Australian Journal of Crop Science AJCS
AJCS 6(2):316-325 (2012) ISSN:1835-2707

Differential proteomic expressions between superior and inferior spikelets of rice in response to varied nitrogen treatments

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Abstract

Grain-filling and molecular mechanisms of *Indica* rice jinhui No.809 (large-panicle type) were investigated. By keeping the total N supply constant and varying the early and late growth stage fertilizer application ratios, changes in the protein expressions of the rice superior and inferior spikelets were determined. The two N fertilization treatments were traditional and modern nitrogen applications (TNA and MNA). Using 2-DE and MALDI-TOF/MS, 38 proteins were identified to show differential expressions in response to the different N treatments. MNA appeared to promote protein up-regulation in the inferior spikelets, including cell respiration related proteins (e.g. fructose-bisphosphate aldolase, phosphoglycerate mutase, glyceraldehyde-3-phosphate dehydrogenase, malic oxidoreductase, succinate dehydrogenase, transketolase, phosphoglucomutase, and some starch synthesis related proteins (e.g., phosphoglucomutase, WRKY, glycolipid transfer and fatty acid hydroxylase) clearly indicating that the increased N supply at the late growth stage was favorable for the grain-filling of inferior spikelets. Moreover, the up-regulated auxin-responsive protein IAA22 and gibberellin response modulator implied that the MNA treatment could increase the content of phytohormones in inferior spikelets. In addition, the proteomic analysis and physiological observations in the present study also elucidated the mechanism underlying the asynchronous grain-fillings between the superior and inferior spikelets of rice.

Keywords: Rice; Superior and Inferior spikelets; Grain filling; Proteomics.
Abbreviations: DAF_Days After Flowering; TNA_Traditional nitrogen application; MNA_Modern nitrogen application; 2-DE_ Two-Dimensional Electrophoresis; PVP_Polyvinylpyrrolidone; TCA_Trichloroacetic Acid; DTT_DL-Dithiothreitol; IEF_ Isoelectric Focusing; MALDI_Matrix Assisted Laser Desorption Ionization; TFA_Trifluoroacetyl; TCA_Tricarboxylic Acid Cycle; PPP_Pentose Phosphate Metabolism Pathway; GLTP_Glycolipid Transfer Protein; CFAH_Cytochrome P450-dependent Fatty Acid Hydroxylase; Glc-6-P_Glucose 6-phosphate; ABA_Abscisic Acid; GA_Gibberellin; IAA_Indole-3-acetic Acid; PA_Polyamines; GABA_G-amino Butyric Acid.

Introduction

A staple crop for more than three billion population worldwide, rice (*Oryza sativa* L.) has been a focused research subject for agriculture scientists (Fageria, 2007, Li et al., 2011). Its grain-filling process, in particular, is of great interest, as it directly relates to the productivity as well as quality of the rice. The genetic and cultivation practices continue to strive for further improvement on rice yield. To increase the yield, some researchers achieved it by breeding the rice to increase its number of spikelets per panicle, which, in turn expands the rice's yield sink capacity (Kato et al., 2007). Although cultivars with large or extra-heavy panicles are available with the unrelenting efforts by the scientists (Yang and Zhang, 2010), the theoretical maximum yield has not been realized due to the new varieties' poor grain-filling characteristics, such as non- or slow-filling of the grains (Ao et al., 2008; Peng et al., 1999; Yang et al., 2002).The degree and rate of grain-filling of rice spikelets differ according to the spikelet's location on a panicle, and thus, the superior or inferior spikelets (Zhu et al., 1988; Iwasaki et al., 1992; Umemoto et al., 1994). The superior spikelets generally bloom earlier than the inferior spikelets, and are located on the apical primary branches, while the inferior spikelets located on the proximal secondary branches. In addition, the superior spikelets usually fill their grains faster and heavier than their counterpart (Yang et al., 2002; Yang and Zhang, 2010). Our earlier work (Guo et al., 2002; Lin et al., 1992) showed that rice, especially which with large panicles, had two filling peaks and two environmentally sensitive phases, attributing to the developmental asynchronism on grain-filling between the superior and inferior spikelets. Studies have shown that the inferior spikelets are more sensitive to the environmental factors than the superior spikelets (Qiu et al., 2004; Wu et al., 2007; Zhang et al., 2009). Low grain-filling rate and grain weight of the inferior spikelets implicating a reduced grain yield often result from a limited carbohydrate supply, especially under stress, such as nutrient deficiency and unfavorable climatic conditions (Lin et al., 1992; Murty and Murty, 1982; Yang et al., 2003; Zhu et al., 1988). However, to date, the biochemistry and physiology of low grain-filling of the inferior spikelets under unfavorable conditions remain unknown. Our proposed hypothesis attributed the filling disparity to the differential expression of relevant proteins in the temporal and space of the two kinds of spikelets. Previous studies showed that N supply significantly affects rice growth, especially on the grain filling stage. (Iwasaki et al., 1992; Ramasamy et al., 1997). N fertilization during the late growth stage closely relates to the leaf metabolism, grain-filling and

seed respiration (Perez et al., 1996; Wopereis et al., 2002; Zhang et al., 2011). For rice, an appropriate reduction of N supply on the basal dressing accompanied by an increase on N during the late growth stage decreases the amount of non-productive tillers, prevents root and leaf senescence, increases photosynthesis, accelerates matter translocation, and improves grain-filling. Therefore, such practice can result in an increased seed-setting percentage, high yield and improved quality on the rice (Juan et al., 2006; Leesawatwong et al., 2005; Perez et al., 1996; Qiu et al., 2004). Zhang et al. (2009) found that N reduction before anthesis followed by N fertilization at anthesis is necessary to increase post-anthesis dry matter accumulation and finally rice grain yield. These results suggested that the N supply at the early growth stage (sowing and tillering) might be reduced, but be increased around anthesis to meet the plant's need during grain-filling. The maneuver might prove to be effective and economically feasible, especially for the large panicles rice varieties. The objectives of this study were to (1) determine the protein expressions for both superior and inferior spikelets at different growth stages when N supplies were altered and (2) understand the molecular regulation and mechanism of the rice's grain-filling. As a first contribution to this subject, the results of a proteomic analysis shown that the N affected the expression of various proteins, which played major roles in the grain-filling process of the rice spikelets.

