Low Temperature Treatment at the Young Microspore Stage Induces Protein Changes in Rice Anthers* 

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Male reproductive development in rice is very sensitive to various forms of environmental stresses including low temperature. A few days of cold treatment (<20 °C) at the young microspore stage induce severe pollen sterility and thus large grain yield reductions. To investigate this phenomenon, anther proteins at the early stages of microspore development, with or without cold treatment at 12 °C, were extracted, separated by two-dimensional gel electrophoresis, and compared. The cold-sensitive cultivar Doongara and the relatively cold-tolerant cultivar HSC55 were used. The abundance of 37 anther proteins was changed more than 2-fold after 1, 2, and 4 days of cold treatment in cv. Doongara. Among them, one protein was newly induced, 32 protein spots were up-regulated, and four protein spots were down-regulated. Of these 37 protein spots, we identified two anther-specific proteins (putative lipid transfer protein and Osg6B) and a calreticulin that were down-regulated and a cystine synthase, a β-6 subunit of the 20 S proteasome, an H protein of the glycine cleavage system, cytochrome c oxidase subunit VB, an osmotin protein homologue, a putative 6-phosphogluconolactonase, a putative adenylate kinase, a putative cysteine proteinase inhibitor, ribosomal protein S12E, a caffeoyl-CoA O-methyltransferase, and a monodehydroascorbate reductase that were up-regulated. Identification of these proteins is available upon request. Accumulation of these proteins did not vary greatly after cold treatment in panicles of cv. Doongara or in the anthers of the cv. HSC55. The newly induced protein named OsCIA cold-induced anther protein (OsCIA) was identified as an unknown protein. The OsCIA protein was detected in panicles, leaves, and seedling tissues under normal growth conditions. Quantitative real time RT-PCR analysis of OsCIA mRNA expression showed no significant change between low temperature-treated and untreated plants. A possible regulatory role for the newly induced protein is proposed.  

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Rice (Oryza sativa L.) evolved in tropical and subtropical areas, and as a result it has the characteristic of being vulnerable to cold weather. In particular, the combination of the low temperature sensitivity and a maximal sensitivity to these temperatures at precise stages of pollen microspore development (1) makes cool midseason temperatures the major environmental limitation (other than soil and water) for growing rice in temperate regions and at high elevations in the tropics (2). During the life cycle of rice, pollen microspore development at the booting stage is known to be the most susceptible to cool weather damage (3). Cold damage at this reproductive stage of rice causes limitation to the yields of temperate-grown rice around the world and can reduce yields to 40% (3, 4). The main cause of this damage is pollen sterility resulting from low temperature damage at the early stage of microspore development (1). The developmental stage most sensitive to the various forms of environmental stress, including cold damage, is just after meiosis. That is tetrad to early microspore phase, or the young microspore stage (5). In cold-treated rice plants, both cytological and histological abnormalities are greater in the anthers than in the pistil or other organs of the flower, and the cold damage can be rescued by artificial pollination with healthy pollen (5). In this process of cold damage, anthers become smaller, and cells in the tapetal layer in the anthers that surrounds and is responsible for transferring nutrients to the developing pollen undergo hypertrophy and eventual breakdown. As a result, the normal development of pollen grains does not occur, and the pollen grains contain little or no starch and are functionally sterile (3, 6). In anthers from cold-treated rice plants, anther respiration decreases, sucrose accumulates in anthers, protein levels drop, and amino acid composition also changes including a large decrease in proline and increase in asparagine (7). Recently it was found that a novel tetrasaccharide Ara3Gal2 (where Ara is arabinose), which closely resembles the glycan chain of arabinogalactan protein, decreases after cold treatment in rice anthers (8). As β-1,3-glucanase, or callase, hydrolyzes the callose wall and releases tetrads (9), Tsuchiya et al. (10) demonstrated that premature dissolution of the callose wall causes male sterility in transgenic tobacco. A decrease in invertase activity in water-stressed rice anthers at the meiosis stage was also found, suggesting that a disturbance in carbohydrate metabolism may be involved in male sterility when anthers are subjected to water stress (11). Kitashiba et al. (12)
demonstrated partial male sterility in transgenic tobacco carrying an antisense gene for an alternative oxidase under the control of a tapetum-specific promoter, suggesting an important role of alternative oxidase in pollen development. It was found that a *Petunia* mutant that abolished pollen flavonoid accumulation and induced male sterility was complemented by a chalcone synthase transgene, indicating an involvement of flavonoid biogenesis in male sterility (13). It is also known that for many rice varieties, top dressing with nitrogen fertilizer at the booting stage exacerbates the problem of cold-induced sterility (14). Again the reasons for these observations are unknown. A recent cDNA microarray analysis of rice anther genes under chilling stress at the microsporogenesis stage revealed as many as 160 cDNAs (of 8987 cDNAs) were up- or down-regulated by chilling during the microspore release stage, including two genes with the DNA transposon Cast away in the 5'-flanking region (15). In that study the authors used Hayayuki, a relatively cold-tolerant japonica rice variety from Hokkaido (16) to identify genes involved in cold tolerance.

In general, a primary effect of chilling is considered to be the phase transition of membrane lipids at critical temperatures (17, 18). It has also been reported that, in rice plants, growth rate and metabolism are already markedly inhibited at temperatures above the chilling temperature in the range of 15–20 °C (19, 20). It is generally recognized that the protein synthesis patterns and mRNA levels change when plants are exposed to cold temperatures (21, 22). In addition, the mechanism for the effects of low temperature stress on growth and the accompanying metabolic changes and the relationship of general cold stress with cold-induced male sterility remain largely unclear. Despite much recent research on the subject, the underlying mechanisms that cause cold-induced male sterility are poorly understood. In particular, it is not known whether the tapetal abnormalities, the tapetal hypertrophy, or the biochemical changes seen are the causes or the consequences of the processes that lead to pollen sterility.

Proteomic analysis using two-dimensional gel electrophoresis (2-DE) has been applied previously to investigate stress-related proteins (23–27) and developmentally regulated proteins (28–31) in plants. To identify the earliest processes of cold-induced male sterility, we focused on the identification of the anther proteins that are affected by cold temperatures at the early stages of microspore development in rice using high resolution 2-DE and mass spectrometry analysis.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All chemicals used were of the highest obtainable grade and are outlined in Refs. 32 and 33 unless stated. Deionized water (Millipore, Bedford, MA) with resistance of greater than 18 megaohm-cm was used throughout.

**Plant Materials, Growth, Cold Treatment, and Sampling**—Cold-sensitive rice cv. Doongara was extensively studied in this project. The semidwarf, indica type Doongara was derived from a cross between tall, long grain cultivar Dawn (Texas) and IR8, a semidwarf cultivar carrying semidwarf-1 that was developed at the International Rice Research Institute in the 1960s (34). Relatively cold-tolerant Hungarian cv. HSC55 was subsequently studied to examine the difference between the responses of the two cultivars to cold temperature treatment. Seeds of rice (O. sativa L. cvs. Doongara and HSC55) were supplied by R. L. Williams, Yanco Agricultural Institute, New South Wales, Australia. Soil used in all the experiments was from the Yanco Agricultural Institute, New South Wales, Australia. Controlled environment facilities were used for rice growth and anther sampling. Rice ( cvs. Doongara and HSC55) seeds were germinated and grown in individual pots (height, 15 cm; diameter, 8.3 cm) in tubs (width, 36 cm; length, 52 cm; height, 30 cm). Nitrogen (15 g/m^2^) was applied as urea to 4-week-old seedlings, and the tubs were flooded to cover 10–20 cm of the shoots in a glasshouse under the following conditions until panicle initiation: 30/20 °C (day/night), 70% relative humidity, and natural day length. Plants under this growth condition had around three to four tillers each. Just after the time of panicle initiation, the plants were transferred into a growth chamber and grown under the same conditions except a day/night cycle of 12/12 h with an average photon flux density of 330 μmol m^−2^ s^−1^ was used.

