J. Agr. Sci. Tech. (2012) Vol. 14: 1365-1378

Agronomic and Physiological Characteristics and Proteomic Expression in Near-isogenic Lines of Bt and Non-Bt Glandless Cotton

X. H. Chen[1], Z. Q. Jin[2], Z. H. Chen [1], S. J. Zhu [1], F. B. Wu[1*]

ABSTRACT

Agronomic and physiological traits and protein expression were compared between non-Bt glandless upland cotton variety-Zhong5629 and its Bt-transgenic near-isogenic lines differing in *cryIAc* gene. Results showed that the Bt isogenic line had higher plant height and rate of effective bolls, more internal bolls but lower yield, than the non-Bt line. The Bt isogenic line had lower chlorophyll content and transpiration rate relative to the non-Bt line, but had higher Chl a/b, F_0 and Fm. Soluble protein content in the Bt isogenic line was significantly higher at boll setting stage (BSS) but lower at initial flowering stage (IFS) compared with the non-Bt line. The Bt isogenic line had significantly higher concentrations of Ca, Mg, Cu, Zn, Mn and Fe at IFS, and P and Cu at BSS, whereas it had lower in concentrations of P, K and B at IFS, and K, S, Zn and Fe at BSS. The Bt-isogenic line exhibited less malondialdehyde content at BSS and lower superoxide dismutase activity at IFS and BSS. Furthermore, proteomic analysis of the two NILs detected 20 differentially expressed proteins. The 4 up- regulated proteins in Bt *vs* non-Bt-isogenic line were attributed to signal transduction, photosynthetic carbon assimilation and defense response, whereas the 16 down- regulated proteins were attributed to signal transduction and protein metabolism.

Keywords: *Bacillus thuringiensis* (Bt), Glandless upland cotton (*Gossypium hirsutum* L.), Near-isogenic line, Two-dimensional electrophoresis

INTRODUCTION

Cotton, one of world's most important economic crops, is cultivated mainly for fibers, and is also the potential source of edible oil and food since every kilogram of fiber production is accompanied by about 1.65 kg of oil- and protein-rich seeds (Gerasimidis *et al.*, 2007). The seed meal is a protein-rich byproduct useful to feed ruminant livestock, but toxic to non-ruminant animals and humans because of the existence of pigment glands of gossypol, a terpenoid aldehyde (Gerasimidis *et al.*, 2007). Breeding glandless cotton is a cost effective approach to considerably reduce or even eliminate gossypol in cotton seeds in order to widen its use for feed and food (Zhu and Chen, 2005). However, a major problem hindering the production and growing areas of glandless cotton is its susceptibility to pests mainly due to the absence of gossypol and other terpenoid aldehydes (Foster *et al.*, 1994). Therefore, it is imperative to develop glandless varieties with high insect-resistance. Cotton (*Gossypium hirsutum* L.) cultivars containing the *Bacillus thuringiensis subsp. kurstaki* (Bt) gene are commercially known as Bollgard® or Bt cotton (Perlak *et al.*, 2001). In China, Bt cotton was widely grown and accounted for more than 38.1% of cotton production in 2009 (Mao, 2010).

Premature senescence of cotton has been occurring at an increasing scale in China, directly influencing both yield and fiber

[1] Department of Agronomy, College of Agriculture and Biotechnology, Zijingang Campus, Zhejiang University, Hangzhou, 310058, P. R. China.
[2] Cixi Research Institute of Agricultural Science, Cixi 315300, P. R. China.
[*] Corresponding author: e-mails: wufeibo@zju.edu.cn

quality (Hao *et al.*, 2011). Based on the changes and genetic development of biochemical traits associated with antioxidant systems such as superoxide dismutase (SOD, EC 1.15.1.1) and peroxidase (POD, EC 1.11.1.7) activities as well as malondialdehyde (MDA) and minerals, soluble sugar and soluble protein, the biochemical assistant breeding technology has been established and the relative selective standards to select promising parents have been developed (Yu *et al.*, 2005) to be used to investigate how Bt gene affects glandless cotton lines in a short time.

Proteomics attempts to study the structure, function, and control of biological systems and processes by the systematic and quantitative analysis of the many properties of proteins. Although research efforts on conventional upland cotton with glandless seeds and foliage glands, and insect resistant transgenic cultivars are widely published (Gaufichon *et al.*, 2010), there is little investigation using 2-DE/MS technique to explore the protein expression imposed by Bt transgene in glandless cotton. Also the physiological characteristics of low gossypol content and insect-resistant transgenic varieties are still less known.

The present work was carried out by comparing a pair of near-isogenic lines (NILs) of Bt and non-Bt glandless upland cottons differing only by the presence of the *cryIAc* gene to: (1) evaluate the effects of Bt gene on agronomic characters such as boll distribution patterns, yield and some physiological traits; (2) identify candidate proteins associated with the insertion of Bt gene using 2-DE coupled with MS. The results can lay important basis and serve as a guide for further protein/gene function research in Bt glandless upland cotton.

MATERIALS AND METHODS

Plant Materials and Experimental Design

Non-Bt glandless cotton variety (Zhong5629) and its Bt-transgenic NIL were grown in a designated area for transgenic crops in the experimental farm of Huajiachi Campus, Zhejiang University, China in 2009. The Bt-isogenic line was derived after five backcrossing of Zhong5629×Zhongzhe905 with non-Bt glandless cotton variety, Zhong5629. Zhongzhe905 was the donor of Bt trait (*CryIAc*). Zhong5629 and its Bt NIL were described as non-Bt and Bt-isogenic lines.

The soil is relatively fertile containing typical nutrient levels of the top 30 cm soil are: total soil N 0.072%, organic C 1.73%, rapidly available P 85.11 mg kg^{-1}, rapidly available K 265.47 mg kg^{-1} and pH 6.43. All seeds were sown in pots on April 20, 2010 and seedlings were transplanted to the field on May 12 with a density of 37500 plants ha^{-1}. A completely random block design with 6 replications was used and the plot size was 27 m^2 (4.5 m×6 m). All plots received 150 kg P_2O_5 ha^{-1} and 150 kg K ha^{-1} and were well-watered through furrow irrigation when necessary. Pesticides were sprayed as required for the non-Bt line. Other conventional practices of cultivation were the same as those used locally.

Measurement of Agronomic Traits

At initial flowering stage (IFS), 10 plants were tagged in each plot, and plant height, fruiting branches, buds, flowers and bolls per plant were investigated in 10 day intervals. In this text, BL refers to the branch position of vertical boll distribution on the plants and BN is the node position of horizontal distribution of bolls on fruiting branches. BL_{1-5}, BL_{6-9}, and BL_{10-15}, represent boll number at each level per plant, i.e. the number of bolls borne by the 1^{st} - 5^{th}, 6^{th} - 9^{th}, and 10^{th} - 15^{th} fruiting branches, respectively, from the bottom to the top of the plant. BN_{1-2} and $BN_{\geq 3}$ represent the bolls per plant on the 1^{st} - 2^{nd}, and $\geq 3^{rd}$ nodes of fruiting branches with position 1 being the closest to the main stem.

