

## Identification of gibberellin acid-responsive proteins in rice leaf sheath using proteomics

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## 1. ABSTRACT

The phytohormone gibberellin acid (GA) controls many aspects of plant development. In this study, we identified proteins that are differentially expressed between the rice (*Oryza sativa* L.) GA-deficient cultivar, Aijiaonante, and its parental line, Nante. Proteins were extracted from rice leaf sheath and examined by 2DGE. Among more than 1200 protein spots reproducibly detected on each gel, 29 were found to be highly up-regulated by GAs in Nante, and 6 were down-regulated by GAs in Aijiaonante. These 35 proteins were identified by MALDI-TOF MS and were classified into three groups based on their putative function in metabolism, stress/defense processes and signal transduction. These data suggest that metabolic pathways are the main target of regulation by GAs during rice development. Our results provide new information about the involvement of GAs in rice development.

## 2. INTRODUCTION

Global cereal production has increased dramatically since the 1960s. The major contributing factor has been the widespread adoption of high-yielding semidwarf varieties of rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) (1). To reduce plant height, so-called “Green Revolution” genes were introduced into the crop: Reduced Height (*Rht*) alleles for wheat and semidwarf (*sd-1*) alleles for rice. These contributed to improvements in the harvest index (the ratio of grain to grain plus straw) and lodging-resistant properties (2). In 1999, *Rht* wheat was shown to encode a repressive regulator that acted in the gibberellin acid (GA) signaling pathway (3). Three years later, *sd-1* rice was shown to harbor a defective gene encoding GA 20-oxidase (GA20ox), a multifunctional enzyme involved in the GA biosynthetic pathways (4-6). The *sd-1* allele from indica rice contains a 383-bp deletion in its genomic sequence at

the *SD1/OsGA20ox2* locus, and the japonica *sd-1* allele contains an amino acid substitution within the highly conserved region of dioxygenase sequences (4, 5). Expression profiles and functional redundancy of the *GA20ox* genes in rice explain the semidwarf phenotype caused by *sd-1* alleles (3). In addition to *SD1/OsGA20ox2*, at least two other *GA20ox* genes have been identified: *OsGA20ox1*, which is expressed in most vegetative and reproductive organs, and *OsGA20ox3*, which is preferentially expressed in the panicles (7-9).

GA controls many aspects of plant development, such as seed germination, shoot elongation, flower differentiation, fruit setting and seed development. Over the last decade, at least ten enzymes have been reported to play a role in the GA biosynthetic pathways of higher plants (10, 11). In addition to *sd-1* cultivars, several GA-deficient dwarf mutants have been isolated in rice (7, 8, 12, 13). Most of these involve loss of function of genes, such as *OsCPS1*, *OsKSI*, *OsKO2*, *OsKAO*, and *OsGA3ox2*, which encode enzymes required for GA biosynthesis. Their dwarf phenotypes can be classified into three categories: (i) null alleles of *OsCPS1*, *OsKSI*, *OsKO2*, and *OsKAO*, which cause severe dwarfism without reproductive development, because they are unique genes encoding enzymes expressed throughout the rice growth cycle (7); (ii) null alleles of *OsGA3ox2* (*d18-AD* and *d18h*), which result in severe dwarfism but normal reproductive organs, because GA3ox is encoded by two genes (*OsGA3ox1* and *OsGA3ox2*) that are mostly responsible for GA3ox activity in reproductive and vegetative organs, respectively (7, 8, 12); and (iii) a weak allele of *OsKO2* (*d35*) that produces similar semidwarf characteristics to *sd-1* alleles (13). From the viewpoint of crop production, the severe dwarf mutants of rice are not suitable for breeding, and the *d35* cultivar produces inferior culm architecture compared with *sd-1*. The *sd-1* alleles therefore have the most important agronomical significance in rice breeding.

Despite the fact that mutant genes in the rice genome were discovered several years ago, the basis (in terms of protein function) of semidwarf traits arising from *sd-1* alleles remains unclear. Because *sd-1* alleles lead to aberrations in GA biosynthesis in the vegetative organs, the semidwarf traits of *sd-1* cultivars are directly related to GA deficiency in corresponding organs (3). Proteomic analysis has shown that the expression of several proteins is modulated by exogenous bioactive GA, GA<sub>3</sub> in the excised rice leaf sheath and root, and in cultured suspension of rice cells (14-18). The major GA-regulated proteins in these reports are metabolic enzymes and stress/defense-related proteins. However, these results cannot represent the real responses of rice to GA, because the materials in these studies were excised rice tissues and were treated with exogenous bioactive GA, but not endogenous GA. In this study, we found that a semidwarf cultivar of Indica rice, Aijiaonante was a spontaneous *sd-1* mutant of its parental line, Nante. To study *in vivo* responses of GA in rice, we therefore used Aijiaonante, Nante and GA<sub>3</sub> treated Aijiaonante to analyze differentially expressed proteins by comparative proteomics. Up to 35 protein spots representing 29 unique proteins were detected to be

regulated by both endogenous and exogenous GA. In addition to proteins involved in general metabolic pathways and stress/defense processes, we identified two putative signal transduction factors, a receptor-like secreted protein kinase, and a protein-protein interaction-mediated protein. Further research on these proteins is needed to understand the biological relationship between the GA-responsive proteins and the *sd-1*-type semidwarf traits in detail.

## 3. MATERIALS AND METHODS

### 3.1. Growing conditions for rice cultivars

A spontaneous semidwarf cultivar of rice, Aijiaonante, and its parental line, Nante, were used. The shelled and surface-sterilized seeds were placed onto solid Murashige and Skoog medium (Sigma Aldrich Inc., USA) at 25°C under a 16-h white fluorescent light/8-h dark cycle in a growth chamber, then transplanted to the field after 2 weeks. The leaf sheaths of 2-week-old seedlings were immediately frozen in liquid nitrogen and stored at -80°C until needed for protein extraction, or fixed with formalin aceto-alcohol (FAA) solution containing 5% (v/v) formalin, 5% (v/v) acetic acid and 50% (v/v) ethanol for microscopic observation. For gibberellic acid (GA<sub>3</sub>) treatment, additional GA<sub>3</sub> (Sigma Aldrich Inc., USA) was applied to the medium of Aijiaonante at a final concentration of 1 μM or 5 μM.

### 3.2. Molecular cloning of *OsGA20ox2*

The total RNA from leaves from Nante and Aijiaonante cultivars was extracted using TRNzol (Tiangen Biotech, Beijing, China) according to the protocol provided. DNase I (Takara Bio, Dalian, China) was used to digest the genomic DNA. The first strand of cDNA was synthesized using 1 μg total RNA with M-MLV Reverse Transcriptase (Promega Corporation, USA). The cDNA fragments were amplified by the gene-specific primers *OsGA20ox2*-FS (5'-GCTCAACACAGCGCTCACT-3') and *OsGA20ox2*-FA (5'-TCGTTCCGTTTCGGTCCGT-3'), which were designed from the cDNA sequences of Nipponbare rice. After cloning into the T-vector, *OsGA20ox2* -T was sequenced by the Bioasia Bio-Asia Diagnostics Co. Ltd., Beijing, China.

### 3.3. Preparation of rice leaf sheath for microscopy

Leaf sheaths were fixed in FAA for 16 h at 4°C, and then dehydrated in a graded ethanol series. After leaf sheaths were cleared in the benzyl-benzoate-four-and-a-half fluid as described by Herr (19), they were observed under a microscope equipped with Nomarski differential interference contrast optics (model IMT-2, Olympus).

### 3.4. Extracting rice leaf sheath proteins

Leaf sheath proteins were extracted using the trichloroacetic acid/acetone precipitation method (20). Briefly, rice leaf sheaths were ground to a fine powder in liquid nitrogen then suspended in pre-cooled 10% trichloroacetic acid in acetone containing 0.07% (v/v) 2-mercaptoethanol, and incubated at -20°C for at least 2 h. The suspension was then centrifuged at 40,000 × g at 4°C for 1 h, and the resulting precipitate was washed with pre-cooled acetone containing 0.07% (v/v) 2-mercaptoethanol.

