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Differences in physiological traits and protein expression between glyphosate-tolerant glandless cotton and its wild type

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ABSTRACT

Physiological traits and proteomic expression were compared between *EPSPS*-G6 transgenic glyphosate-tolerant glandless upland cotton (GT) and its non-transgenic wild type (non-GT). GT showed higher net photosynthetic rate at boll setting stage (BSS) than non-GT, with higher stomatal conductance (g_s), intercellular CO_2 concentration (Ci) and transpiration rate (E) at the beginning of boll open stage (BBOS), but lower in Ci at BSS. Contents of chlorophyll a, chlorophyll b and chlorophyll a+b in GT were uniformly significantly less than those in non-GT at BSS and BBOS. Insertion of *EPSPS* gene had altered uptake of mineral nutrients: GT demonstrated less concentrations in N, Mg and K, but higher in P, Ca, Fe, K, Cu, Mn and Zn compared with non-GT. Contents of soluble sugar at BSS and BBOS and soluble protein at PFS and BSS in GT were significantly higher than non-GT; but GT had lower soluble sugar content at PFS and soluble protein at squaring stage. GT recorded significantly higher activities in superoxide dismutase and catalase, but lower in peroxidase and ascorbate peroxidase compared with non-GT. Proteomic alteration in leaves of GT vs non-GT was analyzed using 2-DE coupled with mass spectrometry. Eleven differentially expressed proteins were identified, of which 7 and 4 spots being up- and down- regulated, respectively. GT showed up-regulated expression of RuBisCO large subunit, *CP4EPSPS*, and ATP synthase, but down-regulated glutamate-1-semialdehyde-2, 1-aminomutase and manganese-stabilising protein, respectively.

Key words: Active oxygen metabolism, Glandless upland cotton, *Gossypium hirsutum*, Glyphosate-tolerance, Mineral nutrient, Photosynthetic performance, Proteome

Weeds, being a serious threat to the quality and yield of cotton, are one of the main factors affecting cotton production. Promoting use of herbicides, as an efficacious labour-saving measurement, is widely applied in cotton fields. However, due to the conventional varieties being sensitive to such herbicides as glyphosates, herbicides are often phytotoxicity or cause damages to cotton plants. Herbicides-resistant/ tolerant (GT) cotton revolutionized weed control in cotton (Wilcut *et al.* 1996). The introduction of GT cotton minimal crop injury from herbicides application provides producers with greater flexibility in the timing of herbicide applications and also offered a broader spectrum of weed control than other systems on the market (Askew and Wilcut 1999). Therefore, herbicide-tolerant cotton is becoming increasingly prevalent in cotton production, e g herbicide-tolerant cotton has been more widespread in the USA with 78% and 73% planted in 2010 and 2011, respectively (USDA-NASS 2011). However, development and application of herbicide-resistant cotton variety is still limited in China (Ma *et al.* 2010).

Glyphosate (N-phosphonomethyl glycine) is one of the most outstanding widely used organic phosphorus herbicides, which can effectively control annual and perennial grasses and dicotyledonous weeds. Because of its unique nonselective and broad-spectrum, and post-emergent, low-residue and low-cost characteristics, so far it is still the world's biggest-selling herbicide species in the world. Accordingly, breeding new varieties of Roundup Ready crops have been the major research focus concerning herbicide-tolerant because of producer-oriented factors and also its favourable environmental impact of glyphosate molecule (Main *et al.* 2007). The herbicidal action of glyphosate is by chelating with Mn, a cofactor for the 5-enolpyruvylshikimate-3-phosphate (EPSP, E.C. 2.5.1.19) synthase in the Shikimate pathway, to inhibit its metabolism of plants and many microorganisms (Pline-Srnic 2006). Glyphosate-tolerant transgenic cotton lines inserted with *CP4-EPSPS* gene which bypasses the inhibition

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of native EPSPS in the presence of glyphosate, allow sufficient production of aromatic amino acids and secondary metabolites (Nida *et al.* 1996). The creating of new glyphosate resistant plants would increase the available choices for planting and lower the price of genetically modified crop seeds.

In addition, high levels of gossypol, a natural toxin in most cotton varieties, make all of the plant's tissue, including seeds, inedible by humans and most animals. Developing glandless cotton, with cottonseeds free of gossypol, for food and feed applications is a promising possibility. Accordingly, the *aroA* gene *G6* was cloned from *P. putida* and transformed into glandless cotton (refer as GT). The insertion of glyphosate-tolerance gene into glandless cotton was confirmed by molecular analyses and field trials. In this paper, proteomic alteration in leaves of GT vs its wild type non-GT is reported, and characterization of both GT and non-GT glandless cotton lines was compared with respect to mineral uptake, photosynthetic performance and active oxygen metabolism. The characterization and composition data are part of the safety assessment to gain regulatory acceptance of the glyphosate-tolerant lines.

MATERIALS AND METHODS

Non-glyphosate-tolerant glandless cotton variety (non-GT, Zhong 5629) and its glyphosate-tolerant transgenic line (GT) were grown in a designated area for transgenic crops in the experimental farm of Huajiachi Campus, Zhejiang University, China in 2010. A novel *G6* gene from *Pseudomonas putida* (gb: EU169459) that encoded 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) was isolated. The *G6* gene was transfected into glandless cotton Zhong 5629 *via* Agrobacterium-mediated transformation. The insertion of the glyphosate-tolerance gene was confirmed by molecular analyses and field trials. GT exhibits both vegetative and reproductive tolerance to glyphosate, allowing glyphosate to be applied at any growth stage, without risk of boll abortion. Field observation indicates no significant yield response to multiple, topical applications of glyphosate to this GT cotton.