Auricle distance (AD), the distance between the auricle of the flag leaf (last leaf) and that of the penultimate leaf, were used as a non-destructive measurement to gauge the stage of development of the rice panicle and pollen microspore. Correlation of the AD with the developmental stages of the microspore was done by measuring AD of the panicle investigated and classifying the microspheres in the anthers of the spikelets with the microscopic techniques described below. We determined that the AD of −18 to −10 mm for cv. Doongara and −100 to −90 mm for cv. HSC55 corresponds with the tetrads to early stage of microspore development of the top five spikelets from the top three panicle branches as seen in Supplemental Table I. This measurement was used to determine the most temperature-sensitive stage for the start of cold treatment. However, it is important to mention that the correlation of AD with the developmental stages should be used cautiously as many other factors such as growing season, tiller number, and light and temperature conditions can alter the correlation, and this needs to be checked in each batch of grown plants. Cold treatment was performed by moving individual plants in which the AD was about −15 mm for cv. Doongara and −100 to −90 mm for cv. HSC55 into an identical chamber kept at 12 °C, 70% relative humidity with the same illumination and day/night cycle of the control condition. To be able to compare anthers from cold-treated plants with the anthers at the same stage of development of normally grown plants, we used anthers from normally grown plants that had the same AD and developmental stage as the treated plants after the completion of their cold treatment (Supplemental Table I).

**Microscopic Methods for Anther Analysis**—Anthers were removed from the upper 5 spikelets from each of the top three branches of immature panicles with precision forceps and scalpels under a dissection microscope. Then they were stained either by heating in acetocarmine solution (4% carmine in 45% acetic acid, boiled for 1 h under reflux, and then filtered when cooled) or incubated with DAPI (1...
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μg/ml 4,6-diamidino-2-phenylindole in 50% ethanol) or with toluidine blue solution (pH 4.4) without heating (35). They were either prefixed in fixation solution (50% ethanol, 5% acetic acid) if they were to be stored prior to staining and microscopy, or they were stained directly if microscopy proceeded immediately. DAPI-stained samples were visualized under UV light. Alternatively for embedding and sectioning, anthers were fixed at least overnight in fixation solution, dehydrated at 4 °C in increasing concentrations of ethanol (70, 80, 90, and 96% for 1 h each), and infiltrated with Historesin (Leica Instruments, Heidelberg, Germany) at 4 °C on a rotating wheel overnight. Sections of 10 μm were cut on an Ultratec microtome (Reichert-Jung, Vienna, Austria) with a glass knife. Sections were transferred to glass slides, stretched onto the slide surface, and then stained with a drop of toluidine blue on top of each section. The slides were placed on a warming plate at 40 °C for 15 min after rinsing with water several times. Color micrographs of the stained anthers were taken with a Nikon FX35 camera mounted onto a Nikon SMZ-10 stereo microscope using Kodak Ektachrome 100 ASA, EPY 64T or Agfachrome RSX100 color reversal films.

Two-dimensional Gel Electrophoresis—2-DE was carried out in a horizontal electrophoresis system, Multiphor II (Amersham Biosciences), as described previously (33, 36). For the first dimension, IPG gel strips (18 cm) were placed onto Amersham Bioscience ExcelGel Immobiline DryStrip kit in a Multiphor II electrophoresis unit (Amersham Biosciences), and the apparent pI values of the proteins were as- signed after calculating the pl of selected landmark spots on the 2-DE gels using a ruler. This calculation was based on the information provided by the manufacturer that IPG (linear) strips have a linear pH gradient between a given pH range. Gel comparison was done visually examining the spot intensity and subsequently calculating relative spot volume (%Vol, the volume divided by the total volume over the whole gel image, whereas the volume of the spot is the integration of the highest optical density over the area of the spot) of the selected protein spots was calculated by the Melanie program. A protein spot was classified as being differentially displayed if the %Vol varied more than 2-fold between untreated and cold-treated plants in at least three experiments. Thus, we defined these proteins as cold-responsive proteins.

Western Blotting and Amino-terminal Sequencing—Semidry electrophoretic blotting onto PVDF membranes (Bio-Rad) was performed as described previously (33). Selected spots were excised from dried membranes for amino-terminal Edman sequencing. This sequencing was carried out at the Biomolecular Resource Facility of the Australian National University using Edman degradation chemistry on a Procise 494-01 sequencer system (Perkin-Elmer Life Sciences).

MALDI-TOF MS and MS/MS Analysis—Proteins in preparative 2-DE gels were visualized by colloidal Coomassie staining, and selected spots were excised from polyacrylamide gels. Selected spots were in-gel digested and analyzed by MALDI-TOF as described previously (33). Briefly the spots were destained and underwent an in-gel 16-h tryptic digest at 37 °C. The resulting peptides were extracted from the gel with a 50% (v/v) acetonitrile, 1% (v/v) TFA solution. A 1-μl aliquot was spotted onto a sample plate with 1 μl of matrix (α-cyano-4-hydroxycinnamic acid, 8 mg/ml in 50% (v/v) ACN, 1% (v/v) TFA) and allowed to air dry. MALDI mass spectrometry was performed with a Micromass ToFSpec 2E time of flight mass spectrometer. A nitrogen laser (337 nm) was used to irradiate the sample. The spectra were acquired in reflectron mode in the mass range 600–3500 Da. An internal calibration was applied using two trypsin autodigestion peaks at 842.51 and 2211.1 Da.

For tandem mass spectrometry analysis, the in-gel digested spots were analyzed either on a PE SCIEX (Foster City, CA) QSTAR hybrid LC/MS/MS quadrupole TOF system or on a Micromass (Manchester, UK) LC/MS/MS quadrupole TOF system at the Australian Proteome Analysis Facility, Sydney, Australia. Data was acquired over the m/z range 400–1800 Da to select peptides for MS/MS analysis. After peptides were selected, the MS was switched to MS/MS mode, and data were collected over the m/z range 50–2000 Da with variable collision energy settings.

Sequence Analysis, Database Searching, and Peptide Mass Fingerprint (PMF) Analysis—Signal peptides and the other targetting sequences were predicted with the SignalP (37), MITOPROT (38), and TargetP (39) programs. Molecular mass and pl of the theoretical proteins were calculated after deleting the signal peptides. For amino acid sequence homology comparisons, amino-terminal protein sequences were used to search non-redundant protein databases (Swiss-Prot, TrEMBL, and NCBI) using the FASTA (40) and BLAST (41) programs or to search translated rice gene index (OsGI) sequences (www.tigr.org) using WU-BLAST. For protein identification following MS/MS analysis, full or partial amino acid sequences generated by decomposition analysis were used to search non-redundant protein databases as mentioned above. Masses of the full peptide were also considered for the confidence of the matches. In the searches, methionine residues were assumed to be modified to methionine sulfoxide, and cysteine residues were assumed to be reduced and alkylated by iodoacetamide to carboxymidomethylcysteine wherever necessary. Protein identification by PMF was done as described previously (33). Briefly, the list of masses were used to
search against Swiss-Prot and NCBI protein databases available at 129.85.19.192/prowl-cgi/ProFound.exe using the search engine ProFound (42). All peptide masses were assumed as monoisotopic and [M + H]+ (protonated molecular ions). In the searches, methionine residues were assumed to be modified to methionine sulfoxide, cysteine residues were assumed to be reduced and alkylated by iodoacetamide to carboxymethylcysteine wherever necessary, and one missed cleavage site was allowed. For PMF matches, the number of peptides matched (at least four), sequence coverage (at least 20%), mass accuracy (lower than 50 ppm), number of missed cleavages (no more than two single cleavages), number of chemical modifications (no more than two modifications), and Z score (above 1.6) (42) were considered as confident identifications. Z score is the distance to the population mean in unit of standard deviation. For example, a Z score of 1.65 for a search means there are about 5% of random matches that could yield higher Z scores than this search (see Ref. 42 for details). The multiple sequence alignment and the construction of phylogenetic trees were done by using the ClustalW program (43) available at www.ebi.ac.uk/clustalw/

**RESULTS**

**Cold-treated Doongara Plants Showed High Levels of Sterility**—To ensure that the cold treatments used were causing sterility, we tested periods of cold temperature initiated at the young microspore stage after which plants were grown normally, and the effect of the treatments on eventual grains filled was assessed. We focused on the analysis of the upper 15 spikelets because they were reported as the most sensitive spikelets and synchronous in microspore development (6). Anthers at the young microspore stage were chosen as the main tissue and stage of analysis (5). A temperature condition of 12/12 °C day/night was used to maximize the effect of cold stress. By growing rice plants under this temperature condition, considerable sterility was observed in cv. Doongara plants that had a cold treatment that commenced at the young microspore stage on sterility of the rice cvs. Doongara and HSC55. Sterility was determined by counting the number of filled grains of the spikelets from the top three branches. Columns 1–4 represent control and 1, 2, and 4 days of cold treatment of cv. Doongara at 12 °C, respectively. Columns 5 and 6 represent control and 4 days of cold treatment of cv. HSC55 at 12 °C, respectively. Error bars represent standard deviation (n = 40).