On September 15, 50 bolls in the central position of plants were randomly harvested in each plot for the measurements of boll

weight and lint index. During boll opening stage, seed cotton in each plot was harvested, and then seed index, lint yield and lint percentage were calculated after ginning of seed cotton.

Measurement of Chlorophyll a Fluorescence and Photosynthetic Parameters

Photosynthesis and chlorophyll fluorescence parameters were performed with intact fully expanded functional leaves (the 3rd or 4th up-most leaves). Photosynthetic parameters were measured at the beginning of boll opening stage (BBOS, September 5) by using a Portable Photosynthesis System LI-6400 (LI-COR, Lincoln, NE, USA) (Cai *et al.,* 2011). Chlorophyll fluorescence measurements were performed at IFS and boll setting stage (BSS) using pulse-modulated chlorophyll fluorometer (IMAGING-PAM, Walz, Effeltrich, Germany; Cai *et al.,* 2011). Chlorophyll (Chl) content was measured by the method of Arnon (Zhang, 1992).

Determination of SOD, POD Activities and MDA Content

Ten functional leaves collected at IFS and BSS from each plot in three replicates, were used to determine superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7) activities and malondialdehyde (MDA) content according to Wu *et al*. (2003).

Assay of Soluble Sugar, Soluble Protein and Mineral Contents

Soluble protein and sugar content of functional leaves were measured according to Zhang (1992). Total nitrogen was quantified according to the micro-Kjeldahl method using Kjeflex K-306 (BUCHI Labortechnik AG, Flawil, Switzerland). P content was determined by phosphorus vanadium molybdate yellow colorimetric method, and other minerals were determined by ICP Optical Emission Spectrometer (Thermo iCAP 6000 SERIES ICP-OES, Waltham, England).

One-way ANOVA was carried out with Data Processing System (DPS) statistical software package (Tang and Feng, 1997). The Duncan's Multiple Range Test was applied to evaluate significant differences between two isogenic lines at $P \leq 0.05$.

Proteome Analysis

Total protein extracts from functional leaves were prepared essentially according to the phenol extraction method described by Carpentier *et al*. (2005) with minor modification (Bah *et al.,* 2010*).* Two-dimensional gel electrophoresis, mass spectral analysis and protein identification were performed as reported previously (Bah *et al.*, 2010). For each sample, two independent protein extracts and at least three 2-DE analyses of each protein extract were performed. Fold increase and decrease in Bt *vs*. non Bt line were calculated as Bt/non-Bt and non-Bt/Bt 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 ($p < 0.05$).

RESULTS

Agronomic Traits

Plant height was significantly higher in the Bt-transgenic glandless cotton isogenic line than the non-Bt one (Figure 1), however, this was reversed for buds per plant, regardless of plant growing stages. At boll opening stage, plant height of the Bt-isogenic line was 9.2% higher than that of the non-Bt-isogenic line, while reproductive nodes per plant was 26.6% less in the Bt-isogenic line respectively (Table 1).

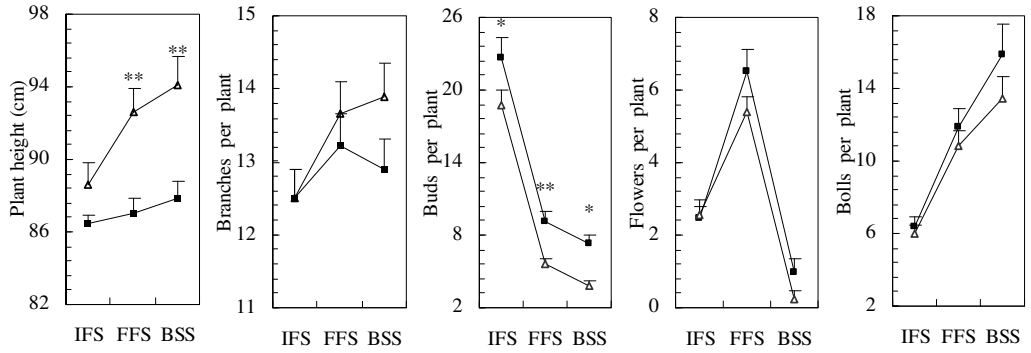

Figure 1. Comparison of the dynamics of growth parameters between Bt transgenic glandless cotton (Bt isogenic line, Δ) and its non-Bt isogenic line (■). Means ± SE (n = 10), * and **, significant at 0.05 and 0.01 levels, respectively. IFS= initial flowering stage; FFS=full flowering stage; BSS=boll setting stage.

Table 1. Comparison of plant height, yield and boll distribution on the plants between Bt and non-Bt glandless isogenic line at boll opening stage.

NILs	Plant height (cm)	Seed cotton (g plant $^{-1}$)	Fruiting branches per plant	Vertical boll distribution on the plants			Horizontal distribution of bolls on fruiting branches	
				BL_{1-5}	BL_{6-9}	BL_{10-15}	BN_{1-2}	$BN_{\geq 3}$
Non-Bt isogenic line	85.5*	78.6*	13.4	7.4	6.7	4.1	5.5	12.7*
Bt isogenic line	93.4	61.5	13.0	7.2	4.8	3.6	7.8*	7.5

*, Significant at 0.05 level between the two NILs (n=10). BL_{1-5}, BL_{6-9}, and BL_{10-15}, represent the bolls per plant on the 1st – 5th, 6th - 9th, and 10th - 15th fruiting branches from the bottom to the top of the plant. BN_{1-2} and $BN_{\geq 3}$ represent the bolls per plant on the 1st - 2nd, and ≥3rd nodes of fruiting branches, position 1 is the closest to the main stem.

There was no difference between the two NILs in terms of bolls at the lower levels (BL_{1-5}) (Table 1), however, at the middle and upper parts of plants (BL_{6-9} and BL_{10-15}), thw non-Bt line gained more bolls. Seed cotton per plant was 21.8% lower significantly ($P<0.05$) in the Bt-isogenic line than the non-Bt-isogenic line (Table 1), but no differences were observed in boll weight, bolls per plant, lint percent, seed index, or fiber length (data not shown).

Photosynthetic Parameters

Leaf fresh weight based Chl contents differed significantly between the two NILs at IFS, but not at BSS (Figure 2 a-c). When calculating these contents on a leaf area basis, Chl a, Chl b and Chl a+b contents of the Bt-isogenic line were 8.9%, 6.6% and 10.3% less; but Chl a/b was higher than that in the non-Bt line at BSS. Transpiration rate (E) in the Bt-isogenic line was 11.0% higher than that in the non-Bt line at BBOS, but no significant difference was found in net photosynthetic rate (Pn), stomatal conductance (g_s) or intercellular CO_2 concentration (Ci) (Table 2).

Images of the initial fluorescence (F_0) showed 5.2% (IFS) and 10.8% (BSS) higher values in the Bt-isogenic line than the non-Bt-isogenic line (Figure 3 e, f, E, F, and h). The maximal fluorescence (F_m) at BSS was

Table 2. Photosynthetic parameters in functional leaves of the two NILs at the beginning of boll opening stage.

