Proteomic analysis revealed nitrogen-mediated metabolic, developmental, and hormonal regulation of maize (Zea mays L.) ear growth

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Received and revised 6 February 2012; accepted 30 May 2012

Abstract

Optimal nitrogen (N) supply is critical for achieving high grain yield of maize. It is well established that N deficiency significantly reduces grain yield and N oversupply reduces N use efficiency without significant yield increase. However, the underlying proteomic mechanism remains poorly understood. The present field study showed that N deficiency significantly reduced ear size and dry matter accumulation in the cob and grain, directly resulting in a significant decrease in grain yield. The N content, biomass accumulation, and proteomic variations were further analysed in young ears at the silking stage under different N regimes. N deficiency significantly reduced N content and biomass accumulation in young ears of maize plants. Proteomic analysis identified 47 proteins with significant differential accumulation in young ears under different N treatments. Eighteen proteins also responded to other abiotic and biotic stresses, suggesting that N nutritional imbalance triggered a general stress response. Importantly, 24 proteins are involved in regulation of hormonal metabolism and functions, ear development, and C/N metabolism in young ears, indicating profound impacts of N nutrition on ear growth and grain yield at the proteomic level.

Key words: C/N metabolism, hormonal metabolism, maize ear, nitrogen deficiency, nitrogen oversupply.

Introduction

As one of the major cereal crops, maize plays an essential role in maintaining worldwide food security (Pinstrup-Andersen et al., 1999; Lobell et al., 2008). Maize plants enter the silk-ing stage when any silk outgrows ear husks and the number of fertilized ovules is determined at this stage (Hanway, 1963). Sink strength of the maize ear is also primarily determined at the silking stage when cobs accumulate a large amount of amino acids such as glutamine and asparagine (Seebauer et al., 2004). Mutation of glutamine synthetase isoenzymes reduces the kernel number and kernel size (Martin et al., 2006). Inefficient asparagine transport to developing kernels significantly reduces kernel production (Martin et al., 2006). Kernel set and number are largely dependent on assimilate availability at flowering (15 d before to 15–20 d after silking) (Hawkins and Cooper, 1981; Tollenaar et al., 1992; Cirilo and Andrade, 1994a).

Sufficient nitrogen (N) nutrients are required for amino acid metabolism, ear growth, and dry matter accumulation in maize kernels (Hirel et al., 2001). At the reproductive phase, N availability affects assimilate partitioning between vegetative and reproductive organs and N metabolism in young earshoots (Czyzewicz and Below, 1994). Due to extreme sensitivity of kernel set to environmental stresses during silking, the spikelet number on each ear is significantly reduced under various abiotic stresses (Kiniry, 1985; Cirilo and Andrade, 1994b). N deficiency reduces the kernel number and dry matter accumulation, and causes a 14–80% decrease in grain yield as a result (Uhart and Andrade, 1995b).
Since the Green Revolution, the increase of maize grain yield has relied heavily on extensive application of N fertilizers, although breeding efforts and agricultural management have also helped (Tilman, 1998; Hirel et al., 2001; Evenson and Gollin, 2003). However, the usage of N fertilizers is usually not optimal and causes worldwide challenges facing farmers and scientists. On the one hand, N deficiency remains a major limiting factor in promoting maize production in many undeveloped areas in Africa (Vanlauwe et al., 2001). On the other hand, N oversupply causes a series of environmental problems including N run-off, leaching, or soil acidification in certain rapidly developing areas such as the intensively cultivated North China Plain (Liu and Diamond, 2005; Zhao et al., 2006; Ju et al., 2009; Guo et al., 2010). Given highly dynamic amino acid conversion between the cob and kernels at silking, and rapid protein and carbohydrate accumulation in pollinated flowers in this process, it is imperative to understand the proteomic mechanism of how different N regimes affect ear growth at key developmental stages, especially the silking stage.

Materials and methods

Maize growth

Field experiments were conducted on a calcareous soil at Shangzhuang Experimental Station, China Agricultural University, Beijing. Twelve adjacent plots with the same fertility were utilized as control (optimal N fertilization: 250 kg ha\(^{-1}\); ON), and high N (farmer’s N fertilization: 365 kg ha\(^{-1}\)); HN) plots (Supplementary Table S1 available at JXB online), and each treatment had four replicates. ON was calculated as plant N uptake minus soil N supply according to the field experiments in the previous 2 years. N fertilization was determined according to farmers’ practice. The physical and chemical properties of experimental soil were as follows: extracted mineral N 29.3 kg ha\(^{-1}\), pH (H\(_2\)O) 7.9, soil density 1.4 g cm\(^{-1}\), Olsen-P 7.1 mg kg\(^{-1}\), NH\(_4\)OAc-extracted K 117.5 mg kg\(^{-1}\), and organic matter 7.3 g kg\(^{-1}\).