New Zealand White rabbits using purified protein as antigen, and immunodetection was done as described previously (45).

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**RT-PCR and Real Time PCR**—Total RNA from rice panicles and anthers was extracted using the RNeasy plant minikit (Qiagen) according to the manufacturer’s instructions. cDNA synthesis was done using 5 μg of total RNA for each sample. RNA was treated in 1× buffer with 2 units of DNase I (Ambion, Austin, TX) added to the reaction and incubated for 30 min at 37 °C. The reaction was stopped by adding 1 μl of 25 mM EDTA followed by a 15-min incubation at 65 °C. One microliter of 5 μM oligo(dT)18 was added to the reaction, and it was incubated for 10 min at 70 °C and then chilled on ice. First strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) or water (for the non-reverse transcriptase control). PCR for cloning was done using the primers OsCIA_F (5′-GTT GAC ATG TCG ACG GTG ACC TC-3′) and OsCIA_R (5′-GAC TCG AGT CGA CAT CGA CAT CGA-3′).

The following real time PCR primers were designed using Primer Express software (Applied Biosystems): forward 5′-AGA GAG GTG GTC CAG GCA AA-3′ and reverse 5′-TCC TTG ACC TTG CCA ATG GT-3′ for OsCIA and forward 5′-TCC TTC ATG AAT CAT GGA ACT GTG ATG G-3′ and reverse 5′-ACA GCC CTG GGC GCA T-3′ for the actin gene (GenBank™ accession number X15865 was used for normalization). PCR was carried out in a total volume of 20 μl containing a 0.2 μM concentration of each primer and 1× SYBR green PCR Master Mix (PE Applied Biosystems). Reactions were amplified in an ABI PRISM 7700 thermocycler as follows: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, and 60 °C for 1.5 min. Normalization was done as described previously (44) against the rice actin gene. Two biological and two technical repeats were done for each treatment. For each sample, a no RT enzyme reaction was performed also in two replicates.

**Southern Blotting**—Genomic DNA was isolated using the PUREGENE DNA isolation kit (Gentra). Ten micrograms of genomic DNA were digested with restriction enzymes electrophoresed through a 0.8% agarose gel and then transferred to Hybond-N membrane (Amersham Biosciences) using an alkaline method. Radiolabeled probes were generated using PCR-amplified template by a Prime-a-Gene labeling system (Promega). Hybridization was done at 60 °C using ExpressHyb (Clontech) solution according to the manufacturer’s instructions.

**Protein Expression, Antibody Production, and Immunodetection**—PCR-amplified fragments were ligated into pET28a (+) vector (Novagen), transformed into BL21(DE3) pLysS competent cells (Invitrogen), and induced with 0.5 mM isopropyl 1-thio-β-d-galactopyranoside (Sigma). Recombinant protein with a carboxy-terminal His6 tag was purified with HIS-Select nickel affinity gels (Sigma) according to the manufacturer’s instructions. Polyclonal antibody was produced in
Cold-treated Doongara Plants Showed Cytological Abnormalities in Anthers—As previous research has demonstrated cytological abnormalities in the tapetal cells due to cold treatment (3, 6), we also examined the effect of the cold treatment we were using on the anthers harvested from the upper 15 spikelets. Under the microscope, cross-sections of cold-treated anthers showed abnormal tapetum and microspore development in the cold-sensitive cv. Doongara. There was severe swelling of tapetal cells known as tapetal hypertrophy observed after cold treatment. Microspores also showed abnormal development (Fig. 2).

Cold Treatment Induced Protein Changes in Doongara Anthers—To study the effects of cold treatment on the protein expression level, we used 2-DE to display and compare all extractable rice anther proteins from cold-treated and untreated plants. To do this, we have performed 1, 2, and 4 days of cold treatment at 12 °C for cv. Doongara and 4 days of cold treatment for cv. HSC55 plants. Anther proteins with and without cold treatment were extracted from microdissected anthers separated by 2-DE. The 2-DE anther protein maps of anther proteins from 4-day cold-treated cv. Doongara plants are shown in Fig. 3 (pH 4–7) and in Fig. 4 (pH 6–11). Melanie 3 image analysis of at least three replicate gels revealed that over 4000 anther proteins were reproducibly resolved in silver-stained gels over a combined pH range of 4–11 and size range of 6–120 kDa (Figs. 3 and 4). Silver-stained gels (pH 4–7) were used for most of the comparisons and quantification, although narrow pH range gels were used for reference and further separation of crowded protein spots (data not shown). Coomassie-stained gels were also generated (except for 2-day cold treatment and its control due to insufficient materials for those typical analyses) and used for comparisons and quantification of the proteins that do not stain well with the silver nitrate method. Silver-stained gels (pH 6–11) were generated for only 4-day cold-treated cv. Doongara plants and their controls. Although the global pattern of anther proteins was largely unaltered (more than 99% of the total protein spots remained unchanged), 37 protein spots were reproducibly detected as changed in their protein levels after 1, 2, and/or 4 days of cold temperature treatment at 12 °C in

![Fig. 2. Effect of cold treatment on cv. Doongara anthers.](image-url)

Fig. 2. Effect of cold treatment on cv. Doongara anthers. The cross-sections of the early and middle stages of microspore development were stained with toluidine blue. A, B, and C are the cross-sections of the anthers from plants that were untreated or that underwent 2 and 4 days of cold treatment at 12/12 °C beginning at the young microspore stage, respectively. D, E, and F show enlarged loculi of A, B, and C, respectively. Swollen tapetal cells are indicated with arrows. Scale bars represent 100 μm for the top panels and 20 μm for the bottom panels.
the cold-sensitive cv. Doongara when compared with their relevant untreated plants as shown in Figs. 3 and 4. All the up- and down-regulated protein spots are shown, although some (spots 25, 34, and 36) were not detected as protein spots on the silver-stained gel (Fig. 3). Selected parts of the gels are highlighted in Fig. 5A to show comparisons of cold-responsive protein spots of rice anthers in cold-treated and untreated plants. Relative protein levels (shown as %Vol) of
these 37 cold-responsive proteins and their changes after different lengths of cold treatment are given in Table I. Examples of protein spot comparison and their correlation with relative expression levels are given in Fig. 5B. One protein spot (spot 37) was newly induced in all cold treatments compared with controls. We named this protein *O. sativa* cold-induced anther protein (OsCIA). Three protein spots (spots 12, 34, and 36) were down-regulated, and 34 protein spots were up-regulated after different days of cold treatment (Figs. 3 and 4 and Table I). For spots 25 and 34, %Vol was measured from Coomassie-stained gels as these spots did not stain well with the silver nitrate method (Figs. 5 and 6).

The Hungarian cv. HSC55 was reported as a relatively cold-tolerant rice cultivar, and this was also proved in our experiments (Fig. 1). We compared 2-DE anther protein maps (pH 4–7, silver-stained and Coomassie-stained) of control and 4-day cold-treated cv. HSC55 plants. Seven protein spots were differentially displayed after cold treatment. Six protein spots (h1, h2, h3, h4, h5, and h7) were up-regulated, and one spot (h6) was down-regulated, as shown in Table I and Supplemental Fig. 1. By gel matching, we found that spot h3 was the same as spot 36 detected in cv. Doongara anther samples. Except for protein spot 11, we did not see any of the changes in protein abundance that occurred in cv. Doongara anthers upon cold treatment. However, spot 26 can be seen but at a much reduced level in the Coomassie-stained gels of panicle samples run for the same pl range and running conditions, indicating an enrichment in the anther tissues (Supplemental Fig. 2).