The protein-containing pellets were then dried under vacuum and dissolved in lysis buffer consisting of 7 M urea, 2 M thiourea, 4% (w/v) 3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid (CHAPS), 20 mM dithiothreitol, and 2% (v/v) Pharmalyte pH 3–10 (GE Healthcare, Piscataway, NJ, USA) at room temperature for 1 h, followed by centrifugation at  $100,000 \times g$  at 4°C for 1 h. Protein concentration was measured using the Bradford dye-binding assay kit (Bio-Rad Laboratories Inc., Hercules, USA), which uses bovine serum albumin as the standard.

### 3.5. Two-dimensional gel electrophoresis of rice leaf sheath proteins

The two-dimensional gel electrophoresis (2DGE) experiments were performed with both analytical (100 µg) and preparative (1 mg) amounts of protein. For the first dimension, isoelectric focusing was conducted using an Ettan IPGphor system (GE Healthcare) according to the user manual. Two 24-cm immobilized pre-cast gradient (IPG) strips (Immobiline™ DryStrip gels, pH 4–7, GE Healthcare) were rehydrated in 450 µl of rehydration buffer (8 M urea, 0.5% w/v CHAPS, 20 mM dithiothreitol, 0.5% v/v IPG buffer pH 4–7) containing the protein samples (100 µg or 1 mg on each strip) in a reswelling tray (Immobiline™ DryStrip Reswelling Tray, GE Healthcare) at room temperature overnight. The reswelled strips were transferred to the IPG strip holder, and the following isoelectric focusing protocol was followed: 100 V for 1 h, 300 V for 1 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 12 h. The resulting focused IPG strips were equilibrated twice for 15 min in 10 ml sodium dodecyl sulfate (SDS) equilibration buffer (6 M urea, 30% w/v glycerol, 2% w/v SDS, and 50 mM Tris-HCl buffer, pH 8.8.). For the first equilibration, 1% w/v dithiothreitol was added to the buffer. For the second equilibration, 2.5% w/v iodoacetamide was added.

After equilibration, the second dimension electrophoresis was performed according to the user manual using an Ettan Dalt six Electrophoresis System (GE Healthcare) and 12.5% SDS-PAGE (polyacrylamide gel electrophoresis, GE Healthcare) gels. Electrophoresis for both analytical and preparative gels was performed at 20°C for 1 h at 2 W/gel and followed about 5 h at 15 W/gel. The analytical gels were silver stained using PlusOne Silver Staining Kit (GE Healthcare) prior to image analysis according to the protocols. The preparative gels were stained with Coomassie Blue R-350 using PhastGel Blue R-350 (GE Healthcare) in preparation for enzymatic digestion of protein spots and subsequent matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). At least three replicates were performed for each sample.

### 3.6. Image analysis of rice leaf sheath proteins

The silver-stained analytical gels were scanned with a scanner (GE Healthcare) at a resolution of 300 dots per inch in the transparency mode. Image analysis was performed using ImageMaster 2D Elite software, version 5.0.1 (GE Healthcare). Individual protein spots were detected automatically, and identified spots were manually checked. The isoelectric point (pI) of the each protein was

determined by comparison of the migration of standard proteins on IPG strips. Relative molecular weight (MW) was determined (using the software) by comparison of the low-MW protein standards that were co-electrophoresed with the protein sample. After identifying one gel as the reference gel, remaining gels were automatically matched to it. The abundance of protein in each spot was estimated as a percentage (%Vol) of the total intensity of all the spots in the gel. Protein spots that showed reproducible changes (in triplicate) between the two rice cultivars were considered to represent differentially expressed genes.

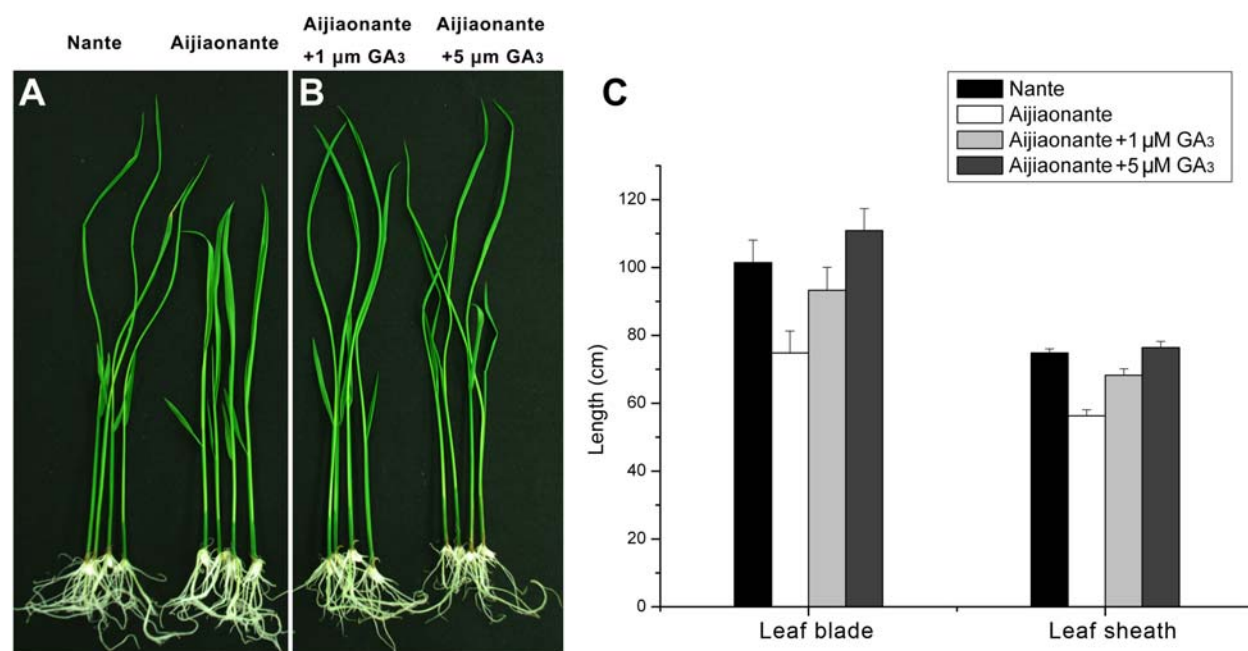
### 3.7. In-gel digestion and MALDI-TOF-MS analysis of rice leaf sheath proteins

The Coomassie-stained protein spots were excised from the SDS-PAGE gels manually. Gel pieces were destained in a solution of 50 mM  $\text{NH}_4\text{HCO}_3$  in 50% v/v acetonitrile, with shaking, for 1 h and then dehydrated in 100% acetonitrile for 15 min. After removal of acetonitrile, dehydrated gel pieces were vacuum dried and rehydrated in 25 mM  $\text{NH}_4\text{HCO}_3$  containing 5 ng/µl trypsin (sequencing grade trypsin, Promega Corporation, Madison, WI, USA) at 4°C for 30 min, followed by incubation at 37°C overnight. Digested peptides were extracted from individual gel slices by bathing in a solution of 0.5% trifluoroacetic acid, with shaking, at 37°C for 1 h. The resulting peptide solution was mixed with an equal volume of 10 mg/ml R-cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid/50% acetonitrile prior to MS analysis. MALDI-TOF MS was performed using a Bruker Reflex III instrument (Bruker-Franzen, Bremen, Germany) in positive ion mode at an accelerating voltage of 20 kV. The obtained peptide mass fingerprints were searched in the NCBI nr database using Mascot (<http://www.matrixscience.com>) with a peptide mass tolerance of  $\pm 100$  ppm and one missed cleavage site. The taxonomic category selected was “rice”; if there was no significant match, “Viridiplantae” (green plant) was used instead.