Maize hybrid DH 3719, a high-yield genotype in Northern China, was sown at the end of April. All 12 plots were overseeded with hand planters and thinned at the seedling stage to a stand of 100 000 plants ha\(^{-1}\). To optimize planting density and ensure sufficient light interception and nutrient uptake, the inter-row distance was 50 cm for wide rows and 20 cm for narrow rows; the intra-row distance was 28 cm. Seeds were only planted interlayingly in narrow rows. Border plots were included on all sides of the experimental field to eliminate marginal effects. Weed only planted interlayingly in narrow rows. Border plots were included on all sides of the experimental field to eliminate marginal effects. Weed only planted interlayingly in narrow rows.

Total N content analysis

All plant samples were heated at 105 °C for 30 min, dried at 70 °C until constant weight, then weighed and ground into powder. Appropriate amounts of ground tissue were analysed for the total N content using a modified Kjeldahl digestion method (Baker and Thompson, 1992).

Proteomic analysis of young maize ears

Total soluble proteins were isolated from ~3.0 g of frozen young ear tissue of maize per biological replicate. Proteins were precipitated and purified following the procedures described by Damerval et al. (1986) and Liu et al. (2006). In brief, proteins were precipitated with 10% (w/v) trichloroacetic acid (TCA)–acetone and recovered by centrifugation. The protein pellet was washed three times with 100% methanol, air-dried for 5 min, and then dissolved in the protein solubilization buffer (7 M urea, 2 M thiourea, 4% (w/v) 3-(cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS), 65 mM N\(_2\)-dithiotreitol (DTT) and 0.5% (v/v) IPG buffer pH 4–7). The insoluble fraction was removed via centrifugation at 14000 g for 70 min, and a 0.5% (v/v) IPG buffer (pH 4–7) from GE Healthcare (Piscataway, NJ, USA) was added into the soluble protein fraction. The protein concentration was assessed using the GE Healthcare 2-D Quant Kit.

Protein samples of 500 µg were loaded into a 24 cm GE Healthcare Strip Hold for active rehydration overnight. The isoelectric focusing electrophoresis (IEF) by the Ettan IPGphor (GE Healthcare) was programmed as follows: 50 V for 1 h, 200 V for 1 h, 200–500 V gradient for 1 h, 500–1000 V gradient for 1.5 h, 1000 V for 2 h, 1000–5000 V gradient for 1.5 h, 5000 V for 1 h, 5000–10 000 V gradient for 1.5 h, 10 000 V for 6 h. DryStrip equilibration and the second dimension 12.5% SDS–PAGE was performed following GE protocols. Proteins were visualized by Coomassie Brilliant Blue (CBB) stain (Neuhoff et al., 1988) for 48 h on an orbital shaker. Stained gels were imaged using the ImageScanner II Imaging System (GE Healthcare). The resulting gel images were analysed with ImageMaster 2D Platinum (GE Healthcare). Proteins were classified as differentially accumulated between different samples when the ratio of their average blot intensities was >1.3 and a t-test for differential accumulation was significant at the 0.05 level.

Protein digestion and peptide treatment were performed following standard methods (Havlíš et al., 2003). Mass spectrometry analysis was performed using an AUTOFLEX II TOF-TOF (ABI-4800, Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. The mass spectrometer was operated under 19 kV accelerating voltage in the reflection mode and an m/ε range of 900–4000. The peptide ions generated by autolysis of trypsin (with m/ε 2163.333 and 2273.434) were used as internal standards for calibration. The list of peptide masses from each peptide map fingerprinting (PMF) was saved for further analysis.

To identify maize proteins that were isolated from 2D gels, tandem mass spectrometry (MS/MS) data were fed to the MASCOT software (Matrix Science) and a customized maize protein database that was built from all annotated proteins from the maize whole-genome sequencing project (www.maizesequence.org, version 5a) was searched. If two or more proteins were identified by MASCOT at the significance level of P < 0.05 for a spot, the hit with best ion score was chosen. To annotate putative functions of identified maize proteins, each maize protein sequence was queried against (blastp with E-value cut-off of 1e-10) all annotated rice (http://rice.plantbiology.msu.edu, version 6.1) and Arabidopsis proteins (www.arabidopsis.org, TAIR9), respectively, and the annotation of the best hit from each of the two species was consulted.

Functional analysis of differentially expressed proteins

Gene Ontology (GO) (http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (http://www.genome.jp/kegg/pathway.html) analyses were performed with the PartiGene program (http://www.nematodes.org/bioinformatics/attnot8r/index.shtml). Annot8r assigns KEGG (gene) pathways and GO (protein) terms based on BLASTX similarity (E-value <10e-5) and known GO annotations (Parkinson et al., 2004; Schmid and Blaxter, 2008). Results for GO were summarized in three independent categories (Biological Process, Cellular Component, and Molecular Function).

DNA/RNA extraction and PCR amplification

Total DNA and RNA samples were extracted from ground maize ear tissue using a DNeasy and RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), respectively. The DNA/RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Agilent Technologies, CA, USA). For PCR, the DNA concentration was adjusted to 200 pg µl\(^{-1}\) with Millipore water.