Protein Spot 34 Is Detected in a Spatially and Temporally Expressed Manner in Rice Anthers—Spot 34 could not be detected in the silver-stained or Coomassie-stained gels of panicle samples of the same developmental stage run over the same pl range under identical conditions, indicating anther-specific expression of the protein (Fig. 6A). In both cvs. Doongara and HSC55, spot 34 could be detected in 2-DE gels (in Coomassie-stained gels only) of anthers at the young microspore stage. To gain insight into the expression pattern of this spot, cv. Doongara anthers at the young microspore ( uninucleate), binucleate, and trinucleate stages were extracted, and their 2-DE protein patterns were compared (Fig. 6A). We observed a decrease in spot 34 protein levels at the binucleate stage, and it became completely undetectable at the trinucleate stage. This stage contains mature pollen, indicating specific accumulation of protein spot 34 in the earlier stages of microspore development in rice anthers.

Identification of Cold-responsive Proteins—Amino-terminal sequences were obtained by Edman degradation analysis for six of 11 cold-responsive protein spots blotted onto PVDF.

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2 R. L. Williams and T. C. Farrel, personal communication.
membranes as shown in Table II. No sequence data could be obtained for protein spots 6, 9, 10, and 11 due to either the amino terminal being blocked or not enough protein being available. Two amino-terminal sequences were generated from spot 25, each matching to two mitochondrial proteins, the H protein of glycine decarboxylase complex and cyto-

| Spot no. | Molecular mass (kDa)/pI | Days | %Vol | Changes in accumulation |
|----------|--------------------------|------|------|-------------------------|
| 1        | 29.5/5.24                | 1    | 0.038/0.006 | † +6                   |
| 2        | 23.5/4.54                | 1    | 0.224/0.058 | † +3                   |
| 3        | 23.2/4.54                | 1    | 0.060/0.023 | † +2                   |
| 4        | 26.7/4.74                | 1    | 0.060/0.023 | † +2                   |
| 5        | 31.3/5.57                | 1    | 0.033/0.014 | † +2                   |
| 6        | 29.0/5.69                | 1    | 0.054/0.018 | † +3                   |
| 7        | 24.7/5.56                | 1    | 0.063/0.009 | † +7                   |
| 8        | 26.5/6.39                | 2    | 0.067/0.033 | † +2                   |
| 9        | 30.1/5.68                | 2    | 0.150/0.050 | † +3                   |
| 10       | 44.5/5.46                | 2    | 0.143/0.046 | † +3                   |
| 11       | 43.8/5.43                | 2    | 0.058/0.025 | † +2                   |
| 12       | 35.8/4.36                | 2    | 0.154/0.309 | † −2                   |
| 13       | 32.8/4.74                | 2    | 0.100/0.041 | † +2                   |
| 14       | 30.9/4.79                | 2    | 0.126/0.047 | † +2                   |
| 15       | 31.1/4.85                | 2    | 0.080/0.027 | † +2                   |
| 16       | 33.1/5.15                | 2    | 0.067/0.018 | † +3                   |
| 17       | 33.4/5.84                | 2    | 0.101/0.05 | † +2                   |
| 18       | 27.6/4.70                | 2    | 0.045/0.021 | † +2                   |
| 19       | 26.9/4.86                | 2    | 0.043/0.014 | † +3                   |
| 20       | 30.6/5.50                | 2    | 0.063/0.027 | † −2                   |
| 21       | 30.1/5.63                | 2    | 0.077/0.038 | † −2                   |
| 22       | 27.9/5.84                | 2    | 0.103/0.050 | † +2                   |
| 23       | 26.8/6.21                | 2    | 0.099/0.049 | † +2                   |
| 24       | 15.4/5.41                | 2    | 0.146/0.042 | † +3                   |
| 25       | 14.7/4.38                | 4    | 1.065/0.377 | † −2                   |
| 26       | 31.9/6.88                | 4    | 0.267/0.131 | † −2                   |
| 27       | 29.1/6.84                | 4    | 0.275/0.080 | † −3                   |
| 28       | 27.5/6.84                | 4    | 0.246/0.041 | † +6                   |
| 29       | 27.5/6.92                | 4    | 0.447/0.088 | † +5                   |
| 30       | 31.1/4.90                | 4    | 0.148/0.057 | † +2                   |
| 31       | 31.4/5.68                | 4    | 0.092/0.022 | † +4                   |
| 32       | 30.8/4.85                | 4    | 0.260/0.116 | † +2                   |
| 33       | 26.7/6.36                | 4    | 0.016/0.008 | † +2                   |
| 34       | 9.7/4.49                 | 1    | 0.520/0.960 | † −2                   |
| 35       | 34.3/4.45                | 1    | 0.112/0.056 | † +2                   |
| 36       | 13.1/5.75                | 4    | 0.120/0.050 | † +2                   |
| 37       | 18.0/5.5                 | 1    | New | † †                   |
|          |                          | 2    | New | † †                   |
|          |                          | 4    | New | † †                   |

* Cold-responsive protein spots were listed with the same identifiers given in Figs. 3 and 4.
* Experimental molecular mass (×10^3) and pl.
* 1, 2, and 4 represents 1, 2, and 4 days of cold treatment at 12 °C, respectively.
* Percent spot volume ratio for a protein from cold-treated and untreated plants.
* Newly induced, up-regulated, and down-regulated protein spots were marked with † †, † †, and † † symbols, respectively, and the relative spot volume ratio changes greater (†) or less (†) than 2-fold or more are given.
* %Vol measured from Coomassie-stained gels as these spots did not stain well with the silver nitrate method.
For these two proteins, matches to database proteins occurred from the amino acids 35 and 55 of the database proteins, respectively. Putative mitochondrial targeting sequences and the cleavage sites were detected for both proteins by using the MITOPROT program (38). High probability scores of 0.99 and 0.91 for export to mitochondria and putative cleavage sites at amino acids 42 and 51, respectively, were given by the program. Amino-terminal sequences of spots 12 and 34 were matched to the amino acid sequences of rice calreticulin and a translation product of the t42 gene from the amino acids 26 and 29, respectively. For both proteins, signal peptides of 25 and 28 amino acids, respectively, were predicted by the SignalP program (37).

Both BLASTP and FASTA searches against the non-redundant NCBI and Swiss-Prot protein databases showed strong homology (about 40% identity) of the translated sequence of the t42 gene (we refer to this protein as T42) to many other plant nonspecific lipid transfer proteins (ns-LTPs). This match was validated by a search against the conserved domain database at NCBI that showed the strongly conserved LTP domain in the sequence (E value, 3e⁻¹²). The LTP domain of T42 can be written as Cₓ₁₀₋₁₅ₓ₁₉₋₂₂ₓ₁₃₋₁₅. A further search of T42 against the translated GenBank™ dbEST using the TBLASTN algorithm gave ~80% identity (with expected value lower than e⁻¹⁰) to anther-specific ESTs from sorghum, goat grass, rye, and wheat with similar molecular weight and pl. Alignment and a phylogenetic tree of T42 from selected sources is given. Lipid transfer proteins from different plant species and translated amino acid sequence of anther ESTs from goat grass and rye are shown. The neighbor-joining tree using percent identities was displayed by the program Jalview based on a multiple sequence alignment generated with the program ClustalW. The scale represents percentage of substitution per site.