Results and Discussion

Protein expressions in superior and inferior spikelets under different N treatments

The inferior spikelet proteins extracted on 7, 14, 21, 28 and 35 DAF under the two N application treatments (i.e., TNA and MNA) were separated by 2-DE to increase resolution (Fig. S1). The superior spikelet proteins extracted on 7, 14 and 21 DAF under the two treatments were also separated by 2-DE (Fig.S2). After removal of pseudo-protein spots, approximately 1000 protein spots per gel could be visualized in p*I* 3–10 and MW 14–116 kD. Since the differences of the protein expressions of 35 proteins (denoted as No. 1–35 in Fig. 1) in the inferior spikelets of the rice raised under either TNA or MNA were greater than 1.5 times, they were considered to be significantly differentially expressed. Meanwhile, 8 proteins, denoted as No. 36–43, were significantly differentially expressed, as detected in the superior spikelets under the same conditions.

In this study, 38 proteins were successfully identified by MS methodology (Table 1). Based on the relative volume of a protein spot, the expression abundance of the protein spot was generated by using Imagemaster 5.0. The dynamic expression abundance changes of these protein spots from both the superior and inferior spikelets are shown in Fig. 2. The identified proteins could be classified into categories including substance metabolism, protein synthesis, cell growth, signal transduction and defense response.

Properties of inferior spikelet protein expression in response to different N treatments

Responses of cell respiration

Our study identified 9 important differentially expressed proteins for the cell respiration in the inferior spikelets with varied N supply. Among them, spots 2, 17, 30 and 35 are associated with glycolysis, spots 8, 22, 24, 29 and 31 with Trichloroacetic Acid (TCA) cycle, and spot 20 with the pentose phosphate metabolism pathway (PPP). Both TNA and MNA induced the low expression abundance of the corresponding proteins (i.e. spots, 8, 17, 24, 29, 30, 31 and 35) in the inferior spikelets on 7 and 14 DAF, and then, up-regulated after 21 DAF. Furthermore, the protein expressions were higher under MNA than TNA after 21 DAF. It suggested that the N increase at the grain-filling stage was conducive to protein up-regulation in the inferior spikelets. Glycolysis and TCA cycle are important in seed development (Fait et al., 2006; Mechin et al., 2007; Xu et al., 2008), and the central carbon metabolism, which includes glycolysis, TCA cycle and PPP, provides energy, cofactor regeneration and building blocks for interconversions and synthesis of metabolites. And, the concentration gradients of metabolites usually act as a signal in the diverse processes regulation (Gutierrez et al., 2007). Thus, our results seemed to show that appropriate increases in N supply at the late growth stage promoted glycolysis, TCA cycle and PPP, and thereby, more energy and building blocks for cell enlargement and starch synthesis in the inferior spikelets could be available.

Responses of starch synthesis

As indicated by the previous reports, one of the important factors relating to poor grain-filling in inferior spikelets was the plant's low starch synthesis (Liang et al., 2001; Zhang et al., 2011; Tang et al., 2009; Ishimaru et al., 2005). In this study, phosphoglucomutase (spot 21) showed a higher expression in the inferior spikelets at 28 and 35 DAF under MNA than TNA. It suggested that appropriate increases in N supply at grain-filling stage could be in favor of phosphoglucomutase up-regulation. Phosphoglucomutase catalyzes the reversible interconversions between glucose 6-phosphate (Glc-6-P) and glucose 1-phosphate (Glc-1-P), and supplies Glc-1-P for AGPase in plastid (Caspar et al., 1985; Fritzius et al., 2001; Tetlow, 2006). Hence, the up-regulated phosphoglucomutase during rice seed development might facilitate Glc-6-P/Glc-1-P pool maintenance, as it is frequently depleted during the starch synthesis in amyloplasts when a supply deficiency occurs (Xu et al., 2008). In addition, the WRKY DNA binding domain containing protein (spot 1), a transcription factor, was up-regulated in the inferior spikelets treated with MNA. Sun et al. (2003) reported that *SUSIBA2* of WRKY protein family was a regulatory transcription factor in starch synthesis involving in the carbohydrate anabolism. Our finding further confirmed the enhancing effect of the appropriate N supply at the late growth stage on starch synthesis, and therefore, an increased seed setting percentage and grain yield for the inferior spikelets.