Clay soil was relatively fertile and the typical nutrient levels for the top 30 cm soil were: total N 0.051%, organic C 1.23%, P 66.25 mg/kg, K 305.18 mg/kg and *p*H 7.71. All seeds were directly sown on April 25 and seedlings at three leaf stage were thinned to 21 667 plants/ha. A completely random block design was used with 12 replicates in each plot at 72 m^2 (6 m×12 m). All plots received 90 kg N/ha, 150 kg P_2O_5/ha and 150 kg K/ha and enough water through furrow irrigation when necessary. Other conventional practices of cultivation were the same as those used locally.

Photosynthetic parameters were performed on intact fully expanded functional leaves (the 3rd or 4th up-most leaves, or the first leaves after de-topping) with 3 replicates, each containing 10 plants (Cai *et al.* 2011), using a Portable Photosynthesis System LI-6400 (LI-COR, Lincoln, NE, USA) at boll setting stage (BSS) and the beginning of boll opening stage (BBOS).

Five functional leaves were sampled each line with 3 replicates at squaring stage (SS), PFS, BSS and BBOS, respectively. Chlorophyll content (Chl a, Chl b, Chl a+b) and total soluble protein and sugar content were measured according to Li (2000). Mineral elements content were determined according to Bao (2008), total nitrogen using Kjeltec™8400 (FOSS, Denmark), Content of P using UV-1450 spectrophotometer (Shimadzu, Japan) and other elements (K, Ca, Mg, Fe, Mn, Zn and Cu) using AA-6300 atomic absorption spectrometry (Shimadzu, Japan). Superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT£¬EC 1.11.1.6) activities and malondialdehyde (MDA) content were determined according to Wu *et al.* (2003) and Chen *et al.* (2010).

Total protein extracts from functional leaves were prepared according to phenol extraction method (Carpentier *et al.* 2005) with minor modification (Bah *et al.* 2010). Protein concentration was determined by standard Bradford assay using bovine serum albumin as standard (Bio-Rad, Hercules, CA, USA). Proteins were separated by two-dimensional gel electrophoresis (2-DE) (Carpentier *et al.* 2005, Bah *et al.* 2010). The protein spots in analytical gels were visualized by silver staining (Shevchenko *et al.* 1996, Bah *et al.* 2010). For each sample, two independent protein extract and at least three 2-DE analyses each protein extract were performed.

To analyze the pattern of expressed proteins, stained gels were scanned and calibrated using a PowerLook1100 scanner (UMAX), followed by analysis of protein spots using GE HealthCare Software (Amersham Biosciences). Spot detection was realized without spot editing. The spots were quantified using the % volume criterion. Only those with significant and reproducible changes ($P<0.05$) were considered to be differentially accumulated proteins in relative abundance between non-GT and GT lines. The target protein spots were automatically excised from the stained gels and digested with trypsin using a Spot Handling Workstation (Amersham Biosciences). Peptide mass fingerprint data were matched to the NCBInr database using profound program under 50 ppm mass tolerance (Bah *et al.* 2010).

Data were processed via the Data Explorer software and proteins were unambiguously identified by searching against a comprehensive non-redundant sequence database (NCBInr) using the MASCOT software search engine (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS). The search parameters were as follows: (1) peptide quality of 800-4000 Da, mass tolerance for the fragment ion of 0.25 Da; (2) a minimum of seven matching peptides; (3) one missed cleavage; (4) taxonomy: viridiplantae (green plants); and (5) allowed modifications, carbamidomethylation of Cys (complete) and oxidation of

Met (partial). Moreover, only matches with over 90% sequence identity and a maximum e-value of 10^{-10} were considered. Fold increase and decrease in GT transgenic line vs non-GT were calculated as GT/non-GT and non-GT/GT for up- and down- regulated proteins, respectively. For single-peptide identified proteins, positive/negative proteins were assigned when it was shown that the regulation factors were above 1.5 ($P < 0.05$).

Statistical analyses were performed with Data Processing System (DPS) statistical software package version 12.5 (Tang 2010) using one-way ANOVA followed by the Duncan's Multiple Range Test (SSR) to evaluate significant difference between the iso-lines at $P \leq 0.05$.

RESULTS AND DISCUSSION

Effects of EPSPS gene on various photosynthetic parameters and soluble sugar and protein contents

At boll setting stage (BSS), net photosynthetic rate (Pn) of GT increased by 11.6%, intracellular CO_2 concentration (Ci) decreased by 6.1% (Fig 1a, c), compared with non-GT, which suggested CO_2 was absorbed by the leaf, rather than being released by the leaf, and absorbed CO_2 was consumed by photosynthesis (Sankaranarayanan *et al.* 2010). There was no significant difference in stomatal conductance (g_s) and transpiration rate (E) between the two lines (Fig 1b, d). Conversely, at the beginning of boll open stage (BBOS), GT exhibited significantly higher g_s (28.4%), Ci (14.8%) and E (20.6%) than non-GT except Pn value (Fig 1).