The primer set 5’-AGAGTTTGATCCTGGCTCAG-3’ and 5’-TGAGACTACTTTGTAGACATT-3’ was used to amplify a 1500 bp 16S rDNA fragment (Lane, 1991). The primer set 5’-AGGAATTCGACG AAGGGCA-3’ and 5’-GTGCGGCCCAGACATCTAAG-3’ was used for PCR.
used to amplify a 325 bp 18S rDNA fragment (Rotem et al., 2007). Potential *Ustilago maydis*, *Fusarium sublutinans*, and *Cochliobolus carbonum* infection were analysed through PCR amplification using the corresponding gene-specific primer sets 5'-GAACCTTTCTGGCC TCTTTT-3' and 5'-CTTGTTITCGTCCGTAC-3' (Xu et al., 1999), 5'-GCCCCTCAAGAGGCGAAA-3' and 5'-GTCAGACACAGAC AATGGGC-3' (Möller et al., 1999), and 5'-CCGCGTTC GAGGTGGTA-3' and 5'-GATGTCGAGGTTGGAAAC-3' (Multani et al., 1998), respectively.

The expression levels of two key genes in jasmonic acid (JA) synthesis, 12-oxo-phytodienoic acid reductase 7 (OPR7) and 12-oxo-phytodienoic acid reductase 8 (OPR8) (Zhang et al., 2005), were analysed using quantitative real-time PCR. The primer set 5'-TCTCAACGTCTCCAGCAG-3' and 5'-CGTAGGACACCAGGTCAGC-3' was used to amplify a 236 bp OPR7 fragment, with the primer set 5'-GTACGGGGCAGCGGAGTC-3' and 5'-AAGCCTTCTGCCCAGTATC-3' for a 249 bp OPR8 fragment.

All PCR experiments were run in a PTC-200 thermocycler (MJ Research, Waltham, MA, USA), strictly following procedures described in the corresponding references.

**Results**

*N* deficiency negatively regulated ear growth

The ear size of *N*-deficient (LN) plants was significantly smaller than that of optimal *N* supplied (ON) plants (Fig. 1A). The dry weight of the cob and grain of LN ears was also significantly lower than that of ON ears (Fig. 1B, 1C), thus grain yield of LN plants significantly decreased compared with that of ON plants (Fig. 1D). On the other hand, *N* oversupply (HN) had no obvious effect on ear growth and grain yield (Fig. 1D).

Sink strength of the maize ear is determined at the silking stage—a key developmental stage pre-determining kernel growth and grain yield (Cantarero et al., 1999). Next the *N* content, and soluble protein and biomass accumulation in young ears at the silking stage were analysed. As shown in Fig. 2, the immature ear of LN plants had approximately seven times less dry weight (Fig. 2B) and *N* content (Fig. 2C) than that of ON plants. In contrast, the soluble protein concentration of young ears had no significant variation among treatments (Fig. 2D).

Forty-seven proteins differentially accumulated in young maize ears under different *N* treatments

Although quantitative variation of total soluble protein concentration was not detected in young ears among different *N* treatments, *N* deficiency or oversupply may cause differential accumulation of a subset of proteins (Johansson et al., 2001). To dissect proteomic variation in young maize ears caused by *N* treatments, proteins were separated by 2D electrophoresis and then the nature of differentially accumulated proteins was determined with matrix-assisted desorption ionization-time of flight (MALDI-TOF/TOF) MS analysis. More than 1300 protein spots per gel were visualized using CBB staining (Fig. 3). The molecular weights of these proteins ranged from 14 kDa to >100 kDa, with a pI range from 4 to 7. Among 50 protein spots that showed significant differential accumulation in young ears of maize plants between treatments, it was possible to identify 47 proteins by searching annotated proteins from the maize whole-genome assembly using the MALDI-TOF/TOF MS data. Forty proteins were derived from LN–ON ear comparison, with the other seven proteins from HN–ON ear comparison. Notably, both spots 351 and 420 were identified as the ubiquitin C-terminal hydrolase (GRMZM2G017086_P01), and spots 1255 and 1257 were identified as the protein elongation factor (GRMZM2G040369_P01). It was hard to determine whether two spots were the same protein because they might contain proteins translated from the same gene with different
post-translational modifications or were highly similar proteins that belong to the same family. Therefore, each spot was counted separately. These 47 proteins fell into two major functional categories: 28 proteins in metabolism, 17 proteins in plant defence, and two proteins with unknown functions as listed in Table 1.

In comparison with ON ears, LN ears had six newly detected proteins, three proteins undetected, and the other 31 differentially regulated proteins comprised 13 up-regulated and 18 down-regulated proteins (Table 1, Fig. 3).

N oversupply had less effect on protein differential accumulation in young maize ears. Two proteins were up-regulated in young HN ears, and five proteins were down-regulated, compared with those in ON ears (Table 1, Fig. 3).

