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RESEARCH ARTICLE

Effects of glyphosate on soybean metabolism in strains bred for glyphosate-resistance

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Abstract To produce high quality, glyphosate-resistant soybeans, we crossed Jinda 73 and glyphosate-resistant RR1 (Roundup Ready First Generation) (RR1) resulting in 34 hybrid strains. To determine the effects of glyphosate on soybean metabolism, we grew the two parents upto the seedling stage, and measured chlorophyll, soluble sugar, malondialdehyde (MDA), relative conductivity and proline. Then, we treated the plants with glyphosate and measured the same factors again. Results showed that the chlorophyll content of Jinda 73 and RR1 decreased after spraying glyphosate. Glyphosate increased the level of soluble sugar, MDA, relative conductivity and proline in Jinda 73, but had no significant effect on RR1. We determined glyphosate resistance of the parents and the 34 hybrid, offspring strains by documenting the growth response in the field after treatment with glyphosate. Results showed that 29 hybrid, offspring strains have complete glyphosate resistance. Polymerase chain reaction (PCR) shows that the strains which have complete resistance to glyphosate have imported the CP4 5-enolpyhruvylshikimate-3- phosphate synthase (*CP4 EPSPS)* gene successfully. We selected three high quality, glyphosate-resistant strains (F_7-3, F_7-16 and F_7-21), which had higher protein and oil levels as compared with Jinda 73.

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Introduction

Soybean has high nutritional value, is rich in protein, fat and trace elements and contains all nine essential amino acids. Weeds growing in soybean fields compete with soybeans for sunlight, water and nutrients, therefore agricultural herbicides are widely used to control weeds (Dill 2005). The most commonly used herbicide is the organophosphate (Bernal et al. 2012). Glyphosate, as one of the most popular organophosphate, effectively kills annual and perennial grasses, trees, shrubs and broadleaf weeds (Dun et al. 2007; Healy-Fried et al. 2007). Glyphosate is adsorbed to soil particles where it is digested by various microbes (Pessagno et al. 2008; Yu et al. 2005; Zelaya et al. 2011), therefore it is inactive or low-active in the soil (Cerdeira and Duke 2006), and is safe for people and animals (Anton et al. 1993). Since glyphosate kills broadleaf plants (Dill et al. 2008), it kills weeds as well as soybeans or other plants with no glyphosate resistance (Gimsing et al. 2004; Pessagno et al. 2008; Wang et al. 2004). Glyphosate treatment reduces soybean yield (Gianessi 2005; Jiang et al. 2012; Schonbrunn et al. 2001; Xu et al. 2003), therefore breeding soybeans which are resistant to glyphosate and also produce high quality harvests is an important goal for soybean breeding (Coalova et al. 2014; Haberhauer et al. 2000; Pipke and Amrhein 1988; Wan et al. 1989).

Soybeans that are resistant to glyphosate survive after treatment with glyphosate because of genetic modification for 5-enolpyhruvylshikimate-3- phosphate synthase (EPSPS), the target enzyme of glyphosate. Glyphosate

targets the shikimate pathway enzyme, EPSPS, which is essential for plant life because it is necessary for the synthesis of phenylalanine, tryptophan and tyrosine. Plants without the genetic modification die when treated with glyphosate (Shinabarger and Braymer 1986; Xu et al. 2003). Glyphosate resistant plants have a modification that makes this enzyme insensitive to glyphosate (Funke et al. 2009). In the United States, Monsanto introduced Roundup Ready (RR) transgenic soybeans in 1994 and commercial planting began in 1996 (Clive 2007). In RR transgenic soybeans, the cauliflower mosaic virus (CaMV) 35 S promoter and nopaline synthase (NOS) terminator are inserted in the soybean (*Glycine max*) genome as a plamid, along with the modified *CP4 EPSPS* gene (Ow et al. 1986; Smart et al. 1985). In soybeans, glyphosate resistance can be determined by detection of the *CP4 EPSPS* gene, CaMV35 promoter and NOS terminator (Zhu et al. 2005).

