open at dehiscence. Papini et al. [12] showed that the degradation of tapetal cells in two angiosperms (Lobivia rauschii and Tillandsia albida) shows cytological features characteristic of PCD when studied by electron microscopy. These include shrinkage

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Abbreviations: APX, ascorbate peroxidase; CIMS, cold-induced male sterility; PCD, programmed cell death
of the cell, condensation of chromatin, swelling of the endoplasmic reticulum, and the persistence of mitochondria. In addition, Wang et al. [13] observed oligonucleosome-sized DNA cleavage (DNA laddering) in barley anthers at the end of the unicellular stage of pollen development. DNA fragmentation mainly occurred in the loculus wall cells, tapetum cells and filament cells, but was absent or infrequently observed in the microsperes of developing anthers in situ [13]. Degeneration and death in a number of anther tissues results ultimately in anther rupture and dispersal of pollen grains [10]. Interestingly, environmental stresses and diseases can also induce apoptosis [14, 15]. It is not known what kind of changes occur at the protein level in mature anthers of rice as part of normal development and how cold damage at the early stage of anther development effects this process. Here we describe a proteomic approach to investigate changes which occur at the trinucleate stage as a result of cold temperature treatment at the early stage. Although these changes may not necessary correlate well with or indicate early causes of CIMS, such an investigation will, nevertheless, contribute to the understanding of the whole picture of CIMS in rice.

2 Materials and methods

2.1 Chemicals

All chemicals used were of the highest obtainable grade and are outlined in [16]. MilliQ-quality water (Millipore, Bedford, MA, USA) with resistivity of greater than 18 MΩ cm was used.

2.2 Plant materials and growth

The relatively cold sensitive Australian rice cultivar, Dooonga, was grown under controlled environment conditions as previously described [17]. Four weeks after sowing, urea (15 g/m²) was applied as a nitrogen fertilizer and the plants were flooded. Prior to the panicle initiation stage the plants were transferred to a growth chamber maintained at a temperature of 20–30°C (12 h day/12 h night cycle, 70% relative humidity and a photon flux density of 330 μmol/m²/s⁻¹).

2.3 Stage determination, cold temperature treatment and sampling

Determination of different stages of anther development were done by using parameters such as auricle distance, days before heading, anther length and cytological staining with acetocarmine and 4′,6-diamidino-2-phenylindole as previously described [17, 18]. For the pollen viability test, anthers and pollen grains were stained with iodine potassium iodide (I₂-KI) solution (12 mM I₂, 130 mM KI). Round, filled and deep-color stained pollen was counted as fertile [22]. Cold temperature treatment was performed by moving individual plants whose auricle distance was about −15 mm (young microspore stage anthers), into an identical chamber kept at 12°C, 70% relative humidity, with the same illumination and day/night cycle as the control conditions. Auricle distance is measured as the distance between the ligule of the flag leaf and the penultimate leaf and is expressed in mm. When the flag leaf has not emerged from the leaf sheath, the auricle distance value is negative. After four days of cold treatment, the plants were moved back to the control growth conditions until harvest. Anthers of control and cold treated plants were harvested one day before anthesis from the spikelets of the top three primary branches. At the time of harvest, these anthers were at the trinucleate stage (pollen grains with three nuclei).

2.4 Extraction of rice anther proteins

The anther proteins were extracted using the TCA-acetone precipitation method as previously described [17]. The protein concentration of the supernatant was determined using the Bradford dye-binding assay (Bio-Rad, Hercules, CA, USA) with a series of concentrations of BSA as the standards.

2.5 2-DE, gel staining and image analysis

2-DE was carried out in a horizontal electrophoresis system, Multiphor II (Amersham Biosciences, Uppsala, Sweden) as previously described [17]. For the first dimension, IPG gel strips (24 cm x 3 mm, pH 4–7 linear; Amersham Biosciences) were used. 100 μg Total proteins for silver-stain, 50 μg for Coomassie stain were loaded onto rehydrated IPG strips and focused for 280 kVh. For the second dimension SDS-PAGE, ExcelGel SDS gels (12–14% gradient; Amersham Biosciences) were used. Silver and Coomassie staining were performed as described [17]. Stained gels were scanned at 600 dots per inch resolution using a UMAX Astra-2400S scanner (UMAX Technologies, Fremont, CA, USA). Silver-stained gels were scanned using transparency mode while Coomassie-stained gels were scanned using reflective mode with an opaque white background. Spot detection and gel comparisons were performed using Melanie 3 software (Swiss Institute of Bioinformatics, Geneva, Switzerland). The relative M, of the proteins were determined by comigration of protein standards using Melanie 3 software. The apparent pI of the proteins were determined by calculating migration of the protein spots on 24 cm IPG (linear) strips using a ruler.
3 Results