## 4. RESULTS

### 4.1. Semidwarf phenotype of the rice GA-deficient cultivar, Aijiaonante

Semidwarfism is one of the most valuable traits in rice breeding because semidwarf cultivars are more resistant to damage by wind and rain and are associated with consistently increased yields. As shown in Figure 1A and C, Aijiaonante displayed characteristic semidwarf phenotypes, compared to its parental line, Nante. When treated with 1 µM  $\text{GA}_3$ , the length of the leaf sheath and blade of Aijiaonante was restored (Figure 1B and C). Matsukura *et al.* (21) have reported that the application of  $\text{GA}_3$  promotes the elongation of parenchyma cells in the leaf sheaths of rice seedlings. We therefore investigated cell morphology in Aijiaonante. The epidermal and parenchyma cells in the second leaf sheath of Aijiaonante exhibited the same arrangement and shape as in Nante (Figure 2A), whereas the cell length was reduced (Figure 2B). The mean length of epidermal and parenchyma cells of Aijiaonante plants was  $152 \pm 43$  µm and  $21 \pm 5$  µm, respectively; this corresponds to 75–80% of those for Nante plants ( $190 \pm 45$  µm and  $28 \pm 6$  µm, respectively).



**Figure 1.** Morphological characterization of the *indica* rice *sd-1* mutant, Aijiaonante. (A) Comparison of Aijiaonante with its parental variety, Nante, at the 2-week-old seedling stage. (B) and (C) Comparison of the effect on promoting elongation of the second leaf blade and sheath with continuous treatment with exogenous GA<sub>3</sub> at two different concentrations. Error bars represent standard deviation from the mean (n = 5).

Our results therefore indicate that semidwarfism in Aijiaonante rice is mainly caused by deficiency in cell elongation rather than in cell division.

#### 4.2. Genetic analysis of Aijiaonante

The cDNA sequences of SD1/OsGA20ox2 were amplified by reverse transcription-polymerase chain reaction from RNA isolated from Nante and Aijiaonante. Primers were designed according to the cDNA sequence of *Oryza sativa* (japonica cultivar-group) in GenBank. Sequence analysis revealed that the PCR product amplified from Nante was similar to the sequence from GenBank. The cDNA sequence amplified from Aijiaonante contained a 2-bp deletion at position 389, located within the coding region. Transcription of this sequence is predicted to yield a truncated, nonfunctional polypeptide with the first 129 amino acid residues identical to GA20ox followed by the additional 88 residues transcribed in the wrong reading frame (Figure 3). The results confirmed that Aijiaonante was indeed a rice *sd-1* mutant.

#### 4.3. Differential expression of rice leaf sheath proteins in Aijiaonante and Nante

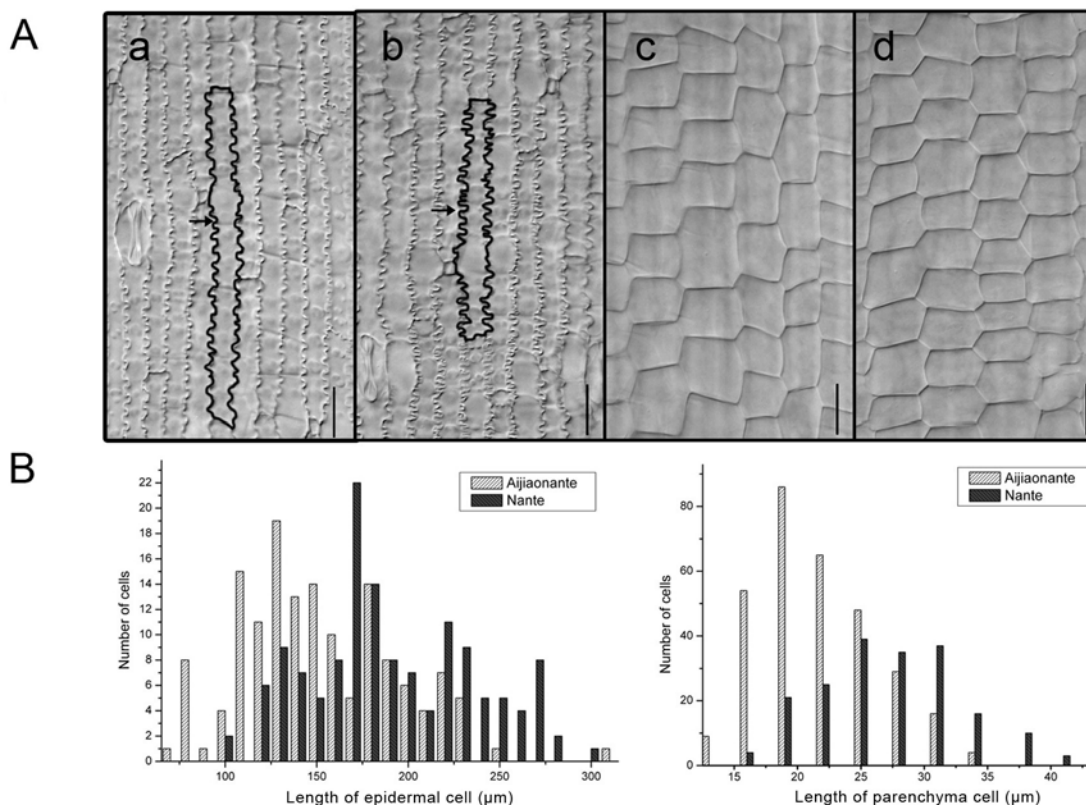
##### 4.3.1. Detection of GA-responsive proteins

Rice leaf sheath proteins were extracted from 2-week-old seedlings of Nante and Aijiaonante (Figure 1A) and Aijiaonante treated with two different concentrations of GA<sub>3</sub> (Figure 1B). In total, four protein samples were examined using 2DGE. A set of twelve 2DGE gels (i.e., three gels for each of the four protein samples) were compared using image analysis software. More than 1200 protein spots were detected on silver-stained 2DGE gels

(Figure 4), and most of the spots separated with the same electrophoretic mobility between gels. Only a few spots displayed altered accumulation patterns. First, the protein profiles of Nante and Aijiaonante samples were analyzed to identify differences between the *sd-1* mutant and its parental line. Next, we compared the accumulation of corresponding protein spots between the 1  $\mu\text{M}$  and 5  $\mu\text{M}$  GA<sub>3</sub>-treated Aijiaonante samples. Proteins that exhibited GA<sub>3</sub> concentration-dependent accumulation patterns were considered to be the only reliable indicators of GA-responsive proteins; other protein spots that changed in an inconsistent manner were considered “false” and were thereby eliminated. Using this selection criterion, 35 protein spots were identified; these proteins were therefore considered to play a role in the semidwarf phenotypes displayed in Aijiaonante. Of these 35 spots, 29 were up regulated by GA (Figure 4, 5); 26 spots (spots n1–n25, and n28) showed increased protein levels, and 3 spots (n26, n27, n29) revealed protein induction by GA. Six protein spots, a1–a6 (Figure 4, 5), were down-regulated by GA, accumulating in Aijiaonante more than in Nante. The GA<sub>3</sub> concentration-dependent accumulation pattern of these proteins is shown in Figure 6.

