SCIENCE CHINA
Life Sciences

• RESEARCH PAPER •

November 2012 Vol.55 No.11: 984–992
doi: 10.1007/s11427-012-4390-z

Protein differential expression in the elongating cotton (*Gossypium hirsutum* L.) fiber under nitrogen stress

WANG YouHua†, ZHENG Mi†, GAO XiangBin & ZHOU ZhiGuo*

College of Agriculture, Nanjing Agricultural University, Nanjing 210095, China

Received July 29, 2012; accepted September 1, 2012

Nitrogen (N) is an essential macronutrient and an important factor limiting agricultural productivity. N deficient or excess conditions often occur during the cotton growth season and incorrect N application may affect cotton fiber yield and quality. Here, the influence of N stress on the cotton fiber proteome was investigated by two-dimensional gel electrophoresis and mass spectrometry. The results indicated that N application rate affects nitrogen accumulation in fiber cells and fiber length. The proteins differentially expressed during N stress were mainly related to plant carbohydrate metabolism, cell wall component synthesis and transportation, protein/amino acid metabolism, antioxidation and hormone metabolism. The most abundant proteins were C metabolism-related. Ten days post anthesis is a critical time for fiber cells to perceive environmental stress and most proteins were suppressed in both N deficient and N excess conditions at this sampling stage. However, several N metabolism proteins were increased to enhance N stress tolerance. Excess N may suppress carbohydrate/energy metabolism in early fiber development much like N deficiency. These results have identified some interesting proteins that can be further analyzed to elucidate the molecular mechanisms of N tolerance.

cotton, fiber, protein, nitrogen, stress

Citation: Wang Y H, Zheng M, Gao X B, *et al*. Protein differential expression in the elongating cotton (*Gossypium hirsutum* L.) fiber under nitrogen stress. Sci China Life Sci, 2012, 55: 984–992, doi: 10.1007/s11427-012-4390-z

Upland cotton (*Gossypium hirsutum* L.) is an important economic plant and its development is significantly influenced by genetics, environmental conditions and management practices [1,2]. Generally, nitrogen (N) is the most important inorganic nutrient for cotton plants [3]. Under- or over-fertilization of cotton results in loss of lint yield, delayed crop maturity or impaired cotton fiber quality [4]. Therefore, efforts to investigate the N response in upland cotton are of fundamental importance for cotton production.

Although the number of studies on the influence of N on cotton genes is limited, large-scale studies have been carried out in Arabidopsis [5–7]. The N-responsive genes identified were mainly related to glycolysis, amino acid biosynthetic breakdown, energy production, metabolism, gluconeogenesis and hormone metabolism. However, there are limitations encountered at the DNA/RNA level mostly due to post-translational modifications and the large differences between mRNA and protein turnover [8]. Proteomic analysis has become an efficient approach to study the molecular tolerance mechanisms of plants to N stress. Studies have been conducted on tolerance to N deficiency/excess in wheat [8–10], the Chlorophyceae [11], rice [12] and maize [13]. Such research should provide an understanding of the regulation mechanisms of N-responsive proteins, such as the proteins that involved in fatty acid biosynthesis, central metabolism, signal transduction and their dynamic changes in response to N stress in the research of Zhao *et al.* [14]. There have been no similar studies conducted on cotton fiber development. Fortunately, the elongating cotton fiber

†Contributed equally to this work
*Corresponding author (email: giscott@njau.edu.cn)

proteome has been successfully examined and the fiber growth mechanisms illustrated in detail, providing some insight into the N tolerance of cotton fibers [15–18].

Here, we studied the temporal changes of the cotton fiber proteome under N stress (both deficiency and excess) with NuCOTN 33B as the plant material. The objectives of this study were to screen for proteins that were differentially regulated and explore the molecular basis of the cotton fiber response to N stress.

1 Materials and methods

1.1 Plant materials, growth, nitrogen treatment and sampling

Pot experiments were conducted in the summer of 2009 at the experimental station of Nanjing Agricultural University in Nanjing (N32°02′ and E118°50′), Jiangsu Province, China. Cotton seeds (cv. NuCOTN 33B) were sowed on 25 April 2009. When the seedlings had three true leaves, individual healthy, uniform plants were transplanted into plastic pots, 55 cm high and 60 cm wide, filled with 40 kg of soil. The yellow-brown soil (Dystrudept) was collected from the 0–30 cm topsoil layer at the experimental station. The soil profile at a depth of 20 cm of the plastic pots was 17.8 g kg^{-1} organic matter, 0.9 g kg^{-1} total N, 74.6 mg kg^{-1} available N, 37.1 mg kg^{-1} available P, and 214.0 mg kg^{-1} available K.

The three N treatment levels were 0 kg hm^{-2} (N0), 240 kg hm^{-2} (N1), 480 kg hm^{-2} (N2), equivalent to 0, 4.5, 9.0 g per pot, respectively (hereafter these concentrations are noted as N0, N1 and N2). N1 represents normal nitrogen application, N0 nitrogen starvation and N2 nitrogen excess. N fertilization was split into two equal applications of urea, once before transplanting and again at the early flowering stage, for each treatment.

Cotton flowers were labeled at anthesis, and the bolls were collected from the first node positions on the 6–8 sympodial branches. Cotton boll samples were collected at 9:00–11:00 a.m. for proteome analysis. The fibers were excised from the bolls with a scalpel and were immediately put into liquid nitrogen and stored at −80°C prior to protein extraction. To ensure that the differences detected in the proteomes were independent of the environment, two completely independent sets of plants were included in this proteome study.

1.2 Determination of plant N accumulation and fiber length

N content was measured in samples of each boll and fiber using the Kjeldahl method [19]. Fibers were straightened in a water stream and the average fiber length was measured [20]. Eight to 10 ovules were measured per treatment.

1.3 Protein extraction from fiber tissue, two-dimensional polyacrylamide gel electrophoresis, and image/data analysis

A total of 18 independent samples were extracted, two samples at each developmental stage, e.g., 10, 15, 20 days post anthesis (DPA) for each treatment (N0, N1, N2). Cotton fiber samples were subjected to phenol extraction followed by methanolic ammonium acetate precipitation, according to the method described by Yao *et al.* [17]. The protein concentration in each sample was determined by the Bradford method using BSA as the standard. We rehydrated IPG strips (17 cm, pH 3–10, NL; Amersham Bioscience, Uppsala, Sweden) with 80 μg proteins (for silver staining) and the stained gels were scanned and analyzed using the PDQuest software (v7.1, Bio-Rad). Only spots of high quality were chosen for further analysis. Spot quantity normalization was conducted in the 'total quantity of valid spots' mode and spot analysis was performed using two standards: the Student's *t*-test mode with a significance of 95% ($P<0.05$), and a change of at least 1.5-fold in abundance of at least one of the developmental stages during the nitrogen treatment. The correlation coefficient estimate was analyzed using JMP 5.1 (SAS Institute).