Studies have shown that in drought conditions the activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in first generation RR plants are altered (Yuan et al. 2010), which indicates that RR1 soybeans have poor resistance for drought. In other studies, results have shown that glyphosate affected photosynthesis and biomass production in RR1 and RR2 soybeans (Zobiole et al. 2010). If glyphosate-resistant soybean varieties are crossed with regionally superior, drought-resistance, and high quality varieties (Dhir and Widholm 1992), the hybrid, offspring soybeans should be high quality and herbicide resistant (Crockett et al. 2000). In order to produce soybeans which have glyphosate-resistance, resistance for drought, and high protein and oil content, we crossed a glyphosate-resistant RR1 with Jinda 73, which has good drought resistance (Zhang and Li 2005) and high protein content.

Glyphosate has a significant detrimental effect on many physiological and biochemical processes. Glyphosate reduces the efficiency of photosynthesis, increases the degradation of chlorophyll, inhibits chlorophyll function, the synthesis of carotene, iron reductase activity, auxin transduction, and increases auxin oxidation (Gomes et al. 2014; Ozturk et al. 2008; Vivancos et al. 2011). In this study, we measured the effect of glyphosate on Jinda 73 and RR1 by measuring chlorophyll content, soluble sugar, malondialdehyde (MDA), relative conductivity and proline content before and after treatment with glyphosate, factors which can reflect the influence of glyphosate on photosynthesis and plant resistance to stress. We also measured glyphosate resistance in the field, detected the glyphosate resistance gene, and measured protein and oil content in 34 hybrid, offspring strains. The purpose of the study was to cross glyphosate resistant soybeans with high protein soybeans, to produce strains that are resistant to glyphosate, and have high protein content.

Materials and methods

Materials

We used Jinda 73 as the female parent and a first generation glyphosate-resistant soybean, RR1, as the male parent. We crossed the parents, and obtained 34 hybrid strains of the F_7 generation. When plants reached seedling stage, we treated half of each parent group with 1% glyphosate, and measured physiological factors in treatment and control groups.

Field treatment with glyphosate

Research was conducted at Shanxi Agricultural University in Jinzhong, Shanxi, China (37°4′N, 112°6′E). All cultivars of each experiment were produced in a common environment in May 6, 2016. We divided a field into three districts using a randomized block design. We planted Jinda 73, RR1 and their 34 hybrid strains of the F_7 generation in a randomized block of three replications. Each block was designed with 2 rows, a length of 5 m and a width of 65 cm for each row, and a space of 15 cm between each plant. The soil of the block is a type of loam soil with moderately loose, good water and fertilizer retention capacity. Previously, this field was a bare earth (flat dirt, nothing planted). During soybeans growing, the field was irrigated regularly. We treated one district with 1% glyphosate, the second district served as a control with no treatment and the third district was used to measure physiological indexes. A week after spraying, plants from all districts were observed for glyphosate resistance by measuring plant survival rate.

The determination of chlorophyll

We collected the first trifoliolate leaves at the vegetative-1 (V1) stage, ground them and extracted chlorophyll using ethanol. We determined chlorophyll levels at 663 and 645 nm absorbance values. The concentration of chlorophyll a was: 12.7*OD663 and 2.69*OD645 and the concentration of chlorophyll b was: 4.68*OD663 and 22.9*OD645. The seeds of the soybeans were collected to determine the content of glyphosate and AMPA.

The determination of soluble sugar

We collected the first trifoliolate leaves at the V1 stage and extracted the soluble sugar in a boiling water bath. The absorbance of the sample was determined at 620 nm. We calculated soluble sugar of one mL of solution using the equation: soluble sugar (μg) × the volume of extract

(mL) × the dilution ratio/[the volume of the sample fluid (mL) × the sample weight (g) × 10^6] × 100.

The determination of MDA content

We collected the first trifoliolate leaves at the V1 stage and ground them in the presence of trichloroacetic acid to homogenize the samples. Then we transferred liquid and the residues in the mortar to a 10 mL centrifuge tube. The liquid in the centrifuge tube was centrifuged at a speed of 3000 r/s for 10 min. The absorbance values of MDA are 532 nm and 600 nm and were determined with 0.6% thiobarbituric acid (TBA) as a reference (Botsoglou et al. 1994). We calculated the concentration of MDA using the equation: MDA quality molar concentration (nmol/g) = (A532–A600) × VT × V1/(0.155 × W×V2). VT: the total volume of the reaction liquid (4 mL). V1: the volume of extract (10 mL). W: fresh weight of sampled (g). V2: the volume of the determined extract (2 mL).