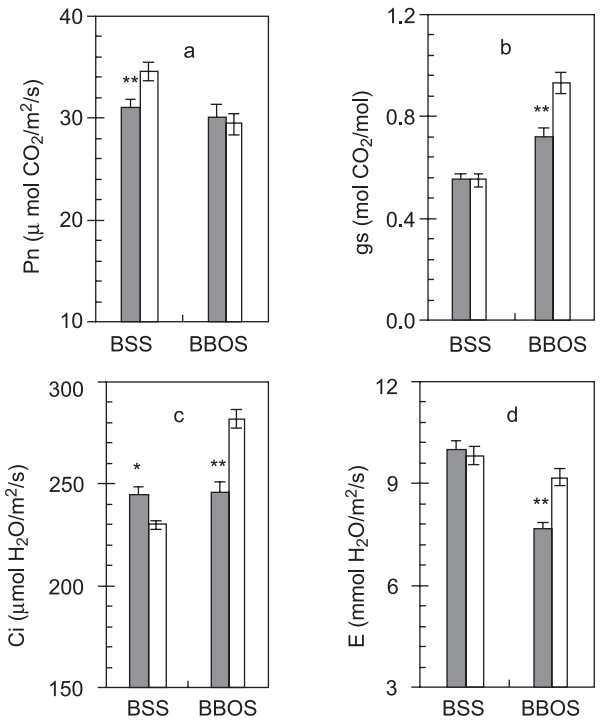

Fig 1 Comparison of photosynthetic parameters in the functional leaves between glyphosate-tolerant (GT, □) and its wild type (non-GT, ■) glandless cotton. Values are means ± SE (n=10). Pn, net photosynthetic rate; Ci, intercellular CO_2 concentration; g_s, stomatal conductance; E, transpiration rate. BSS= boll setting stage; BBOS= at the beginning of boll open stage. * and ** indicate significant difference at P= 0.05 and 0.01 respectively

At SS, no difference was found in Chl a, Chl b and Chl a+b content based on leaf fresh weight or leaf area between the two lines (Fig 2). However, GT showed significantly less Chl a, Chl b, and Chl a+b contents than non-GT at BSS and BBOS. Concerning Chl a/b (Fig 2d, h), GT was similar with non-GT at BBOS.

Significantly positive correlations was observed between g_s and Ci, soluble sugar and soluble protein, Chl a and Chl b, and Chl a and Chl a+b in glandless cotton lines at both BSS and BBOS (Table 1). However, there was a significant negative correlation between E and Chl contents. Synchronous alteration trends of contents of total Chl and soluble protein together with Pn decline were observed from BSS to BBOS. In green leaves, the process of leaf senescence is mostly characterized by a loss in total chlorophyll (Lim *et al.* 2007, Akbarian *et al.* 2011). Our results demonstrated that GT's senescence accelerated, especially at BBOS, indicating that application of nitrogen fertilizer in the late growth stage for GT glandless cotton be more important than non-GT to prevent senescence.

Effects of EPSPS gene on mineral nutrients in leaves

Compared with non-GT at SS, PFS, BSS, BBOS, insertion of *EPSPS* gene markedly decreased contents of N by 10.1%, 17.6%, 27.4%, 16.3%, and Mg by 13.6%, 11.7%, 4.8%, 19.3% in GT, respectively (Fig 3a, d), but increased P (4.5%, 3.1%, 38.9%, 13.3%), Ca (4.0%, 2.5%, 37.4%, 63.8%), Mn (11.2%, 15.8%, 31.5%, 34.3%) and Zn (412.7%, 1.5%, 177.9%, 188.5%). In addition, at SS Cu concentration in GT was 5.0% higher than non-GT (Fig 3g), while at PFS GT had 14.9% higher K and 4.6% lower Cu concentrations (Fig 3c, g). In contrast, at BSS GT exhibited 16.5% lower K and 26.3% higher Cu concentrations (Fig 3c, g). At BBOS, K concentration in GT significantly decreased by 40.5% but Fe and Cu increased by 7.7% and 6.4% (Fig 3c, f, g), respectively. However, *EPSPS* gene showed no significant effect on P, K, Ca and Fe at SS; P, Ca, Fe and Zn at PFS, and Mg concentrations at BSS (Fig 3b-f, i). Decreased N and K in GT may indicate that GT glandless cotton cultivation needs more application of N and K.

To further assess the relationships of nine elements in the functional leaves concentrations glandless cotton lines at four stages, their correlations were analyzed and shown in Table 2. Significantly positive correlation between Fe and Mn, Ca and Zn were detected. However, a significant negative correlation occurred between N and Ca, K and Fe, Mg and Ca, and Mg and Zn.