3.1 Anther and pollen maturation are affected by cold temperature treatment at the early stage

Anthers at the trinucleate stage from untreated and cold temperature treated (at the young microspore stage) plants were examined microscopically. Different types of abnormal anthers were observed in cold treated samples including irregular shaped small, white or light yellowish anthers. The pollen grains in these anthers were either deformed or round without any iodine stained content. Figure 1 shows examples of anthers from cold temperature treated and untreated Doongara plants. In the anthers of cold treated plants, there were either no iodine stained pollen grains, indicating no starch deposition or only small portions of the pollen grains were stained due to a reduction in the numbers of starch filled pollen grains. There were also cases in which starch accumulation in pollen grains ranged from undetectable to fully engorged. To study the later consequences of cold treatment on the protein level, 2-DE was used to display and compare proteins of rice anthers at the trinucleate stage from cold treated and untreated plants.

3.2 Differentially displayed proteins of the trinucleate stage anthers were observed after cold temperature treatment at the young microspore stage

We used 2-DE to display and compare rice anther proteins from control Doongara plants and plants harvested approximately 10 days after the end of four days of cold temperature treatment. A 2-DE protein map of anther proteins from cv Doongara plants cold treated for four days is shown in Fig. 2. Melanie 3 image analysis of at least three replicate gels revealed over 3000 anther proteins at the trinucleate stage were reproducibly resolved in silver-stained gels over a pH range of 4–7 and a size range of 6–120 kDa. Silver-stained gels were used for most of the comparisons and quantification. Coomassie-stained gels were also used for comparisons and quantification of the proteins that did not stain well (e.g. negatively stained) with the silver nitrate method used. While the global pattern of anther proteins was largely unaltered (approximately 98% of the total protein spots remained unchanged), 70 protein spots were reproducibly detected as changed more than two-fold in their protein levels after four days of cold temperature treatment at 12°C in cold sensitive cv Doongara plants compared with untreated plants at a corresponding developmental stage as shown in Fig. 2. Among them, 12 protein spots were newly-induced, 47 were up-regulated and 11 protein spots were down-regulated by cold treatment at the early microspore stage. Selected parts of the gels are highlighted in Fig. 3 to show comparisons of cold responsive protein spots in rice anthers from cold treated and untreated plants.
Figure 2. 2-DE protein map of proteins from anthers at the trinucleate stage in cv Doongara plants that were cold treated for four days at the young microspore stage. Protein spots were assigned arbitrary identifiers. IEF in the first dimension was on 24 cm IPG strips with a linear gradient ranging from pH 4–7 loaded with 100 μg of extracted anther protein. For the second dimension 12–14% T SDS-PAGE gels were used. Proteins were visualized by silver-staining. Newly induced, up-regulated and down-regulated protein spots are marked with green, blue and red circles, respectively. Some of the differentially displayed proteins are enlarged in Fig. 3.

Figure 3. Comparison of some of the proteins that were differentially displayed by cold treatment at the early stage. Protein spot numbers are as in Fig. 2 and Table 1. All enlargements are from silver-stained gels. Differentially displayed proteins are indicated with arrows.