Fig. 6. Temporal and spatial expression of the T42 protein and its alignment and phylogenetic tree with other plant ns-LTPs. A, temporal and spatial expression of T42 protein. 2-DE gel (pH 4–7, Coomassie-stained) regions containing T42 protein (spot 34) of Doongara panicle at the auricle distance of ~15 mm (panel 1) and anther at the uninucleate (panel 2), binucleate (panel 3), and trinucleate stages (panel 4) are shown. B, alignment of T42 protein with other plant LTPs and with translated amino acid sequence of anther ESTs from goat grass (BQ840880) and rye (BF429311) are shown. This was done using the ClustalW program. Signal peptide is shown at the amino-terminal end of the T42 protein sequence. Eight conserved cysteines are indicated with asterisks; phenylalanine 115 that replaced tyrosine of which the side chain divides the hydrophobic cavity into two parts in other ns-LTPs is indicated with an arrow. C, a phylogenetic tree representing alignment of T42 protein from selected sources is given. Lipid transfer proteins from different plant species and translated amino acid sequence of anther ESTs from goat grass and rye are shown. The neighbor-joining tree using percent identities was displayed by the program Jalview based on a multiple sequence alignment generated with the program ClustalW. The scale represents percentage of substitution per site.
Identification of Cold-responsive Proteins in Rice Anthers

Cold-responsive rice anther proteins identified by amino-terminal and MS/MS sequencing in cv. Doongara

Protein spots are listed with the same identifiers given in Figs. 3 and 4.

| Spot no. | Molecular mass (kDa)/pI | Sequences | Matching identity | Accession no. | Molecular mass (kDa)/pI |
|----------|-------------------------|-----------|-------------------|---------------|------------------------|
| 8        | 26.5/6.39               | DVFASATER,^a SPSPLL Пар,^a EYIDLRKD^a | β-6 subunit of 20 S proteasome | O64464 | 24.3/6.43 |
| 12       | 35.8/4.36               | EVFFOKEFDGWESR | Calreticulin^f | Q9SLY8 | 45.6/4.48 |
| 24       | 15.4/5.41               | ALCAEHNVHLTVPSAK^a | 40S ribosomal protein S12E | Q8H2J8 | 14.8/5.33 |
| 25a      | 14.7/4.38               | STVLGDKYSSHE | 15-kDa H protein of glycine decarboxylase complex (fragment)^/ | Q9FW99 | 7.1/8.98 |
| 25b      | 14.7/4.38               | SGDKPATVEDVMPIA | Cytochrome c oxidase subunit Ib^/ | P92683 | 12.7/4.44 |
| 31       | 31.4/5.68               | SGTTTTXTLTHHRT | Osmotin protein homologue^/ | AAX95344 | 21.3/5.39 |
| 34       | 9.7/4.49                | AVQCQGVMQLMAPCM, DAAAGFPAVDFSR^d | Translated product of t42 gene^/ | CAA01672 | 9.4/4.49 |
| 36       | 13.1/5.75               | AFALL^e | Anther-specific protein Osg6B | Q40662 | 13.2/9.42 |
| 37       | 18.0/5.5                | XISSNKPGDKPI, SLSPVVLVN,^e LVNPV,^e SNYNTPTSGAIDHQ | Unknown protein | AAP52230 | 16.3/5.22 |

Species) ns-LPTs based on percentage identity are given in Fig. 6, B and C.

One to three sequence tags were obtained for five of the eight protein spots analyzed by MS/MS analysis (Table II). Seventeen cold-responsive protein spots were analyzed by MALDI-TOF MS, and eight of them could be identified by PMF analysis (Table III). Seven other protein spots (spots 2, 6, 9, 10, 18, 26, 31, and 35) could not be identified due to either an incomplete genome sequence being available, a poor mass spectrum being generated, or post-translational modifications of the mature proteins in vivo. The matches obtained from MS/MS and amino-terminal sequencing analysis for protein spots 8 and 25 were confirmed with PMF analysis.

OsCIA, a Novel Protein, Was Suppressed in Rice Anthers under Normal Conditions but Induced by Cold Treatment—As described in Table I, spot 37 (OsCIA) was the only cold-induced protein spot detected after all cold treatment. We examined the panicle proteome of cv. Doongara and the anther proteome of the relatively cold-tolerant cv. HSC55 by 2-DE protein differential display. In the cv. Doongara panicle proteome, a protein spot (p8) was detected at the same position as spot 37 from the cv. Doongara anther proteome by matching 2-DE gels for the pH ranges of 4–7 and 5–6 (data not shown). Interestingly this panicle protein spot did not change in its protein level upon cold treatment (Fig. 7). Further evidence of the shared identity of these two protein spots was obtained by amino-terminal Edman sequencing (for the panicle protein, the sequence was AISSNKPGDKPI) and MALDI-TOF analysis. The protonated masses of the peaks were 1777.02, 2239.11, and 3100.31 for spot 37 and 1777.02, 2239.11, and 3100.31 for spot p8, respectively. At least it is evident that these two protein spots (37 and p8) share similar molecular mass, pl, PMF pattern, and amino-terminal sequence. OsCIA was detected in cv. Doongara anthers at a high level after as little as 1 day of cold treatment at 12 °C. However, upon restoring normal growth conditions after cold stress that lasted for 4 days from the young microspore stage, the level of OsCIA started to decrease, and it had disappeared by the trinucleate stage (data not shown). We detected a protein spot (h8) in cv. HSC55 in the same position as spot 37 by gel comparison and gel matching that did not change upon cold treatment (data not shown). However, we identified h8 as a putative small heat shock protein (sHSP) by MS/MS analysis (partial sequence obtained was PGAYAFVVDM, which matched to a rice putative 18-kDa heat shock protein, GenBankTM accession number XP_550428). Considering any possible mismatching, we also analyzed other cv. HSC55 anther protein spots close to spot h8 from the narrow range (pH 5–6) 2-DE proteome map of cv. HSC55 by MALDI-TOF without finding any PMF pattern similar to that of spot 37 (data not shown).