GT also exhibited significant 6% lower leaf soluble

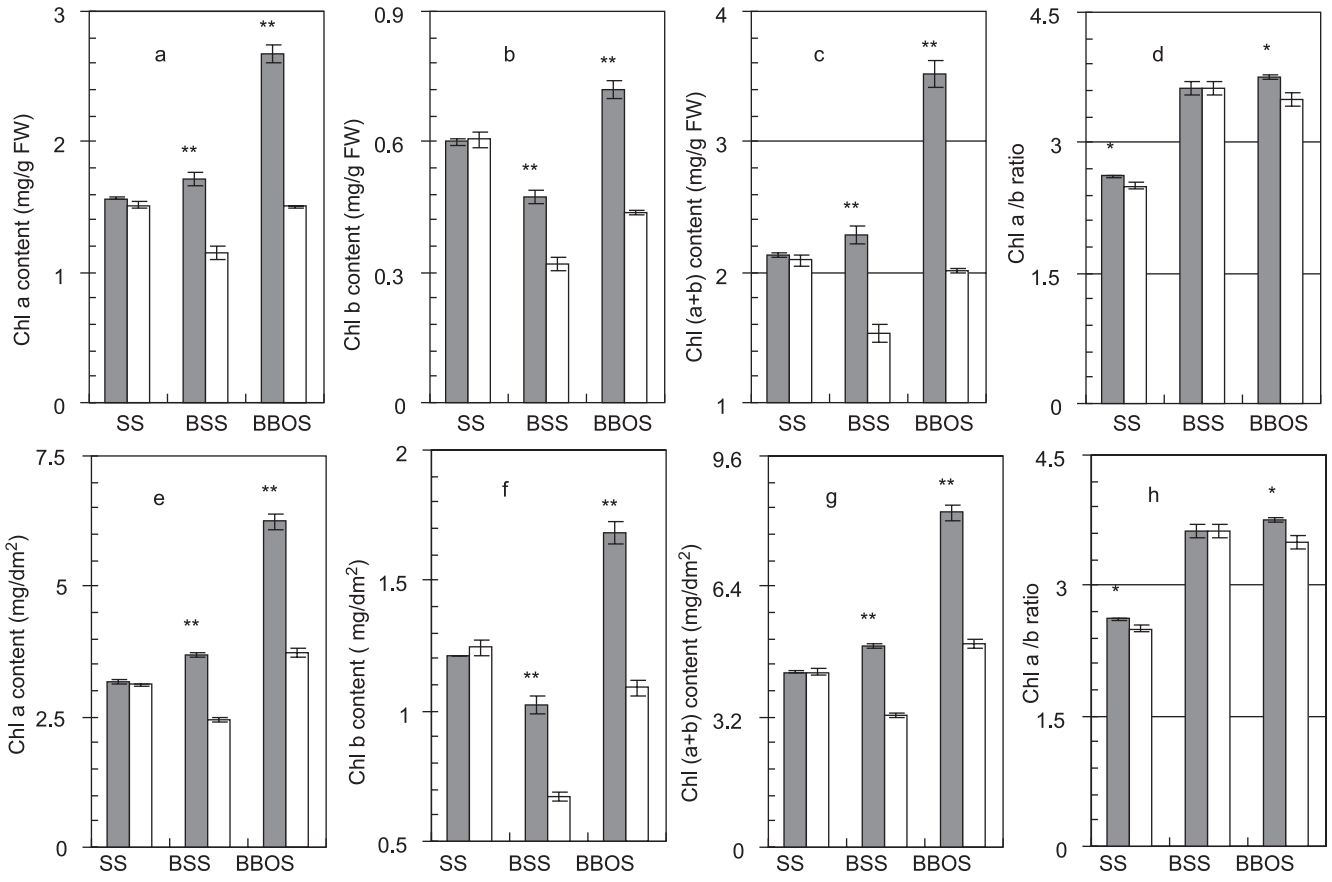

Fig 2 Comparison of chlorophyll content (Chl a, Chl b, Chl, Chl a/b ratio) in functional leaves (Up, expressed on a fresh weigh basis; Down, expressed on a leaf area basis) at SS, BSS and BBOS between glyphosate-tolerant (GT, □) and its non-GT (■) glandless cotton. Values are means ± SE (n = 3). * and ** indicate significant difference at *P*=0.05 and 0.01 respectively. SS= Squaring stage; BSS= boll setting stage; BBOS= at the beginning of boll open stage

sugar at PFS and 56.0% lower protein content at SS than non-GT (Fig 3k, l). Contrarily, soluble sugar in GT at BSS and BBOS increased by 18.3% and 5.95% in comparison to non-GT, respectively. Also, soluble protein in GT at PFS and BSS was significantly increased by 18.8% and 38.2% accordingly when compared with the non-GT.

Effects of EPSPS gene on activities of antioxidant enzymes and MDA content

Generally an important change associated with leaf senescence was enhanced accumulation in MDA content, accompanied by improved activities of antioxidative enzymes, such as SOD, POD, CAT and APX (Palma *et al.* 2006). Increases in GT were 21.5% (PFS) and 20.2% (BBOS) for SOD (Fig 4a), 49.8% (PFS) and 16.3% (BBOS) for CAT activities (Fig 4c), and 44.7% (SS), 22.6% (BSS) and 13.4% (BBOS) for MDA content (Fig 3j), compared with non-GT. However, *EPSPS* gene significantly resulted in decrease of POD, and APX activities relative to non-GT (Fig 4b, d). The activities of SOD and CAT in GT were much higher than non-GT, but POD and APX activities markedly lower. These results suggest POD and APX were a less efficient H_2O_2 scavenger than SOD and CAT upon insertion of *EPSPS*-G6 gene. Significant increase (*P*=0.05) in activities of SOD and CAT in GT, with increased MDA content, relative to non-GT (Fig 4a, c and 3j) also indicated *EPSPS-G6* gene stimulated lipid peroxidation and accelerating senescence.

Effects of EPSPS gene on proteome profiles and differential proteins

Total proteins were resolved into approximately 1976 spots (ranging from1822 to 2229) in 24-cm SDS-polyacrylamide gels (Fig 5), where 7 and 4 proteins were up- and down-regulated in GT vs non-GT, respectively (Table 3, Fig 6). Theses 11 protein spots were selected for Matrix Assisted Laser Desorption Ionization, Time-of-Flieght, Time-of-Flieght (MALDI-TOF-TOF) MS analysis, and identified with significant difference in protein expression levels and present in sufficient amounts to be visible on a coomassie-stained preparative gel and were excised for mass spectrometric analysis. Their spectra analysis and further protein identification by MS and data bank analysis identified