3.3 Identification of differentially displayed proteins

Sixty-five of the differentially displayed proteins were analyzed by MALDI-TOF and PMF after excision from colloidal Coomassie-stained 2-DE gels and digestion with trypsin. Of these, 18 protein spots were identified and are listed in Table 1. Among them, six protein spots were matched to two cytosolic ascorbate peroxidases that have 83% identity to each other. Four protein spots (27, 36, 38 and 39) were from the gene ascorbate peroxidase (APX)B (TrEMBL accession number Q9FE01). Spot 27 was down-regulated and the rest (protein spots 36, 38 and 39) were up-regulated. Two protein spots, one up-regulated (protein spot 42) and one down-regulated (protein spot 29), were from another gene (TrEMBL accession number P93404). Previously, four isoforms of APX were observed in the anthers of cv Doongara at the young microspore stage, but these isoforms showed no changes upon immediate cold temperature treatment (unpublished data) although they represent one of the most abundant proteins in anthers [17]. Two other proteins, an endosperm lumenal binding protein (TrEMBL accession number O24182, spot 5) and a triosephosphate isomerase (Swiss-Prot accession number P48494,
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3.4 Evidence that cold stress at the young microspore stage enhances and induces partial protein degradation in rice anthers at the trinucleate stage

Of the identified proteins, we observed seven to have much smaller $M_i$ when compared with those of the matched proteins, indicating possible cleavage or partial degradation. Table 2 highlights the $M_i$ and $pI$ of the breakdown products and their matching proteins. The PMF analysis of these proteins also showed that the sequences covered by matched peptides were either in the $N$- or $C$-terminal regions of the matched proteins only, further confirming possible cleavage or partial degradation. Examples of two breakdown products are illustrated in Fig. 4. A heat shock protein 70 (HSP70) (Fig. 2) was identified by gel matching to the 2-DE map of rice cv Doongara anther proteins at the young microspore stage [17]. The HSP70 had an observed $M_i$ of 63.2 kDa. Its PMF coverage map is given in Fig. 4 (A, left panel). Protein spot 33 ($M_i$, 30.1 kDa), a newly-induced protein (Fig. 2 and Table 1), was also matched to the same HSP70 protein, but only to the $N$-terminal end of the complete sequence with 14% coverage, indicating possible partial degradation or cleavage of HSP70 in trinucleate anthers after cold temperature treatment at the young microspore stage (Fig. 4 and Table 2). A $\beta$-expansin was identified by gel matching to a 2-DE map of rice cv Doongara anther proteins at the young microspore stage [17]. The $\beta$-expansin had an observed $M_i$ of 29.1 kDa. Its PMF coverage map is given in Fig. 4B (left panel). An up-regulated protein, spot 62 ($M_i$, 13.1 kDa; Fig. 4 and Table 2), was also matched to the same $\beta$-expansin protein, but only to the $C$-terminal end of the complete sequence with 20% coverage of the whole protein, indicating partial degradation of $\beta$-expansin in trinucleate anthers after cold temperature treatment at the young microspore stage. Two differentially dis-