OsCIA proteins (spot 37) from five gel pieces and PVDF blots of rice anther 2-DE from cold-treated plants were pooled together for MS/MS analysis and Edman sequencing, respectively. MS/MS analysis generated two partial sequences from two different peptides with overlapping sequences and overlapping masses (Table II and Fig. 8A). Subtraction of the masses of the amino acids valine and proline from the mass of the carboxyl-terminal fragment of peptide_A is equal to the mass of the carboxyl-terminal fragment of peptide_B (Fig. 8A).
| Spot no. | Molecular mass (kDa)/pI | Matching identity | Accession no. | PM (P/I)/S%C | Matched peptides | Z score | Molecular mass (kDa)/pI |
|----------|-------------------------|-------------------|--------------|--------------|-----------------|---------|------------------------|
| 7        | 24.7/5.56               | Cysteine synthase | Q9XEA8       | 11 (28)/42%  | IGYSMITDAEKEGLTPGK AFGAEVLTDPLLGMK MPNSYIQQFNAPANPK IYETTGPEIWK IYGVEPTESAILSGGRPGPHK VAQRPENK VAQRPENKKG GKLVVVVFPSFGER LVVVFPSFGER YLSSVLFESIK VDILVSGIGTTGVTGTCGK SPSPLLLPAR LSVGYNILTR GCVFTYDAVSSYER MSCPAMAQLLSNLYLKK FFPYAFNLGGLDSEGK TGYSQAQGTSALMPVDQNLK GDWWYENNGGTCVAJAGADYCVAADTR | 2.25 | 34.3/5.35             |
| 8        | 26.5/6.39               | β-6 subunit of 20S proteasome | O64484 | 7 (26)/54% | IGYSMITDAEKEGLTPGK AFGAEVLTDPLLGMK MPNSYIQQFNAPANPK IYETTGPEIWK IYGVEPTESAILSGGRPGPHK VAQRPENK VAQRPENKKG GKLVVVVFPSFGER LVVVFPSFGER YLSSVLFESIK VDILVSGIGTTGVTGTCGK SPSPLLLPAR LSVGYNILTR GCVFTYDAVSSYER MSCPAMAQLLSNLYLKK FFPYAFNLGGLDSEGK TGYSQAQGTSALMPVDQNLK GDWWYENNGGTCVAJAGADYCVAADTR | 2.26 | 24.3/6.43             |
| 11       | 43.8/5.43               | Monodehydroascorbate reductase | Q9SXX0 | 19 (30)/53% | IGYSMITDAEKEGLTPGK AFGAEVLTDPLLGMK MPNSYIQQFNAPANPK IYETTGPEIWK IYGVEPTESAILSGGRPGPHK VAQRPENK VAQRPENKKG GKLVVVVFPSFGER LVVVFPSFGER YLSSVLFESIK VDILVSGIGTTGVTGTCGK SPSPLLLPAR LSVGYNILTR GCVFTYDAVSSYER MSCPAMAQLLSNLYLKK FFPYAFNLGGLDSEGK TGYSQAQGTSALMPVDQNLK GDWWYENNGGTCVAJAGADYCVAADTR | 2.4  | 46.6/5.3              |
| 17       | 33.4/5.84               | Putative adenylyl kinase | Q9SNJ4 | 8 (26)/37% | IGYSMITDAEKEGLTPGK AFGAEVLTDPLLGMK MPNSYIQQFNAPANPK IYETTGPEIWK IYGVEPTESAILSGGRPGPHK VAQRPENK VAQRPENKKG GKLVVVVFPSFGER LVVVFPSFGER YLSSVLFESIK VDILVSGIGTTGVTGTCGK SPSPLLLPAR LSVGYNILTR GCVFTYDAVSSYER MSCPAMAQLLSNLYLKK FFPYAFNLGGLDSEGK TGYSQAQGTSALMPVDQNLK GDWWYENNGGTCVAJAGADYCVAADTR | 1.8  | 35.3/8.97             |
| 20       | 30.6/5.50               | Putative 6-phosphogluconolactonase | Q69NG5 | 7 (27)/41% | IGYSMITDAEKEGLTPGK AFGAEVLTDPLLGMK MPNSYIQQFNAPANPK IYETTGPEIWK IYGVEPTESAILSGGRPGPHK VAQRPENK VAQRPENKKG GKLVVVVFPSFGER LVVVFPSFGER YLSSVLFESIK VDILVSGIGTTGVTGTCGK SPSPLLLPAR LSVGYNILTR GCVFTYDAVSSYER MSCPAMAQLLSNLYLKK FFPYAFNLGGLDSEGK TGYSQAQGTSALMPVDQNLK GDWWYENNGGTCVAJAGADYCVAADTR | 2.36 | 29.0/5.46             |
| 23       | 26.8/6.21               | Cysteine proteinase inhibitor | Q5NB11 | 5 (24)/29% | IGYSMITDAEKEGLTPGK AFGAEVLTDPLLGMK MPNSYIQQFNAPANPK IYETTGPEIWK IYGVEPTESAILSGGRPGPHK VAQRPENK VAQRPENKKG GKLVVVVFPSFGER LVVVFPSFGER YLSSVLFESIK VDILVSGIGTTGVTGTCGK SPSPLLLPAR LSVGYNILTR GCVFTYDAVSSYER MSCPAMAQLLSNLYLKK FFPYAFNLGGLDSEGK TGYSQAQGTSALMPVDQNLK GDWWYENNGGTCVAJAGADYCVAADTR | 2.07 | 27.3/6.40             |
Edman sequencing was done for the first 12 amino acids of the protein and for the 15 amino acids of the major peptide after tryptic digestion. Searches for the Edman sequences or MS/MS sequences of the OsCIA against the non-redundant protein database matched the protein to an unknown protein in rice cv. Nipponbare (Table II). The match for the amino-terminal sequence was from amino acid 47 suggesting the mature protein has a leader (or target) peptide cleaved off from the precursor protein. The corresponding gene for the unknown protein in cv. Nipponbare is located on chromosome 10. We cloned the corresponding gene (both genomic DNA and cDNA) in cv. Doongara (cDNA was deposited in GenBankTM under accession number DQ116755), and this identified a 738-bp-long intron interrupting two exons. When compared with cv. Nipponbare, there were three nucleotide substitutions in two exons (Fig. 8). Interestingly there was no substitution in the intron. As a result of single nucleotide substitutions, serine 61 is replaced by leucine, lysine 118 is replaced by asparagine, and lysine 189 is replaced by glutamine at the protein level in cv. Doongara (Fig. 8).

Southern hybridization was performed under standard conditions (65 °C) to estimate OsCIA gene copy number in rice cultivars Doongara and Nipponbare. Single bands were detected with four different restriction enzymes (BamHI, EcoRI, HindIII, and PstI). These results suggest that OsCIA is encoded by single gene copy in the genome of Doongara and Nipponbare (Supplemental Fig. 3). However, there were some differences in the hybridizing fragment sizes between Doongara and Nipponbare digested with BamHI and PstI (Fig. 10) indicating some differences in the flanking sequences of these two cultivars.

Analysis of the OsCIA amino acid sequence using Peptide-Mass (au.expasy.org/tools/peptide-mass.html) and MS/MS and MALDI-TOF data indicated that the major peptide 1777.02 [M + H]+ (from MALDI-TOF) is the same as peptide_A, 889.00 [M + 2H]2+ (from MS/MS), and the amino acid sequence derived from this peptide is SISPVVLVN-PVPVDGER. This peptide was a result of a chymotryptic cleavage by trypsin. The amino acid sequence of peptide_B, 821.13 [M + 3H]3+ (from MS/MS), is IDVLPFSISPVVLVN-PVPVDGER (the same sequence obtained from peptide_A is

### TABLE III—continued

| Spot no. | Molecular mass (kDa)/pI | Matching identity | Accession no. | PM (PI)/S%C | Matched peptides | Z score | Molecular mass (kDa)/pI |
|----------|-------------------------|------------------|--------------|-------------|-----------------|---------|------------------------|
| 24       | 15.4/5.41 | S ribosomal protein S12E | Q8H2J8       | 5 (22)/46%  | SSAHDGLVK       | 2.1     | 14.8/5.33  |
| 32       | 30.8/4.85 | Caffeoyl-CoA O-methyltransferase | Q9XGP7      | 14 (27)/678% | EYFDLGLPVIK | 2.29    | 28.4/4.85  |

a Experimental molecular mass (kDa) and pl.
b Swiss-Prot or TrEMBL accession numbers are given.
c Number of peptides matched (PM) and number of peptides identified (PI)/percent sequence coverage.
d Z score is the distance to the population mean in unit of standard deviation. For example, a Z score of 1.65 for a search means there are about 5% of random matches that could yield higher Z scores than this search (see Ref. 42 for details).
e Theoretical molecular mass (kDa) and pl of the matched proteins.

Edman sequencing was done for the first 12 amino acids of the protein and for the 15 amino acids of the major peptide after tryptic digestion. Searches for the Edman sequences or MS/MS sequences of the OsCIA against the non-redundant protein database matched the protein to an unknown protein in rice cv. Nipponbare (Table II). The match for the amino-terminal sequence was from amino acid 47 suggesting the mature protein has a leader (or target) peptide cleaved off from the precursor protein. The corresponding gene for the unknown protein in cv. Nipponbare is located on chromosome 10. We cloned the corresponding gene (both genomic DNA and cDNA) in cv. Doongara (cDNA was deposited in GenBank™ under accession number DQ116755), and this identified a 738-bp-long intron interrupting two exons. When compared with cv. Nipponbare, there were three nucleotide substitutions in two exons (Fig. 8). Interestingly there was no substitution in the intron. As a result of single nucleotide substitutions, serine 61 is replaced by leucine, lysine 118 is replaced by asparagine, and lysine 189 is replaced by glutamine at the protein level in cv. Doongara (Fig. 8).

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underlined), and this was a result of tryptic digestion alone. The mass of peptide_B matches correctly to the above sequence in which serine 61 has been substituted by leucine in cv. Doongara (Figs. 7B and 8).