##### 4.3.2. Identification of GA-responsive proteins

The 35 protein spots were excised from the gels and digested with trypsin. Tryptic peptides were extracted and identified by peptide mass fingerprinting using MALDI-TOF MS and database searching. Using this approach, the 35 protein spots were identified as arising from 29 known or putative rice proteins (Table 1), including four isoforms of ribulose-1,5-bisphosphate



**Figure 2.** Cell morphology of the second leaf sheath of 2-week-old seedlings of Nante and Aijiaonante. (A) Epidermal and parenchyma cell morphology of Nante (a and c) and Aijiaonante (b and d). Scale bars = 25 μm. (B) Distribution of the length of epidermal (left) and parenchyma (right) cells of Nante and Aijiaonante.

carboxylase/oxygenase (RuBisCO) large subunit (LSU), two isoforms of heat shock protein 70 (HSP 70), two RuBisCO activases, a proteasome subunit, a receptor-like protein kinase, a putative ankyrin-like protein, several defense/stress-related proteins, and some enzymes involved in basic metabolism. Several of these proteins were identified in multiple different spots. The most abundant proteins were predominantly important metabolic enzymes: two up-regulated spots (n8 and n14) were identical to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); one down-regulated spot (n17) and one up-regulated spot (a6) were matched to a 24-kDa subunit of nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase (complex I); eight up-regulated spots (n9, n10, n11, n24, n25, n26, n28 and n29) were identified as four different isoenzymes of RuBisCO LSU, each with a distinct isoelectric point and apparent molecular mass. This phenomenon is due to protein heterogeneity arising from post-translational modification and/or truncation or fragmentation during proteolysis. In addition to these heterogeneous protein spots, two up-regulated spots (n2 and n3) were identified as isoforms of HSP 70, and four up-regulated spots (n16, n19, n21 and n22) were identified as isoforms of ascorbate peroxidases (APX). In addition to metabolic enzymes and chaperons, we also identified a domain of unknown function 26 (DUF26)-containing protein (spot n18), the UV excision repair protein Rad 23 (spot a3), a putative ankyrin-like protein (spot a4), and two unknown proteins (n12 and n27).

## 5. DISCUSSION

### 5.1. The genetic basis for semidwarf characteristics of Aijiaonante

Most of the GA-deficient dwarf cultivars isolated in rice are unsuitable for crop breeding because they are associated with abnormal grain development or severe dwarfism (7). The *sd-1* mutant, however, exhibits semidwarf phenotypes in stems and leaves but normal floral and grain development, thus making it suitable for crop improvement. As a spontaneous Indica rice mutant, Aijiaonante was the first semidwarf cultivar developed and broadly adopted in China in the 1960s. We amplified cDNA sequences of *OsGA20ox2* by RT-PCR from Aijiaonante and its parental line, Nante. Sequence analysis revealed that the amplified cDNA from Nante was identical with that of Japonica rice, whereas the sequence from Aijiaonante contained a 2-bp deletion in the coding region. The mutation was predicted to produce a nonfunctional enzyme, having the first 129 residues of GA20ox intact but with the subsequent 88 residues transcribed in the wrong reading frame (Figure 3). These data suggest that Aijiaonante is indeed a rice *sd-1* mutant that exhibits impaired GA biosynthesis in its leaf sheath, leaf blade and stem.

### 5.2. GA-responsive proteins in rice leaf sheath

From differential analysis of the proteomes of the *sd-1* mutant and its parental line, we showed that 35 of

**Table 1.** Identification of GA-responsive proteins based on MALDI-TOF MS analysis

Spot ID	Protein Identification	Accession Number	$M_r/pI$ Th <sup>1</sup>	$M_r/pI$ Obs <sup>2</sup>	Score
n1	RuBisCO activase small isoform precursor	AAX95414	52.4/5.5.9	51/5.0.1	80
n2	Heat shock 70 kDa protein	ABG22608	65.8/4.9.7	47/5.0.0	65
n3	Heat shock 70 kDa protein	CAA47948	71.4/5.1.7	46/4.8.5	74
n4	Sedoheptulose-1,7-bisphosphatase precursor	NP_001052293	42.7/5.6.4	42/5.0.3	85
n5	Phosphoribulokinase precursor	NP_001047825	45.2/5.6.8	42/5.1.8	103
n6	RuBisCO activase	ABG22613	38.7/5.3.6	41/5.3.1	81
n7	Enolase	ABB46862	51.9/5.7.2	41/5.8.8	191
n8	Glyceraldehyde-3-phosphate dehydrogenase	NP_001053139	36.9/6.3.4	39/6.0.8	87
n9	RuBisCO large chain	CAG34174	53.3/6.2.3	36/6.3.0	167
n10	RuBisCO large chain	CAG34174	53.3/6.2.3	32/6.7.1	117
n11	RuBisCO large chain	CAG34174	53.3/6.2.3	32/6.4.4	106
n12	Hypothetical protein	NP_001042256	31.3/6.9.9	30/5.9.9	66
n13	Glyoxalase I	BAB71741	32.9/5.5.1	29/5.3.1	86
n14	Glyceraldehyde-3-phosphate dehydrogenase (fragment)	NP_001053139	36.9/6.3.4	29/5.6.8	128
n15	Putative 4,5-DOPA dioxygenase extradiol	NP_001044983	30.6/5.7.0	29/5.8.2	78
n16	Thylakoid-bound ascorbate peroxidase	NP_001066305	33.7/6.7.3	30/6.0.1	64
n17	NADH-ubiquinone oxidoreductase 24 kDa subunit	NP_001056000	24.3/5.5.6	28/5.6.9	69
n18	Receptor-like protein kinase DUF26	NP_001054135	27.9/5.0.1	27/4.7.8	92
n19	Ascorbate peroxidase	EAZ05251	26.7/5.1.9	27/5.0.3	94
n20	Putative triosephosphate isomerase	NP_001042016	27.3/5.3.8	26/5.2.0	188
n21	Putative ascorbate peroxidase	NP_001049769	27.3/5.4.2	25/5.2.7	115
n22	Ascorbate peroxidases	EAY89537	27.3/5.3.1	25/5.1.5	99
n23	Glutathione S-transferase GSTF15	NP_001048884	25.8/6.6.7	24/5.8.8	80
n24	RuBisCO large chain precursor	BAF06408	23.0/8.2.4	23/5.9.2	63
n25	RuBisCO large chain precursor	BAF06408	23.0/8.2.4	21/5.4.1	79
n26	Putative RuBisCO large chain	ABB47308	40.8/8.5.1	21/6.2.5	86
n27	Hypothetical protein	CAE54541	22.0/6.8.5	20/6.2.4	80
n28	Putative RuBisCO large chain	ABB47308	40.8/8.5.1	14/5.0.9	113
n29	RuBisCO large chain	NP_039391	53.4/6.2.2	14/5.3.4	70
a1	Glycine dehydrogenase P protein	Q69X42	105.2/5.8.1	102/6.1.9	86
a2	Sucrose-UDP glucosyltransferase 2	Q7G7N0	93.4/5.9.4	96/5.9.9	75
a3	Putative RAD23 protein	NP_001057334	43.1/4.7.0	52/4.9.2	82
a4	Putative ankyrin-like protein	BAD23133	43.2/6.1.2	43/6.1.7	84
a5	Isoflavone reductase-like protein	NP_001041776	33.5/5.6.9	36/5.6.9	66
a6	NADH-ubiquinone oxidoreductase 24 kDa subunit	NP_001056000	24.3/5.5.6	28/5.8.3	92

<sup>1</sup>Theoretical  $M_r$  and  $pI$ , <sup>2</sup>Observed  $M_r$  and  $pI$ 

1200 detected protein spots were differentially expressed reproducibly in Aijiaonante compared with Nante and GA<sub>3</sub>-treated controls. Twenty-nine proteins (n1–n29) were down-regulated, whereas only six (a1–a6) were up-regulated (Figure 4, 5, 6). On the basis of their hypothetical function, these proteins can be grouped into three categories, namely those associated with metabolism, stress/defense, and signal transduction.