The determination of relative conductivity

We collected the first trifoliolate leaves at the V1 stage and ground them to extract leaf filtrate. We determined the relative conductivity before and after boiling using an HQ14d electrical conductivity meter (HACH, USA). We calculated relative conductivity using the equation: relative conductivity (100%) = the conductivity before boiling/the conductivity after boiling × 100%.

The determination of proline concentration

The proline concentration of proline was determined according to the previous study (Abraham et al. 2010). We collected the first trifoliolate leaves at the V1 stage, measured 100 mg for each reaction and snap froze in liquid nitrogen. We added 3% sulfosalicylic acid solution (5 µL/mg fresh weight) and ground the plant material. We centrifuged the tubes at 3000 r/s for 10 min. In a separate tube, we prepared the reaction mixture: 100 µL of 3% sulfosalicylic acid, 200 µL glacial acetic acid, 200 µL acidic ninhydrin. To this we added 100 µL of the supernatant of the plant extract and mixed well. We incubated the tubes at 96 °C for 1 h and terminated the reaction by placing the tubes on ice. We then added one mL toluene to the reaction mixture to facilitate extraction of proline. We centrifuged the samples for 20 s, and left on the bench for 5 min to facilitate separation of organic and water phases. We removed the chromophore containing toluene to a fresh tube and measured absorbance at 520 nm using toluene as a reference.

Detection analysis for genetic modification

We collected plant leaves from each of the 34 hybrid strains when the soybeans reached seedling stage, for DNA extraction. The primer sequences, names, targets, lengths and Genebank numbers are listed in Table 1. We attempted to detect soybean agglutinin (lectin), the CaMV35S promoter, *CP4 EPSPS* and the NOS terminator.

The determination of protein and oil content

The male parent, RR1, female parent, Jinda73, and the 34 strains of the F_7 generation were planted in a field. All soybean seeds were harvested at natural maturity. We used the Infratec ™ 1241 Grain Analyzer V5.00 quality analyzer (FOSS North America, Eden Prairie, MN, USA) to determine the content of protein and fat. We selected three samples randomly in every district of the field for three replicates.

The determination of glyphosate and aminomethyl phosphoric acid (AMPA)

We collected soybean seeds at natural maturity and determined the content of glyphosate and AMPA in Jinda 73, RR1, and their 34 hybrid, offspring strains in the F_7 generation. We measured the content of glyphosate and AMPA using high performance liquid chromatography (HPLC) according to the method described by Zhang et al. (Zhang et al. 2013). In brief, the soybean seeds were homogenized in ultrapure water and methylene chloride, and then 8 mL of the homogenate was centrifuged for 10 min at 9000 r/min. The upper water layer of centrifuge tubes was transferred and mixed with 0.5 mL of acidity regulator (potassium di-hydrogen phosphate, hydrochloric acid and methanol). After fully mixing, the solution was purified with cation exchange resin, rinsed by eluent, and dried at 60 °C. The obtained solid was reconstituted in ultrapure water, mixed with buffer solution of pH 10.5, derivatised with DPCS-Cl for 25 min at 70 °C, filtered through a 0.22 µm membrane filter and then injected on HPLC. The fluorescence intensities were monitored at excitation and emission wavelengths of 318 nm and 440 nm, respectively.

Statistical analyses

We performed statistical analyses using SPSS version 20.0 (IBM Corporation, New York, NY, USA). The results were expressed as mean ± standard deviation (SD). Student's *t* test was used to compare means between two groups. Analysis of variance (ANOVA) was used to compare

Table 1 Primer sequence and length of product

Detect target	Primer name	Primer sequence	Product length (bp)	Genebank number
CaMV35S promoter	35S-1	5′TCATCCCTTACGTCAGTGGAG3′	165	I08076
	35S-2	5′CCATCATTGCGATAAAAGAAA3′		
NOS terminator	NOS-1	5′GAATCCTGTTGCCGGTCTTG3′	180	I08076
	NOS-2	5′TTATCCTAGTTTGCGCGCTA3′		
CP4-EPSPS	CP4-EPSPS-1	5′GCAAATCCTCTGGCCTTTCC3′	146	I43998
	CP4-EPSPS-2	5′CTTGCCCGTATTGATGACGTC3′		
Agglutinin	Lectin-1	5′CTTCGCCGCTTCCTTCAAC3′	436	K00821
	Lectin-2	5′GAGTCCCGTGGCAGCAGAG3′		

values in more than two groups. A p value < 0.05 was considered statistically significant.