NILs	Non-Bt isogenic line	Bt isogenic line
Pn (μmol CO_2 $m^{-2}s^{-1}$)	22.40	23.37
Ci (μmol CO_2 mol^{-1})	260.33	258.33
g_s (mol H_2O $m^{-2}s^{-1}$)	0.56	0.54
E (mmol H_2O $m^{-2}s^{-1}$)	7.68	6.83

*, significant at 0.05 level.

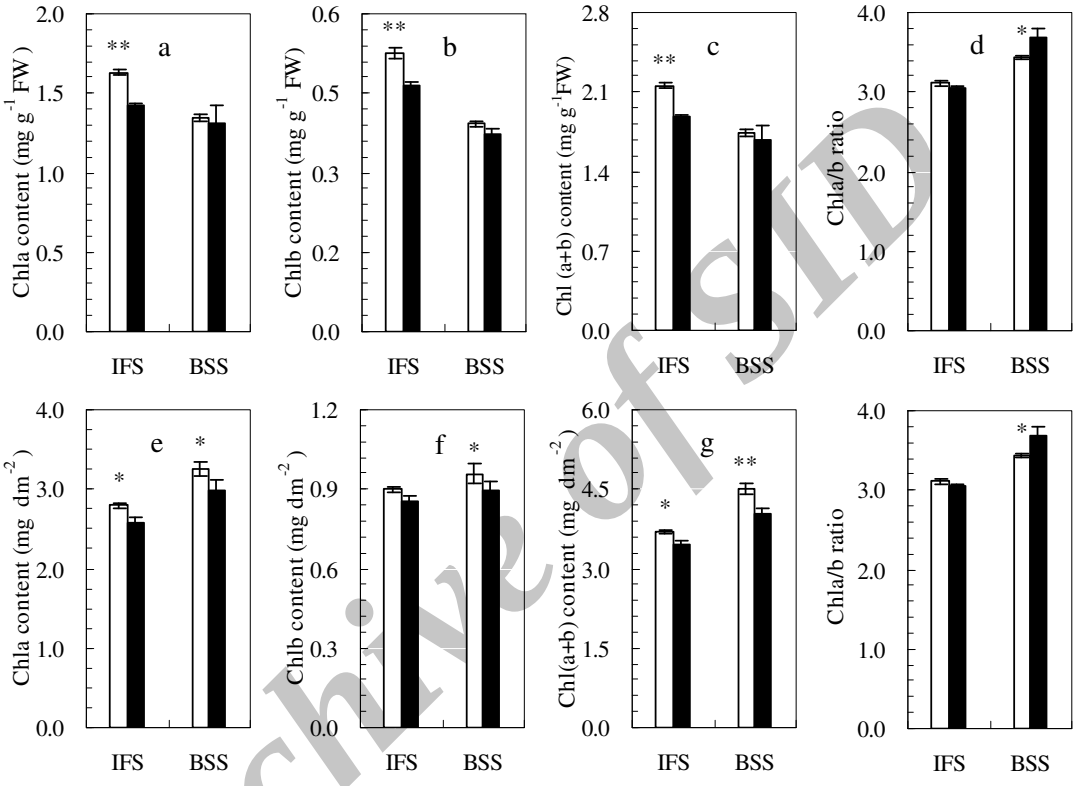

Figure 2. Comparison of chlorophyll content (Chl a, Chl b, Chl, Chl a/b ratio) in functional leaves (Up, expressed on a fresh weight basis; Down, expressed on a leaf area basis) at IFS and BSS between Bt (■) and its non-Bt (□) isogenic lines, Means ± SE, n = 3, *, ** significant at 0.05 and 0.01, respectively.

5.6% higher in the Bt-isogenic line than the non-Bt line (Figure 3 c, d, C, D and i). No significant difference in maximum efficiency of photosystem II photochemistry (F_v/F_m ratio) was observed (Figure 3 a, b, A, B and g).

Minerals, Soluble Sugar and Protein Contents

The Bt-isogenic line exhibited decreases by 8%, 24%, and 8% in B, K, and P at IFS, and decreased by 24%, 12%, 9%, and 16% in Fe, K, S, and Zn concentrations at BSS, respectively, relative to the non-Bt-isogenic line (Table 5). However, the Bt-isogenic line increased by 10%, 32%, 42%, 16%, 12% and 24% in Ca, Cu, Fe, Mg, Mn and Zn concentrations at IFS, and increased Cu and P contents by 15% and 7% at BSS, respectively. Soluble protein contents were 21.6% lower at IFS but 17.2% higher in the Bt-isogenic line than the non-Bt line at BSS (Figure 4b). No significant differences were observed in soluble sugar and N contents