Responses of lipid metabolism

Glycolipid transfer protein (GLTP)-like protein (spot 3) and cytochrome P450-dependent fatty acid hydroxylase (CFAH) (spot 33) related to lipid metabolism were detected and identified in the inferior spikelets at the early grain-filling stage. GLTP, a cytosolic protein catalysing glycolipids transfer between intracellular membranes (Rao et al., 2004), was not detected in the inferior spikelets at the early grain-filling stage prior to 14 DAF. Its expression was considerably increased on 21 and 28 DAF. CFAH, which catalyzes the enzymatic reactions of hydroxy-fatty acid production, showed a similar expression pattern in the inferior spikelets. Both GLTP and CFAH were up-regulated under MNA, in comparison to TNA. Rice seeds accumulate lipids rapidly at the early grain-filling stage (Choudhury and Juliano, 1980; Ichihara et al., 2003). The lipids are mainly stored in aleurone cells (Krishnan and Dayanandan, 2003), which might benefit the aleurone cell proliferation at the grain-filling phase that follows (Kvaale and

Table 1. Proteins identified by MS (MALDI –TOF/MS)

Spot No[a]	Aceesion No[b]	Protein name	MW[c]	PI[d]	Score[e]	Coverage[f]
1	Q2QYK0	WRKY DNA binding domain containing protein	25061	8.73	54	23%
2	Q2RA00	Fructose-bisphosphate aldolase	41808	6.07	62	28%
3	Q6K1Q5	Glycolipid transfer protein-like	22687	6.1	53	44%
4	Q19BJ6	Caffeic acid 3-O-methyltransferase	40066	5.41	75	41%
5	Q8SB85	Putative leucine-rich repeats containing protein	32122	5.69	59	20%
6	Q8GSY5	Putative 2-oxoglutarate-dependent oxygenase	41607	5.91	60	21%
7	Q2QM44	F-box domain containing protein	41802	7.99	62	32%
8	Q7XLP7	Isocitrate lyase and phosphorylmutase family protein	41636	5.66	107	37%
9	Q6ENY9	Ankyrin-like protein	26400	7.11	57	24%
10	O22385	Glycine-rich protein	16017	7.82	62	36%
11	Q69QD5	Putative chaperonin 21	25479	5.97	59	29%
12	Q93WM2	Glutathione transferase	24226	5.39	65	21%
13	Q9LWU9	Putative gibberellin response modulator	65806	6.02	69	16%
14	A5X3J8	polyphenol oxidase	63758	10.76	114	24%
15	Q6H5M8	Putative polyamine oxidase	54960	5.45	56	15%
16	Q5ZD09	ATP-binding Cassette Transporter protein	29573	9.22	64	30%
17	Q5QMK7	phosphoglycerate mutase	60980	5.42	119	44%
18	Q6Z7L1	Heat shock 70 kDa protein	73081	5.49	231	45%
19	Q84LK3	Betaine-aldehyde dehydrogenase	55446	5.36	112	40%
20	Q0DEU8	Transketolase	73973	5.44	79	27%
21	Q10E00	Phosphoglucomutase, cytoplasmic 2	63138	5.40	86	38%
22	Q8W317	NADH-ubiquinone oxidoreductase 75 kDa subunit	82148	5.86	138	30%
23	Q93WM3	Asparaginyl-tRNA synthetase	62948	5.68	105	27%
24	Q0JJQ7	Malic oxidoreductase family protein	65639	8.59	94	23%
25	Q84VE0	phenylalanine ammonia-lyase	72859	6.11	117	30%
26	Q7Y1H8	Auxin-responsive protein IAA22	28623	6.61	63	16%
27	Q6Z875	26S protease regulatory subunit 6B homolog	46597	5.75	124	43%
28	Q6H660	Heat shock protein STI	65159	6.03	91	32%
29	Q0D8R4	Succinate dehydrogenase	69494	6.61	122	27%
30	Q6Z9G0	Glyceraldehyde-3-phosphate dehydrogenase	53963	6.57	112	33%
31	Q10LR5	Fumarate hydratase 1	53991	6.93	128	31%
33	Q5JN64	putative cytochrome P450-dependent fatty acid hydroxylase	34685	6.6	62	39%
34	Q2QWY2	Thylakoid-bound ascorbate peroxidase	33652	6.73	82	55%
35	Q8H3Q7	Xylose isomerase	53803	5.42	137	46%
37	A2ZIZ8	Zn-finger-like, PHD finger domain containing protein	26351	4.81	59	27%
38	Q8HCN9	Ribosomal protein S4	42070	10.5	62	21%
39	Q6ESG5	Splicing coactivator subunit-like	36615	10.50	67	37%
40	Q945W3	Glutathione S-transferase N-terminal domain containing protein	21042	9.67	58	48%

Note: a: Numbers correspond to those on 2D gel in Fig.1. b: Accession number in Swiss-prot, c: Theoretical molecular weight

Olsen, 1986), as well as the formation of starchy endosperm's periphery cells (Becraft and Asuncion-Crabb, 2000). The results obtained from our study suggested that appropriate increases in N at the late growth stage could significantly aid the protein accumulation associated with lipid metabolism that are favorable for cell proliferation and starchy synthesis in the inferior spikelets.

Responses of phytohormones and signal transduction

Our study showed a higher expression of the auxin-responsive protein IAA22 (spot 26), which belongs to Aux/IAA family and can be induced by auxin, in the inferior spikelets under MNA than TNA on 21 DAF when grain-filling started. This suggested that an appropriate N supply at the late growth stage could help regulate the phytohormone level to generate sufficient auxin for the inferior spikelets. The gibberellin response modulator (spot 13) that participates in gibberellin signaling was also found in the inferior spikelets. This protein had low expressions on 7 and 14 DAF, but the expressions began to increase after 21 DAF. On 28 and 35 DAF, higher expressions were detected with MNA than TNA. It is generally believed that phytohormones are the key regulators in rice seed development. The IAA and GA signaling and/or the interactions between the signal pathways are essential for normal seed development, and they may be involved in starch synthesis (Xu et al., 2008; Yang et al., 2001). GA was reported to regulate the expression of alcohol dehydrogenase in barley seeds (Macnicol and Jacobsen, 2001), and the cross-talk between ABA and GA signaling was found in rice seeds (Xie et al., 2006). Thus, GA was believed to be associated with the regulation of the metabolic switches during the normal eed