1.4 In-gel digestion, protein identification, database search and expression clustering

We cut the matching silver-stained protein spots out of the 2DE gels, digested the protein with trypsin (Promega, Madison, WI, USA) and extracted the peptide mixtures. MALDI-TOF mass spectrometry and tandem TOF/TOF mass spectrometry were carried out on a time-of-flight Ultraflex II mass spectrometer (Bruker Daltonics, Bremen, Germany). Peptide mass maps were acquired in the positive reflection mode, averaging 800 laser shots per MALDI-TOF spectrum and 800 shots per TOF/TOF spectrum; the resolution was 15000–20000. The Bruker calibration mixtures were used to calibrate the spectrum to a mass tolerance within 0.1 Da. Each acquired mass spectrum (m/z range of 700–4000) was processed using the software FlexAnalysis v2.4 (Bruker Daltonics). The peak detection algorithm was SNAP (Sort Neaten Assign and Place); S/N threshold: 3; Quality Factor Threshold: 50. The trypsic auto-digestion ion picks (trypsin, MH+842.509, trypsin, MH+2211.104) were used as internal standards. Matrix and/or auto-proteolytic trypsin fragments, or known contaminant ions, such as keratins, were excluded. The resulting peptide mass lists were used to search the NCBInr database with Mascot (v2.1.03) in the automated mode. The following search parameters were used: significant protein MOWSE score at $P<0.05$, a minimum mass accuracy of 100 ppm, trypsin as enzyme, 1 missed cleavage site allowed, alkylation of cysteine by carbamidomethylation as the fixed modification and oxidation of methionine as the variable modification,

similarity of pI and the specified molecular mass, and a minimum sequence coverage of 15%.

SOTA (self-organizing tree algorithm) clustering was performed on the log-transformed *n*-fold induction expression values across three time points using the MultiExperiment Viewer software (The Institute for Genomic Research). The details of this method are described in Bhushan *et al.* [21]. The data were recorded in terms of *n*-fold expression with respect to the control expression value. The data sets were then log-transformed to base 2 to level the scale of expression and to reduce noise. The clustering was done with Pearson correlation as distance, 10 cycles and a maximum cell diversity of 0.8.

2 Results

2.1 Changes in fiber length

To evaluate the effect of N on fiber development, the N accumulation of bolls and fibers and fiber length in the corresponding bolls were analyzed under the different N treatments. The data showed that N accumulation in the boll and fiber at 10 and 15 DPA was not significantly different among the three treatments, but there was a difference at 20 DPA ($P<0.05$) (Figure 1). Boll nitrogen accumulation was greater in N1 (58.6 mg, $P<0.05$) than in N0 (53.5 mg) and N2 (55.0 mg), and similarly, fiber length in N1 was higher ($P<0.05$) than in either N0 or N1 (Figure 1). This suggested that appropriate N accumulation in the boll and fiber would benefit fiber length development, and greater or lesser N accumulation would have an adverse effect.

2.2 Proteomic analysis

Temporal changes (10, 15, 20 DPA) in the cotton fiber proteome were visualized by 2-DE. Triplicate gels were run for each sample with two biological replicates. The gels that showed high reproducibility were used for further analysis. A total of ~560 protein spots across pH values 3–10 were repeatedly detected in the silver-stained gels (Figure 2). Most proteins migrated to pI values between 3.5 and 9.5, ranging from 12–105 kD in *Mr*. The spot-to-spot correlation coefficients between gels in the same treatment varied from 0.69 to 0.92, with all being significant at $P<0.0001$. Spot abundance in the three replicates was not significantly different.

We found 67 proteins that showed more than 1.5-fold differences in at least one test period in response to N variation by MS/MS analysis. The expression patterns of the proteins changed as fiber development progressed (Table 1, Figure 4). In response to N increase, some proteins, such as spots 5303 and 8319, first increased (10 DPA) and then decreased, while some proteins, such as spots 6702 and 8707, decreased steadily.

2.3 Identification/functional classification of the differentially expressed proteins

The N-responsive proteins were digested with trypsin and the peptides were analyzed using MALDI-TOF/TOF MS coupled with database searching. In total, 67 proteins were successfully identified (Table 3), 45 of which had been reported in upland cotton. Functional categories were assigned to all identified proteins with the Gene Ontology tool (http://www.geneontology.org). They were classified into seven functional categories including carbohydrate/energy metabolism (16 spots), protein metabolism (9 spots), cell wall modification (7 spots), amino acid metabolism (4 spots), cytoskeleton formation (13 spots), cell response/signaling (7 spots), redox homeostasis (8 spots) and hormone metabolism (3 spots) (Figure 3).

2.4 Expression profile of N-responsive proteins

To achieve a comprehensive overview of the expression profiles in terms of protein function, cluster analysis was conducted for the 67 proteins. As shown in Figure 4, at 10

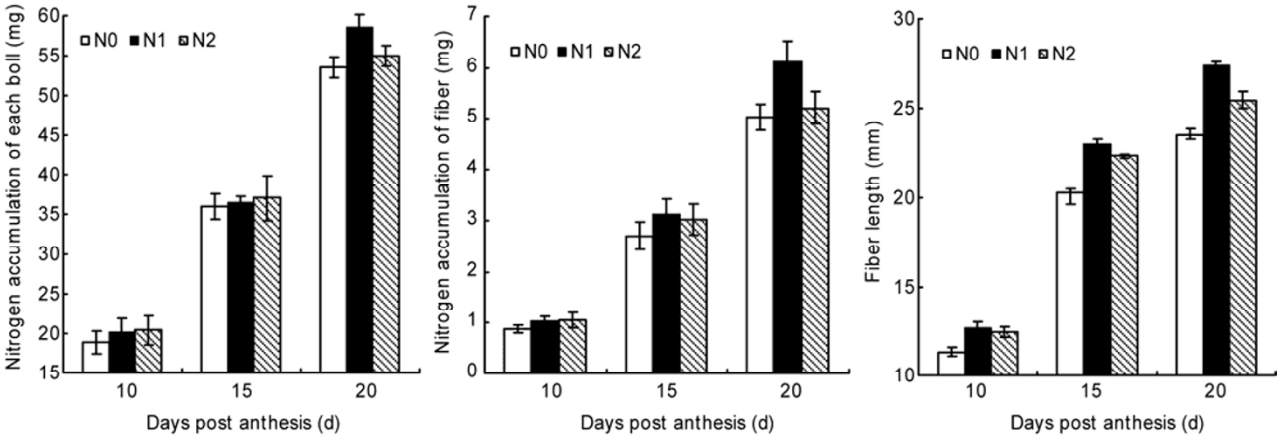

Figure 1 Changes in fiber length and nitrogen accumulation in cotton bolls and fibers. N0, no N fertilizer applied; N1, 240 kg N hm^{-2} applied; N2, 480 kg N hm^{-2} applied.