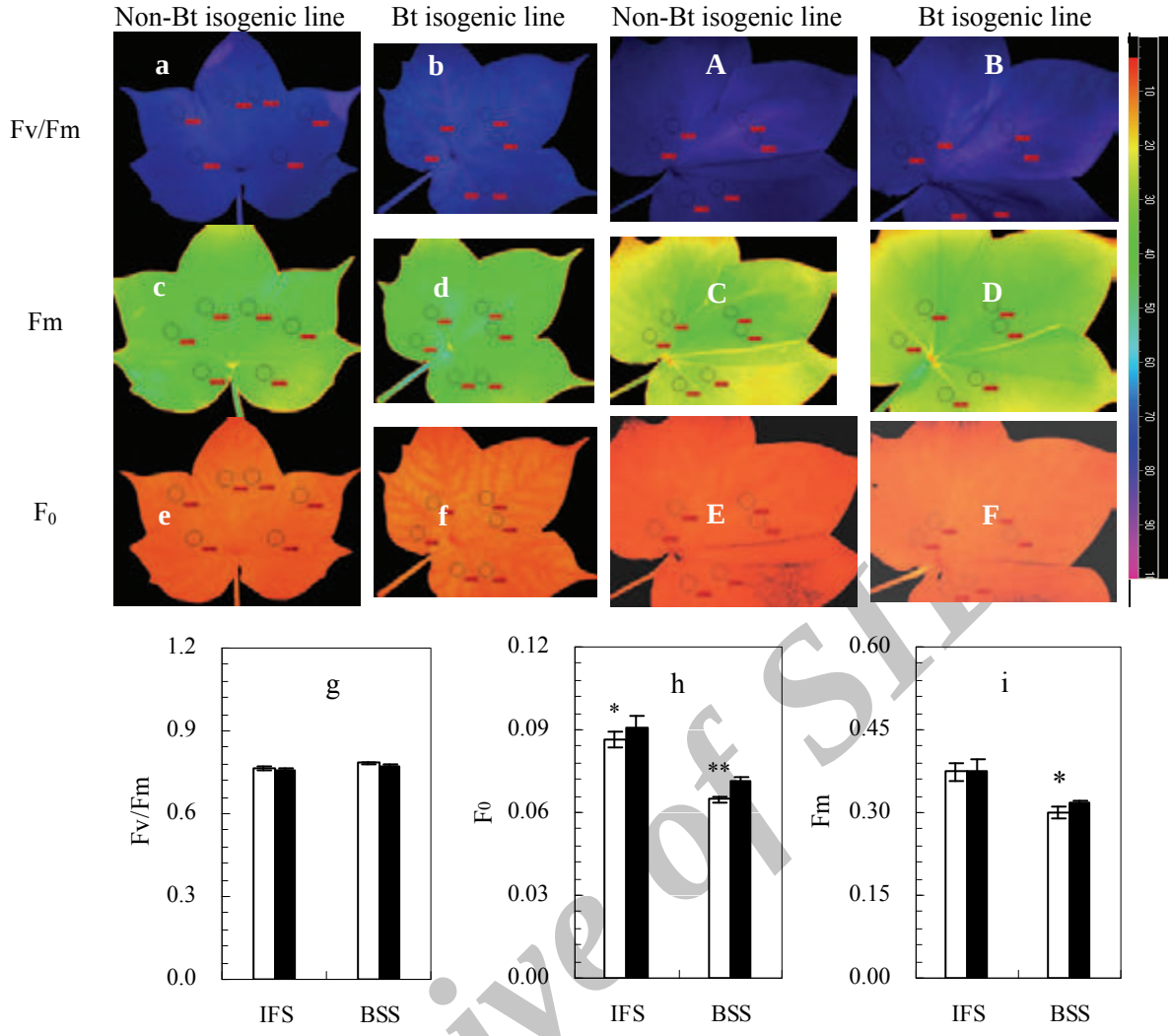

Figure 3. Comparison of chlorophyll fluorescence parameters and emission spectra image showing F_v/F_m, F_m, F_0, at flowering stage (a-f) and boll setting stage (A-F) between Bt isogenic line(■) and its non-Bt isogenic line(□). F_v/F_m, maximal quantum yield of photo system II photochemistry. F_0, minimal fluorescence yield of a dark-adapted leaf. F_m, maximal fluorescence yield of a dark-adapted leaf. The rainbow bar on the right shows the scaling.

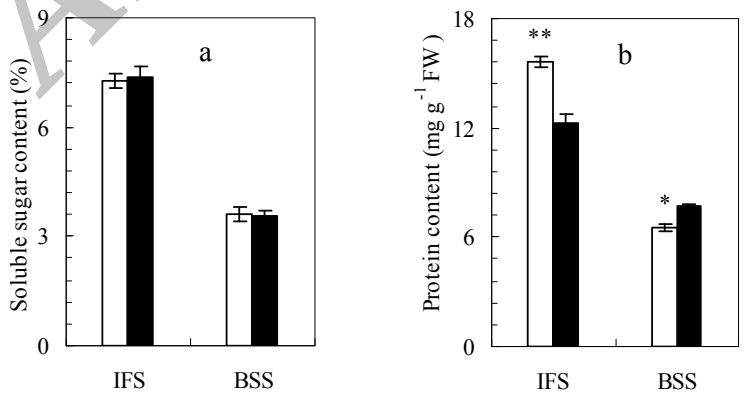

Figure 4. Comparison of soluble sugar and soluble proteins contents in functional leaves between Bt isogenic line (■) and its non-Bt isogenic line (□) Means ± SE, n = 3, *, ** significant at 0.05 and 0.01, respectively.

between the two isogenic lines.

SOD and POD Activities and MDA Contents

The Bt-isogenic line exhibited 6.6% and 9.9% lower SOD activity than the non-Bt line at IFS and BSS, respectively (Figure 5a). No difference in POD activity was observed between the two isogenic lines. Also, a significant difference was found in MDA content levels only at BSS, that of the Bt-isogenic line being 11.9% lower than non-Bt line (Figure 5c).

Proteome Profiles and Differential Proteins

Protein spots, visualized by silver staining (Figure 7), were resolved into approximately 1783 spots (ranging 1589-1920) on 2-DE gels (isoelectric focusing pH range, 4-7; size, 24 cm). Importantly, 20 differentially expressed proteins were identified with high confidence. Their spectra analysis and further protein identification by MS and data bank analysis identified 4 up-regulated proteins (U1-U4) and 16 down-regulated

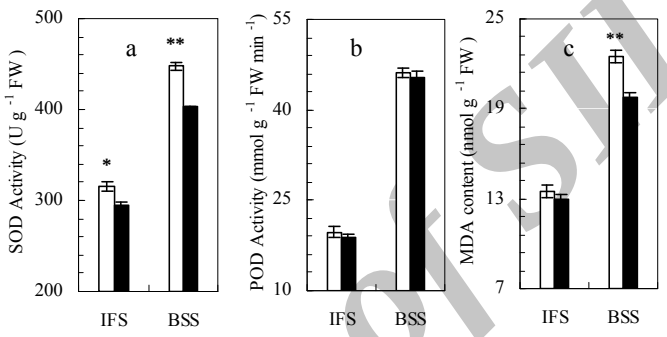

Figure 5. Comparison of SOD, POD activities and MDA content in functional leaves between Bt isogenic line (■) and its non-Bt isogenic line (□). Means ± SE, n = 3

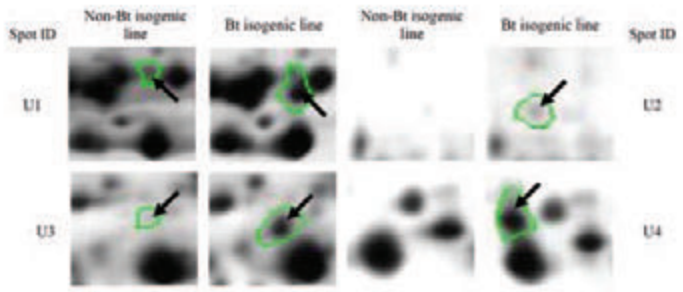

Figure 6. The 'spot view' of proteins up-regulated in Bt isogenic line functional leaves compared to its non-Bt isogenic line at full flowering stage.

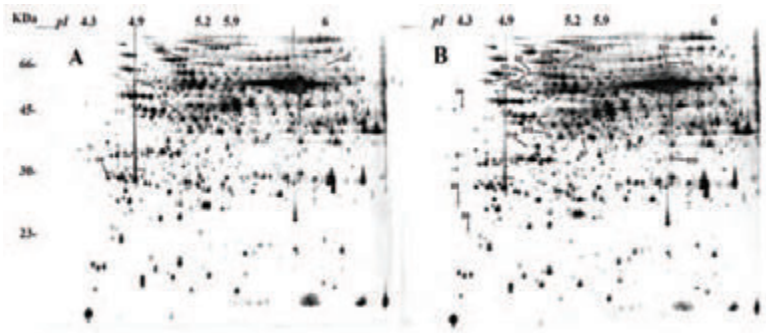

Figure 7. Representative 2-DE maps of proteins extracted from Bt-transgenic glandless cotton (A) and its non-Bt isogenic line (B) functional leaves at full flowering stage. Labeled proteins were found to be up-regulated (U) or down-regulated (D) Bt-transgenic glandless cotton vs. its non-Bt isogenic line and were analyzed by LC-MS/MS analysis.