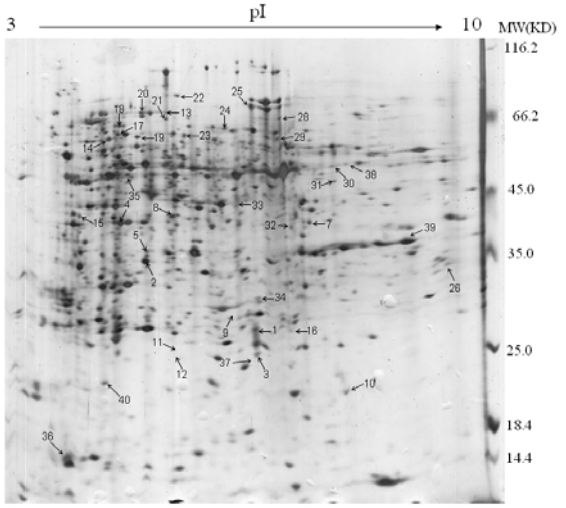

Fig 1. Silver stained 2D-gel of proteins extracted from rice spikelets. Differentially regulated protein spots in response to different N treatments are indicated with arrows. See Table 1 for detailed information on corresponding proteins. Proteins (150 mg) were separated in the first and second dimensions by IEF tube gel (17 cm×0.02 cm) followed by 12% SDS–PAGE then visualized by silver staining. See Supplementary for all 2D-gels of rice superior and inferior spikelets under TNA and MNA treatments. The changes in protein spots were calculated with Image-Master 5.0 software. For details see Materials and methods.

development. The present study also found that the phytohormones played an important role in rice grain-filling, and that appropriate increases in N supply at the late growth stage were favorable for the IAA and GA regulation in controlling the metabolic activity in the inferior spikelets.

Responses of other proteins

Three proteins involving the cellular growth，namely, 26S protease regulatory subunit 6B homolog (spot 27), F-box domain containing protein (spot 7) and glycine-rich protein (GRP) (spot 10), were up-regulated in the inferior spikelets when the rice was treated with MNA. These proteins are considered important for various cellular and developmental events in rice, according the previous reports (Moon et al., 2004, Craig and Tyers, 1999, Ringli et al., 2001), which indicated that appropriate increases in N supply during rice's late growth stage was favorable for cell enlargement of inferior spikelets. The proteins involving protein synthesis, such as ankyrin-like protein (spot 9) and chaperonin 21 (spot11), had higher expressions in the inferior spikelets under MNA than TNA. A similar result was also found with the expression pattern of the proteins involved in amino acid metabolism, such as asparaginyl-tRNA synthetase (spot 23) and phenylalanine ammonia-lyase (spot 25). It suggested that both protein synthesis and amino acid metabolism in the inferior spikelets could be regulated by N supply, especially in the late growth stage of the rice plant.

Protein expression properties of superior spikelets in response to different N treatments

In this study, 9 differentially expressed proteins were detected in the superior spikelets, and 4 of them were successfully identified. Ribosomal protein S4 (spot 38) is the molecular machine of protein synthesis. PHD domain containing protein (spot 37) undertakes various functions, including protein interaction and recognizing specific methylation password of histone (Ma et al., 2008). The two proteins had higher expression at 21 DAF under MNA than TNA, indicating that the increased N supply enhanced protein synthesis. The splicing coactivator subunit-like protein (spot 39) was probably involved in the nucleic acid metabolism of grain and the post-transcriptional modification of gene expression adjustment. Our tests also showed a higher expression at 21 DAF under MNA than TNA. The expression of glutathione S-transferase (spot 40), an enzyme helps protect plants from electrophilic xenobiotics and endogenous toxic compounds, decreased gradually as the grain-filling proceeded in the superior spikelets. It decreased, however, more slowly with MNA than TNA. This suggested that the increased N supply could improve the anti-adversity ability of the superior spikelets during the grain-filling process.

Different expression patterns of proteins in response to different N treatments in superior and inferior spikelets

The proteomic results obtained from our study showed that more differentially expressed proteins were detected in the inferior than the superior spikelets (i.e., 35 vs. 9 proteins) under different N treatments. Moreover, the expression of these proteins did not vary significantly in the entire course of grain-filling for the superior spikelets (data not shown). Since most of these proteins played important roles in the grain-filling, the results indicated that changes in N supply at the late growth stage would bring about more significant effect on the inferior than the superior spikelets.

The 1000-grains dry weight of the rice also showed that, at a constant total N supply, changes in the N application ratio from the early tillering stage to the grain-filling stage did not significantly affect the grain-filling on the superior spikelets, but did on the inferior spikelets (Fig. 3). It implied that the grain-filling of the inferior spikelets could be regulated artificially as a means for yield improvement, especially for the large-panicle type rice.

Differential temporal and spatial expressions of proteins in superior and inferior spikelets

In this study, the proteins in the superior and inferior spikelets exhibited different temporal and spatial expression patterns. In the superior spikelets, the expressions of those proteins, which played important roles in the course of grain-filling, such as carbon metabolism (spots 2, 8, 17, 24, 29, 30, 31 and 35), cell growth (spot 7 and 27) and amino mechanism (spot 23), were high on approximately 7 or 14 DAF, but decreased on 21 DAF. On the other hand, in the inferior spikelets, those proteins that displayed distinctive expression profiles showed no or low expressions during the same phases. It suggested that, at the initial stage of grain-filling, the superior spikelets had an active cell enlargement and starch synthesis, while the inferior spikelets had a low level of substance metabolism. Moreover, most of these proteins were up-regulated in the inferior spikelets on 21 DAF, suggesting that the physiological filling activity tended to increase after 14 DAF. The differences in the protein expression patterns between the superior and inferior spikelets were highly consistent with their dry weight accumulation patterns (Fig. 3). In the superior spikelets, the filling speed reached a peak at 14 DAF, and the dry matter accumulation peaked at 21 DAF. The grain-filling of the inferior spikelets only began to accelerate on 21 DAF, reached the peak at 28 DAF, and then leveled off until 35 DAF at time