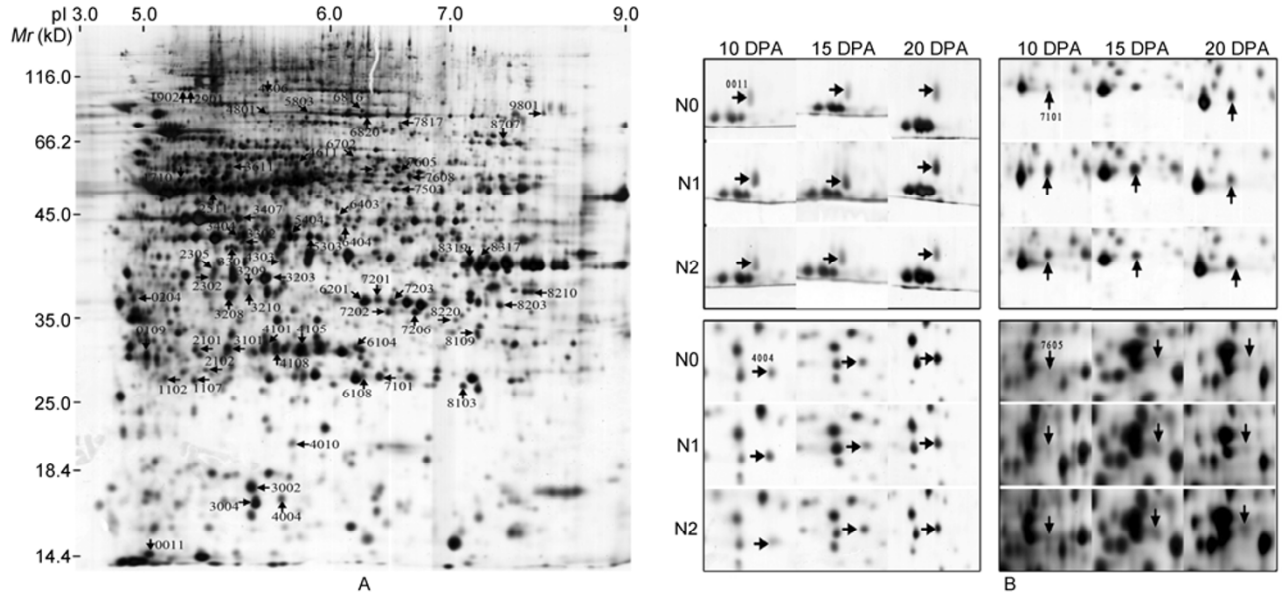

Figure 2 Protein 2-DE analysis under nitrogen deficient (N0), excess (N2) and control (N1) conditions. In IEF, 80 μg of proteins was loaded onto pH 3–10 IPG strips (17 cm, nonlinear), SDS-PAGE was performed with 10.8% gels. The spots were visualized by silver straining. Quantitative image analysis revealed 67 spots that changed in abundance significantly (P<0.05) in at least one sampling time. A, Representative gel of total cotton fiber proteins, arrows indicate the 67 proteins that were differentially expressed between the different N application treatments. B, Selected differentially expressed proteins between N0, N1 and N2 at 10, 15 and 20 DPA.

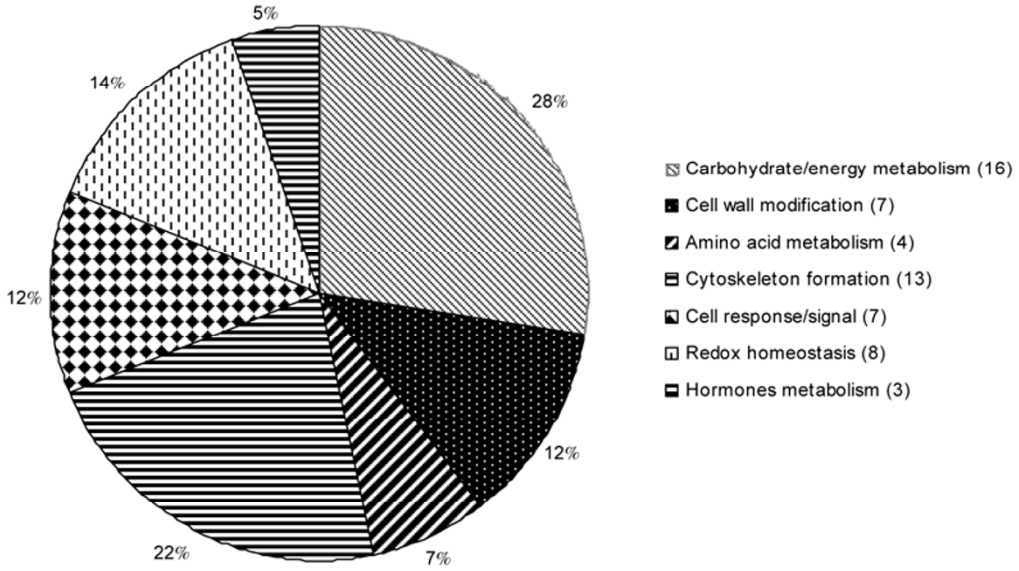

Figure 3 Functional classification of the 67 identified differentially expressed proteins.

and 15 DPA most of the differentially expressed proteins were down-regulated under both N0 and N2, although more proteins were down-regulated in N0 than in N2.

Under N0 conditions, proteins involved in protein/amino acid metabolism or carbohydrate/energy metabolism generally decreased sharply at 10 and 15 DPA. Proteins related to cytoskeleton formation generally decreased slightly at 10 and 15 DPA and then returned to control levels at 20 DPA. Proteins related to cell responses/signaling generally decreased at 10 DPA and then increased at 20 DPA. These results indicate that proteins related to protein/amino acid metabolism, carbohydrate/energy metabolism and cytoskeleton formation were adversely affected by N deficiency at the early stages of fiber development (Table 1).