### Table 1. Differentially displayed rice anther proteins at the trinucleate stage identified by PMF analysis

| Spot no. | Protein change | $M_i$ ($\times 10^3$) /$pI$ | Protein matched to | Access. no. | PM/%SC | Z score | $M_i$ ($\times 10^3$) /
pI |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>↓</td>
<td>65.9/5.25</td>
<td>Endosperm luminal binding protein</td>
<td>O24182</td>
<td>13/30%</td>
<td>2.31</td>
<td>73.7/5.3</td>
</tr>
<tr>
<td>6</td>
<td>↓</td>
<td>62.7/5.39</td>
<td>Putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase</td>
<td>Q94DV7</td>
<td>6/18%</td>
<td>1.98</td>
<td>60.1/5.53</td>
</tr>
<tr>
<td>17</td>
<td>↑</td>
<td>33.7/5.51</td>
<td>Putative soluble inorganic pyrophosphatase</td>
<td>Q94817</td>
<td>5/31%</td>
<td>NI</td>
<td>22.3/5.72</td>
</tr>
<tr>
<td>26</td>
<td>↑</td>
<td>25.8/5.17</td>
<td>Triosephosphate isomerase</td>
<td>P48494</td>
<td>9/61%</td>
<td>2.44</td>
<td>29.6/5.7</td>
</tr>
<tr>
<td>27</td>
<td>↑</td>
<td>27.2/5.26</td>
<td>Ascorbate peroxidase</td>
<td>Q9FE01</td>
<td>12/65%</td>
<td>2.37</td>
<td>27.2/5.20</td>
</tr>
<tr>
<td>29</td>
<td>↓</td>
<td>27.0/5.70</td>
<td>Ascorbate peroxidase</td>
<td>P93404</td>
<td>11/56%</td>
<td>2.22</td>
<td>27.2/5.42</td>
</tr>
<tr>
<td>31</td>
<td>↑</td>
<td>28.0/6.00</td>
<td>Glycogen phosphorylase H isozyme</td>
<td>Q9FPE6</td>
<td>9/15%</td>
<td>1.9</td>
<td>91.5/7.35</td>
</tr>
<tr>
<td>33</td>
<td>↑↑</td>
<td>30.1/5.26</td>
<td>Heat shock protein 70</td>
<td>Q40693</td>
<td>7/14%</td>
<td>2.25</td>
<td>71.5/13.5</td>
</tr>
<tr>
<td>34</td>
<td>↑</td>
<td>27.5/6.24</td>
<td>Aldolase C-1</td>
<td>Q42476</td>
<td>7/27%</td>
<td>0.74</td>
<td>38.8/8.35</td>
</tr>
<tr>
<td>36</td>
<td>↑</td>
<td>23.7/4.71</td>
<td>Ascorbate peroxidase</td>
<td>Q9FE01</td>
<td>9/48%</td>
<td>2.33</td>
<td>27.2/5.20</td>
</tr>
<tr>
<td>38</td>
<td>↑</td>
<td>25.0/4.84</td>
<td>Ascorbate peroxidase</td>
<td>Q9FE01</td>
<td>11/58%</td>
<td>2.36</td>
<td>27.2/5.20</td>
</tr>
<tr>
<td>39</td>
<td>↑</td>
<td>23.3/5.09</td>
<td>Ascorbate peroxidase</td>
<td>Q9FE01</td>
<td>10/54%</td>
<td>2.31</td>
<td>27.2/5.20</td>
</tr>
<tr>
<td>42</td>
<td>↑</td>
<td>24.4/5.27</td>
<td>Ascorbate peroxidase</td>
<td>P93404</td>
<td>10/50%</td>
<td>2.31</td>
<td>27.2/5.42</td>
</tr>
<tr>
<td>51</td>
<td>↑↑</td>
<td>19.9/6.36</td>
<td>UDP-glucose pyrophosphorylase</td>
<td>Q9M4X0</td>
<td>7/20%</td>
<td>NI</td>
<td>51.7/5.46</td>
</tr>
<tr>
<td>55</td>
<td>↑↑</td>
<td>16.9/6.65</td>
<td>Putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase</td>
<td>Q94DV7</td>
<td>7/18%</td>
<td>NI</td>
<td>60.1/5.53</td>
</tr>
<tr>
<td>57</td>
<td>↑</td>
<td>16.1/5.67</td>
<td>Hypothetical protein</td>
<td>Q9SNF2</td>
<td>6/35%</td>
<td>1.94</td>
<td>19.0/5.94</td>
</tr>
<tr>
<td>62</td>
<td>↑</td>
<td>13.1/5.74</td>
<td>$\beta$-expansin</td>
<td>Q9LD01</td>
<td>5/20%</td>
<td>2.22</td>
<td>28.6/6.34</td>
</tr>
<tr>
<td>63</td>
<td>↑</td>
<td>13.2/6.01</td>
<td>Nucleoside diphosphate kinase 1</td>
<td>Q07661</td>
<td>4/32%</td>
<td>1.85</td>
<td>16.9/6.30</td>
</tr>
</tbody>
</table>

a) Experimental $M_i$ ($\times 10^3$) and $pI$

b) For proteins which have no Swiss-Prot accession numbers, the TrEMBL accession number is given

c) Number of peptides matched/percentage sequence coverage

d) See [19] for an explanation of the Z score. NI, not identified by Profound using default parameters, but identified by Masslynx searches

e) Theoretical $M_i$ ($\times 10^3$) and $pI$

Newly induced, up-regulated and down-regulated protein spots are indicated with ↑↑, ↑ and ↓, respectively.
Table 2. Partially degraded proteins induced at the trinucleate stage