Table 2. Grain yield and yield components for different N treatments

Nitrogen application treatment	Effective panicles·hm^{-2} (×10^4)	Grains/panicle	Seed setting rate (%)	1000-grain weight (g)	Grain yield (kg/hm^2)
TNA	216.75	225.14	80.21	25.62	10003.22
MNA	217.59	236.44★	85.33★	26.78★	11710.59★

★Significant at the 0.05 probability level.

of harvest. Thus, the grain-filling of the superior spikelets occurred much earlier than the inferior spikelets. That was consistent with the proteomic results, as well as, the previous reports (Guo et al., 2002; Lin et al., 1992; Yang and Zhang, 2010; Yang et al., 2006). For the first time, our study explained, at the proteomic level, the asynchronous filling phenomenon of rice's superior and inferior spikelets. The findings indicated that the increases in N supply at the late growth stage would be important for the development of inferior spikelets on a large-panicle rice plant.

Grain yield and yield components under different N treatments

The increase in N supply in the late growth stage, or change of N application ratio from TNA to MNA, was shown to be conducive to the dry matter accumulation in the inferior spikelets spikelets and grain-filling speed, or the seed setting percentage and grain weight. Data on the rice yield showed that the MNA was superior to the TNA treatment (Table 2). In other words, an improper N fertilization could result in lower grain yield. The proteomic results also demonstrated the protein up-regulation in the inferior spikelets due to appropriate N application. The important role of fertilization during the late growth stage on grain-filling of the inferior spikelets, and consequently, the rice yield, seemed evident.

Correlation between mRNA and protein levels

The transcriptomic techniques, such as *qRT-PCR,* are a powerful tool for profiling mRNA expression. In the present study, changes in gene expression of 5 genes under different N treatments were investigated at the mRNA level by using *qRT-PCR* with 3 biological replications. The genes and the sequence of the forward and reverse primers are summarized in Table S1. In order to confirm the accuracy of our proteomic results, we examined the 5 genes expressed in the inferior, as well as, the superior spikelets (Fig. 4). In the inferior spikelets, the expression profiles of 4 genes (4/5) at the mRNA level were consistent with those of the corresponding proteins, both showing the upward trends under the MNA treatment. This, at the mRNA level, confirmed that appropriate N increases at the late growth stage would benefit the grain-filling for the inferior spikelets. On the other hand, in comparing the mRNA and proteomic analysis results, some discrepancies were found. For example, in the superior spikelets, the 5 genes showed either upward or downward trend under different N treatments at the mRNA level, but no differential expression was evident. The discrepancies indicated that the post-transcription and post-translation events might have occurred in the relevant RNA and protein expression process in the superior spikelets. Additionally, in certain cases, the translation products of a same mRNA could be different, due to varying translation and degradation efficiencies or post-translation modifications.

Materials and methods

Plant materials and treatments

The experiments were carried out at the Experimentation Station of Fujian Agriculture and Forestry University, Fuzhou, Fujian, China (119.280E, 26.080 N) during the rice growing season (June–November). A large-panicle rice cultivar, Jinhui No. 809 (*Oryza sativa* Indica), was used in the study. The seeds were grown in the nursery field. The seedlings at the 5-leaf stage were transplanted with a spacing of 0.15×0.15 m and one seedling per hill. Each plot was 4×4 m that received a total fertilization of 225 kg N ha^{-1}, 112.5 kg P_2O_5 ha^{-1}, and 180 kg K_2O ha^{-1}. The phosphorus fertilizer was used for the basal, and the potassium for the top dressing. In the present study, two different N fertilizer application ratios were set as the treatment. The TNA treatment was applied in 3 sessions, i.e., 60% before transplanting, 30% at tillering and 10% at panicle initiation. The MNA treatment was applied in 4 sessions, i.e., 30% before transplanting, 30% at tillering, 30% at panicle initiation and 10% at anthesis. The soil of the field was sandy loam with available N, phosphorus, and potassium at 190.6, 126.6 and 201.6 mg kg^{-1}, respectively. The experiment was repeated three times.

Sampling of rice spikelets

Five hundred panicles that headed on the same day were chosen and tagged for each treatment. Approximately 60 labeled panicles were sampled every 7 d from the flowering stage to maturity. Fifty of the sampled panicles were immediately cryogenically frozen in liquid nitrogen for subsequent protein extraction and total RNA determination. The remaining 10 panicles were used to study the grain-filling dynamics. The samples of superior and inferior spikelets were collected according the previous report (see also Zhang et al., 2009). Due to the asynchronous filling properties of the superior and inferior spikelets, the former were sampled 3 times (i.e., 7, 14 and 21 DAF) and the latter 5 times (i.e., 7, 14, 21, 28 and 35 DAF) for protein extraction and proteomic determination. This was designed with a 2-DE approach.

Determination of rice dry matter and grain yield

The superior and inferior spikelets on each of the 10 sampled panicles were separated, blanched in an oven at 105°C for 15 min, and dried at 80°C to a constant weight. At the ripened stage, 10 rice plants were dried to a constant weight to determine biomass weight and grain yield with 3 replications for each sampling.