Under N2 conditions, proteins involved in cytoskeleton formation generally decreased at 10 DPA, returned to control levels at 15 DPA and increased at 20 DPA. Proteins related to carbohydrate/energy generally remained at control levels at all three sampling times. Proteins involved in protein/amino acid metabolism generally stayed at control lev-

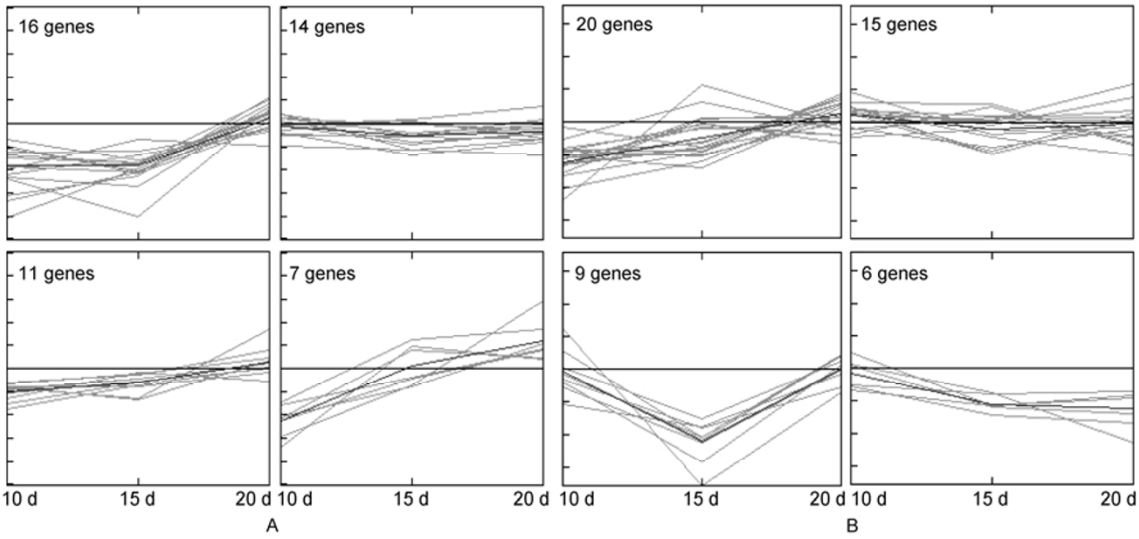

Figure 4 Trend clustering of the differentially expressed proteins in cotton. A, Protein changes in fiber cells under N0 conditions (0 kg N hm^{-2}). B, Protein changes in fiber cells under N2 conditions (480 kg N hm^{-2}).

els at 10 DPA, decreased at 15 DPA and then returned to control levels at 20 DPA. Proteins involved in redox homeostasis generally stayed at control levels at 10 DPA and decreased at both 15 and 20 DPA (Table 1).

3 Discussion

Our results showed that nitrogen deficiency (N0) or excess (N2) leads to changes in nitrogen accumulation in cotton bolls and fibers. N0 and N2 also severely affected the fiber yield (data not shown) and quality. Similar observations were made in cotton plants by Hearn and Constable [22] and it has been observed that nutrient stress decreases lint yield, particularly of late-season fruit (bolls), and may decrease fiber length, strength and micronaire [3]. Over-fertilization also reduces fiber quality [23].

Using comparative proteomics, we successfully identified 67 proteins responsive to N levels. Clustering data showed that N0 and N2 had different effects on protein expression. Although some proteins showed the same patterns of change, there were still differences between N0 and N2, suggesting that cotton fibers may take different but effective measures to deal with N deficiency or excess during fiber development. The differentially expressed proteins we identified in cotton fibers revealed a complex network underlying nitrogen responses, showing that plants exhibit sophisticated adaptive mechanisms to cope with variations in soil N availability.

3.1 Signal transduction-related proteins

The proteins or components involved in signal transduction that were differentially expressed in this study were a glycine-rich RNA-binding protein (GRPs, s4004), protein phosphatase pp1 regulatory subunit, a putative protein (s3210), calmodulin (s0011), two 14-3-3 proteins (s4010, s9801), MYB10 (s6104), and a guanine nucleotide-binding protein subunit beta-like protein (s8203). GRPs have been reported to bind cell wall-associated kinases to form a signalosome complex for environmental stress responses [24]. 14-3-3 proteins are phosphoserine-binding proteins that regulate the activities of a wide array of targets and play an important role in responses to biotic and abiotic stresses [25]. In addition, 14-3-3 proteins act hand-in-glove to control diverse target proteins in C:N metabolism [26]. MYB proteins generally serve as transcriptional factors [27] and MYB-like gene expression was found to be increased under N deprivation in *Arabidopsis* [28]. G-proteins have been shown to participate in signal transduction pathways and are involved in responses to various environmental stresses in plants [29]. Calmodulin serves as an intracellular Ca^{2+}-receptor and mediates the Ca^{2+} regulation of cyclic nucleotides and Ca^{2+} transport [30]. In both N deficient (N0) and N excess (N2) conditions, these signal transduction-related proteins or protein subunits were all decreased at 10 DPA. However, at 15 or 20 DPA the effects of N stress on these proteins differed. These results indicate that 10 DPA is a critical time for fiber cells to perceive environmental stress and many major signaling proteins are likely to be decreased in both N deficient or N excess conditions.