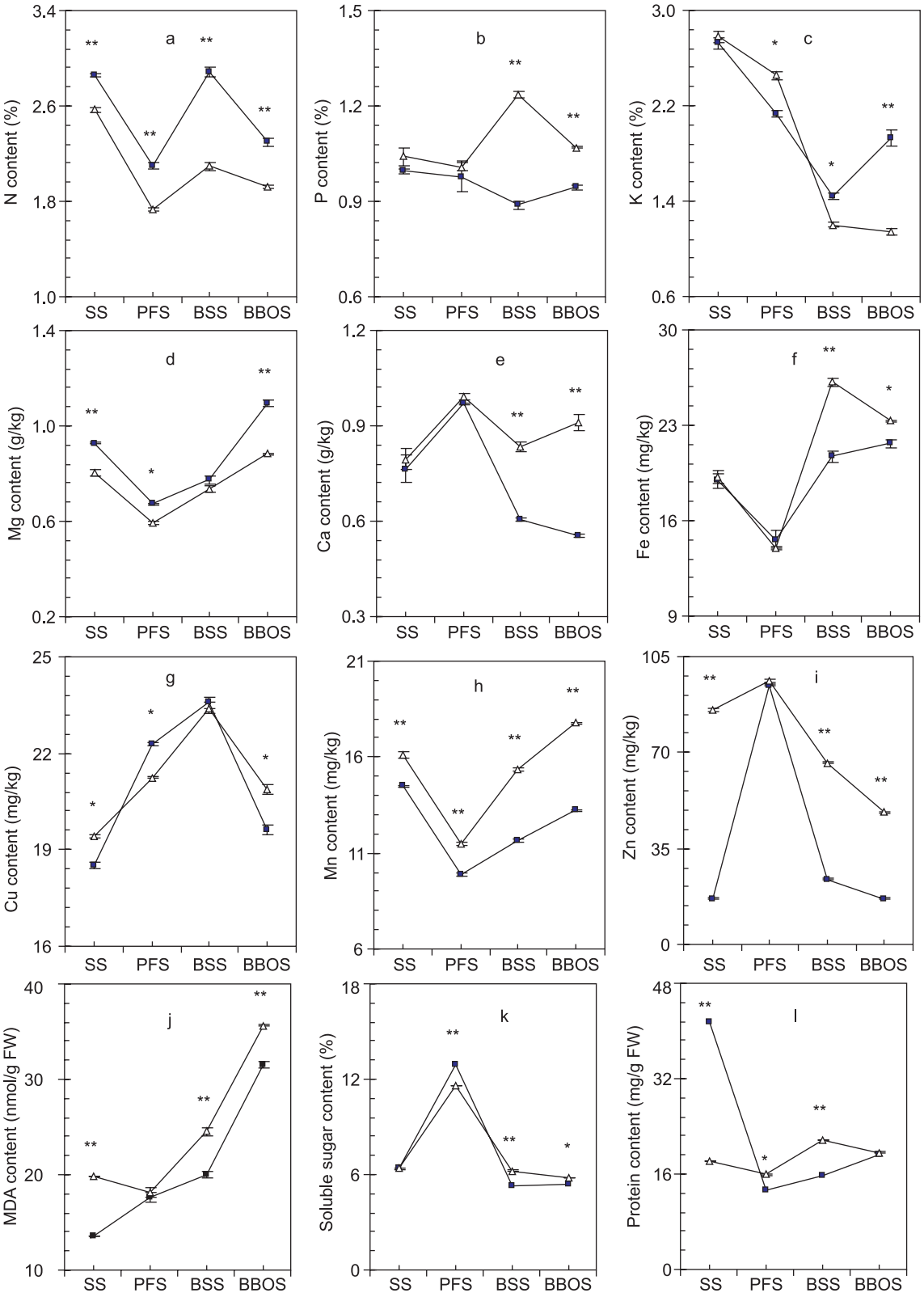

Fig 3 Comparison of mineral concentrations, MDA, Soluble sugar and soluble protein between glyphosate-tolerant (GT, □) and its non-GT (■) glandless cotton. Data are means ± SE (n = 3). * and ** denote significant difference at *P*=0.05 and 0.01 respectively. SS= squaring stage; PFS=peaking of flower stage; BSS=boll setting stage; BBOS=at the beginning of boll open stage

Table 1 Correlations among the 13-parameters at BSS and BBOS in glandless cotton lines

Parameter	Pn	g_s	Ci	E	Soluble protein	Soluble sugar	Chl a mg/g	Chl b mg/g	Chl a+b mg/g	Chl a/b ratio	Chla mg/dm	Chlb mg/dm	Chl a+b mg/dm
Pn	1												
g_s	–0.726	1											
Ci	–0.726	0.940*	1										
E	0.519	–0.434	–0.141	1									
Soluble protein	0.487	0.177	–0.027	–0.16	1								
Soluble sugar	0.669	0.025	–0.032	0.305	0.89	1							
Chl a mg/g	–0.602	0.173	–0.029	–0.876	–0.304	–0.705	1						
Chl b mg/g	–0.656	0.236	0.041	–0.881	–0.314	–0.713	0.998**	1					
Chl a+b mg/g	–0.613	0.177	–0.02	–0.871	–0.317	–0.715	1.000**	.998**	1				
Chl a/b ratio	0.122	–0.488	–0.707	–0.573	–0.06	–0.369	0.713	0.662	0.705	1			
Chl a mg/dm^2	–0.682	0.317	0.11	–0.915*	–0.25	–0.663	0.989**	0.995**	0.989**	0.618	1		
Chl b mg/dm^2	–0.733	0.386	0.187	–0.913*	–0.256	–0.663	0.975*	0.987**	0.976*	0.555	0.997**	1	
Chl a+b mg/dm^2	–0.693	0.325	0.122	–0.911*	–0.261	–0.671	0.988**	0.995**	0.988**	0.608	1.000**	0.998**	1