Protein sample preparation

One g of a superior or inferior spikelet sample was mixed with

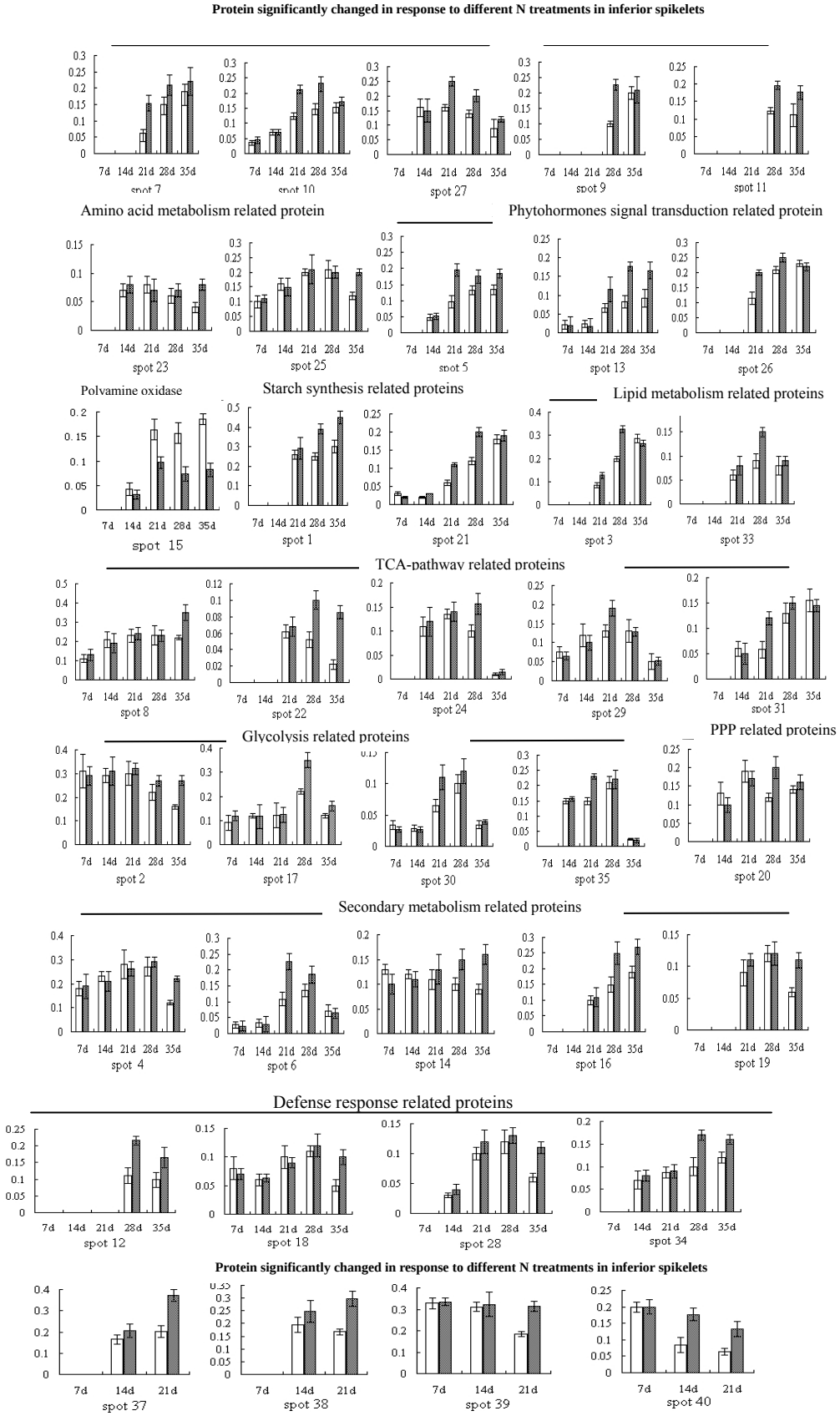

Fig 2. Changes in expression abundance of differential protein spots from superior and inferior spikelets in rice under different N application ratios. The spot numbers are the same as those specified in Fig. 1 and Table 1. Histogram displays changes in intensity of protein spots between TNA and MNA treatments, and bars represent spot intensity with relative volume divided by total volume over the whole image, according to Image-Master 5.0 software description. □ Expression abundance under TNA**;** ▩ Expression abundance under MNA. Values are the means ± SE of protein volumes of gels from three independent experiments.

0.5 g PVP, ground to fine powder in a mortar, and subsequently, immersed in liquid nitrogen. The sample was suspended in

precooled, 10% TCA with acetone containing 0.07% β-mercaptoethanol and kept at -20°C overnight. The thawed sample was then centrifuged at 15, 000 rpm for 30 min at 4°C. After decanting the supernatant，the precipitate was washed with precooled, 100% acetone containing 0.07% β-mercaptoethanol followed by centrifugation at 15, 000 rpm for 30 min at 4°C. These procedures were repeated approximately every 8 h until the supernatant was achromatic. Then, the precipitate was dried under vacuum to yield sample pellet. The dried pellet was dissolved in a lysis buffer containing 8 M urea, 4% CHAPS, 40 mM Tris and 65 mM DTT. The mix was homogenized by using ultrasound for 20-25 min, followed by centrifugation at 15, 000 rpm for 15 min. The supernatant was collected and stored at -80°C for proteomic analysis. The protein concentration was determined using a Bradford assay with BSA (bovine serum albumin) as the standard (Garrels, 1983).