3.2 C/N cycling-related proteins

In a leaf proteomics study by Bahrman *et al.* [9], it was reported that most protein spots identified under nitrogen deficiency were involved in carbon metabolism. We found

Table 1 Differentially expressed proteins identified by PMF or MS/MS[a)]

Spots No.	Accession No.	Protein name	Protein *n*-fold change[a]			PM[b]	SC (%)[c]	Theoretical mass (kD) /pI	Experimental mass (kD) /pI	Species
			10 DPA N0/N2	15 DPA N0/N2	20 DPA N0/N2					
Carbohydrate/energy metabolism										
1206	CAD31714	fructokinase-like protein	0.63*/0.87	0.40*/1.39*	1.60*/1.70*	10	31	26.2/5.03	35.9/4.92	*Cicer arietinum*
6404	ACJ11715	fructokinase	0.90/1.36	0.98/1.16	1.12/1.56*	34	71	35.2/5.28	43.9/6.60	
2101	ACJ11723	Triosephosphate isomerase	0.64*/0.12	0.82*/0.60	1.76*/1.70*	17	50	27.4/6.00	36.5/4.27	*Gossypium hirsutum*
5303	BAA33802	cytosolic phosphoglycerate kinase 1	1.88*/1.32	0.65*/0.55*	0.34*/0.45*	17	40	42.8/5.70	43.0/5.85	*Gossypium hirsutum*
6702	ACJ11747	pyruvate decarboxylase	0.25*/0.86*	0.52*/0.95	0.61*/0.55	25	48	60.7/6.18	744/6.17	*Gossypium hirsutum*
7605	ACJ11739	dihydrolipoamide dehydrogenase, putative	0.55*/1.56*	0.40*/1.40*	1.23*/2.22*	36	62	41.2/6.93	70.7/641	*Gossypium hirsutum*
6610	ACJ11739	dihydrolipoamide dehydrogenase, putative	0.47*/0.86	0.69*/0.95	1.88*/0.78*	17	36	41.2/6.93	67.9/656	*Gossypium hirsutum*
8319	ACJ11728	glyceraldehyde-3-phosphate dehydrogenase	1.30*/1.51*	0.45*/0.47*	0.62*/0.59*	36	81	37.0/7.06	40.0/7.35	*Gossypium hirsutum*
8707	ABY25305	pyrophosphate-dependent phosphofructokinase alpha subunit	0.21*/0.73*	0.40*/0.62*	0.78*/0.63*	18	29	68.0/6.71	81.8/8.19	
0204	AAK73692	succinate dehydrogenase subunit 3	0.85*/0.74*	0.25/0.95	1.11/0.77*	25	63	22.3/10.00	34.4/3.31	*Gossypium hirsutum*
4801	EEF30445	NADH-ubiquinone oxidoreductase, putative	0.50*/1.16	0.22*/0.21*	0.92/2.30*	27	32	81.6/6.56	94.9/5.71	*Ricinus communis*
8220	O23948	V-type proton ATPase subunit E	1.36*/2.00*	2.50*/2.44*	2.17*/2.26*	16	49	27.2/6.50	32.5/7.10	*Gossypium hirsutum*
7503	Q01915	ATP synthase subunit alpha,mitochondrial	0.57*/0.40*	0.57*/1.04	0.88*/1.25	25	51	55.5/6.23	54.5/6.76	*Glycine max*
1710	P29685	ATP synthase subunit beta, mitochondrial	1.12/1.00	0.68*/0.56*	1.58*/1.59*	17	36	60.3/5.95	56.4/5.29	*Hevea brasiliensis*
1107	YP_514682	ATP synthase F0 subunit 1	0.65*/0.89	0.60*/0.56*	1.31/1.21	–	17	55.5/5.85	25.7/5.40	*Oryza sativa Indica Group*
1902	AAF23260	Transitional endoplasmic reticulum ATPase	1.65*/1.36	0.45*/0.38*	0.78/1.26	19	31	90.1/5.13	109.4 5.36	*Arabidopsis thaliana*
Protein metabolism										
8210	ACG33889	DNA-binding protein	0.23*/0.35*	0.60*/0.47*	1.69*/1.56	12	30	41.9/6.07	35.2/8.67	*Zea mays*
1215	YP_538924	ribosomal protein S2	1.97*/0.99	0.59*/0.36*	1.76*/0.70*	16	38	26.9/9.80	32.0/3.75	*Gossypium hirsutum*
6108	YP_538957	ribosomal protein S18	0.26*/1.43*	0.30*/0.28*	1.29*0.66*	21	86	12.0/11.96	25.3/6.30	*Gossypium hirsutum*
0109	ACI15344	NAC domain protein NAC3	0.69*/1.12	0.70*/0.67	1.14/0.38*	14	38	34.1/6.54	28.0/3.89	*Gossypium hirsutum*
7101	ABO41851	putative protein disulfide isomerase	0.28*/0.83	0.30*/0.69*	1.37*/1.59*	34	68	55.9/4.95	57.3/5.03	*Gossypium hirsutum*
2102	XP_002516232	groes chaperonin, putative	0.34*/0.70	0.29*/0.16*	1.88*/1.21	–	38	26.5/8.89	26.1/5.14	*Ricinus communis*
3004	ABW89469	low molecular weight heat shock protein	1.48*/1.46*	1.65*/1.76*	1.73*/1.02	–	40	175.0/6.62	29.4/5.45	*Gossypium hirsutum*
1102	EEF42124	proteasome subunit beta type, putative	0.92/0.63*	1.43/1.38	2.32/1.40	7	25	23.1/5.17	25.1/5.12	*Ricinus communis*
3101	EEF48777	proteasome subunit alpha type, putative	0.94/0.54*	0.53*/0.46*	0.78*/1.74*				31.3/5.51	*Ricinus communis*
Cell wall modification										
6816	BAA88905	sucrose synthase	0.54*/0.76*	0.59*/0.50*	1.52*/1.29	22	20	92.6/5.91	117.5/6.40	*Citrus unshiu*
2511	ACJ11711	UDP-D-glucose pyrophosphorylase	0.96/0.80	1.09/1.51*	0.64*/0.60*	–	57	51.4/5.62	61.4/5.16	*Gossypium hirsutum*
4101	ACJ11713	UDP-L-rhamnose synthase	0.53*/1.22*	0.74/0.56*	1.38/1.12	21	46	33.9/5.73	30.1/5.52	*Gossypium hirsutum*
6820	XP_002525709	5-methyltetrahydropteroyltriglutamate-Homocysteine methyltransferase, putative	1.78*2.22*	1.23/1.34*	0.50*/0.67*	–	18	84.8/6.09	100.4/6.35	*Ricinus communis*
6201	ACJ14023	S-adenosylmethionine synthetase	0.75*/0.96	0.69*/0.96*	0.59*/0.75*	3	75	70.5/9.46	34.0/6.36	*Helianthus annuus*
3407	AAA32868	S-adenosylmethionine synthetase	0.55*/0.45*	0.35/0.45*	1.56*/1.32	–	2	43.6/5.50	45.3/5.55	*Arabidopsis thaliana*
3301	ACJ11716	adenosine kinase	0.91/1.85	0.37*/0.44*	1.88*/1.68	30	46	37.8/5.47	42.7/5.27	*Gossypium hirsutum*

(*To be continued on the next page*)

(*Continued*)