Pn, Net photosynthetic rate; Ci, intercellular CO_2 concentration; g_s, stomatal conductance; E, transpiration rate. chlorophyll content (Chl a, Chl b, Chl, Chl a/b ratio; mg/g, expressed on a fresh weigh basis; mg/dm^2, expressed on a leaf area basis). * and ** indicate significant difference at P= 0.05 and 0.01 respectively

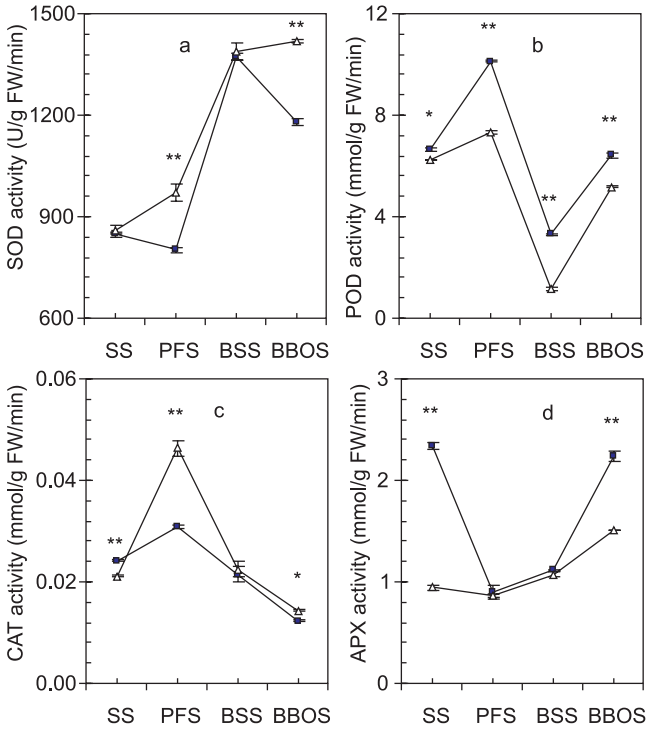

Fig 4 Comparison of SOD, POD, CAT and APX activities in functional leaves between glyphosate-tolerant (GT, □) and its non-GT (■) glandless cotton. Data are means ± SE (n = 3). * and ** denote significant difference at P=0.05 and 0.01, respectively. SS= squaring stage; PFS=peaking of flower stage; BSS=boll setting stage; BBOS=at the beginning of boll open stage

7 up-regulated proteins (Table 3 and Fig 5A). Of the 7 up-regulated proteins identified in GT, spots U1 and U2 corresponding to large subunits of RuBisCo and U3 were identified as 50S ribosomal protein L21, chloroplast/L21 proteins respectively. Ribulose 1, 5-bisphosphate carboxylase/oxygenase (RuBisCO, E.C. 4.1.1.39) usually catalyzes the

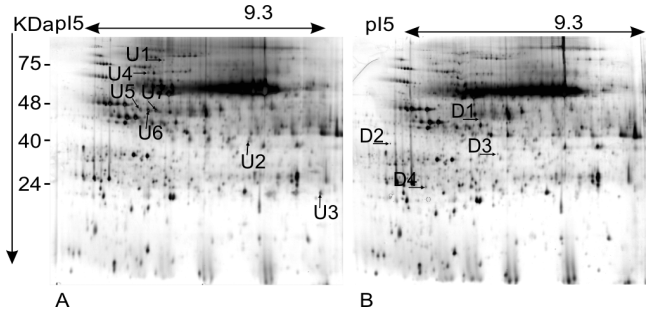

Fig 5 Representative 2-DE maps of proteins extracted from glyphosate-tolerant GT (A) and non-GT (B) glandless cotton functional leaves at the peak of flowering stage (PFS). Labeled proteins were found to be up-regulated (U) or down-regulated (D) GT glandless cotton (A) vs. non-GT (B) and were analyzed by LC-MS/MS analysis

Table 2 Correlations among the nine-element concentrations at four stages in glandless cotton lines

Parameter	N	P	K	Mg	Ca	Fe	Cu	Mn	Zn
N	1								
P	–0.428	1							
K	0.244	–0.312	1						
Mg	0.382	–0.208	–0.058	1					
Ca	–0.687*	0.413	0.121	–0.717*	1				
Fe	0.134	0.532	–0.692*	0.475	–0.436	1			
Cu	–0.206	0.187	–0.680*	–0.574	0.123	0.179	1		
Mn	0.009	0.56	–0.221	0.408	–0.035	0.688*	–0.352	1	
Zn	–0.609	0.341	0.236	–0.798**	0.804**	–0.485	0.238	–0.198	1

* and ** indicate significant difference at P= 0.05 and 0.01 respectively

Table 3 Proteins significantly expressed with up (ID/down-regulation (D) in functional leaves of glyphosate-tolerant (GT) vs non-GT cotton at the peak of flowering stage