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein change</th>
<th>Protein</th>
<th>Matching identity</th>
<th>PM/%SC</th>
<th>(M_r(\times 10^3))/p</th>
<th>Residual protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>↑</td>
<td>28.0/6.00 Glycogen phosphorylase</td>
<td>9/15%</td>
<td>91.5/7.35</td>
<td>C-terminus</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>↑↑</td>
<td>30.1/5.26 Heat shock protein 70</td>
<td>7/14%</td>
<td>71.1/5.13</td>
<td>N-terminus</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>↑</td>
<td>27.5/6.24 Aldolase C-1</td>
<td>7/27%</td>
<td>38.8/8.35</td>
<td>N-terminus</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>↑</td>
<td>19.9/6.36 UDP-glucose pyrophosphorylase</td>
<td>7/20%</td>
<td>51.7/5.46</td>
<td>C-terminus</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>↑↑</td>
<td>16.9/6.65 Putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase</td>
<td>7/18%</td>
<td>60.1/5.53</td>
<td>N-terminus</td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>↑</td>
<td>13.1/5.74 β-expansin</td>
<td>5/20%</td>
<td>28.6/6.34</td>
<td>C-terminus</td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>↑</td>
<td>13.2/6.01 Nucleoside diphosphate kinase 1</td>
<td>3/26%</td>
<td>16.9/6.30</td>
<td>C-terminus</td>
<td></td>
</tr>
</tbody>
</table>

a) Experimental \(M_r(\times 10^3)\) and p/
b) Number of peptides matched/ percentage sequence coverage
c) Theoretical \(M_r(\times 10^3)\) and p/

Newly-induced and up-regulated protein spots are indicated with ↑↑ and ↑, respectively.

Figure 4. Percentage sequence coverage maps of breakdown products. The sequence coverage maps were generated from Profound [19] search results. Relative lengths of matched peptides are indicated with short bars and combined as shown on the bottom of the panels. Observed \(M_r\) of intact proteins and their breakdown products are indicated with long bars on the top of the boxes. Predicted signal peptide is indicated by SignalP.

played proteins matched to a putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (Tables 1 and 2). One of them, spot 6 (down-regulated) had a very similar \(M_r\) and p/ to that of the matched protein. Another spot 55 (newly-induced) had a much lower \(M_r\), indicating partial degradation or cleavage. No further work has been conducted to correlate the degradation rate of putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (spot 6) with the accumulation rate of the newly-induced protein spot (55). However, the partial degradation or cleavage of the putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase to a stable breakdown protein product was clear.

4 Discussion

The main cause of sterility at the booting stage (just before anthesis) is the indehiscence of anthers that is due to incomplete ripening of pollen grains [3, 21]. However, this is a downstream effect, a consequence, not the primary cause of cold damage. It is not known which pathways or genes expressed at the later stage are affected by an early cold treatment that leads to the incomplete ripening of pollen grains. Determination of which pathways are affected by cold temperature treatment may be valuable in understanding CIMS. Anthers of cold affected rice had an abnormal appearance and their pollen grains did not stain with iodine potassium iodide suggesting that they might not be fertile (Fig. 1). Although the viability of these engorged pollen grains has not been tested in vitro, staining with iodine potassium iodide has been used to demonstrate the viability of pollen grains [22]. Thus, this study demonstrates that anthers and pollen grains at the trinucleate stage are affected by cold temperature treatment at the young microspore stage.

4.1 Most anther proteins are unaltered by early cold treatment

Initially we extracted anther proteins at the trinucleate stage with or without cold temperature treatment at the early stage and separated and compared them on 1-D
interested in pollen maturation or why the cold temperature causes alteration in the regulation and/or in the process of post-translational modification. Interestingly, none of the abundantly expressed late stage proteins (approximately 2%) to be differentially displayed.