Results

The effect of glyphosate on the content of chlorophyll in Jinda 73 and RR1

The concentration of total chlorophyll is the sum of the concentration of chlorophyll a and chlorophyll b. The content of chlorophyll a, b and a + b continually decreased in Jinda 73 leaves after glyphosate treatment from the first day of treatment as compared to the control group of Jinda 73 which received no glyphosate treatment. The difference was statistically significant between groups from day three after treatment to day five after treatment ($p < 0.05$, Fig. 1). At day five, the content of chlorophyll a, b and a + b had decreased to 1.0667, 0.3191, 1.3858 mg/g, respectively. At day six, the leaves of Jinda 73 treated with glyphosate were dry, bleached and dead, which indicated that glyphosate had significantly damaged chlorophyll.

The content of chlorophyll a, b and a + b declined in RR1 treated with glyphosate as compared to the RR1 control. The difference was statistically significant from day three to day 18 (all $p < 0.05$, Fig. 1). However after 18 days, the content of chlorophyll began to increase. From 19 to 30 days, there was no statistically significant difference between the content of chlorophyll a, b and a + b between the RR1 plants treated with glyphosate and RR1 control ($p > 0.05$, Fig. 1).

The effect of glyphosate on the soluble sugar content of Jinda 73 and RR1

In Fig. 2a, the results show that the content of soluble sugar in Jinda 73 treated with glyphosate increased from day one to day five. The difference between the treatment and the control groups were statistically significant from day three to day five ($p < 0.01$, Fig. 2a). At day six, the leaves of Jinda 73 treated with glyphosate were dry, bleached and dead. There was no statistically significant difference in the content of soluble sugar between the RR1 plants treated with glyphosate and the RR1 control plants ($p > 0.05$).

The effects of glyphosate on MDA content in Jinda 73 and RR1

As shown in Fig. 2b, the content of MDA in Jinda 73 treated with glyphosate increased from day one to day five and the difference was statistically significant from day three to day five compared to Jinda 73 control ($p < 0.01$, Fig. 2b). At day six, the leaves of Jinda 73 treated with glyphosate were dry, bleached and dead. There was no statistically significant difference in the content of MDA between the RR1 group treated with glyphosate and the RR1 control from day one to day six ($p > 0.05$).

The effect of glyphosate on relative conductivity in Jinda 73 and RR1

We determined the effect of glyphosate on plants by measuring the relative conductivity. As shown in Fig. 2c, the relative conductivity in Jinda 73 treated with glyphosate increased from day one to day five and the differences were statistically significant from day three to day five as compared to the untreated control Jinda 73 plants ($p < 0.01$, Fig. 2c). The relative conductivity increased from approximately 20% at day one to 75.47% at day five. At day six, the leaves of Jinda 73 treated with glyphosate were dry, bleached and dead. There was no statistically significant difference in relative conductivity between the RR1 plants treated with glyphosate and the RR1 control group from day one to day six ($p > 0.05$).