### 5.2.1. GA-responsive proteins involved in metabolic pathways

Four metabolic pathways are relevant to the GA-responsive proteins we identified (Figure 7). The first of these is the Calvin cycle, which converts CO<sub>2</sub> and water into organic compounds (carbon fixation) via a series of reactions in the stroma of chloroplasts. As shown in Figure 7, two enzymes in this pathway, sedoheptulose-1,7-bisphosphatase (SBPase; EC 3.1.3.3.7.; spot n4) and phosphoribulokinase (PRK; EC 2.7.1.1.9.; spot n5), were up-regulated by GA. From our results, several spots (n9, n10, n11, n24, n25, n26, n28 and n29) accumulated reproducibly in the proteomes of Nante and GA<sub>3</sub>-treated Aijiaonante (Figure 6). These spots were identified as fragments of RuBisCO LSU, which has previously been found to accumulate in rice under stress conditions (e.g., drought and/or the presence of ozone) or with phytohormone treatment, such as jasmonate and abscisic acid (22–25). Despite the accumulated fragments of RuBisCO

LSU, the protein spot corresponding to intact RuBisCO LSU exhibited no detectable alteration in any of the proteomes examined (Figure 4). This indicates that although GA may have a marked effect on RuBisCO LSU metabolism, they appear to not affect the abundance of intact RuBisCO LSU. In addition to photosynthetic enzymes, isoenzymes of another essential enzyme in the Calvin cycle, RuBisCO activase, were found to correspond to two GA-up-regulated spots (n1 and n5) (Figure 4, 5, 6). RuBisCO activase plays an important role in activating RuBisCO by carbamylation at the active site (26, 27). The identified enzymes in the Calvin cycle were all up-regulated by GA, except for RuBisCO LSU, indicating that GA enhances photosynthetic efficiency in rice seedlings.

The second related metabolic pathway is the photorespiratory cycle. As shown in Figure 7, this involves the cooperation of three organelles: chloroplast, peroxisome and mitochondria. Glycine dehydrogenase (GLDC; EC 1.4.4.2.) catalyzes the conversion of glycine to serine in mitochondria. Here, GA down-regulated spot a1, which corresponds to mitochondrial GLDC. This indicates that photorespiration might be repressed when the level of bioactive GA is increased, and be promoted in GA-deficient conditions. GA may therefore enhance photosynthesis by up-regulating the level of enzymes involved in the Calvin cycle and by down-regulating those involved in photosynthesis.

## Identification of gibberellin acid-responsive proteins in rice leaf sheath

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Japonica	MVAEHPTPPQPHQPPMDSTAGSGIAAPAAAACDLRMEPKIPEPFVWPNGDARPASAAELDMPVVDVGV	70	
NT	MVAEHPTPPQPHQPPMDSTAGSGIAAPAAAACDLRMEPKIPEPFVWPNGDARPASAAELDMPVVDVGV	70	
AJNT	MVAEHPTPPQPHQPPMDSTAGSGIAAPAAAACDLRMEPKIPEPFVWPNGDARPASAAELDMPVVDVGV	70	
*****			
Japonica	LRDGAEGRLRRAAQVAAACATHGFFQVSEHGVDAAALARAALDGASDFFRLPLAEKRRARRVPGTVSGYT	140	
NT	LRDGAEGRLRRAAQVAAACATHGFFQVSEHGVDAAALARAALDGASDFFRLPLAEKRRARRVPGTVSGYT	140	
AJNT	LRDGAEGRLRRAAQVAAACATHGFFQVSEHGVDAAALARAALDGASDFFRLPLAEKRRARRVPGTVSGYT	137	
***			
Japonica	SAHADRFASKLPWKETLSFGFHDRAAAPVVADYFSSTLGPDPFAPMGRVYQKYCEEMKELSLTIMELLELS	210	
NT	SAHADRFASKLPWKETLSFGFHDRAAAPVVADYFSSTLGPDPFAPMGRVYQKYCEEMKELSLTIMELLELS	210	
AJNT	-----LHQRPRRPLRLQ-----APME-----	153	
*****			
Japonica	LGVERGYREFFADSSSIMRCNYYPPCPEPERTLGTGPHCDPTALTILLQDDVGGLEVLVDGEWRPVSPV	280	
NT	LGVERGYREFFADSSSIMRCNYYPPCPEPERTLGTGPHCDPTALTILLQDDVGGLEVLVDGEWRPVSPV	280	
AJNT	-----	153	
*****			
Japonica	PGAMVINIGDTFMALSNRGYKSLHRAVVNQRRERRSLAFLCPREDRVVRPPPSAATPQHYPDFTWADL	350	
NT	PGAMVINIGDTFMALSNRGYKSLHRAVVNQRRERRSLAFLCPREDRVVRPPPSAATPQHYPDFTWADL	350	
AJNT	-----GDPLRLRPRR-----RRPRRRRLQLQHPR-----PRLRANGEGVPEVLRGDE	196	
*****			
Japonica	MRFTQRHYRADTRTLDAFTRWLAPPAADAAATAQVEAAS	389	
NT	MRFTQRHYRADTRTLDAFTRWLAPPAADAAATAQVEAAS	389	
AJNT	GAVADDHG-----TPGAESGRGARLL---	217	

**Figure 3.** Amino acid sequence alignment of OsGA20ox2 from the three cultivars: Japonica, Nante (NT) and Aijiaonante (AJNT). Compared to the sequences of Japonica and Nante, the deduced amino acid sequence of Aijiaonante includes the first 129 residues of OsGA20ox2 followed by an additional 88 residues that are transcribed using the wrong reading frame.

The third metabolic pathway is glycolysis. As shown in Figure 7, three enzymes that catalyze a series of reactions for conversion of triosephosphate into pyruvate were up-regulated by GA: triosephosphate isomerase (TIM; EC 5.3.1.1.; spot n20), GAPDH (EC 1.2.1.1.2.; spots n8 and n14), and enolase (EC 4.2.1.1.1., spot n7). TIM catalyzes the interconversion of two isomeric triosephosphates, which are also involved in chloroplastic carbon fixation and cytosolic glycolysis. The TIM identified here (accession no.: NP\_001042016) is a cytosolic protein, indicating it is responsible for glycolysis but not carbon fixation. GAPDH identified in spots n8 and n14 had a *pI* of 6.0.8 and MW 39 kDa for n8 (close to theoretical values of *pI* 6.3.4 and MW 37 kDa) and *pI* 5.8.8 and MW 29 kDa for spot n14. This suggests that spot n14 may be a proteolytic fragment of GAPDH. In addition to these up-regulated proteins, a down-regulated protein (spot a1) was identified as an early glycolytic enzyme, sucrose synthase (SuSy; EC 2.4.1.1.3.), responsible for the breakdown of sucrose and for unloading sucrose from the phloem.