Spot ID	Protein	Accession no.	Molecular weight (Da)	pl	Fold increase (+) or decrease (GT vs. non-GT)	Ammo acid sequence coverage %	Number of peptides matched	Score	Putative function
Ul	Ribulose -1,5-bisphosphate carboxylase /oxygenase large subunit *[Maerua kirkii]*	gil46325906	53079	6	+2.348	20	10	164	Metabolism/energy
	Ribulose -1,5-bisphosphate carboxylase/oxygenase large subunit *[Reseda alba]*	gil7240436	52433	6.22		24	9	154	Metabolism/energy
U2	Ribulose -1,5-bisphosphate carboxylase/oxygenase large subunit *[Trichomanes ankersi* i]	gil37728139	44738	6.45	+1000000	19	6	140	Metabolism/energy
	Ribulose -1,5-bisphosphate carboxylase/oxygenase large subunit *[Pogostemon cablin]*	gil349048	50571	6.1		17	5	137	Metabolism/energy
U3	SOS ribosomal protein L21, chloroplast / CL21 (RPL21) [*Arabidopsis thaliana]*	gil15219695	24195	9.32	+1000000	12	4	83	Structural constituent of ribosome, RNA binding
U4	VAR2 (VARIEGATED 2); ATP-dependent peptidase/ ATPase/ metallopeptidase/ zinc ion binding [*Arabidopsis thaliana]*	gil30684767	74282	6	+1.912	22	14	155	Metallopeptidase activity, ATP - dependent peptidase activity, ATPase activity, zinc ion binding
	FtsH-like protein Pftf precursor [*Nicotiana tabacum]*	gil4325041	74507	6		20	12	118	Thylakoid - localized AAA (ATPases associated with diverse cellular activities) -family protein

Continued

Table 3 *Concluded*

Spot ID	Protein	Accession no.	Molecular weight (Da)	pl	Fold increase (+) or decrease (GT vs. non -GT)	Ammo acid sequence coverage %	Number of peptides matched	Score	Putative function
US	*CP4EPSPS [Glycine max]*	gil18266432	47699	5.13	+1000000	14	5	91	Glyphosate tolerant
	5-enol-pyruvylshikimate-3-phospate synthase class 2 precursor [*Agrobacterium* sp. CP4]	gil62318479	55732	5.98		12	5	89	Glyphosate tolerant
U6	*CP4EPSPS [Glycine max]*	gil18266432	47699	5.13	+12.1551	40	17	91	Glyphosate tolerant
	5-enol-pyruvylshikimate-3-phospate synthase class 2 precursor [*Agrobacterium* sp. CP4]	gil62318479	55732	5.98		30	15	89	Glyphosate tolerant
U7	*CP4EPSPS [Glycine max]*	gil18266432	47699	5.13	+3.60304	33	12	91	Glyphosate tolerant
	5-enol-pyruvylshikimate-3-phospate synthase class 2 precursor [*Agrobacterium* sp. CP4]	gil62318479	55732	5.98		24	10	89	Glyphosate tolerant
Dl	GSA1 (glutamate -1-semialdehyde 2,1 -aminomutase) [*Arabidopsis thaliana]*	gil15242822	50737	6.43	–1000000	14	7	64	Glutamate - 1 - semialdehyde 2,1 -aminomutase activity
	GSA2 (glutamate-1-semi-aldehyde 2,1 -aminomutase 2); catalytic/glutamate-1-semialdehyde 2,1-amino-mutase/pyridoxal phosphate binding/ transaminase *[Arabidopsis thaliana]*	gil15229018	50452	7.01		15	8	64	Glutamate -1-semi-aldehyde 2,1 - aminomutase activity, pyridoxal phosphate binding, transaminase activity, catalytic activity
D2	Photosystem II protein 33kD [*Spinacia oleracea]*	gil224916	26759	5.01	–1000000	22	3	94	Manganese - stabilising protein/ photosystem II polypeptide
	Chloroplast photosynthetic oxygen-evolving protein 33 kDa subunit [*Nicotiana benthamiana]*	gil61697115	35374	5.61		16	3	91	Manganese - stabilising - protein/ photosystem II polypeptide
D3	Os04g0490800 *[Oryza sativa (Japonica* cultivar -group)]	gil115459134	39811	6.75	–1.54949	15	6	104	2-phosphoglycolate phosphatase, eukaryotic family protein;
	4-nitrophenylphosphatase [*Zea mays*]	gil226491816	39501	5.46		8	3	86	Phosphoglycolate phosphatase
D4	Chalcone isomerase [*Gossypium hirsutum*]	gil295687229	23419	4.86	–1.74003	42	6	292	Chalcone isomerase
	Chalcone isomerase [*Petunia × hvbrida*]	gi/299889035	23505	48		10	2	111	Chalcone isomerase