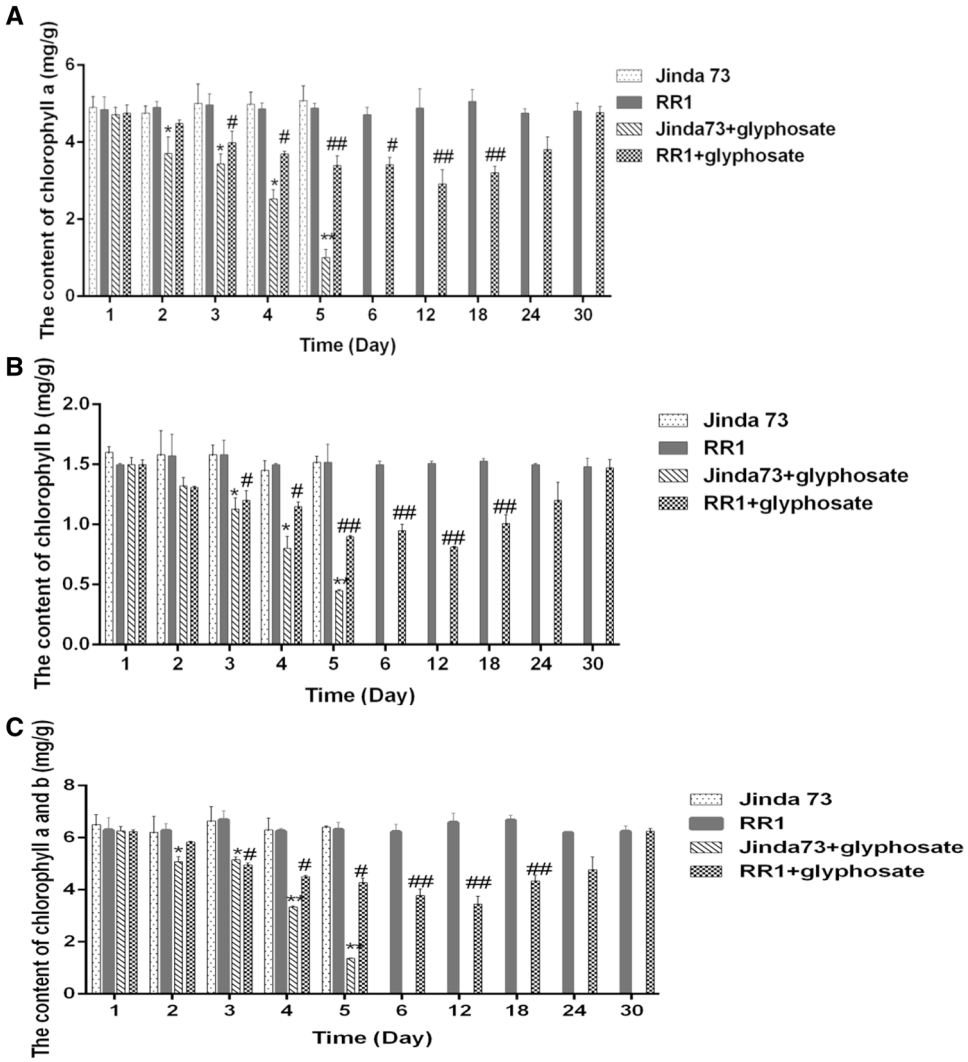

Fig. 1 The determination results of chlorophyll content after treating with glyphosate. The content of chlorophyll a, chlorophyll b, and chlorophyll a + b detected for Jinda 73 and RR1. **a** The content of chlorophyll a after treating with glyphosate from 1 to 30 days. **b** The content of chlorophyll b after treating with glyphosate from 1 to 30 days. **c** The total content of chlorophyll a and b from 1 to 30 days. $^{*}p < 0.05$ compared to Jinda 73 groups. $^{**}p < 0.01$ compared to Jinda 73 groups. $^{\#}p < 0.05$ compared to RR1 groups. $^{\#\#}p < 0.01$ compared to RR1 groups

The effect of glyphosate on proline content in Jinda 73 and RR1

Our results show that the content of proline in Jinda 73 treated with glyphosate increased from day one to day five and the differences were statistically significant from day three to day five as compared to Jinda 73 plants in the untreated control group ($p < 0.01$, Fig. 2d). The concentration of proline reached 283.0126 μg/g at day five from approximately 25 μg/g at day one. At day six, the leaves of Jinda 73 treated with glyphosate were dry, bleached and dead. There was no statistically significant difference in the content of proline between the RR1 plants treated with glyphosate and the RR1 untreated control group from day one to day six ($p > 0.05$).

Glyphosate resistance of parents and 34 hybrids

The glyphosate resistance of the parents and the 34 hybrids were observed in the field. Glyphosate resistance was defined as plant survival rate. The results showed that RR1 (P_m-33) is a glyphosate resistant strain and Jinda 73 (P_f-34) has no glyphosate resistance. Among the 34 hybrids, 29 strains have glyphosate resistance as shown in Table 2.