(D1-D16) in the Bt-isogenic line *vs* the non-Bt line (Tables 3 and 4), and representative images of spot changes are shown in Figures 6 and 8.

DISCUSSION

Comparisons of yields of isogenic Bt and non-Bt lines generally come out in favor of Bt cultivars. For example, Mac Griff *et al*. (2003) reported that lint yields were 99-107% greater in Bt than conventional varieties in 5 years of trials in the USA, during 1998 to 2002. Yield gains of 5.8–10% have been reported in China (Pray *et al.*, 2002). However, in our present study, 21.8% and 26.9% of seed cotton and lint yield losses were recorded in the Bt-isogenic line compared with its non-Bt line (Table 1). It is proved that the advantages of Bt varieties are described as being the consequence of better pest protection, which dramatically reduces boll loss (Ihrig and Mullins, 2001). In addition, it is important that Bt transgenic glandless line with potential use in breeding programs for insect resistance be coupled with the selection of desirable yield component. Cotton yield is particularly affected by insect attack because major pests feed preferentially on the fruiting structures which are normally shed after injury.

Boll distribution patterns may explain the cause of yield differences and are useful for assessing pest damage and crop management effects (Kerby and Buxton, 1981). In this study, boll distributions on plants showed that the Bt-isogenic line produced more bolls at positions 1 and 2 (BN_{1-2}) on fruiting branches but the trend was reversed from position 3 ($BN \geq 3$), with the non-Bt line producing more bolls than the Bt-isogenic

Table 3 . Up-regulated expressed proteins in functional leaves of Bt isogenic line vs. non-Bt isogenic line at full flowering stage.

Spot ID	Protein name	Accession number	MW(Da)	pI	Fold increased (Bt vs non-Bt)	Protein score C. I. %	Amino acid sequence coverage %	Number of peptides matched	Putative function
U1	Rof1 [*Arabidopsis thaliana*]	gi|1354207	61400.4	5.25	3.4	100	22.9	10	Signal transduction
	Rof1 (rotamase fkbp 1) [*Arabidopsis thaliana*]	gi|1865104403	62872.2	5.47		100	21.9	10	Signal transduction
U2	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Gossypium hirsutum*]	gi|912089910	53247.7	6.00	5.1	100	49.2	17	Photosynthetic carbon assimilation
	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Gossypium hirsutum*]	gi|167369	52007.1	6.09		100	49.8	17	Photosynthetic carbon assimilation
U3	Os02g0621700 [*Oryza sativa* (japonica cultivar-group)]	gi|115447367	45062.7	5.98	4.7	100	35.3	11	Catalytic activity, ATP binding and ligase activity
	Hypothetical protein [*Vitis vinifera*]	gi|147782941	45372.8	5.80		100	24.1	7	Unknown classified
U4	Core-neo fusion protein [*Hepatitis C virus replicon* I377/NS2-3'UTR]	gi|5441832	30710.4	4.90	1000000	100	33.5	12	Selectable marker
	Core-neo fusion protein [*Hepatitis C virus replicon* I389/NS2-3'UTR]	gi|5441838	31195.6	4.99		100	65.4	12	Selectable marker

MW: molecular weight ; pI. isoelectric point ; CI%: cross confidence interval %. Over 95% represents high confidence identification.

Table 4 . Down-regulated expressed proteins in functional leaves of Bt isogenic line vs its non-Bt isogenic line at full flowering stage.

Spot ID	Protein name	Accession number	MW(Da)	pI	Fold decreased (Bt vs non-Bt)	Protein score C. I. %	Amino acid sequence coverage %	Number of peptides matched	Putative function
D1	Rof1 (rotamase fkbp1) [*Arabidopsis thaliana*]	gil186510403	62872.2	5.47	1000000	100	23.3	10	Signal transduction
	Rof1 [*Arabidopsis thaliana*]	gil1354207	61400.4	5.24		100	22.9	11	Signal transduction
	Unnamed protein product [*Vitis vinifera*]	gil157332851	64028.6	5.11		100	22.1	10	Unknownin classified
D2	Unnamed protein product [*Vitis vinifera*]	gil157343535	65292	4.93	1.85	100	18.3	9	Unknown classified
	Protein phosphatase 2A [*Nicotiana tabacum*]	gil1568511	65395.1	5.03		97.7	23. 9	9	65kD regulatory subunit
	Hypothetical protein [*Yarrowia lipolytica*]	gil50543746	139225.2	5.82		70.2	18.0	16	Unknown classified
D3	Unnamed protein product [*Vitis vinifera*]	gil157343535	65292	4.93	1.56	100	32.4	12	Unknown classified
	Unknownn [*Populus trichocarpa*]	gil118484366	65546.1	4.92		100	34.1	13	Unknown classified
	Protein phosphatase 2A [*Nicotiana tabacum*]	gil1568511	65395.1	5.03		100	28.7	11	65kD regulatory subunit
D4	Protein phosphatase type 2A regulator [*Arabidopsis thaliana*]	gil15230896	65556.1	5.01	1.99	100	30.7	11	Protein phosphatase type 2A regulator activity
	Unnamed protein product [*Vitis vinifera*]	gil157343535	65292	4.93		100	27.1	10	Unknown classified
	PP2AA2 (protein phosphatase 2A subunit A2); protein phosphatase type 2A regulator [*Arabidopsis thaliana*]	gil79313513	60927.5	5.13		100	23.5	7	Protein phosphatase type 2A regulator activity
	Translationally-controlled tumor protein homolog								Calcium binding and
D5	[*Saccharomyces cerevisiae*]	gi60944439	19026.6	4.37	2.41	100	42.4	5	Microtubule stabilization
	Unknownn [*Populus trichocarpa*]	gil118482407	19055.5	4.46		100	42.9	4	Unknown classified
	Unknownn [*Populus trichocarpa*]	gil118481497	19387.6	4.38		100	41.9	4	Unknown classified
D6	Unnamed protein product [*Vitis vinifera*]	gil157340763	38061.3	4.54	2.05	99.99	18.4	5	Unknown classified
	TGB12K interacting protein 2 [*Nicotiana tabacum*]	gil29826242	37319	4.46		97.9	22.4	5	Mediate protein-protein interactions
	Ankyrin-repeat protein HBP1 [*Nicotiana tabacum*]	gil13310811	37294	4.45		97.9	22.3	5	Mediate protein-protein interactions
D7	Unnamed protein product [*Vitis vinifera*]	gil157336073	37879.2	5.61	1.51	100	27.9	8	Unknown classified
	Replication factor C 37 kDa subunit [*Oryza sativa* (Japonica Group]	gil107988806	37402.3	5.32		100	21.3	5	DNA replication and repair mechanisms
	Os12g0176500 [*Oryza sativa* (japonica cultivar-group)]	gil115487618	41052	5.46		100	23.7	6	Replication factor C small subunit
D8	Unnamed protein product [*Vitis vinifera*]	gil157343779	27826.9	4.52	1000000	100	18.8	3	Unknown classified
	Os07g0614500 [*Oryza sativa* (japonica cultivar-group)] Hypothetical protein Os1_025931 [*Oryza sativa* (indica cultivar-group)]	gil115473331	24847.3	4.36		100	14.0	4	Translation elongation factor activity
		gil125559163	27746.7	4.54		100	21.3	4	Unknown classified