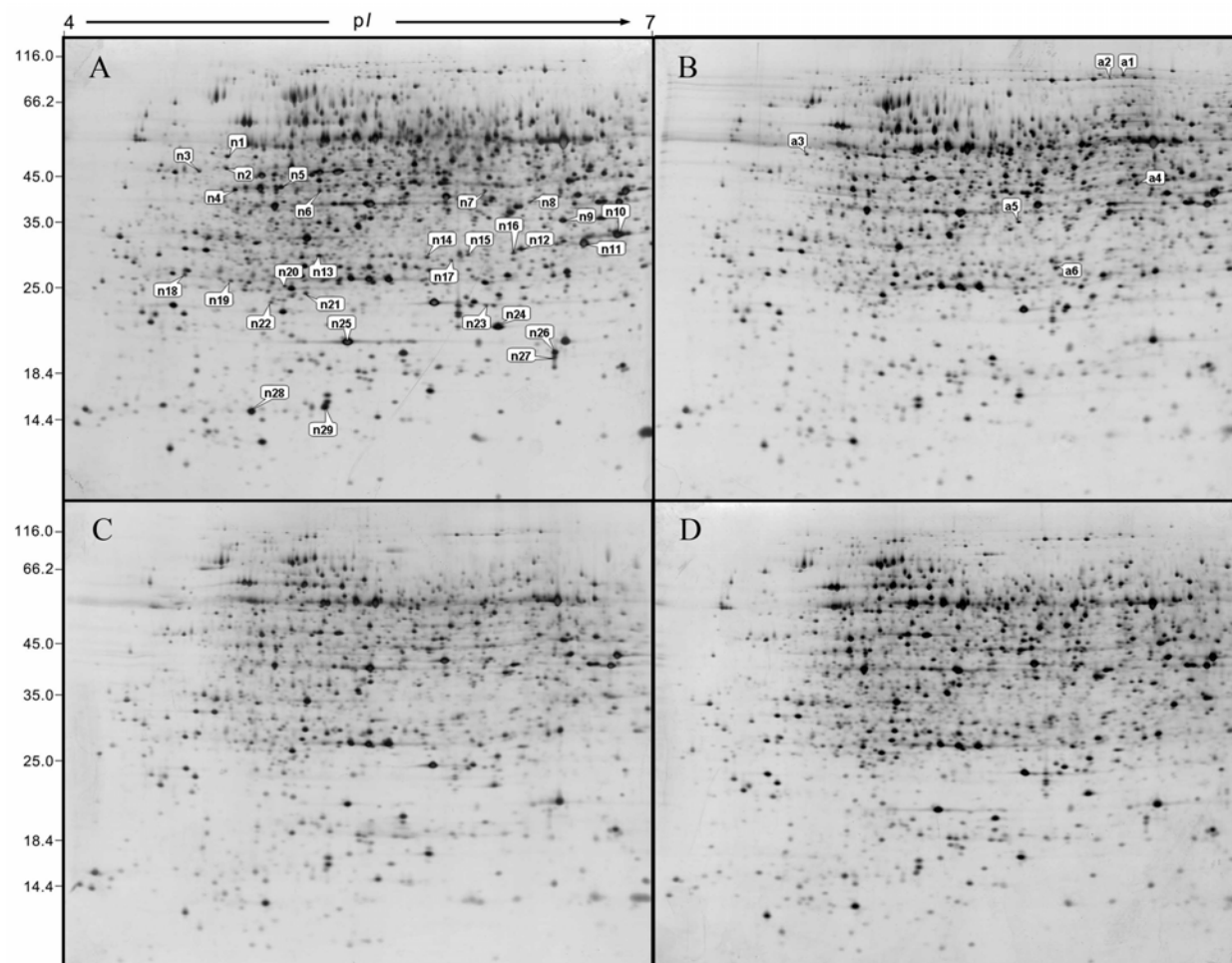
The fourth metabolic pathway identified is the mitochondrial electron transport chain. Spots n17 and a6 were identified as the same protein, a 24-kDa subunit of Complex I (EC 1.6.5.3.), which couples the oxidation of NADH and the reduction of ubiquinone and provides the

input to the respiratory chain, as shown in Figure 7. The two spots, n17 and a6, had the same MW (28 kDa), but exhibited different isoelectric points of 5.6.9 and 5.8.3, respectively. In the *sd-1* mutant, the 24-kDa subunit had shifted from n17 to a6, and then underwent the reverse shift upon treatment with exogenous GA<sub>3</sub> (Figure 5, 6). These results indicate that GA may regulate the protein by post-translational modification, such as phosphorylation, which changes the properties of the protein (28) and usually leads to activation or deactivation of function. Further experiments are needed to confirm whether GA activate or suppress the activation of the 24-kDa subunit of Complex I, and what type of post-translational modification occurred. Glycolysis and oxidative phosphorylation are the main energy-producing metabolic pathways. In total, our data suggest that the energy required to regulate GA-related cellular processes, such as cell elongation and division, arises from the regulation of the above-mentioned enzymes, including the alteration of accumulation profiles and/or post-translational modification patterns.

### 5.2.2. Stress/defense-related GA-responsive proteins

It is clear from our proteomic analysis that expression of several predicted stress/defense-related proteins e.g., APX, glutathione *S*-transferase (GST), and isoflavone reductase-like (IFR-like) protein, was modulated by GAs in the rice leaf sheath, in agreement with published





**Figure 4.** 2DGE analysis of GA-regulated proteins. Protein samples (80  $\mu$ g) were separated using 2DGE (pI 4–7) and then silver stained. Comparison of differentially expressed proteins for the *sd-1* mutant, Aijiaonante, (A) and the parental line, Nante (B). Labels on gels indicate GA-up-regulated proteins for Aijiaonante (A) and GA-down-regulated proteins for Nante (B). (C) and (D) Gels of 1  $\mu$ M (C) and 5  $\mu$ M (D)  $\text{GA}_3$ -treated Aijiaonante.

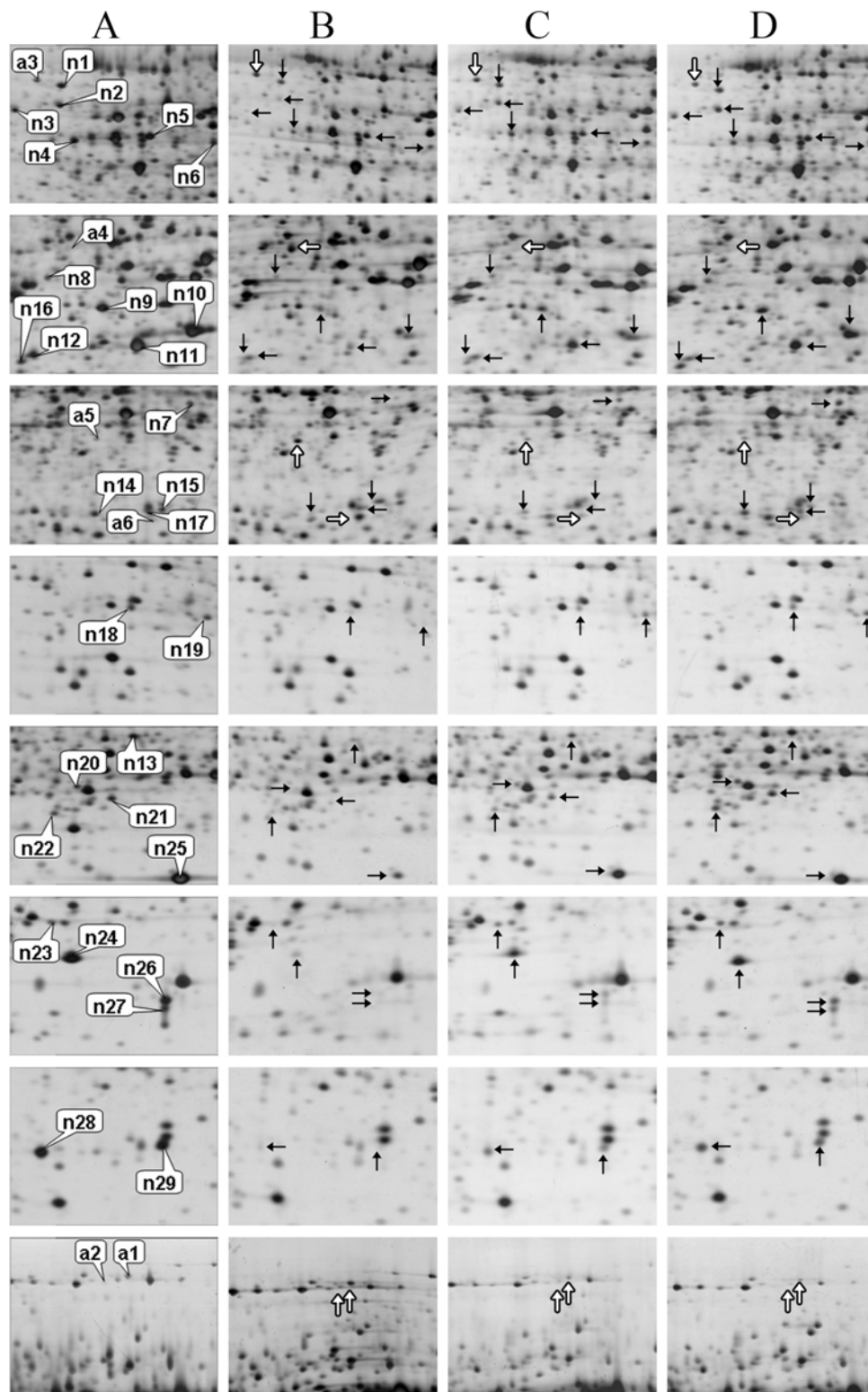
results (17). The up-regulated spot n23 was identified as GST, which is responsible for detoxification reactions of xenobiotics and may be induced by oxidative stress (29, 30). In addition, four up-regulated spots (n16, n19, n21 and n22) were identified as APX isoenzymes: spot n16 was identified as a thylakoid-bound APX and the others as putative cytosolic APXs. APX isoenzymes play an important role in the removal of hydrogen peroxide that is generated as a by-product of metabolism in chloroplasts and the cytosol. In chloroplasts, thylakoid-bound APX is an essential enzyme for photosynthesis, because a mutant version exhibits reduced photosynthetic activity and biomass accumulation in wheat (31). The function of cytosolic APXs is more complex. It has been reported that cytosolic APX may be involved in a pathogen response in tobacco (32). Furthermore, cytosolic APX1 in Arabidopsis plays a key role in maintaining the stability of chloroplast and mitochondrial APX, as the entire chloroplast antioxidative system breaks down in APX1-deficient Arabidopsis plants (33). Here, the up-regulation of APX and GST by GA suggests their role in detoxification