Spots No.	Accession No.	Protein name	Protein *n*-Fold change[a] 10 DPA N0/N2	15 DPA N0/N2	20 DPA N0/N2	PM[b]	SC (%)[c]	Theoretical mass (kD) /pI	Experimental mass (kD) /pI	Species
Amino acid metabolism										
2305	ACJ11722	2-nitropropane dioxygenase	0.59*/0.54	0.32*/1.12	1.74*/0.39*	29	38	36.1/5.32	38.9/5.23	*Gossypium hirsutum*
3404	ACJ11724	glutamine synthase	1.05/1.21	0.57*/1.36	0.73*/1.34*	12	35	39.1/5.77	44.5/5.33	*Gossypium hirsutum*
4806	AAG52429	putative aminopeptidase	2.18*/0.56*	0.28*/0.20	0.40*/0.55*	–	–	108.1//5.97	100.8/5.64	*Arabidopsis thaliana*
3611	XP_479171	alanine aminotransferase	1.23/1.56*	1.01/1.23	0.86/1.22	15	58	59.51 /5.95	59.5/5.51	*Oryza sativa*
Cytoskeleton formation										
3208	AAP73452	actin	1.34*/2.19	0.69*/1.12	0.88*/1.30	21	54	41.9/5.44	33.8/5.30	*Gossypium hirsutum*
3103	AA08622	actin	0.53*/0.82	0.74*/0.92	1.18/0.92	11	25	41.9/5.32	33.6/5/18	*Phalaenopsis hybrid cultivar*
7608	AAP73454	actin	0.29*/1.74*	0.60*/1.14	1.05/1.03	13	42	41.9/5.23	67.2/6.61	*Gossypium hirsutum*
4308	ABD66506	actin depolymerizing factor 4	0.10*/0.43*	0.23*/0.68*	1.19/1.02	21	51	16.6/6.84	37.6/5.65	*Gossypium hirsutum*
8317	AAD50629	alpha-tubulin	1.72*/2.41*	1.11/1.95*	1.51*/0.98	12	58	14.1/4.49	40.1/7.83	*Gossypium hirsutum*
3209	AAP83637	katanin	0.46*/0.62*	0.42*/0.63*	1.64/1.98	29	47	57.3/7.60	54.3/7.90	*Gossypium hirsutum*
7202	AAR13288	Anx1	0.20*/1.42*	0.35*/1.33*	1.66*/0.47*	17	53	36.1/6.19	33.2/6.54	*Gossypium hirsutum*
7206	AAB67994	annexin	0.39*/0.79*	0.36*/0.86*	1.54*/1.66*	37	80	35.9/6.38	33.4/6.64	*Gossypium hirsutum*
5803	AAC33305	fiber annexin	0.51*/0.98	0.45*/0.40*	0.39*/1.26	27	66	36.2/6.34	97.1/5.85	*Gossypium hirsutum*
7201	AAC33305	fiber annexin	1.34*/2.22*	0.55*/0.70*	0.68*/1.03	32	80	36.2/6.34	34.4/6.46	*Gossypium hirsutum*
7203	AAC33305	fiber annexin	1.68*/2.24*	0.53/0.61*	0.91/1.10	29	76	36.2/6.34	34.0/6.56	*Gossypium hirsutum*
3302	AAD48019	Rab GTP-binding protein Rab11b	0.57*/0.48*	0.40*/0.64*	1.25*/1.62*	39	71	24.8/5.13	42.0/5.31	*Gossypium hirsutum*
2901	P54774	Cell division cycle protein 48 homologue	1.51*/0.78	0.34*/0.31*	0.54*/1.48	33	51	89.8/5.18	109.4/5.38	*Glycine max*
Cell response/signal										
4004	ACJ11730	glycine-rich RNA-binding protein	0.22*/0.21	1.74*/1.97	1.38*/1.06	35	100	17.0/7.82	15.5/5.61	*Gossypium hirsutum*
3210	XP_002523203	protein phosphatases pp1 regulatory subunit, putative	0.73*/0.84	0.69*/1.21	0.63*/0.96	–	18	17.4/6.66	35.3/5.41	*Ricinus communis*
0011	ES831951	calmodulin	0.56*/0.78*	0.48*/0.56*	0.78/0.56*	30	86	16.5/4.64	16.1/5.28	*Gossypium hirsutum*
4010	ABY65001	14-3-3b protein	0.32*/.045*	0.78/0.33*	1.32/2.19*	18	79	30.3/4.72	21.5/5.94	*Gossypium hirsutum*
9801	ABY65002	14-3-3c protein	0.43*/0.54	0.98*/0.97*	0.62*/1.67*	17	65	29.6/4.79	86.4/0.00	*Gossypium hirsutum*
6104	ABR01222	MYB10	0.38*/0.69*	0.31*/0.35*	1.29*/0.83	21	65	32.1/9.00	30.0/6.31	*Gossypium hirsutum*
8203	O24076	Guanine nucleotide-binding protein subunit beta-like protein	0.39*/0.66*	1.63*/1.21*	1.30*/1.92*	15	40	36.0/7.07	34.9/8.02	*Medicago sativa*
Redox homeostasis										
3002	ACJ11720	peroxiredoxin	0.35*/0.75*	0.65*/0.91*	1.20*/0.74*	–	92	17.3/5.58	15.1/5.46	*Gossypium hirsutum*
8103	gi|3219353	manganese superoxide dismutase	0.52*/0.64*	0.50*/0.71*	1.49*/1.39*	12	57	22.1/8.54		*Gossypium hirsutum*
4108	ABR18607	cytosolic ascorbate peroxidase 1	0.67*/1.38*	0.40*/0.54*	1.74*/1.52*	13	53	27.7/5.93	29.1/5.61	*Gossypium hirsutum*
4105	AB2402321	benzoquinone reductase	0.41*/1.13	0.43*/0.45*	0.86/0.51*	11	58	21.6/6.09	30.7/5.78	*Gossypium hirsutum*
4101	AAF29773	glutathione S-transferase	0.52*/1.72*	0.74*/0.56*	1.38/1.12	11	35	32.1/8.45	30.2/8.20	*Gossypium hirsutum*
5404	DT546041	glutathione reductase	0.72*/1.45*	1.12/1.46*	0.66*/0.54*	21	70	53.8/5.75	42.9/5.75	*Gossypium hirsutum*
6403	AAF31416	aldehyde dehydrogenase	0.63*/1.13	0.73*/1.67*	0.86*/1.15	16	100	81.5/10.17	51/4/6.12	*Gossypium hirsutum*
2305	ACJ11722	2-nitropropane dioxygenase	0.54*/1.52*	0.36*/0.82	1.49*/1.98*	29	38	36.2/5.32	39.7/5.48	*Gossypium hirsutum*
Hormones metabolism										
8109	AAW80969	gibberellin 20-oxidase	0.28*/0.25*	0.24*/0.25*	0.82*/0.33*	16	52	43.7/6.67	31.0/8.30	*Gossypium hirsutum*
7817	ABA01488	tryptophan decarboxylase	0.30*/0.41*	0.75/0.70	1.52*/1.43*	26	63	41.1/6.17	79.5/6.25	*Gossypium hirsutum*
4611	ACM79256	cytokinin oxidase/dehydrogenase	0.33*/0.52*	0.76*/0.42*	0.70*/0.67*	32	63	59.7/8.63	60.9/5.77	*Gossypium hirsutum*