PCR detection results of glyphosate resistant gene

To detect the glyphosate resistant gene in the 34 offspring and both parents, we performed PCR. To determine whether the extraction of the genomic DNA was successful, we performed PCR amplification on the lectin gene as a control in each of the 36 samples. Figure 3a shows a relatively clear band at 436 bp in all samples. The PCR amplification results for the CaMV35S promoter gene shown in Fig. 3b indicates that 32 stains, including male parent RR1 (P_m-

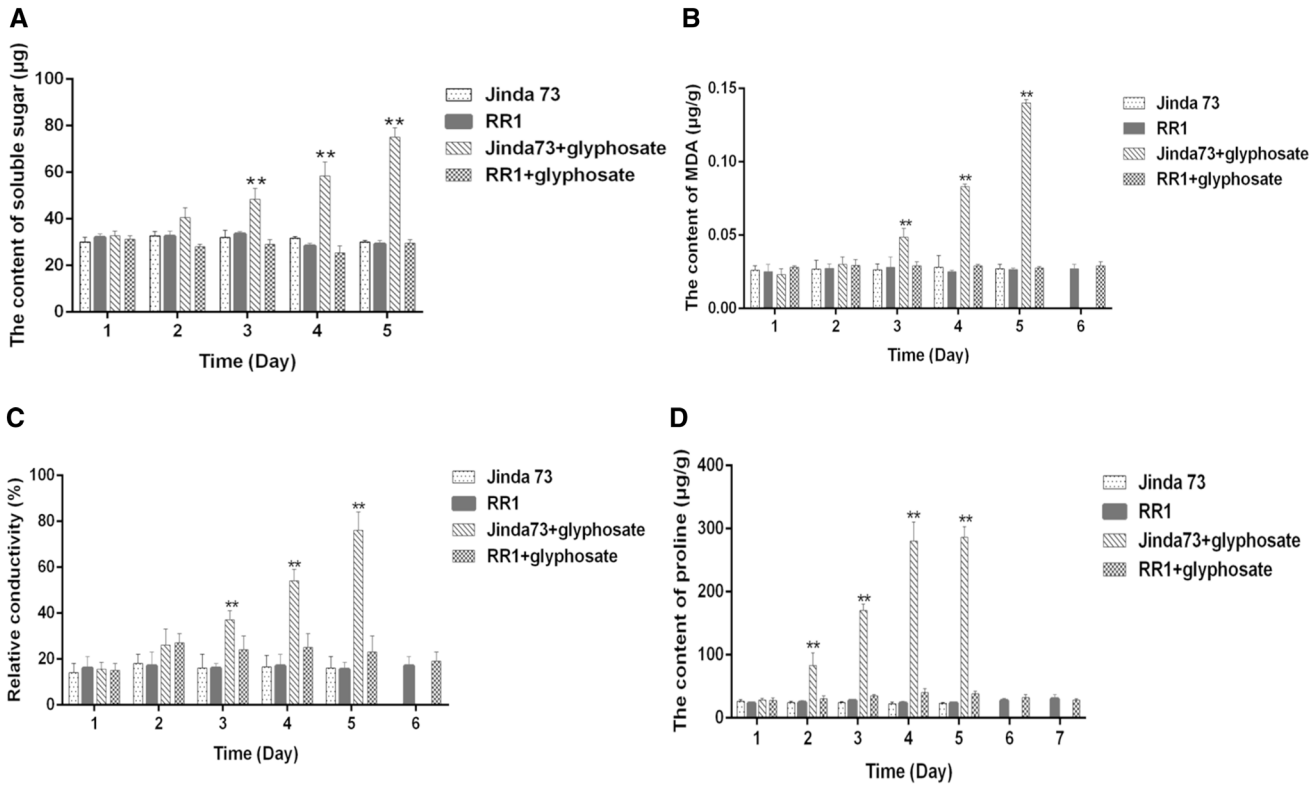

Fig. 2 The changes of soluble sugar, MDA, conductivity and proline after treating with glyphosate for Jinda 73 and RR1. **a** The content of soluble sugar from day one to day five for Jinda 73 and RR1 after trreating with glyphosate. **b** The content of MDA for Jinda 73 and RR1 from day one to day six after treating with glyphosate. **c** The changes of relative conductivity for Jinda 73 and RR1. **d** The changes of proline concentration from day one to day seven for Jinda 73 and RR1 after treating with glyphosate. $^{**}p < 0.01$ compared to Jinda 73 groups

33), successfully imported CaMV35S and strains F_7-19, F_7-30, F_7-31, and P_f-34 (the female parent Jinda 73) did not. The PCR results for *CP4 EPSPS* shown in Fig. 3c indicate that only 29 strains successfully imported the *CP4 EPSPS* gene as shown by the 146 bp band. Samples F_7-13, F_7-19, F_7-30, F_7-31, F_7-32 and P_f-34 (the female parent) did not. Thus we concluded these six strains have no glyphosate resistance. The PCR results for the NOS terminator shown in Fig. 3d indicated that 29 strains successfully imported the NOS terminator as seen by the 180 bp band. Samples F_7-4, F_7-10, F_7-13, F_7-19, F_7-31, F_7-32 and P_f-34 (the female parent) did not.