processes, such as photosynthesis and glycolysis, or in stress/defense responses which might be regulated by GA.

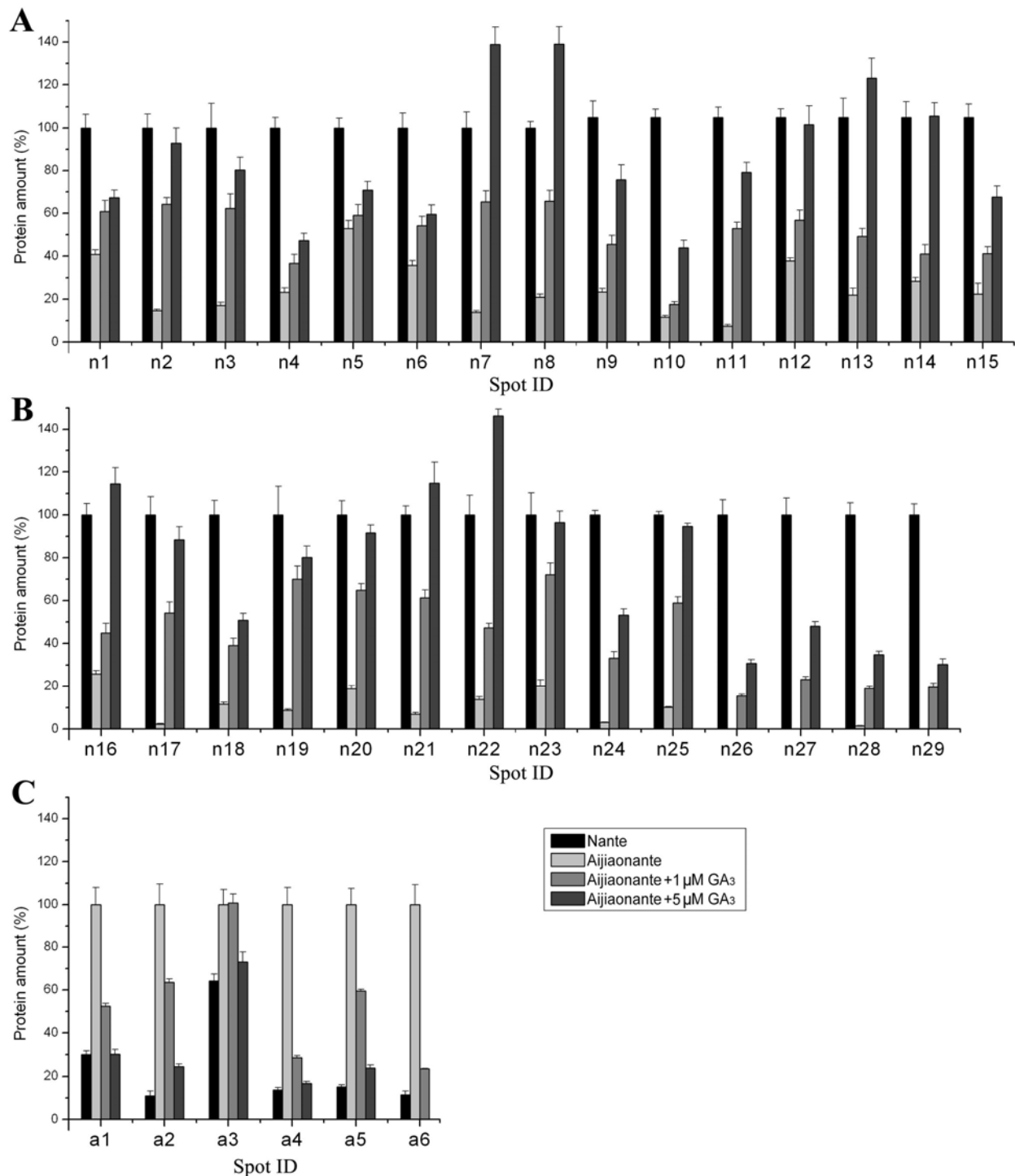
Additionally, the down-regulated spot a5 was identified as IFR-like protein. IFR is a key enzyme in isoflavonoid phytoalexin metabolism and accumulates in response to pathogen attack, fungal elicitors, and drought stress (23, 34–36). IFR-like proteins act in isoflavone reductase, or other similar reductase reactions. For example, two tobacco IFR-like proteins are involved in lignan biosynthesis and nicotine metabolism (37). From our proteomic analysis, we were unable to determine the function of the IFR-like protein identified as spot a5, but it clearly plays an important role in GA-modulated cellular processes; it accumulated in the *sd-1* mutant and was down-regulated in the  $\text{GA}_3$ -treated Aijiaonante, as shown in Figure 5 and 6.

Another down-regulated protein spot, a3, was identified as the putative RAD23 protein. RAD23 is involved in DNA damage recognition during nucleotide





**Figure 5.** Differentially expressed protein spots for Nante, Aijiaonante and GA<sub>3</sub>-treated Aijiaonante. Column (A), Nante; column (B), Aijiaonante; column (C), 1  $\mu$ M GA<sub>3</sub>-treated Aijiaonante; and column (D), 5  $\mu$ M GA<sub>3</sub>-treated Aijiaonante. GA<sub>3</sub> up-regulated and down-regulated proteins are indicated by solid and outline arrows, respectively.