Table 4 . continued

Spot ID	Protein name	Accession number	MW(Da)	pI	Fold decreased (Bt vs non-Bt)	Protein score C. I. %	Amino acid sequence coverage %	Number of peptides matched	Putative function
D9	Putative protein disulfide isomerase [*G.raimondii*]	gi|133902301	55503.1	4.92	1.79	100	27.3	11	Protein folding catalyst and chaperone activity
	Putative protein disulfide isomerase [*G. hirsutum*]	gi|133902323	55534.1	4.95		100	24.8	10	Protein folding catalyst and chaperone activity
	Putative protein disulfide isomerase [*G.arboreum*]	gi|133902308	55571.2	4.97		100	21.0	8	Protein folding catalyst and chaperone activity
D10	Ribulose bisphosphate carboxylase large subunit[*Rosecheria melanochaetes*]	gi|80968346	48335.2	6.23	1000000	100	49.4	13	Photosynthetic carbon assimilation
	Ribulose bisphosphate carboxylase large subunit[*Clinostigma savoryanum*]	gi|80968350	48327.2	6.23		100	41.8	11	Photosynthetic carbon assimilation
	Ribulose bisphosphate carboxylase large subunit[*Calyptrocalyx albertisianus*]	gi|80968334	48311.2	6.23		100	49.7	14	Photosynthetic carbon assimilation
D11	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*G. hirsutum*]	gi|91208910	53247.7	6.00	1000000	100	49.4	18	Photosynthetic carbon assimilation
	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*G. hirsutum*]	gi|167369	52007.1	6.09		100	46.5	17	Photosynthetic carbon assimilation
	Ribulose bisphosphate carboxylase large chain precursor[*G. hirsutum*]	gi|116242747	52667.3	5.91		100	46.5	17	Photosynthetic carbon assimilation
D12	RuBisCO subunit binding-protein beta subunit precursor; chaperonin, 60 kDa [*A. thaliana*]	gi|10178220	63118.3	5.73	2.25	100	30.2	11	ATPase activity
	RuBisCO large subunit-binding protein subunit beta, chloroplast precursor / 60 kDa chaperonin subunit beta [*Pisum sativum*]	gi|25506277	62945.3	5.85		100	30.6	12	ATPase activity
	ATP binding / protein binding / unfolded protein binding [*A. thaliana*]	gi|79537402	63202.2	5.73		100	28.2	10	Protein folding, cellular protein metabolic process
D13	Carbonic anhydrase isoform 2 [*G. hirsutum*]	gi|4754915	34579.7	7.11	2.31	100	58.0	12	Interconversion of CO_2 and HCO_3^-
	Carbonic anhydrase isoform 1 [*G. hirsutum*]	gi|4754913	34831.9	6.94		100	57.5	11	Interconversion of CO_2 and HCO_3^-
	Carbonic anhydrase [*G. hirsutum*]	gi|20502881	35284.0	6.96		100	56.8	12	Interconversion of CO_2 and HCO_3^-
D14	Core-neo fusion protein [*Hepatitis C virus replicon* I377/NS2-3'UTR]	gi|5441832	30710.4	4.92	1.5124	100	33.5	12	Neomycin/kanamycin resistance
	Core-neo fusion protein [*H. C virus replicon* I389/NS2-3'UTR]	gi|5441838	31195.6	4.99		100	65.4	12	Neomycin/kanamycin resistance
	Aminoglycoside-3'-O-phosphotransferase [*Escherichia coli*]	gi|116006943	29029.5	4.64		100	70.5	11	Neomycin/kanamycin resistance
D15	Unnamed protein product [*Vitis vinifera*]	gi|157360513	35799.1	4.99	1.96	100	35.3	9	unknown classified
	Unknown [*Populus trichocarpa*]	gi|118481185	39944.4	5.71		100	22.0	6	Unknown classified
	Unknown [*Picea sitchensis*]	gi|116788802	40750.7	5.35		99.901	16.4	5	Unknown classified
D16	Hypothetical protein [*Vitis vinifera*]	gi|147770841	23473.8	4.93	1.57	100	48.0	9	Unknown classified
	Unknown [*Populus trichocarpa*]	gi|118486174	37858.1	5.78		100	46.5	9	Unknown classified
	Unnamed protein product [*Vitis vinifera*]	gi|157360462	38080.1	5.32		100	36.9	8	Unknown classified

line (Table 1). Non-Bt lines have often produced more fruiting sites over a longer time as described by Stewart *et al*. (2001). Our results suggested that Bt glandless cotton confers a resistance advantage from the onset of fruit development via producing slightly earlier for better fruit retention at the first sites.

The low chlorophyll content in Bt-isogenic line plants (Figure 2) may be associated with a rapid growth of leaf and be attributed to the destruction of chlorophyll pigments and instability of pigment protein complex. Leaf soluble protein is considered to be an indirect estimator of photosynthetic activity (Udayasoorian and Prabakaran, 2010). The Bt-isogenic line showed a significantly lower soluble protein at IFS, but higher at BSS (Figure 4) when compared with the non-Bt line, which may indicate a more rapid decrease in Pn as plants matured, and reflected more bolls at BN_{1-2} (Table 1) in the Bt-isogenic line than the non-Bt one.

Cotton has a high requirement for K, and K deficiency can dramatically reduce lint yield and fiber quality. In our study, the Bt-isogenic line displayed lower accumulation of many important mineral nutrients such as K, B, Fe, Zn and S (Table 5). This could have a negative impact on growth and development of transgenic glandless cotton, requiring further research work. As a major scavenger, SOD catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen, for protecting cells against oxidative damage. Less membrane damage was evident by 12% lower MDA content in the Bt-isogenic line indicating that the insect-resistant cultivar had a higher capacity for decomposition of H_2O_2 generated by biotic and abiotic stresses in comparison with the non-Bt line. However, further investigation of antioxidant defense system response to Bt insertion is needed to gain a deeper insight.

The 2DE/MS method was used to investigate proteomic responses to Bt gene insertion and further explore the mechanisms underlying Bt-specific and anti-insect

Table 5. Mineral nutrients contents in functional leaves of Bt and non-Bt isogenic lines.at initial flowering stage (IFS) and boll setting stage (BSS).

Growth stage	NILs	Macroelements (g kg^{-1} DW)						Microelements (mg kg^{-1} DW)				
		N	P	K	Mg	Ca	S	Fe	B	Zn	Cu	Mn
IFS	non-BT isogenic line	33.36	5.93*	10.58*	3.90**	14.81*	8.11	123.0*	22.74*	54.28*	7.71**	97.72*
	BT isogenic line	31.55	5.64	9.73	4.53	16.31	8.48	174.83	20.90	67.22	10.18	109.01
BSS	non-BT isogenic line	33.7	2.36*	10.64*	7.67	29.821	10.14**	227.67**	36.80	93.17**	5.57*	124.79
	BT isogenic line	32.76	2.52	9.40	7.87	29.89	8.57	173.66	35.33	41.43	6.39	126.25

* and **, significant at 0.05 and 0.01 levels, respectively (n=3).