Functional analysis indicated that N regimes affected multiple biological processes

To explore further the potential functions of differentially regulated proteins, the KEGG database (Nakao et al., 1999) was searched and it was found that 14 differentially regulated proteins were involved in 11 different biological pathways (Table 2). Ascorbate peroxidase (spot 157, 152), ascorbate peroxidase 1 (spot 153), and glutathione dehydrogenase (spot 159) were involved in ascorbate and aldarate metabolism. Chloroplast heat shock protein 70 (spot 1276) and heat shock protein 81-4 (spot 1250) were involved in plant-pathogen interaction. Interestingly, three proteins were involved in multiple biological pathways. The glutathione dehydrogenase was involved in glutathione, and ascorbate and aldarate metabolism. The lipoxygenase (spot 50) was involved in linoleic and α-linolenic acid metabolism. The ketol-acid reductoisomerase (spot 491) was involved in pantothenate and CoA biosynthesis, and valine, leucine, and isoleucine biosynthesis. The lipoxygenase and ketol-acid reductoisomerase were also involved in other uncharacterized metabolic pathways. Additionally, ATP synthase (spot 431), tRNA synthetase (spot 353), and T-complex protein (spot 430) were involved in secondary metabolite biosynthesis, aminoacyl-tRNA biosynthesis, and protein processing in the endoplasmic reticulum, respectively (Table 2).

GO analysis indicated that 37 differentially regulated proteins were involved in 21 different biological processes, such as responses to stresses, metabolic processes, oxidation reduction, and so forth (Supplementary Table S2 at JXB online; Fig. 4A). Seven proteins were involved in response to cold stress (spot 69, 185), oxidative stress (spot 332), a series of other abiotic stresses (spot 77, 157, 159, 1276), and fungal symbiosis (spot 159) (Supplementary Table S2; Fig. 4A). Twenty-nine proteins were located in 14 cellular components including the cytoplasm, chloroplast, and endoplasmic reticulum (Supplementary Table S2; Fig. 4B), and 39 proteins had molecular functions related to hydrolysis, ligation, GTP binding, nucleic acid binding, oxidoreductase activity, and so on (Supplementary Table S2; Fig. 4C).

Detection of pathogen infection and OPR gene expression

Considering 17 proteins related to plant defence, PCR analysis was performed to detect potential bacterial or fungal infection using 16S (27F/1492R) and 18S rDNA primer sets (Kowalchuk et al., 1997; Yang et al., 2001). As shown in Fig. 5A it was possible to amplify both 16S and 18S products from all samples under different N treatments (Fig. 5A). It was further tested whether maize ear samples were infected by three ear preferential fungal pathogens: U. maydis (Xu et al., 1999), F. sublutinans (Möller et al., 1999), and C. carbonum (Multani et al., 1998). The result indicated that none of these three fungal pathogens was present in the maize ear samples (Fig. 5B).
Notably, five proteins related to JA metabolism had differential accumulation under different N treatments, indicative of potential alteration in JA synthesis. Therefore, the expression levels of two key genes mediating JA synthesis, OPR7 and OPR8 (Zhang et al., 2005), were tested using quantitative real-time PCR. As shown in Supplementary Fig. S1 at *JXB* online, N deficiency down-regulated OPR7 and OPR8 expression at the silking stage; whereas N oversupply up-regulated OPR7 and OPR8 expression.
Table 2. Fourteen proteins participated in 11 metabolic pathways according to KEGG analysis

| Pathway ID | Pathway title                           | Protein accession no. | Putative annotation |
|------------|-----------------------------------------|-----------------------|---------------------|
| ko00053    | Ascorbate and aldarate metabolism       | GRMZM2G140667_P01     | Ascorbate peroxidase |
| ko04626    | Plant-pathogen interaction              | GRMZM2G079668_P01     | Chloroplast heat shock protein 70 |
| ko00591    | Linoleic acid metabolism                | GRMZM2G109130_P01     | Lipoxxygenase^d       |
| ko00592    | Alpha-Linolenic acid metabolism         | GRMZM2G109130_P01     | Lipoxxygenase^d       |
| ko00770    | Pantothenate and CoA biosynthesis       | GRMZM2G004382_P01     | Ketal-acid reductoisomerase^d |
| ko00290    | Valine, leucine, and isoleucine biosynthesis | GRMZM2G004382_P01   | Ketal-acid reductoisomerase^d |
| ko01100    | Uncharacterized metabolic pathways      | GRMZM2G109130_P01     | Lipoxxygenase^d       |
| ko00480    | Glutathione metabolism                 | GRMZM2G035502_P01     | Glutathione dehydrogenase (ascorbate)^d |
| ko01110    | Biosynthesis of secondary metabolites   | GRMZM5G829375_P01     | ATP synthase          |
| ko00970    | Aminoacyl-tRNA biosynthesis             | GRMZM2G083836_P01     | tRNA synthetases class II |
| ko04141    | Protein processing in endoplasmic reticulum | GRMZM2G434173_P01   | T-complex protein     |

^a Pathway ID: number in KEGG (Kyoto Encyclopedia of Genes and Genomes).  
^b Protein accession no.: identification of predicted protein based on the maize whole-genome sequencing project.  
^c Putative annotation: corresponding to those in Table 1.  
^d Proteins involved in multiple metabolic pathways.