2D- PAGE

The protein samples were further separated by 2D-PAGE using the IEF tube gels for the first dimension and SDS-PAGE for the second dimension separation. The IEF tube gels (17×0.02 cm) were prepared containing 8 M urea, 3.5% acrylamide, 2% NP-40 and 2% ampholines (G.E. Healthcare, Uppsala, Sweden; the ratio of pH 3.0-10.0 : pH 5.0-8.0 was 1:3 for a nonlinear gel). A series of electrophoresis was performed at 200, 300, 400, 500 and 600 v for 30 min, 800 v for 16 h and 1000 v for 6 h. After the first-dimensional run, the gels were incubated twice in an equilibration buffer (0.05 M Tris-HCl, pH 6.8, 2.5% SDS, 10% v/v glycerol and 5% 2-mercaptoethanol) for 15min. The second dimension electrophoresis was performed on SDS-PAGE comprising a 5% stacking and a 10% separating gel. After the electrophoresis, the gels were stained with silver staining. The silver stained gels were then scanned in an Imagescan and analyzed by using the Image-master 5.0 software (Bio-Sciences, G.E. Healthcare, Uppsala, Sweden)

In-gel digestion with trypsin and extraction of peptides

In-gel protein digestion was performed as described previously (wang,et al 2011). Prior to mass spectrometric analysis, the protein digestion extracts (tryptic peptides) were resuspended in 5 µl of 0.1% trifluoroacetic acid. The peptide samples were mixed (1:1 ratio) with a matrix consisting of a saturated solution of α-cyano-4-hydroxy-trans-cinnamic acid in 30% acetonitrile containing 0.1% trifluoroacetic acid. The aliquots of 1 µl were, then, spotted onto MALDI plates.

Proteins identification and classification

The peptide mass spectra were obtained on a Bruker UltraFlex III MALDI TOF mass spectrometer (Bruker Daltonics, Karlsruhe, Germany). The obtained MS spectra per spot were combined and submitted to MASCOT search engine (V2.3, Matrix Science, London, U.K.) by Biotools 3.1 (Bruker Daltonics) and searched with the following parameters: the NCBI (National Center for Biotechnology Information) in SwissProt (http://www.matrixscience.com/search_form_select.html), taxonomy of rice (*Oryza satica),* trypsin of the digestion enzyme, one missed cleavage site, carbamidomethyl as a fixed modification of cysteine and oxidized methionine as a variable modification, MS tolerance of 100 ppm, the known contaminant ions (keratin) were excluded. To denote a protein as an unambiguous identification the following criteria were used: coverage of the mature protein by the matching peptides must reach a minimum of 15%, and at least 5 independent peptides should match. The identified proteins were grouped into individual categories according to their functions as documented in EBI (http://www.ebi.ac.uk/InterPro) and NCBI databases.

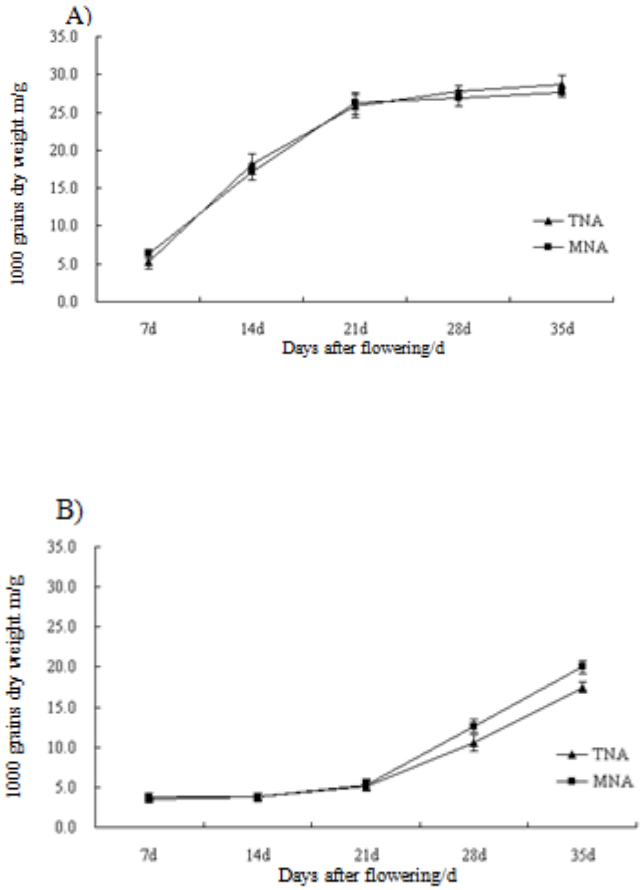

Fig 3. Grain dry weight of superior (A) and inferior (B) spikelets of rice under TNA and MNA treatments. Vertical bars are ± S.E. of the mean (n =3) where these exceed the size of the symbol.

RNA extraction and RT-PCR analysis

Real-time fluorescence quantitative PCR (RT-PCR) was employed to confirm the proteiomic analysis result. The total RNAs extracted from the grain samples were found to be identical with those for the proteome analysis in 3 separate extractions by Trizol reagent (Invitrogen, Life Technologies). The primer pairs were designed by the Beacon Designer 2 software (Table S1). The gene expression was assayed by using the Mastercycler ep realplex real-time PCR system (eppendorf) and iQ SYBR Green Supermix kit (Tagene). The reaction conditions (in 20 mL) were optimized by changing the primer concentration and annealing temperature to minimize primer-dimer formation and increase PCR efficiency. The PCR