a) a, Increase or decrease in the stress treated plants (N0/N2) compared with the controls (N1); b, number of mass values matched; c, sequence coverage; *, treatment effect was significant at $P<0.05$.

similar results; many of the proteins whose expression changed under N stress were involved in C metabolism.

The cotton boll is a typical sink organ where glycolysis and the tricarboxylic acid (TCA) cycle not only provide energy but also produce C skeletons for morphogenesis. In this study, nine proteins involved in glycolysis were differentially expressed under N stress, fructokinase (s1206, s6404), triosephosphate isomerase (s2101), cytosolic phosphoglycerate kinase 1 (s5303), pyruvate decarboxylase (s6702), dihydrolipoamide dehydrogenase, two putative proteins (s6610, s7605), glyceraldehyde-3-phosphate dehydrogenase (s8319), and a pyrophosphate-dependent phosphofructokinase alpha subunit (s8707). Two proteins related to the TCA cycle were differentially expressed, succinate dehydrogenase subunit 3 (s0204) and a putative NADH-ubiquinone oxidoreductase (4801). There were also five proteins identified as ATP synthase subunits (s8220, s7503, s1710, s1107 and s1902), which are involved in energy metabolism.

In this study, most of the identified C or energy metabolism related proteins were down-regulated at 10 and 15 DPA under N0 (Table 1), suggesting that carbohydrate metabolism was suffocated by N deficiency. In N2, these proteins were also down-regulated at 10 DPA, suggesting that N excess also decreased carbohydrates/energy metabolism in early fiber development.

We only identified three differentially expressed proteins involved in amino acid metabolism. They were glutamine synthase (GS, s3404), a putative aminopeptidase (s4806) and alanine aminotransferase (AlaAT, s3611). AlaAT is involved in both C and N metabolism in plants [31], and converts pyruvate and glutamate to alanine and 2-oxoglutarate. Our data showed that AlaAT-like protein (s3611) was up-regulated under N stress, and similar results were observed in *Panicum miliaceum* [32] and maize [33]. GS catalyzes the incorporation of ammonium into glutamine and over-expression of GS increases photosynthetic productivity and growth under N deficiency [34]. Thus, the up-regulation of GS and AlaAT may enhance the N stress tolerance of the cotton fiber.

An aminopeptidase-like protein (s4804) appeared to be decreased under N stress. Aminopeptidases are able to cleave peptides at the N-terminus. The first and best studied plant aminopeptidase is leucine aminopeptidase, which can play a regulatory role in responses to environmental stress [35]. Thus, the down-regulation of the aminopeptidase-like protein suggests that the plant's environmental response ability may be reduced in N stress.

3.3 Hormone homeostasis-related proteins

Hormones play important roles in many plant physiological processes including the control of nutrient homeostasis, for example, auxin can increase the accumulation of potassium transporters under potassium-limited conditions [36]. Three hormone metabolism-related proteins were identified in this research, cytokinin oxidase/dehydrogenase (CKO/CKX, s4611), tryptophan decarboxylase (TDC, s7817) and gibberellin 20-oxidase (s8109). GA 20-oxidase promotes active GA biosynthesis in cotton fibers and the up-regulation of the *GhGA20ox1* gene promotes fiber initiation and elongation [37]. TDC can convert tryptophan into tryptamine, channeling primary metabolites into IAA biosynthesis. CKO/CKX is a flavoenzyme, which irreversibly inactivates cytokinins by severing the isoprenoid side chain from the adenine/adenosine moiety. In both N0 and N2, these three proteins were decreased at 10 and 15 DPA, suggesting that the hormone balance may be perturbed in either N deficient or excess conditions.

4 Conclusion

Both N deficiency and N excess can reduce nitrogen accumulation in fiber cells and decrease fiber length. The proteins differentially expressed in N deficiency or excess were mainly related to plant carbohydrate metabolism, cell wall component synthesis and transportation, protein/amino acid metabolism, antioxidation, and hormone metabolism. The most abundant proteins were C metabolism-related. Ten DPA is a critical time for fiber cells to perceive environmental stress and most proteins were decreased in both N deficient and N excess conditions at this sampling stage. Under N stress, the expression of several N metabolism proteins was increased to enhance N stress tolerance, but the plant's environmental response ability may be reduced and the hormone balance may be perturbed. N excess decreased carbohydrate/energy metabolism in early fiber development much like N deficiency.

We are grateful to Prof. Liu JinYuan from Laboratory of Molecular Biology and MOE Laboratory of Protein Science, Department of Biological Sciences and Biotechnology, Tsinghua University, for his guidance in the protein extraction. This work was supported by the National Natural Science Foundation of China (Grant No. 30971735) and the Specialized Research Funds for the Doctoral Program of Higher Education (Grant No. 200803070017).