Discussion

N deficiency inhibited young ear growth and biomass accumulation

N supply affects N allocation, carbon assimilation, and biomass accumulation in grain (Cazetta et al., 1999; Below et al., 2000; D’Andrea et al., 2008). N deficiency negatively mediates a series of growth indexes related to maize development, such as biomass production, kernel set, and grain yield (Lemcoff and Loomis, 1986; Jacobs and Pearson, 1991; Uhart and Andrade, 1995a; Pandey et al., 2000). Among these indexes, grain yield is largely dependent on kernel set and biomass accumulation (Andrade et al., 2002). Kernel number is predominantly determined by the maize growth rate during the critical period for kernel set (30 d at silking) (Uhart and Andrade, 1995a, b; Andrade et al., 2002). N deficiency shortens the anthesis–silking interval [the average number of days between maize tassel (male) flowering and the first visible silk on the maize ear (female) (Bänziger et al., 2002)] and decreases kernel number and grain yield (Bänziger et al., 2002). In the present study, insufficient N supply inhibited biomass accumulation in maize ear at the silking stage (Fig. 2A, 2B), resulting in a significant decrease in cob biomass (Fig. 1B), kernel biomass (Fig. 1C), and grain yield (Fig. 1C). N oversupply slightly increased biomass accumulation in the ear at silking (Fig. 2B), and had no obvious effect on biomass accumulation in the cob and kernel at harvest (Fig. 1B, 1C).

N nutritional imbalance triggered a stress response that also responded to many other external stimuli

GO analysis indicated that seven differentially regulated proteins upon N nutritional imbalance also responded to other abiotic stresses, and these proteins were chloroplast heat shock protein 70 (spot 1276), ascorbate peroxidase (spot 157), glutathione dehydrogenase (ascorbate) (spot 159), NADP-dependent oxido-reductase (spot 332), glycine-rich RNA-binding protein (spot 69, 185), and an RNA recognition motif-containing protein (spot 77) (Table 3). Heat shock proteins are up-regulated by a variety of stresses including drought, salinity, cold, and hot stresses (Swindell et al., 2007). Cytosolic ascorbate peroxidase 1 is related to drought and heat stress responses (Mittler and Zilinskas, 1994; Koussevitzky et al., 2008). The increase in ascorbate peroxidase activity is involved in plant defence against ozone or sulphur dioxide (Kubo et al., 1995). Glutathione dehydrogenase (ascorbate) responds to UV-B radiation stress and plant–fungi symbiosis (Jiménez et al., 1998; Costa et al., 2002), and NADP-dependent oxido-reductase responds to high light stress via photo-protection signalling in plants (Phee et al., 2004). Proteins with RNA-binding domains have important roles in response to cold and oxidative stresses (Carpenter et al., 1994; Albà and Pagès, 1998; Martin et al., 2006). In addition to the ascorbate peroxidase (spot 157) and glutathione dehydrogenase (ascorbate) (spot 159), N nutritional imbalance resulted in significant differential accumulation of four other proteins, the alcohol dehydrogenase (spot 419), ascorbate peroxidase (spot 152), ascorbate peroxidase 1 (spot 153), and pyruvate decarboxylase (spot 424) that are involved in the hypoxic response (Matton et al., 1990; Christie et al., 1991; Dolfurus et al., 1997; Biemelt et al., 1998; Jiménez et al., 1998; Conley et al., 1999; Costa et al., 2002; Bailey-Serres and Chang 2005). The present result was in agreement with the previous argument that a variety of abiotic stresses such as Fe deficiency, salt stress, and drought stress induced the hypoxic stress response (Mittler and Zilinskas, 1994; Hernandez et al., 1995; Karpinski et al., 1997; Dionisio-Sese and Tobeita, 1998; Donnini et al., 2011).

Five proteins had roles in response to biotic stresses (Table 3). Lipoxigenase was a key enzyme implicated in the fungus–seed interaction (Wilson et al., 2001). Cinnamoyl-CoA reductase
plays a unique role in the defence response by promoting lignin biosynthesis and binding to the small GTPase (Lauvergeat et al., 2001; Kawasaki et al., 2006). Unexpectedly, N deficiency also down-regulated the expression level of a DNA complex protein that is associated with the bacterial-transferred (T) DNA, facilitating entry of DNA into the host cell nucleus (Zeng et al., 2006). Similarly, another nucleo-cytoplasmic transporter that transports diverse proteins into the nuclei also had decreased expression under N deficiency (Jiang et al., 1998). Further, a putative clathrin adaptor subunit that plays important roles in plant endocytosis was also down-regulated under N deficiency (Holstein, 2002).