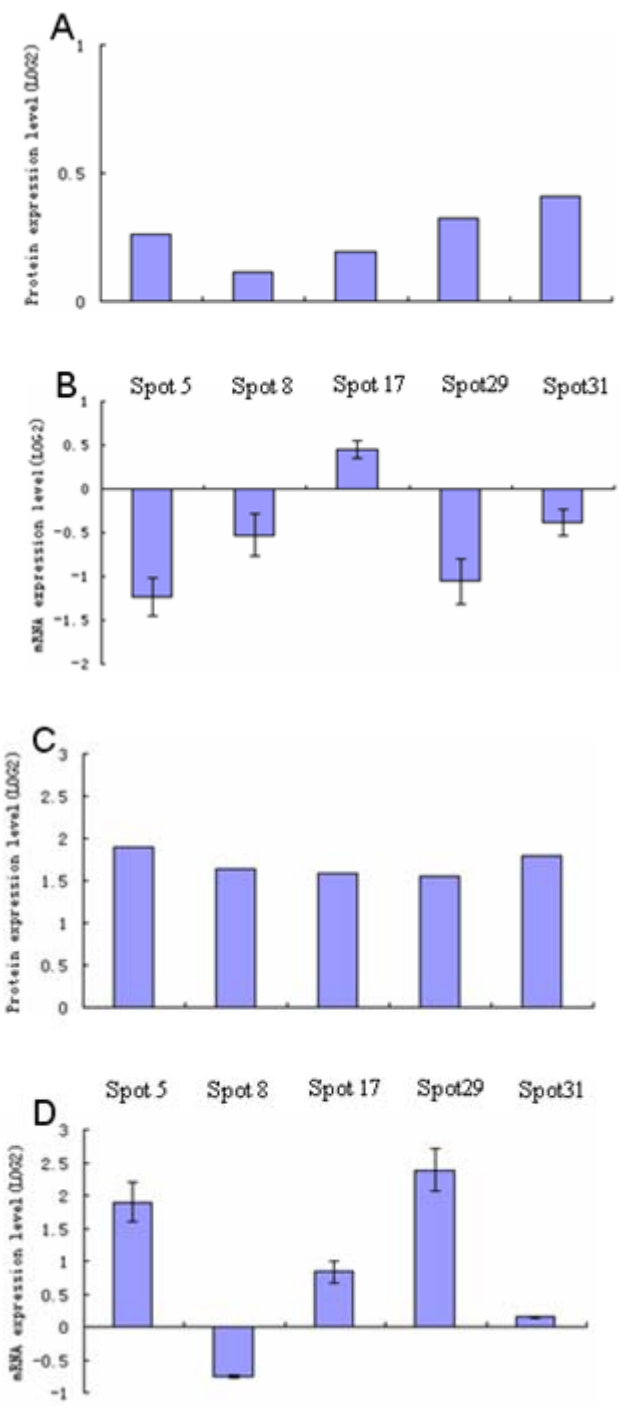

Fig 4. Comparison of 5 genes at mRNA level and proteins in superior and inferior spikelets under different of N treatments (TNA and MNA). Expression level is expressed as average abundance of protein/mRNA in superior and inferior spikelets under MNA treatment divided by that under TNA treatment. (A) Expression level of 5 corresponding proteins in superior spikelets on 14 DAF. (B) mRNA expression level of 5 genes in superior spikelets on 14 DAF. (C) Expression level of 5 corresponding proteins in inferior spikelets (spots 5, 29 and 31 on 21 DAF, spot 17 on 28 DAF, and spots 8 on 35 DAF). (D) mRNA expression level of 5 genes in inferior spikelets (spots 5, 29 and 31 on 21 DAF, spot 17 on 28 DAF, and spot 8 on 35 DAF).

profile used included 1 min at 95°C, (10 s at 95°C, 45 s at 54°C, 10 s at 72°C)×50, and 1 min at 72°C followed by recording the melting curve. The absence of primer-dimer or nonspecific product accumulation was checked by a melting curve analysis. Each run included the standard dilutions and negative reaction controls. The mRNA expression level of each gene of interest, as well as, the housekeeping gene β-actin was determined in parallel. Results were expressed as normalized ratio of mRNA level of each gene over the housekeeping gene using the difference between the threshold cycle values or △△Ct method (Livak and Schmittgen, 2001). The △Ct values for each individual target gene were calculated, and the average △Ct for the housekeeping gene was treated as an arbitrary constant in calculating △△Ct values for all samples.

Statistic analysis

All physiological data were analyzed as a completely randomized design with three replications. Protein volume (%) obtained from the Image-master 5.0 software (G.E. Healthcare, Bio-Sciences, Uppsala, Sweden) was analyzed as a randomized design. Replications were used to determine the significance of differences on the protein volume between the two N treatments. All data were processed using Microsoft Excel 2003 and Statistical Analysis System (SAS, V. 8.1, SAS Institute, Cary, NC).

Conclusion

In conclusion, MNA could increase the rice yield without increasing the total N supply. The grain-filling of superior spikelets could be stably expressed in responses to different N supplies; changes in N application ratio mainly affected the grain-filling of inferior spikelets, and implicated seed setting rate and 1000-grain weight, thereby altering grain yield performance. Based on the evidence shown, the mechanisms of N in facilitating the gain-filling of the inferior spikelets might include: (1) promoting the expression of cell respiration related proteins, such as fructose-bisphosphate aldolase, phospho-glycerate mutase, glyceraldehyde-3-phosphate dehydrogenase, malic oxidoreductase family protein, succinate dehydrogenase,t ransketolase, and phosphoglucomutase, which could provide more assimilate materials and energy for the grain filling; (2) regulating the phosphoglucomutase, WRKY protein, glycolipid transfer protein and cytochrome P450-dependent fatty acid hydroxylase that favorable the starch synthesis; and, (3) improving the expression abundance of auxin-responsive protein IAA22 and gibberellin response modulator that are favorable for the phytohormones synthesis. The findings, were also for the fist time, explained the asynchronous filling properties of superior and inferior spikelets which were attributed to differential expression of proteome level in temporal and space.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 30871494), Doctoral Disciplines Foundation of Education Ministry, China (No. 200803890006), Provincial Natural Science Foundation of Fujian, China (No.2009J01060) and Department of Education Science and Technology Project of Fujian, China (No. JK2011014).

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