4.2 Differentially displayed anther proteins were detected and identified

While the global expression pattern of anther proteins was largely unaltered, 70 anther proteins were observed as differentially displayed following cold temperature treatment for four days at the young microspore stage in cv Doongara after comparison of many independent experiments. Sixty-five out of 70 of the differentially displayed proteins were analyzed by MALDI-TOF and eighteen of them (26%) could be identified by PMF analysis. The enzyme APX (EC 1.11.1.11) is involved in detoxification of activated oxygen species by scavenging hydrogen peroxide generated in plants during metabolism and under stress. APX was reported to be involved in the tolerance of the cell to low temperature stress in rice [24]. At least six different isoforms of two APX gene products were found in the anthers at the trinucleate stage. Of the isoforms of the gene APXB (TrEMBL accession number Q9FE01), one protein spot was down-regulated and three were up-regulated. Of another gene product (TrEMBL accession number P93404), one protein spot was down-regulated and the other was up-regulated (Table 1 and Fig. 4). Accumulation of isoforms of both cytosolic APX gene products was either increased or decreased by cold temperature treatment, suggesting changes in gene expression and/or in the process of post-translational modification. However, it was not clear whether the different isoforms have specific functions in regard to pollen maturation or why the cold temperature causes alteration in the regulation and/or in the process of post-translational modification. Interestingly, four isoforms of these APXs were previously identified in the young microspore stage anthers and found to be unaltered by cold treatment after one, two or four days of cold treatment at the young microspore stage (unpublished data), suggesting a later effect of cold temperature on the expression of APX. Another example of changes in the regulation of isoforms is triosephosphate isomerase (Swiss-Prot accession number P48494). An isoform of this gene product was identified in the 2-DE map of anther proteins at the trinucleate stage by gel matching (Fig. 2). This isoform was not altered more than two-fold in its accumulation after cold treatment. However, another isoform of this gene, protein spot 26, was increased more than two-fold in its accumulation at the trinucleate stage by cold treatment at the young microspore stage.

4.3 Enhancement of protein partial degradation after cold treatment

At least seven protein products were observed to be partially degraded or cleaved (Table 2). They were all either up-regulated (five protein spots) or newly-induced (two protein spots) by cold temperature treatment. This demonstrates an enhancement and induction of partial protein degradation in the anthers at the trinucleate stage by cold treatment at the young microspore stage. This protein degradation/cleavage was highly selective only targeting a small subset of proteins. It is also important to state that protein degradation was not due to sampling, protein extraction or 2-DE procedures. There are three pieces of supporting evidence for this. First, the protein degradation phenomenon was not detected in anthers harvested at the early stages of microspore development (unpublished data) although the anther-sampling procedures were the same. Second, there was no difference in sampling of anthers from control (untreated) and cold temperature treated plants. Third, the protein extraction and 2-DE procedures for control and cold treated samples were conducted at the same time under the same conditions. Thus, the difference in protein accumulation was independent of sampling and extraction procedures.

It is not known how these proteins are partially degraded in the anthers of normally developing plants at the trinucleate stage or after exposure to cold stress at the young microspore stage. Further studies are essential to fully understand this phenomenon of protein degradation. N-terminal sequencing of these breakdown products for example, may indicate exact sites of cleavage and antibody probing may reveal how levels of intact proteins and breakdown products change in response to cold temperature treatment. However, our attempt to sequence N-terminal ends of several partially degraded proteins by Edman microsequencing was not successful most likely due to the insufficient amount of material pro-
vided (data not shown). Protein degradation is often so rapid that detecting partial breakdown products is difficult [25]. In contrast, the protein degradation or cleavage observed in rice anthers at the trinucleate stage after cold treatment is specific degradation or partial cleavage to a significantly accumulated, stable product. Protein degradation occurs during PCD in events such as leaf, flower, ovary and fruit senescence and xylogenesis [25, 26]. However, the protein degradations involved in PCD are complete degradations of proteins into individual amino acids and short peptides [26]. There is no report that PCD in male gametophyte development involves protein degradation with the exception that some maize inbreds show a progressive loss of ubiquitin and ubiquitin-conjugated conjugates during pollen maturation [27]. The most conserved degradation system, ubiquitin-mediated protein degradation, has not been demonstrated in pollen, although elements of the system are present in virtually all gymnosperm and angiosperm pollens examined [28].

There is evidence that some proteins can be partially degraded. For example, NF-κB, a human transcriptional activator involved in the defence response is synthesized as a 105 kDa precursor (p105) that is processed into a 50 kDa mature form (p50) by proteolytic removal of the C-terminal half of the molecule [29, 30]. p50 resides in the cytoplasm under unstressed conditions as a ternary complex with p65 and IκB, an inhibitory protein that masks the nuclear localization signal of the p50/p65 heterodimer. Activation by a number of defence signals, such as tumor necrosis factor α, triggers the selective destruction of the IκB subunit; the rest of the NF-κB complex (p50/p65) then enters the nucleus to transcriptionally activate a number of defence related genes. Both the processing of precursor p105 and the removal of IκB require ubiquitin conjugation and the 26S proteasome [29, 30]. Thus, the ubiquitin-mediated protein degradation system also involves partial degradation or cleavage of some proteins.