Maize ear may be easily infected by bacteria or fungi in air under field conditions (Leifert et al., 1994). Endophytic bacteria could be ubiquitous in most plants in the field without showing pathogenicity (Fisher et al., 1992; Sessitsch et al., 2002), and endophytic fungi may just colonize in maize cobs or seeds without visible damage at harvest (Fisher et al., 1992). The presence of 16S and 18S rDNA was detected in maize ear samples of three different treatments. However, it was not possible to detect ear preferential fungal infection (Fig. 5B). Previously, a large number of proteins related to plant defence or diseases were detected in root mucilage even if all maize plants were healthy and grown in an axenic environment (Ma et al., 2010). Therefore, it was concluded that nitrogen nutritional imbalance, rather than bacterial or fungal pathogen infection, resulted in significant differential accumulation of proteins related to plant defence in the present study. Sequential ubiquitination and deubiquitination regulates gene expression and is involved in multiple cellular processes (Wilkinson, 2000; Henry et al., 2003; Zhang, 2003). Up-regulation of ubiquitin C-terminal hydrolase (spot 351, 420) may decrease ubiquitin levels in the ear in response to N deficiency via cleaving residual peptides from the C-terminus of ubiquitin, suggesting that deubiquitination might regulate plant response to N nutritional imbalance (Pickart and Rose, 1985; Takami et al., 2007).

Together, N nutritional imbalance triggered a general stress response involving many non-specific responders that are also able to respond to many other abiotic and biotic stresses (Table 3).

### N nutritional status had comprehensive effects on hormonal metabolism and functions, ear development, and C/N metabolism

In addition to proteins that showed differential expression in response to N deficiency or oversupply, 24 annotated proteins were implicated in regulation of hormonal metabolism and functions, ear developmental programmes, and C/N metabolism at silking (Table 3). N deficiency negatively affects IAA (indole-3-acetic acid) metabolism, transport, and reception. IAA-Ala conjugate hydrolase catalyses hydrolysis of amide-linked conjugates of IAA (IAA-Ala), giving rise to active IAA (Rampey et al., 2004). Therefore, IAA-Ala conjugate hydrolase plays an essential role in regulating multiple plant developmental processes including germination and flowering (Davies et al., 1999; Rampey et al., 2004). N-1-naphthylphthalamic acid-binding aminopeptidase is a putative negative regulator of polar auxin transport (Bernasconi et al., 1996; Murphy et al., 2000). Interruption of auxin efflux by N-1-naphthylphthalamic acid-binding protein restrained maize growth and development (Murphy et al., 2000). Auxin-binding proteins are auxin receptors localized at the plasmalemma of outer epidermal cells of the coleoptiles, leaf rolls, and mesocotyls of maize (Löhler and Klämbt, 1985;
Shimomura et al., 1986). Down-regulation of IAA-Ala conjugate hydrolase (spot 365) and auxin-binding protein (spot 113), together with up-regulation of N-1-naphthylphthalamic acid-binding protein (spot 1246), suggested that N deficiency inhibits ear growth through antagonizing auxin production, transport, and functions. Additionally, β-glucosidase 13 is implicated in the hydrolysis of conjugated gibberellins (Schliemann, 1984), activation of cytokinin (Brzobohatý et al., 1993), and abscisic acid (ABA) metabolism (Matsuzaki and Koiwai, 1986), and protects young plant tissue against herbivores or other insect pests (Czjzek et al., 2001) via catalysing aryl and alkyl β-d-glucoside hydrolysis. The above hormonal regulation may decrease carbohydrate allocation towards the growing ear, and reduce final grain yield as a result (Ray et al., 1983; Daie et al., 1986; Reed and Singletary, 1989).

Five proteins, Tasselseed2 (spot 20, 21), lipoxygenase (spot 50), and dienelactone hydrolase (spot 172, 1332), may affect ear development via mediating JA signalling under N deficiency or oversupply. First, Tasselseed1 encodes a plastid-targeted lipoxygenase that mediates JA biosynthesis (Acosta et al., 2009). Sex determination protein Tasselseed 2, a homologue of Tasselseed1 (DeLong et al., 1993), plays a pivotal role in

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**Fig. 4.** GO annotation (categories and the percentage of the protein numbers in a single category to the total) of 47 differentially regulated proteins: biological processes (A), cellular components (B), and molecular functions (C). Thirty-seven proteins were involved in 21 different biological processes, 29 proteins were located in 14 cellular components, and 39 proteins were predicted to have 23 different molecular functions.

**Fig. 5.** PCR detection of 16S rDNA, 18S rDNA, and ear preferential fungal infection. (A) PCR amplification of 16S (lanes 1–5) and 18S (lanes 6–10) rDNA. Lane M, DNA marker; lane 1, the positive control using Escherichia coli DNA; lane 2–4, 16S rDNA amplification using LN, ON, and HN DNA samples, respectively; lane 5, negative control using Millipore water; lane 6, positive control using a DNA sample extracted from the rhizospheric soil in our field; lanes 7–9, 18S rDNA amplification using LN, ON, and HN DNA samples, respectively; lane 10, negative control using Millipore water. (B) PCR detection of Fusarium subglutinans (lane 1–3), Ustilago maydis (lane 4–6), and Cochliobolus carbonum (lane 7–9) in maize ear samples with different nitrogen supplies.