An InterPro domain search for OsCIA predicted a bipartite-type (two basic domains separated by a spacer) nuclear localization signal just before the match to the amino-terminal sequence of spot 37 (Fig. 8B) and a chaperone Hsp20 domain at the carboxyl terminus (expected value of $2 \times 10^{-10}$). BLAST searching against the non-redundant GenBank™ protein database identified some proteins with low homologies to OsCIA. The best match was a rice hypothetical protein (33% identical in 192 amino acids, expected value was $9 \times 10^{-10}$, GenBank™ accession number BAD29104) and many small heat shock proteins (second highest match was to Arabidopsis heat shock protein 21 chloroplast precursor; expected value was $1 \times 10^{-9}$, 30% identical in the last 117 amino acids, GenBank™ accession number P31170). However, searches against TIGR EST database identified significant matches (with expected values lower than $3 \times 10^{-30}$ and with minimum identities of 47% for the complete sequence) to the monocots wheat (TIGR accession number TC144210), sorghum (TIGR accession number TC110249), maize (TIGR accession number TC251654), and barley (TIGR accession number TC144210). None of them had a predicted domain or any homology to the heat shock proteins except maize, which had a poorly predicted α-crystallin heat shock protein domain and a low homology to small heat shock proteins. All of these genes are annotated as unknown except for the wheat TC244520, which is annotated as a protein related to cell cycle regulation and aging. Alignment of the OsCIA protein with other monocot homologues is given in Fig. 9.

OsCIA mRNA Is Expressed under Normal Conditions and Is Not Changed by Cold Treatment—Using primers designed to include introns in RT-PCR, a 600-bp fragment was amplified from cold-treated and untreated anthers as well as untreated panicles of cv. Doongara as shown in Fig. 10A. Gel analysis showed that the mRNA levels of all three tissues were similar.

To quantify mRNA levels, we used quantitative real time RT-PCR to quantify OsCIA mRNA from cold-treated and untreated anthers of cv. Doongara; results showed no significant difference between them (Fig. 10B). We also screened cv. Doongara mature leaves and seedlings by RT-PCR and detected OsCIA mRNA expression in all the samples (data not shown).

Anti-OsCIA Antibody Cross-reacts with Monocot and Dicot Leaf Proteins—We expressed carboxyl-terminal His$_6$-tagged OsCIA protein in Escherichia coli (Supplemental Fig. 4) and
purified the recombinant protein using immobilized-metal affinity chromatography. The recombinant protein was identified as OsCIA by Edman sequencing (data not shown). Polyclonal antibodies were raised in rabbits. Using the anti-OsCIA antibody, we screened seedlings of several monocots (rice and maize) and dicots (*Arabidopsis thaliana* and *Medicago truncatula*) and detected strong single 18- or 22-kDa bands in Western blots (Fig. 11).

**DISCUSSION**

Previous studies identified some physiological and cytological characteristics of cold damage at the reproductive stage of plant development, leading to male sterility in rice (1, 3, 5). This research indicated the most cold-susceptible organ (which is the anther), the most sensitive stage (which is from tetrad to early microspore stage), and the effect of different days of cold treatment on the sterility of the plant (1, 5, 6). In our experimental system, we extended these observations to the cold-sensitive cultivar Doongara. This enabled us to focus on anthers of the upper spikelets from the top three branches of the panicle at the young microspore stage and use a 12 °C cold treatment as it gives very high sterility to the spikelets of interest.

**Most of the Anther Proteins Were Unaltered by Cold Treatment**—Greater than 4000 rice anther proteins were resolved,
and cold temperature treatments of 1, 2, and 4 days were extensively studied to reveal a small subset of proteins that are cold-responsive. Although the global expression pattern of anther proteins was largely unaltered after cold treatment, 35 proteins were observed as altered after comparison of many independent experiments. Eleven of these cold-responsive proteins have been identified previously, and they can be accessed at the ANU-2DPAGE site (semele.anu.edu.au/2d/2d.html). Previously we have identified 75 protein spots from rice anthers at the young microspore stage (33), and they can be accessed at the ANU-2DPAGE site. We did not detect changes in protein levels of most of these protein spots upon cold treatment except for protein spots 17 (a putative adenylate kinase) and 23 (a cysteine proteinase inhibitor). For instance, ascorbate peroxidase, which was reported to be involved in the tolerance of the cell to low temperature stress in rice (46), did not vary two-fold or more in its protein level upon cold treatment (data not shown). This was also true for some proteins such as heat shock protein 70, heat shock protein 60, and enolase, which are required for survival under stress conditions in plants (47). Furthermore these were some of the most abundant proteins in the rice anther proteome regardless of cold treatment, indicating either a high level of pre-formed stress defenses in the rice anther cells or a separate role for these proteins in microspore development.

**Cold-responsive Anther Proteins Were Detected and Identified**—Thirty-seven anther proteins were detected as cold-responsive proteins by 2-DE after 1, 2, and/or 4 days of cold treatment at the young microspore stage in cv. Doongara. We analyzed 20 of the cold-responsive proteins by amino-terminal sequencing, MALDI-TOF analysis, and MS/MS analysis and were able to identify 13 proteins. An anther-specific protein similar to lipid transfer proteins (spot 34); a known anther-specific protein, Osg6B (spot 36); and a calreticulin (spot 12) were down-regulated. A cysteine synthase (spot 23), an osmotin protein homologue (spot 31), and a caffeoyl-CoA O-methyltransferase (spot 32) were up-regulated. There were two proteins in one spot (spot 25); both were up-regulated.

We observed only the β-6 subunit of the 20 S proteasome as differentially displayed due to 2 and 4 days of cold treatment. In eukaryotes, the proteasome is the major non-lysosomal protease in the energy-dependent degradation of intracellular proteins, such as abnormal proteins and proteins that turn over rapidly (48). They have a role to play in controlling cellular processes, such as metabolism and the cell cycle, through signal-mediated proteolysis of key enzymes and regulatory proteins. They also operate in the stress response by removing abnormal proteins (49). From the up-regulation of a subunit of 20 S proteasome, it could be inferred that an increased rate of protein turnover is occurring in response to the cold stress.

Calreticulin is a calcium-binding protein that is located in the endoplasmic reticulum. Calreticulin is proposed to have several functions including a role in Ca²⁺ binding and storage, Ca²⁺ signaling, cell adhesion, gene expression, and chaperone function (50). In *A. thaliana* a calreticulin mRNA is accumulated abundantly in a set of subepidermal cells near the abaxial surface of the anther prior to dehiscence, and the authors suggested that calreticulin may be involved in the dehiscence process (51). Cold-induced male sterility is associated with poor anther dehiscence (52, 53). We observed that calreticulin is down-regulated in cv. Doongara anthers following 2 and 4 days of cold treatment at 12 °C. Although there is no evidence about the accumulation of rice calreticulin in the specific cells of the anther that are involved in dehiscence, we hypothesize that the down-regulation of calreticulin may be related to increased indehiscence, which contributes to increased sterility.

It is reported that caffeoyl-coenzyme A O-methyltransferase (CCoAOMT) is the key element of an alternative pathway for lignin biosynthesis in zinnia cells during tracheary element formation (54), and more recently it was proposed to be involved in phenylpropanoid metabolism in tobacco (55). We observed a CCoAOMT that is up-regulated after 4 days of cold treatment but not after 1 or 2 days of cold treatment in cold-sensitive rice cultivar. CCoAOMT may be involved in exine formation during pollen maturation, a process that is closely related to phenylpropanoid and lignin metabolism (56). Further study will be needed to determine whether up-regulation of CCoAOMT expression by cold treatment plays a role in the cold-induced male sterility.

The amino-terminal sequence match for the COXVb in this study supports the prediction that the N-terminal region of (~50 amino acids) is a precursor for mitochondrial targeting proposed by aligning the rice COXVb amino acid sequence with that of human COXVb (57). COXVb is a small subunit of cytochrome c oxidase also located in the mitochondria. COX catalyzes the oxidation of ferrocytochrome into ferricytochrome c (58). Chilling temperatures (above 0 °C) have been reported to lower expression and activity of COX in the mitochondrial inner membranes of a chilling-susceptible genotype of maize (59). However, we saw an opposite effect of 4 days of cold treatment (12 °C) on rice anthers in cold-sensitive cv. Doongara. The timing of the changes of the proteins discussed above suggests that these changes are later consequences of the earlier lowered temperature, which ultimately leads to sterility of the pollen in the rice anthers.