### 4.3.2 Glycogen phosphorylase (spot 31)

The breakdown product (spot 31) of glycogen phosphorylase (TriEMBL accession number Q9FPE6) was detected in rice anthers at the trinucleate stage and its accumulation was increased by cold temperature treatment at the young microspore stage. Glycogen phosphorylase (also called α-1,4-glucan phosphorylase or starch phosphorylase, EC 2.4.1.1) is present in all organisms capable of forming starch or glycogen. The enzyme catalyzes the reversible liberation of glucose-1-phosphate from α-1,4-glucan chains. Depending on the relative concentrations of orthophosphate and glucose-1-phosphate, the enzyme can act both to degrade and synde-size glucan. In mammalian tissue it is generally accepted that phosphorylase participates in the degradation of glycogen and its activity is regulated by covalent and nonco-valent mechanisms [33]. The in vivo function of plant phosphorylases is less well understood and similar control mechanisms have not been described in plants. Glycogen phosphorylase plays a central role in the mobilization of carbohydrate reserves in a wide variety of organisms and tissues [34]. Phosphorylases from plants, animals and prokaryotes occur as dimers or tetramers [35]. The primary structure of phosphorylases is well conserved, but the enzymes differ in their monomer size, kinetic properties, glucan specificity and intracellular location [36]. Higher plant tissues contain two types of phosphorylase isozyme. One type, designated Pho2 (also called H isozyme), is localized in the cytosol and has an apparent monomer size of 90 kDa [36]. It exhibits a very high affinity for large, highly branched glucans, such as glycogen. In contrast, Pho1 (also called L isozyme) is located in the plastid, and has a monomer size of 110 kDa. It prefers maltodextrin to branched polyglucans as a substrate [36]. It was reported that in sweet potato roots a Pho1 starch phosphorylase is regulated by proteolysis [37, 38]. Proteolytic cleavage of this 110 kDa Pho1 iso-
zyme resulted in a group of 50 kDa proteins. Interestingly, these partially degraded/cleaved proteins have a higher level of activity than the complete Pho1 protein. Near the N-terminus of the protein, there were two distinctive destruction sites for ubiquitination, indicating partial degradation of Pho1 through the ubiquitin-mediated protein degradation system [37]. This was also supported by the high abundance of ubiquitin and proteasome in sweet potato roots and contamination of Pho1 with proteasome subunits during purification of the protein [37]. We detected a breakdown product (spot 31) of glycogen phosphorylase H isozyme in rice anthers at the trinucleate stage and its accumulation was increased by cold temperature treatment at the young microspore stage. No studies have been carried out to investigate if this partially degraded product was active in vivo. However, it can be suggested from the existence of proteolytic regulation of some glycogen phosphorylases and its increased activity after degradation, that the partially degraded product (spot 31) of glycogen phosphorylase H isozyme may have a role in starch biogenesis in rice anthers. It is also possible that partial degradation of the glycogen phosphorylase H isozyme may alter its activity/function and hence cause incomplete starch formation and utilization, which may lead to infertility of pollen grains.

4.3.3 Regulation of protein synthesis and accumulation

The concentration of individual cellular proteins is determined by a balance between the rates of synthesis and degradation, which in turn are controlled by a series of regulated biochemical mechanisms. In many proteomic studies, the terms up-regulation and down-regulation are used to describe the increase and decrease in protein accumulation, respectively. However, these terms should be used with caution because accumulation of breakdown products does not indicate up-regulation of the intact proteins.

4.4 Concluding remarks

In this study we successfully employed 2-DE- and MS-based proteome analysis techniques as tools to investigate protein changes in rice anthers upon cold temperature treatment. Our results provide useful information about the effect of early cold treatment on rice anther maturation. We also demonstrated that cold treatment at the young microspore stage enhances and induces selective partial degradation of proteins in rice anthers at the trinucleate stage.

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5 References