Lanes 1, 4, 7, with LN DNA samples as the template; lanes 2, 5, 8, with ON DNA samples as the template; and lanes 3, 6, 9, with HN DNA samples as the template. LN, N deficiency; ON, optimal N fertilization; HN, N oversupply.
determining the sexual fate of maize floral meristems (DeLong et al., 1993; Irish, 1996), presumably via affecting JA signalling (Acosta et al., 2009). N deficiency down-regulated the expression level of Tasselseed2 (spot 21), which might induce female flowers on the tassel; while N oversupply up-regulated Tasselseed2 (spot 20) expression to suppress this developmental abnormality. Secondly, the lipoxigenase plays an important role in converting linolenic acid to 12-oxo-phytodienoic acid, a biosynthetic precursor of JA (Vick and Zimmerman, 1983). N deficiency might modulate JA levels in the developing maize ear through increasing lipoxigenase (spot 50) accumulation.

Thirdly, the dienelactone hydrolase has two conserved domains that are involved in regulation of methyl jasmonate functioning under environment stresses (Benedetti et al., 1998). Further, as shown in Supplementary Fig. S1, nitrogen deficiency may reduce the JA level in maize ear at the silking stage via down-regulating OPR7 and OPR8 expression levels, in contrast to up-regulating the OPR7 and OPR8 expression levels under nitrogen oversupply (Staswick et al., 1992), indicating intricate effects of N deficiency and oversupply on ear development partially via modulating the JA signalling pathway (Katsir et al., 2008).

| Functional category | Accession no. | Putative annotation |
|---------------------|--------------|---------------------|
| A general stress response | GRMZM2G107968_P01 | Chloroplast heat shock protein 70 |
| | GRMZM2G114066_P01 | Ascorbate peroxidase |
| | GRMZM2G035502_P01 | Glutathione dehydrogenase (ascorbate) |
| | GRMZM2G328094_P01 | NADP-dependent oxidoreductase |
| | GRMZM2G042118_P01 | Glycine-rich RNA-binding protein |
| | GRMZM2G009448_P01 | Glycine-rich RNA-binding protein |
| | GRMZM2G080603_P01 | RNA recognition motif containing protein |
| | GRMZM2G006953_P02 | Nuclear transport factor 2B |
| | GRMZM2G017086_P01 | Ubiquitin C-terminal hydrolase |
| | GRMZM2G017086_P01 | Ubiquitin C-terminal hydrolase |
| | GRMZM2G442658_P02 | Alcohol dehydrogenase |
| | GRMZM2G087186_P01 | Pyruvate decarboxylase |
| | GRMZG2137839_P01 | Ascorbate peroxidase |
| | GRMZM2G054300_P01 | Ascorbate peroxidase 1 |
| | GRMZM2G109130_P01 | Lipoxigenase |
| | GRMZM2G035555_P01 | Cinnamoyl-CoA reductase family |
| | GRMZM2G434173_P01 | T-complex protein |
| | GRMZM2G042089_P01 | Putative clathrin adaptor complexes medium subunit |
| Hormone metabolism and functions | GRMZM2G009077_P01 | IAA-Ala conjugate hydrolase/metalloprotease |
| | GRMZM2G169095_P01 | N-1-naphthylphthalamic acid binding/aminopeptidase |
| | GRMZM2G116204_P01 | Auxin-binding protein |
| | GRMZM2G069523_P01 | Sex determination protein tasselseed-2 |
| | GRMZM2G069523_P01 | Sex determination protein tasselseed-2 |
| | GRMZM2G109130_P01 | Lipoxigenase |
| | GRMZM2G179301_P02 | Dienelactone hydrolase family |
| | GRMZM2G073079_P01 | Dienelactone hydrolase family protein |
| | GRMZM2G016890_P01 | Beta-glucosidase 13 |
| Ear development | GRMZM2G107114_P01 | Prohibitin2 |
| | GRMZM2G136885_P01 | Beta-c-xylosidase |
| | GRMZM2G167505_P01 | RNA recognition motif-containing protein |
| | GRMZM2G426591_P01 | RNA-binding protein 45 |
| C/N metabolism | GRMZM2G176397_P01 | Aconitate hydratase |
| | GRMZM2G467338_P01 | Aconitate hydratase |
| | GRMZM2G829375_P01 | ATP synthase |
| | GRMZM2G347056_P01 | Methylene-tetrahydrofolate reductase |
| | GRMZM2G004382_P01 | Ketol-acid reductoisomerase |
| | GRMZM2G035502_P01 | Glutathione dehydrogenase (ascorbate) |
| | GRMZM2G021331_P01 | ATP synthase |
| | GRMZM2G171110_P02 | Calmodulin binding/glutamate decarboxylase |
| | GRMZM2G033208_P01 | Transketolase |
| | GRMZM2G179301_P02 | Dienelactone hydrolase family |
| | GRMZM2G073079_P01 | Dienelactone hydrolase family protein |

Functional category: functional classification based on GO analysis and the available literature.
Accession no.: identification of the predicted protein based on the maize whole-genome sequencing project.
Putative annotation: corresponding to those in Table 1.
Proteins with multiple biological functions.