1 Ramey Jr H H. Stress influences on fiber development. In: Mauney J R, Stewart J M, eds. Cotton Physiology. Memphis: The Cotton Foundation, 1986. 351–359
2 Reddy K R, Koti S, Davidonis G H, *et al*. Interactive effects of carbon dioxide and nitrogen nutrition on cotton growth, development, yield, and fiber quality. Agron J, 2004, 96: 1148–1157
3 Read J J, Reddy K R, Jenkins J N. Yield and fiber quality of upland cotton as influenced by nitrogen and potassium nutrition. Eur J Agron, 2006, 24: 282–290
4 Rochester I, Peoples M, Constable G. Estimation of the N fertiliser requirement of cotton grown after legume crops. Field Crop Res, 2001, 70: 43–53
5 Wang R, Tischner R, Gutierrez R A, *et al*. Genomic analysis of the nitrate response using a nitrate reductase-null mutant of *Arabidopsis*. Plant Physiol, 2004, 136: 2512–2522

6 Wang R, Okamoto M, Xing X, *et al*. Microarray analysis of the nitrate response in *Arabidopsis* roots and shoots reveals over 1000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. Plant Physiol, 2003, 132: 556–567

7 Scheible W R, Morcuende R, Czechowski T, *et al*. Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of Arabidopsis in response to nitrogen. Plant Physiol, 2004, 136: 2483–2499

8 Flaete N, Hollung K, Ruud L, *et al*. Combined nitrogen and sulphur fertilisation and its effect on wheat quality and protein composition measured by SE-FPLC and proteomics. J Cereal Sci, 2005, 41: 357–369

9 Bahrman N, Gouy A, Devienne-Barret F, *et al*. Differential change in root protein patterns of two wheat varieties under high and low nitrogen nutrition levels. Plant Sci, 2005, 168: 81–87

10 Bahrman N, Le Gouis J, Negroni L, *et al*. Differential protein expression assessed by two-dimensional gel electrophoresis for two wheat varieties grown at four nitrogen levels. Proteomics, 2004, 4: 709–719

11 Tran N P, Park J K, Lee C G. Proteomics analysis of proteins in green alga *Haematococcus lacustris* (Chlorophyceae) expressed under combined stress of nitrogen starvation and high irradiance. Enzyme Microb Technol, 2009, 45: 241–246

12 Konishi H, Ishiguro K, Komatsu S. A proteomics approach towards understanding blast fungus infection of rice grown under different levels of nitrogen fertilization. Proteomics, 2001, 1: 1162–1171

13 Dhugga K S, Waines J G, Leonard R T. Correlated induction of nitrate uptake and membrane polypeptides in corn roots. Plant Physiol, 1988, 87: 120–125

14 Zhao D H, Li J L, Qi J G. Identification of red and NIR spectral regions and vegetative indices for discrimination of cotton nitrogen stress and growth stage. Comput Electron Agric, 2005, 48: 155–169

15 Graves D A, Stewart J M D. Analysis of the protein constituency of developing cotton fibres. J Exp Bot, 1988, 39: 59–69

16 Turley R, Ferguson D. Changes of ovule proteins during early fiber development in a normal and a fiberless line of cotton (*Gossypium hirsutum* L.). J Plant Physiol, 1996, 149: 695–702

17 Yao Y, Yang Y W, Liu J Y. An efficient protein preparation for proteomic analysis of developing cotton fibers by 2-DE. Electrophoresis, 2006, 27: 4559–4569

18 Yang Y W, Bian S M, Yao Y, *et al*. Comparative proteomic analysis provides new insights into the fiber elongating process in cotton. J Proteome Res, 2008, 7: 4623–4637

19 Feil B, Moser S B, Jampatong S, *et al*. Mineral composition of the grain of tropical maize varieties as affected by pre-anthesis drought and rate of nitrogen fertilization. Crop Sci, 2005, 45: 516–523

20 Gipson J R, Ray L L. Fibre elongation rates in five varieties of cotton as influenced by night temperature. Crop Sci, 1969, 9: 339–341

21 Bhushan D, Pandey A, Choudhary M K, *et al*. Comparative proteomics analysis of differentially expressed proteins in chickpea extracellular matrix during dehydration stress. Mol Cell Proteomics, 2007, 6: 1868–1884

22 Hearn A, Constable G. Irrigation for crops in a sub-humid environment VII. Evaluation of irrigation strategies for cotton. Irrig Sci, 1984, 5: 75–94

23 Singh V, Nagwekar S. Effect of weed control and nitrogen levels on quality characters in cotton. J Indian Soc Cotton Improvement, 1989, 14: 60–64

24 Anderson C M, Wagner T A, Perret M, *et al*. WAKs: cell wall-associated kinases linking the cytoplasm to the extracellular matrix. Plant Mol Biol, 2001, 47: 197–206

25 Chen F, Li Q, Sun L, *et al*. The rice 14-3-3 gene family and its involvement in responses to biotic and abiotic stress. DNA Res, 2006, 13: 53–63

26 Cotelle V, Meek S E M, Provan F, *et al*. 14-3-3s regulate global cleavage of their diverse binding partners in sugar-starved *Arabidopsis* cells. EMBO J, 2000, 19: 2869–2876

27 Gonda T J. The c-Myb oncoprotein. Int J Biochem Cell B, 1998, 30: 547–551

28 Todd C D, Zeng P, Huete A M R, *et al*. Transcripts of MYB-like genes respond to phosphorous and nitrogen deprivation in *Arabidopsis*. Planta, 2004, 219: 1003–1009

29 Bowler C, Chua N H. Emerging themes of plant signal transduction. Plant Cell, 1994, 6: 1529–1541

30 Means A R, Dedman J R. Calmodulin—an intracellular calcium receptor. Nature, 1980, 285: 73–77

31 Miyashita Y, Dolferus R, Ismond K P, *et al*. Alanine aminotransferase catalyses the breakdown of alanine after hypoxia in *Arabidopsis thaliana*. Plant J, 2007, 49: 1108–1121

32 Son D, Jo J, Sugiyama T. Purification and characterization of alanine aminotransferase from *Panicum miliaceum* leaves. Arch Biochem Biophys, 1991, 289: 262–266

33 Muench D G, Christopher M E, Good A G. Cloning and expression of a hypoxic and nitrogen inducible maize alanine aminotransferase gene. Physiol Plant, 1998, 103: 503–512

34 Fuentes S I, Allen D J, Ortiz-Lopez A, *et al*. Over-expression of cytosolic glutamine synthetase increases photosynthesis and growth at low nitrogen concentrations. J Exp Bot, 2001, 52: 1071–1081

35 Chao W S, Gu Y Q, Pautot V, *et al*. Leucine aminopeptidase RNAs, proteins, and activities increase in response to water deficit, salinity, and the wound signals systemin, methyl jasmonate, and abscisic acid. Plant Physiol, 1999, 120: 979–992

36 Claussen M, Lüthe H, Blatt M, *et al*. Auxin-induced growth and its linkage to potassium channels. Planta, 1997, 201: 227–234

37 Xiao Y H, Li D M, Yin M H, *et al*. Gibberellin 20-oxidase promotes initiation and elongation of cotton fibers by regulating gibberellin synthesis. J Plant Physiol, 2010, 167: 829–837