It was proposed that expression of sHSPs correlates with protection against chilling injury in tomato (60). Recent work on maize small HSP transcripts has shown that sHSPs are expressed and/or accumulate in a stage-specific manner during microsporogenesis, suggesting their gene-specific regu-
T42 Is an Anther-specific Plant ns-LTP with a Potential Role in Cold-induced Male Sterility—Spot 34 was down-regulated by cold treatment in the cold-sensitive cv. Doongara but not in the relatively cold-tolerant cv. HSC55. In cv. Doongara, spot 34 was identified as T42 by amino-terminal sequencing and MS/MS analysis. T42 shares moderate homology with other ns-LTPs; therefore, we propose that the t42 gene encodes a protein similar to the nonspecific lipid transfer proteins. ns-LTPs are small (molecular mass close to 9 kDa) and basic (most of the LTPs have an isoelectric point near 9) with typical amino acid residues such as cysteines conserved, and they are abundant proteins in higher plants (62). T42 and the products of rye, sorghum, and goat grass anther-specific ESTs have molecular masses around 9 kDa and contain eight cysteines in positions strongly conserved in all plant LTP families (Fig. 6B). Although T42 and the anther-specific EST products share many similar properties with other plant ns-LTPs, there are some distinct dissimilarities between them. Although most of the LTPs are basic proteins (pI above 9.0), the rice T42 and the other anther-specific LTPs were revealed to be acidic proteins (pI below 4.6). Furthermore the critical amino acid tyrosine 115, the side chain of which divides the hydrophobic cavity into two parts in the normal rice ns-LTP (63), is replaced with phenylalanine in T42 and its anther-specific homologues (Fig. 6B). Interestingly T42 and its anther-specific homologues all have strongly predicted signal peptides that are extremely alkaline (pI above 9.5). These differences in the sequences indicate the existence of a new class of LTPs: anther-specific acidic LTPs. A lipid transfer gene is up-regulated by low temperature in the winter cultivar of barley but not in the spring cultivar (64), suggesting a role for LTPs in cold tolerance in plants. In general, LTPs are capable of binding fatty acids and of transferring phospholipids between membranes in vitro. LTPs from this family contain a signal peptide and are secreted into the wall. A proposed function of ns-LTPs is to transfer phospholipids and galactolipids across membranes, and they may be involved in cutin biosynthesis, surface wax formation, pathogen defense reactions, or the adaptation of plants to environmental changes (62). A few anther-specific ns-LTPs (different from the anther-specific acidic LTPs discussed above) have been identified as expressed in tapetal cells of Brassica napus (65), Lilium henryi (66), and Zea mays (67). Pollen grains contain several lipidic structures, which play a key role in their development as male gametophytes. The elaborate extracellular pollen wall, the exine, is largely formed from acyl lipid and phenylpropanoid precursors, which together form the exceptionally stable biopolymer sporopollenin. It has been demonstrated that LTPs represent the most abundant protein associated with the extracellular wax of sporopollenin (68, 69). Sporopollenin is built from extracellular lipids originating from the tapetum (56, 70), and LTP function has been suggested to be involved in the transfer of these lipids to the microspore (71). The deposition of the exine begins soon after the completion of meiosis II (56) at the time the rice plants become most susceptible to cold damage. The presence of a putative signal peptide indicates that T42 enters into the secretory pathway to then be released into the anther loculus. It is possible that T42 participates in the transport of fatty acids and/or other sporopollenin precursors from the tapetum to the microspore during the early stages of exine formation. Consequently a reduction in the amount of this protein in response to cold would reduce the degree to which the pollen grain can lay down sporopollenin, and this may play a role in the subsequent pollen sterility.

OsCIA Is a Novel Protein That Is Differentially Regulated at the Post-transcriptional Level—We observed a very interesting expression pattern for OsCIA. First, OsCIA was induced in the anthers of a cold-sensitive rice cultivar by a cold treatment that produced a high level of cold-induced sterility (Fig. 1). Second, the OsCIA protein was present in panicle, leaves, and seedlings of the same cultivar under normal growth conditions (Figs. 3, 7A, and 11). Third, OsCIA mRNA was present in anthers, panicles, leaves, and seedlings of cv. Doongara under normal conditions, and cold treatment did not change its accumulation. These findings strongly indicate a post-transcriptional regulation of OsCIA expression, although it is not certain whether the protein product is constantly degraded or its translation is repressed in anther tissues under normal conditions. The results also indicate differential regulation of OsCIA expression in different tissues and specifically anthers versus other tissues.

We propose two potential roles for the OsCIA during anther development. In the first model, we propose that the OsCIA protein may function as part of a negative regulatory pathway for anther development. The OsCIA protein is expressed in other tissues under normal conditions but not in anthers. Environmental stresses such as cold temperature can induce the accumulation of the OsCIA protein in rice anther tissue at the young microspore stage (the most sensitive stage of microspore development to various stresses) of cold-sensitive cv. Doongara. This temporal and spatial expression of OsCIA coincides precisely with the disruption of male gametogenesis by such stresses, suggesting that the OsCIA protein may play a role in the negative regulation of pollen development. A role in regulation is further supported by the presence of predicted bipartite NLS in the leader peptide.
Identification of Cold-responsive Proteins in Rice Anthers

Negative regulation of specific developmental pathways in plants has been reported previously. In Arabidopsis, seedlings of a mutant called pickle continue to express embryonic identity after germination (72). The PICKLE gene encodes a CHD3 chromatin remodeling factor (73, 74), and PICKLE is necessary for repression of embryonic traits in Arabidopsis seedlings (75).

The second model is that the OsCIA protein may function as a chaperone to protect cells during normal plant development as well as under environmental stresses such as cold damage. sHSPs and the structurally related eye lens α-crystallins are ubiquitous stress proteins that exhibit ATP-independent molecular chaperone activity (76). sHSPs are defined by a conserved carboxyl-terminal domain of 90 amino acids, called the α-crystallin domain, which is flanked by a short carboxyl-terminal extension and a variable length, non-conserved amino-terminal arm (77). sHSPs are very efficient at binding denatured proteins, and current models propose that they function to prevent irreversible protein aggregation and solubilization, thereby increasing the stress resistance of cells (76).

A possibility is that the OsCIA protein and/or its yet to be identified associating partners could be engineered to induce developmental regulation in anther tissues of plants and in particular to induce nuclear male sterility. The generation of male-sterile lines has been a major goal of plant molecular biology and has huge potential for application in commercial crop systems where hybrid lines are used.

In conclusion, we detected and identified cold-responsive proteins that are potentially involved in protein synthesis and folding, stress responses, lipid biogenesis, cell wall formation, protein breakdown, and energy metabolism. These functions are all potentially involved in processes that if perturbed may give rise to the effects seen with cold temperature treatment. These would affect mitochondria (and its inner membrane), endoplasmic reticulum, ribosomes, and cell walls (exine formation), all of which have been observed to be affected by cold treatment. In the past, it was suggested that cold-induced male sterility might be caused by disruption of sugar metabolism (3, 8, 11). However, our results indicate that there are a number of additional cell functions that are being varied by cold. We demonstrated that other pathways are affected by low temperature treatment. Furthermore we postulate that down-regulation of T42, which may transfer lipids or other substances from tapetal cells to developing microspores during the early stages of exine formation, could be a contributing factor to cold-induced male sterility in rice.

Finally this research validates the proteomic approach. Its capacity to handle very small amounts of tissue that are pooled to be synchronous at the specific developmental stages and yet deliver identification of a few elements that are varying in a complex population of proteins is most valuable. At a more practical level, the identification of a small set of specific proteins that are cold treatment-responsive in a sensitive cultivar and yet are not (or less) cold-responsive in a relatively cold-tolerant cultivar indicates that these proteins could be used as molecular markers in marker-assisted breeding for improved cold tolerance in rice and possibly other crops. It also gives insight into microspore development and its response to environmental stresses at the protein level, a result that transcription studies could not have achieved.

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