Table 3. Functional classification of 35 annotated proteins that differentially accumulated under different N treatments.
N oversupply down-regulated prohibitin accumulation (spot 239) to delay ear senescence. Prohibitin may serve as a membrane-bound chaperone that accelerates cellular senescence of plants via misfolding mitochondrial proteins and damaging the mitochondrial membrane (Ahn et al., 2006). Down-regulation of the prohibitin gene also causes smaller flowers and decreases petal size by reducing the cell size or number (Chen et al., 2005), which may partially explain why N oversupply did not increase ear size or kernel number. In contrast, down-regulation of β-d-xylosidase (spot 542) may indicate early onset of ear maturity under N deficiency. β-d-xylosidase participates in xylan or arabinoxylan breakdown to modify the cell wall (Itai et al., 2003). It is expressed at higher levels during fruit development and decreases after the onset of tomato fruit ripening (Itai et al., 2003). Finally, RNA–protein interaction plays important roles in regulating plant development including the flowering process (Schomburg et al., 2001; Lorković and Barta, 2002). N nutrition might affect maize ear development via differential regulation of proteins with an RNA recognition motif (spots 563) and an RNA-binding protein 45 (spot 432).

N deficiency and oversupply had distinct effects on C/N metabolism. N deficiency altered C metabolism via up-regulating several essential enzymes in carbohydrate metabolic pathways including two aconitate hydratases (spots 446, 502), an ATP synthase (spots 431), and a methylenetetrahydrofolate reductase (spot 495). Aconitate hydratase was involved in the tricarboxylic acid cycle (Wendel et al., 1988). ATP synthase, located in the chloroplast thylakoid membrane, is required for the Calvin cycle and crucial in energy supply for cellular reactions (Mccarty, 1992). Methylenetetrahydrofolate reductase (spot 495) participates in folate-mediated one-carbon metabolism in plants (Roje et al., 1999).

As to N metabolism, N deficiency up-regulated ketol acid reductoisomerase accumulation (spot 491) that preferentially synthesizes the branched chain amino acids valine, leucine, and isoleucine in plants (Bryan and Miflin, 1980; Durner et al., 1993). However, N deficiency decreased glutathione dehydrogenase accumulation (spot 159), indicating a negative effect on glutathione, ascorbate, and aldarate metabolism (Table 2) (Crook, 1941).

On the other hand, N oversupply prolonged the ear developmental programme via modifying C/N metabolism, including decreasing ATP synthase (spot 477) activity and down-regulating glutamate decarboxylase. Calmodulin-binding glutamate decarboxylase (GAD; spot 1240) regulates glutamate metabolism and has an essential role in controlling plant development. Inactivation of calmodulin binding of GAD shortens the tobacco stem due to elongation failure of cortex parenchyma cells (Baum et al., 1996). GAD activity in Arabidopsis leaves varies according to N sources, and GAD2 affects nitrogen metabolism by mediating gene expression or RNA stability (Turano and Fang, 1998). N oversupply also modified amino acid and vitamin synthesis via transketolase (spot 589) up-regulation. Transketolase participates in carbon dioxide fixation through the Calvin cycle, and is implicated in glycolysis via providing precursors for nucleotide, aromatic amino acid, and vitamin biosynthesis (Gerhardt et al., 2003).

Rather than opposite regulatory effects, both N deficiency and oversupply down-regulated the protein level of dienelactone hydrolase (Table 1), an enzyme that regulates N metabolism via its Cys–His–Asp catalytic triad (Cheah et al., 1993).

In conclusion, the present field experiments showed that N deficiency reduced both ear size and grain yield. At silking when the sink strength of the maize ear was pre-determined, a consistent decrease was found in N and dry matter accumulation in N-deficient ears. Further proteomic analysis revealed a general stress response triggered by N nutritional imbalance. Importantly, N deficiency or oversupply mediated ear growth via differential regulation of hormonal metabolism and functions, developmental programmes, and C/N metabolism in ears at silking.

Supplementary data
Supplementary data are available at JXB online.

**Figure S1.** The relative gene expression levels of OPR7 (A) and OPR8 (B) in maize ear samples with different nitrogen supplies.

**Table S1.** Fertilization practice at different maize developmental stages.

**Table S2.** The detailed Gene Ontology (GO) analysis of 47 proteins with significant differential accumulation.

Acknowledgements
We thank the State Key Basic Research and Development Plan of China (no. 2007CB109302), the National Natural Science Foundation of China (nos 30671237 and 31172016), and the Innovative Group Grant of the National Natural Science Foundation of China (no. 31121062) for financial support.

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