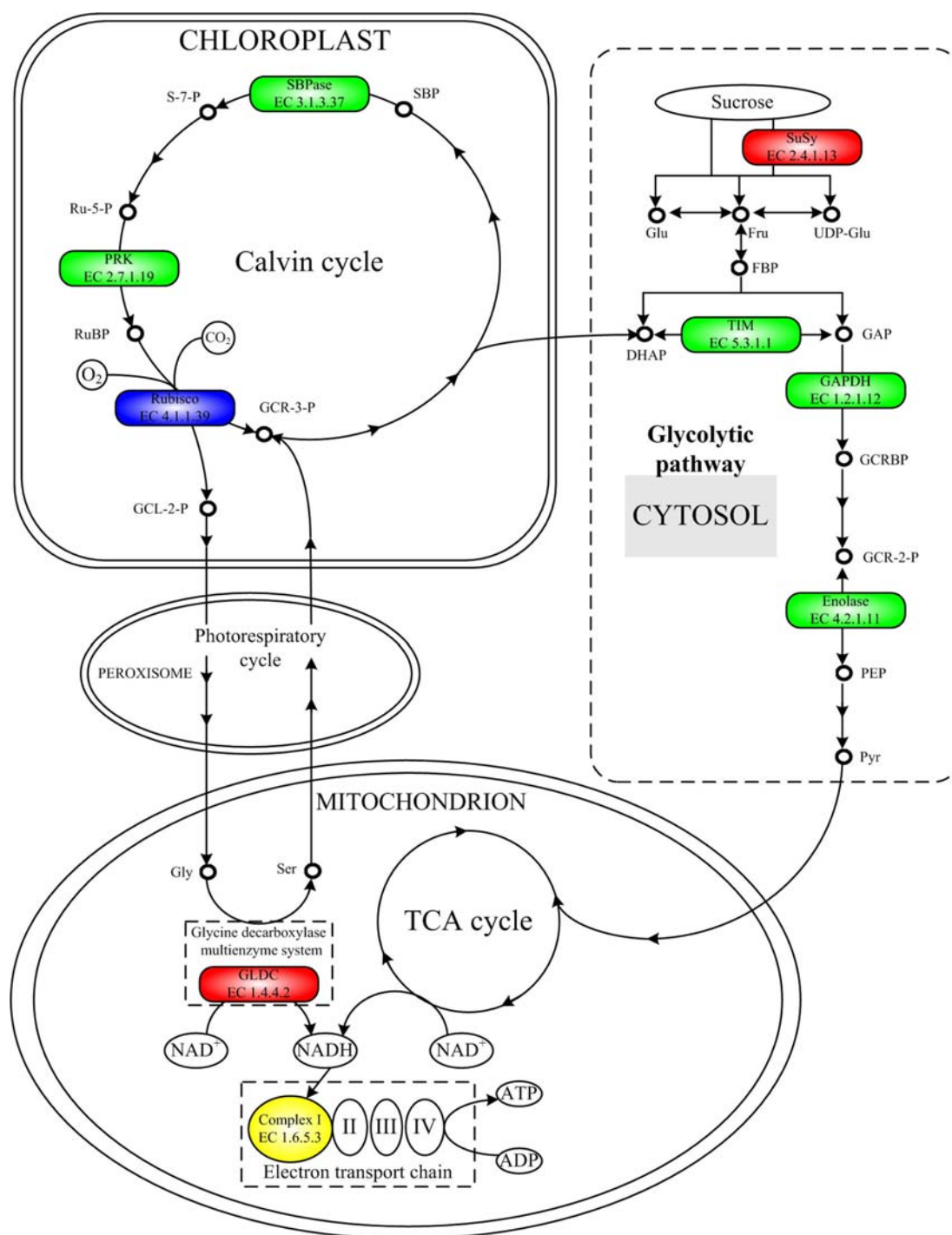


**Figure 6.** Accumulation patterns of 29 GA-up-regulated (A and B) and 6 GA-down-regulated (C) proteins. The amount of protein was determined by measuring the protein spot size and density, using the up- and down- regulated proteins in Nante and Aijiaonante, respectively, as reference 100% values. Error bars represent standard deviation from the mean ( $n = 3$ ).

excision repair in yeast and mammalian cells (38, 39). Although plant homologs of RAD23 have been reported in rice, maize and carrot (39-41), little is known about their function.

### 5.2.3. GA-responsive proteins related to signaling pathways

We identified two proteins that are likely involved in signaling pathways: a putative ankyrin-like



**Figure 7.** Metabolic pathways regulated by GA and associated enzymes. Proteins with differential accumulation patterns are indicated by color: up-regulated proteins, green; down-regulated proteins, red; shifted protein, yellow, means one protein shifted from one protein spot to another; and fragmented protein, blue, means one protein's corresponding spot was detected as fragment. Full names of abbreviations: RuBisCO: ribulose-1,5-bisphosphate carboxylase/oxygenase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; SBP: Sedoheptulose-1,7-bisphosphate; SBPase: Sedoheptulose-1,7-bisphosphatase; S-7-P: Sedoheptulose-7-phosphate; Ru-5-P: Ribulose-5-phosphate; PRK: Phosphoribulokinase; RuBP: Ribulose-1,5-bisphosphate; GCR-3-P: Glycerate-3-phosphate; GCL-2-P: Glycolate-2-phosphate; SuSy: Sucrose-UDP glucosyltransferase; FBP: Fructose-1,6-bisphosphate; DHAP: Dihydroxyacetone phosphate; TIM: triosephosphate isomerase; GAP: Glyceraldehyde-3-phosphate; GCRBP: Glycerate-1,3-bisphosphate; GCR-2-P: Glycerate-2-phosphate; PEP: Phosphoenolpyruvate; Pyr: Pyruvate; GLDC: glycine dehydrogenase

(ANK-like) protein, spot a4, which was down-regulated by GA, and a DUF26-containing protein, spot n18, which was up-regulated by GA. The putative ANK-like protein (Figure 5, 6) contained two domains, the ANK repeat and the tetratricopeptide repeat, both of which are quite common protein motifs known to mediate protein-protein interactions and occur in proteins with diverse biological functions (42-45).

The spot identified as a DUF26-containing protein is a putative receptor-like protein kinase that is predicted to be a secreted protein according to the subcellular protein localization predictor, Protein Prowler Prediction (<http://pprowler.imb.uq.edu.au/index.jsp>). Notably, a nearby spot (n18p), which was unaltered, was also identified as the same protein. As shown in Figure 5, spot n18p exhibited a similar pI and a slightly greater MW compared to n18, (pI values of 4.7.8 and 4.8.0, MW values of 27 kDa and 28 kDa for n18 and n18p, respectively). The difference between these biochemical attributes suggests that n18p corresponds to the unprocessed precursor of the DUF26-containing protein (pI 5.0.1/MW 28 kDa) and n18 corresponds to the processed version (pI 4.8.9/MW 26 kDa). This indicates that the DUF26-containing protein exists as an unprocessed precursor under GA-deficient conditions and is processed (by removal of the signal peptide) when the levels of bioactive GA increase. Further experiments are needed to reveal the function of this protein, which may be involved in GA signaling pathways as a receptor-like protein kinase.

## 6. ACKNOWLEDGMENTS

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**Abbreviations:** GA: gibberellin acid; 2DGE: two-dimensional gel electrophoresis; FAA: formalin-Aceto-Alcohol; IPG: immobilized pre-cast gradient; SDS: sodium dodecyl sulfate; MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; RuBisCO LSU: ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; HSP 70: heat shock protein 70; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; APX: ascorbate peroxidases; SBP: Sedoheptulose-1,7-bisphosphate; SBPase: Sedoheptulose-1,7-bisphosphatase; S-7-P: Sedoheptulose-7-phosphate; Ru-5-P: Ribulose-5-phosphate; PRK: Phosphoribulokinase; RuBP: Ribulose-1,5-bisphosphate; GCR-3-P: Glycerate-3-phosphate; GCL-2-P: Glycolate-2-phosphate; SuSy: Sucrose-UDP glucosyltransferase; FBP: Fructose-1,6-bisphosphate; DHAP: Dihydroxyacetone phosphate; TIM: triosephosphate isomerase; GAP: Glyceraldehyde-3-phosphate; GCRBP: Glycerate-1,3-bisphosphate; GCR-2-P: Glycerate-2-phosphate; PEP: Phosphoenolpyruvate; Pyr: Pyruvate; GLDC: glycine dehydrogenase; ANK: ankyrin; TPR: tetratricopeptide repeat; GST: glutathione *S*-transferase; IFR: isoflavone reductase; DUF26: domain of unknown function 26;

**Key Words** Rice, *sd-1*, Gibberellin acids, Two-dimensional gel electrophoresis, Matrix-assisted laser desorption, ionization time-of-flight mass spectrometry

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