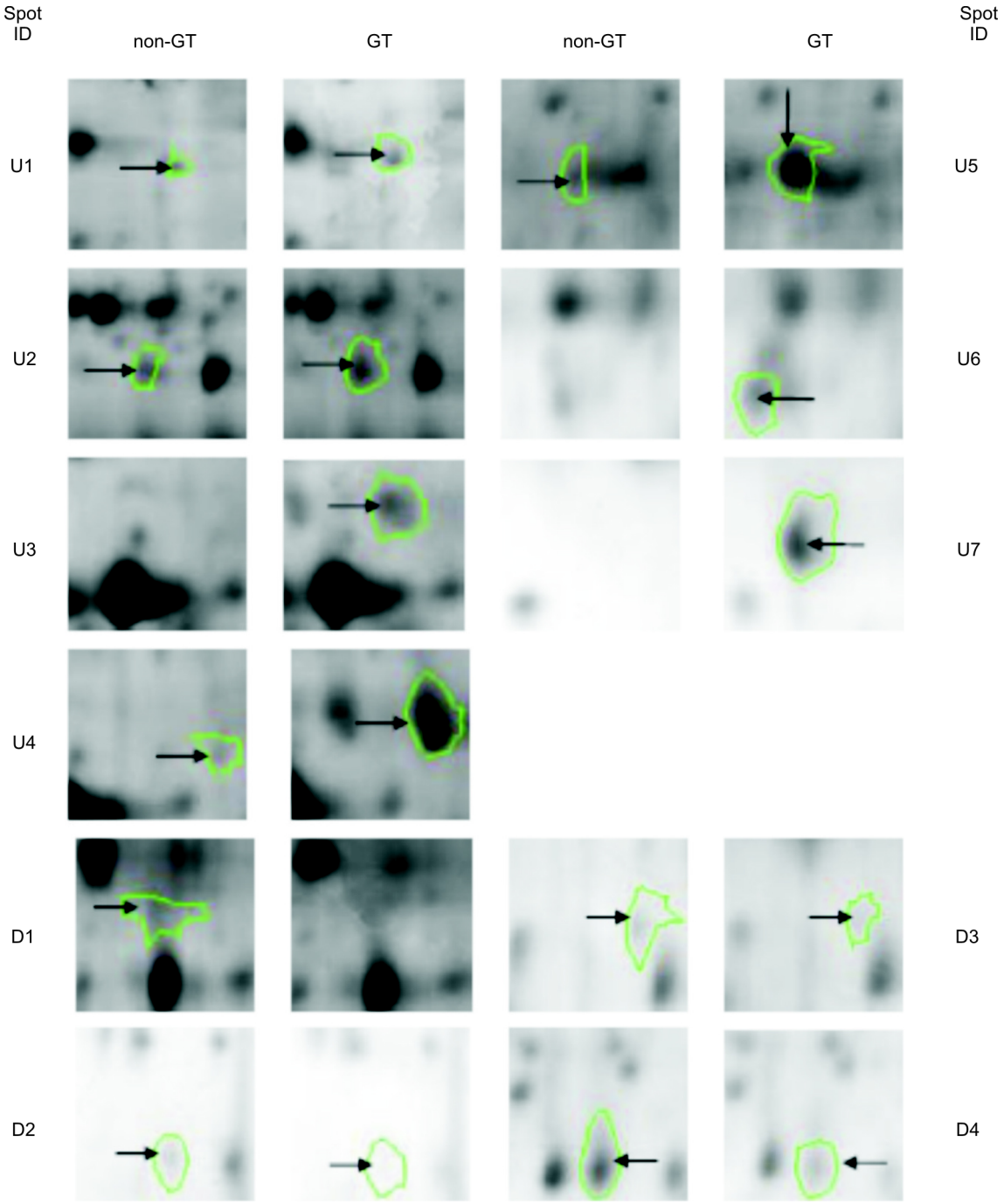

Fig 6 'Spot view' of proteins up (U) /down-regulated (D) in GT functional leaves compared to non-GT at the peak of flowering stage

initial step of carbon metabolism, the fixation carbon dioxide, in photosynthetic eukaryotes. The opposing oxygenase activity of RuBisCo results in synthesis of phosphoglycolate, a molecule of limited use to most organisms. Our results suggest that increase in net photosynthesis of GT cotton line at BSS may be due to the up-regulation of RuBisCo large subunits, which indicated that oxygenation was suppressed and carboxylation improved accordingly. Spots U5-7, *CP4EPSPS*, confer tolerance against the substance glyphosate providing evidence that GT cotton was transgenic line.

Among the 4 down- regulated proteins identified (GT vs non-GT, D1-4, Table 3), D1 was identical to glutamate-1-semialdehyde 2, 1-aminomutase activity. Glutamate-1-semialdehyde 2, 1 aminomutase (EC 5.4.3.8), which locate in chloroplast stroma, chloroplast, chloroplast envelopec, catalysis of the reaction: (S)-4-amino-5-oxopentanoate = 5-

aminolevulinate, cofactor of pyridoxal phosphate in porphyrin biosynthesis process. 5-Aminolevulinic acid (ALA) is the universal precursor of tetrapyrroles, such as chlorophyll and heme. The major control of chlorophyll biosynthesis is at the step of ALA formation. In the chloroplasts, as in *Escherichia coli,* ALA is derived from the glutamate of Glu-tRNA via the two-step C5 pathway. The first enzyme, Glu-tRNA reductase, catalyzes the reduction of Glu-tRNA to glutamate 1-semialdehyde with the release of intact tRNA. The second enzyme, glutamate 1-semialdehyde 2, 1-aminomutase, converts glutamate 1-semialdehyde to ALA. Hence, the down-regulation of D1 may be directly affecting N content reduction in GT at PFS.

D2 may function as manganese-stabilising protein/ photosystem II polypeptide. Manganese Stabilizing Protein (MSP) plays an important role in maintaining the stability and activity of the manganese cluster under physiological conditions and is present in all of the oxygenic photosynthetic organisms (Miyao and Murata 1984, Enami *et al.* 2008). The main function of this protein appears to be to stabilize the cluster of four manganese ions at the catalytic center of the oxygen-evolving complex (Miyao and Murata 1984); D3 was correlated with 2-phosphoglycolate phosphatase, eukaryotic family protein. D4 spots were associated with chalcone isomerase, which also known as chalcone-flavanone isomerase, is a plant enzyme responsible for isomerisation of chalcone to naringenin in the flavonoid biosynthesis pathway (Ralston *et al.* 2005). CHS in *G. hirsutum* is preferential expression in developing fiber (Xiao *et al.* 2007). In our study, spot D4 was blasted to be identical with chalcone isomerase [*G. hirsutum*] and [*Petunia* × *hybrida*] and its drastic down-regulation in GT showed that insertion of *EPSPS* gene imparts herbicide resistance affected flavonoid biosynthesis.

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