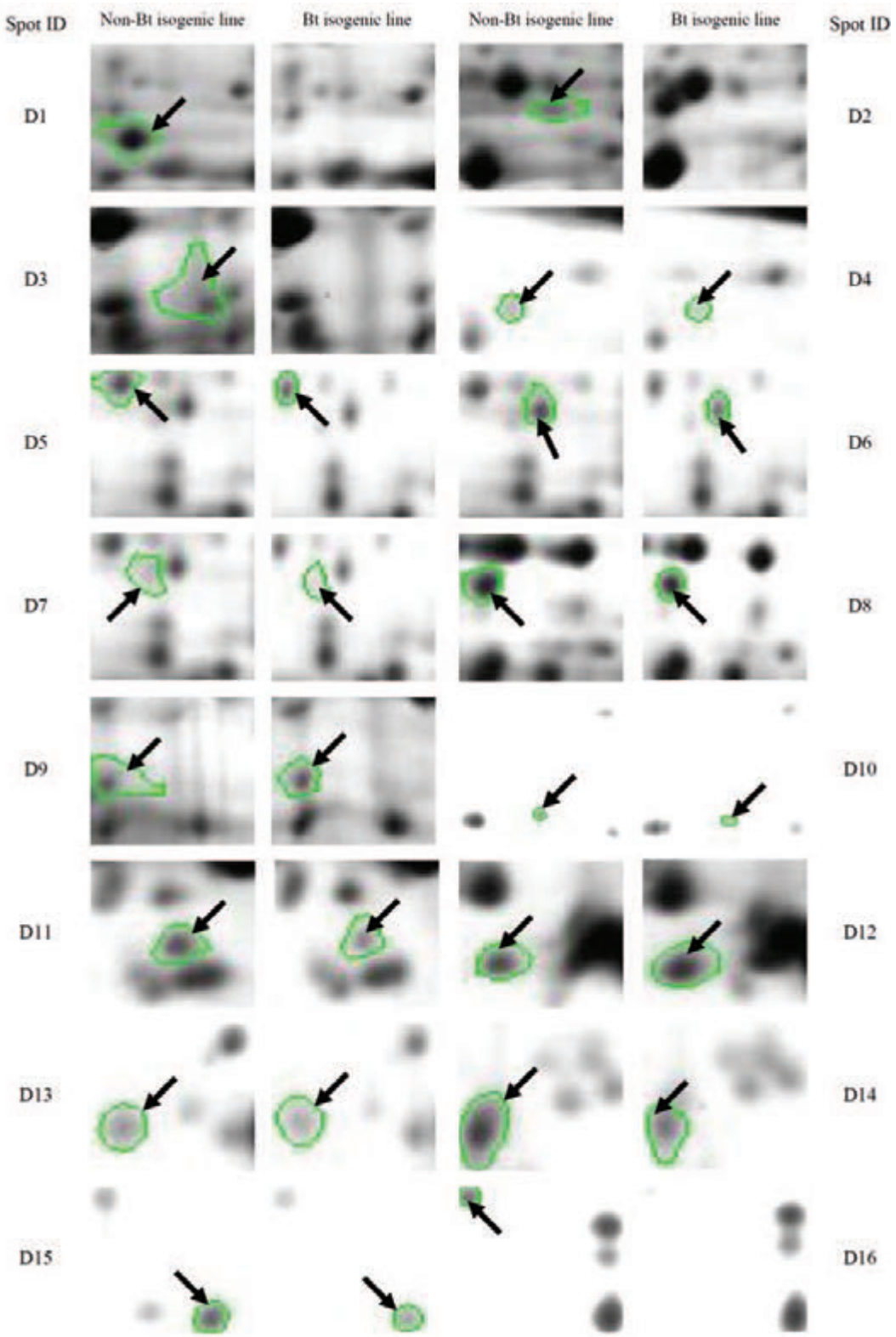

Figure 8. The 'spot view' of proteins down-regulated proteins in Bt-transgenic glandless cotton near isogenic line functional leaves compared to its non-Bt isogenic line at full flowering stage.

strategies. Four up-regulated protein spots were identified in the Bt *vs*. the non-Bt line: a core-neo fusion protein, appeared to be specific to cotton antibiotic binding site previously reported as a selectable marker. The up-regulation of core-neo fusion protein in the Bt-isogenic line highlighted the importance of its immune response against external and environmental conditions during different growth stages. Indeed, MS analysis revealed that many distinct protein spots shared the same protein identity. For example, rof1, signal transduction-like protein was identified in spots U1 and D1; ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit was found in spots U2 and D11; core-neo fusion protein was found in spots U4 and D14, accordingly**.**

RuBisco, the most abundant leaf protein, is degraded and the nitrogen is remobilized during leaf senescence to support the plant function. A prominent effect observed in the present study was the drastic reduction (up to 1000000 fold) of the large subunit of RuBisCO at spots D10-D13 which may explain lower yield of the Bt than the non-Bt line, although U2 demonstrated an up-regulation of RuBisco in the Bt-isogenic line. Carbonic anhydrase (CA EC.4.2.1.1) are zinc-containing enzymes that catalyze the reversible hydration/dehydration of carbon dioxide/bicarbonate, and thus participate in a variety of biological processes that include acid-base balance, CO_2 transfer and ion exchange (Serrano *et al.*, 2007). The 2.3-fold decrease in carbonic anhydrase protein content (D13, Table 4) of the Bt-isogenic line in comparison with the non-Bt line may be due to the fact that CA is a good characteristic of resistance to biotic stresses. Moreover, D15 and D16 protein spots still could not be identified because no detailed annotations were found in databases. This is an inevitable disadvantage for proteomic analysis in species whose genomes have not been fully sequenced (Yang *et al.,* 2008). However, to some extent, their unique 2-DE locations and PMFs were annotated, thus it will be helpful in the identification of these unknown proteins in future studies.

ACKNOWLEDGEMENTS

This work was financially supported by the National R & D Project of Transgenic Crops of Ministry of Science and Technology of China (Major Program, 2009ZXD8001-027B).

REFERENCES

1. Bah, M. A., Sun ,H., Chen, F., Zhou, J., Dai, H., Zhang, G. P. and Wu, F. B. 2010. Comparative Proteomic Analysis of *Typha angustifolia* Leaf under Chromium, Cadmium and Lead Stress. *J. Hazard. Mater*. **184**: 191-203.
2. Cai, Y., Cao, F., Cheng, W., Zhang, G. P. and Wu, F. B. 2011. Modulation of Exogenous Glutathione in Phytochelatins and Photosynthetic Performance against Cd Stress in the Two Rice Genotypes differing in Cd tolerance. *Biol. Trace Elem. Res*. **143**: 1159-1173.
3. Carpentier, S. C., Witters, E., Laukens, K., Deckers, P., Swennen, R. and Panis, B. 2005. Preparation of protein extracts from recalcitrant plant tissues: An Evaluation of Different Methods for Two-Dimensional Gel Electrophoresis Analysis. *Proteomics* **5**: 2497-2507.
4. Foster, D. J., Verhalen, L. M. and Murray, D. S. 1994. Prometryn Tolerance in Glanded Versus Glandless Isolines of Cotton. *Crop Sci*. **34**: 67-71.
5. Gaufichon, L., Reisdorf-Cren, M., Rothstein, S. J., Chardon, F. and Suzuki, A. 2010. Biological Functions of Asparagine Synthetase in Plants. *Plant Sci*. **179**: 141-153.
6. Gerasimidis, K., Fillou, D. T., Babatzimopoulou, M., Tassou, K. and Katsikas, H. 2007. Preparation of an Edible Cottonseed Protein Concentrate and Evaluation of its Functional Properties. *Int. J. Food Sci. Nutr*. **58**: 486-490.
7. Hao, J. J., Liu, H. M. and Ma Q. X. 2011.Genetic Effects and Diagnosis of Premature Senescence of Leaf in Upland Cotton. *Acta Agronomica Sinica*. **37**: 389-396.
8. Ihrig, R. A. and Mullins, W. 2001. A Case of Subthreshold Advantages of Bollgard® Cotton C. P. Dugger, D. A. Richter (Eds.), Proc. Belt. Cot. Prod. Conf., Anaheim, CA, 9–13 January, National Cotton Council, Memphis, TN (CD-Rom computer file).
9. Kerby, T. A., Buxton, D. R. 1981. Competition between Adjacent Fruiting Forms in Cotton. *Agron J* ., **73**: 867-871.
10. Mac Griff, E., Hudgins, J., May, M., Brown, S. M. and May, L. 2003. May Evaluation of Transgenic Versus Conventional Cotton Varieties (1998–2002) D. A. Richter (Ed.), Proc. Belt. Cot. Prod. Conf. Nashville, TN, 6–10 January, National Cotton Council, Memphis, TN (CD-Rom computer file).

11. Mao, S. C. 2010. Report on China's Cotton Production Prosperity 2009. China Agriculture Press, Beijing, China.
12. Perlak, F. J., Oppenhuizen, M., Gustafson, K., Voth, R., Sivasupramaniam, S., Heering, D., Carey, B., Ihrig, R. A. Roberts, J. K. 2001. Development and Commercial Use of Bollgard (R) Cotton in the USA - Early Promises versus Today's Reality. *Plant J*. **27**: 489-501.
13. Pray, C. E., Huang, J., Hu, R. and Rozelle, S. 2002. Five Years of Bt Cotton in China - the Benefits Continue. *Plant J*. **31**: 423-430.
14. Serrano, L., Halanych, K. M. and Henry, R. P. 2007. Salinity-stimulated Changes in Expression and Activity of Two Carbonic Anhydrase Isoforms in the Blue Crab *Callinectes sapidus*. *J. Exp. Bot.* **210:** 2320-2332.
15. Stewart S., Layton D. M., Williams B. M., Ingram R. and Maily D. W. 2001. Response of cotton Plant to Pre-bloom Square Loss. *J. Econ. Entomol*. **94**: 388-396.
16. Tang, Q. Y. and Feng, M. G. 1997. Practical Statistics and Its Data Processing System Statistical Software Package. China Agriculture Press, Beijing, China.
17. Udayasoorian, C. and Prabakaran, C. 2010. Effect of Fertigation on Leaf Proline, Soluble Protein and Enzymatic Activity of Banana. *EJEAF. Che*. **9**:1404-1414.
18. Wu, F. B., Zhang, G. P. and Dominy, P. 2003. Four Barley Genotypes Respond Differently to Cadmium: Lipid Peroxidation and Activities of Antioxidant Capacity. *Environ. Exp. Bot*, **50**: 67-78.
19. Yang, Y. W., Bian, S. M., Yao, Y. and Liu, J. Y. 2008. Comparative Proteomic Analysis Provides New Insights into the Fiber Elongating Process in Cotton. *J. Proteome Res*. **7**: 4623-4637.
20. Yu, S. X., Song, M. Z., Fan, S. L., Wang, W. and Yuan, R. Y. 2005. Biochemical Genetics of Short-season Cotton Cultivars that Express Early Maturity without Senescence. *J. Integr. Plant Biol*. **47**: 334-342.
21. Zhang, X. Z. 1992. Research Methodology of Crop Physiology. Agriculture Press, Beijing, 148-152, 156-157.
22. Zhu, S. J. and Chen, J. X. 2005. Advances in Research on Genetic Analysis of Gossypol Gland and Molecular Breeding of Low-gossypol Cotton. *Chin. Agric. Sci. Bull*. **21**: 57-60.

بررسی خصوصیات زراعی، فیزیولوژیکی و بیان پروتئوم لاین های ایزوژنیک پنبه بدون غده Bt و غیر Bt

ژ. ه. چن، ز. ک. جین، ز. ه. چن، ش. ج. ژو و ف. ب. وو

چکیده

در این تحقیق صفات زراعی و فیزیولوژیکی و بیان پروتئین بین واریته غیرBt پنبه بدون غده مناطق مرتفع با نام Zhong5629 و لاین های ایزوژنیک متفاوت در ژن cryIAc با هم مقایسه شدند. نتایج نشان دادند که لاین Bt ایزوژنیک نسبت به نوع غیرBt آن، ارتفاع بوته و نرخ غوزه موثر و تعداد غوزه داخلی بیشتر اما عملکرد پایین تری داشت. لاین Bt ایزوژنیک مقدار کلروفیل و نرخ تعرق کمتری نسبت به لاین غیر BT داشت، اما مقدار Chl a/b، F_0 و F_m آن بالاتر بود. محتوای پروتئین محلول در لاین Bt ایزوژنیک به طور قابل توجهی در مرحله تشکیل غوزه (BSS)، بالاتر اما در مرحله گلدهی اولیه (IFS) در مقایسه با لاین غیر Bt کمتر بود. در لاین Bt ایزوژنیک به شکل معناداری غلظت کلسیم، منیزیم، مس، روی، منگنز و آهن در IFS ، و فسفر و مس در BSS، بالاتر بود در حالی که غلظت پتاسیم، فسفر و بور در IFS و پتاسیم، گوگرد، روی و آهن در BSS پایین تر بود. لاین Bt ایزوژنیک مقدار کمتری مالون دی آلدئید در BSS و فعالیت کمتر سوپراکسید دیسموتاز در IFS و BSS را از خود نشان داد. علاوه بر این، تحلیل پروتئوم این دو NIL ۲۰ پروتئین متفاوت را شناسایی نمود. ۴ پروتئین با بیان بیشتر در لاین Bt ایزوژنیک نسبت به لاین غیر Bt به هدایت سیگنال ، انرژی سوخت و ساز و پاسخ دفاعی نسبت داده شدند، در حالی که ۱۶ پروتئین با بیان کمتر به هدایت سیگنال و سوخت و ساز پروتئین نسبت داده شدند.