The determination of protein and oil content

Protein and oil content are two important parameters indicative of the quality of soybeans. The results shown in Table 3 indicate that the protein and oil content of female parent, Jinda 73 (P_f-34) is 43.83% and 19.43% respectively. The total protein and oil content is 63.26%. The protein and oil content of male parent RR1 (P_m-33) is 40.5% and 23.47% respectively (Table 3). The total protein and oil content is 63.97%. The protein content of offspring samples F_7-3, F_7-5, F_7-16, F_7-21, F_7-28, F_7-29 and F_7-35 are higher than the male parent (40.5%, Table 3). The protein content of the sample F_7-35 strain was the highest, at 44.1%. The male parent has 23.57% oil content, which is the highest of all 36 samples. Sample F_7-3 has 22.57%, which is the next highest oil content. The total protein and oil content for sample F_7-3 is 65%, which is higher than the 63.97% of RR1. In addition to the F_7-3 sample, the total the protein and oil content of F_7-16 sample and F_7-21 sample were 64.54% and 65.4% respectively, which were higher than both parents (P_m-33 male, and P_f-34 female). Besides, there were no significant differences in performances amongst F_7-3, F_7-16 and F_7-21, because the ANOVA method showed that all p values > 0.05. Therefore, F_7-3, F_7-16 and F_7-21 could be considered as the hybrid strains with high quality in the F_7 generation.

The determination of glyphosate residues in seeds

We determined the amount of glyphosate residue in seeds of the two parents and the 34 hybrid, offspring strains. The concentration of glyphosate in the seeds was from 0.05 to 1.2 mg/kg and the concentration of AMPA, which is the

Table 2 The resistance to glyphosate of parents and offspring

Sample	Survival rate (%)	Sample	Survival rate (%)
F_7-1	100	F_7-19	0
F_7-2	100	F_7-20	100
F_7-3	91	F_7-21	100
F_7-4	0	F_7-22	100
F_7-5	94	F_7-23	100
F_7-6	100	F_7-24	100
F_7-7	100	F_7-25	100
F_7-8	90	F_7-26	100
F_7-9	90	F_7-27	100
F_7-10	100	F_7-28	100
F_7-11	100	F_7-29	94
F_7-12	94	F_7-30	0
F_7-13	11	F_7-31	0
F_7-14	100	F_7-32	0
F_7-15	100	P_m-33	100
F_7-16	100	P_f-34	0
F_7-17	100	F_7-35	100
F_7-18	100	F_7-36	100

The P_m-33 is male parent RR1. The P_f-34 is female parent Jinda 73

most common metabolite of glyphosate, in plants was from 0.07 to 1.5 mg/kg (Data not shown).

Discussion

To produce high quality, glyphosate-resistant soybeans, we crossed Jinda 73 and glyphosate-resistant RR1 which resulted in 34 offspring, hybrid strains. We determined the amount of chlorophyll, soluble sugar, MDA, the relative conductivity and proline concentrations in Jinda 73 and glyphosate-resistant RR1 soybeans before and after treating with glyphosate.

Chlorophyll is essential in photosynthesis that provides matter and energy for plant growth (Jiao et al. 2015). Chorophyll a and b are two types of chlorophyll that exist in the photosystems of green plants (Shimoda et al. 2012). Soluble sugars, as nutrient and metabolite signaling molecules, are critical for plant structure, metabolism, growth, as well as maintenance of the cellular osmotic homeostasis (Chen et al. 2015; Couee et al. 2006; Rosa et al. 2009). MDA is an important product of membrane lipids peroxidation, and is able to crosslink with proteins, amino acids, nucleic acids, and other substances, resulting in poisoning of the cell membrane (Lin et al. 2015). Besides, plenty of studies show that the imposition of adverse environments on plants leads to enhanced membrane peroxidation in leaf tissues, which can increase the leaf MDA content and the relative conductivity (Wang et al. 2012; Xun et al. 2015). Proline is an atypical amino acid, which plays a critical role in mediating osmotic adjustment of plants subjected to drought and salt stress

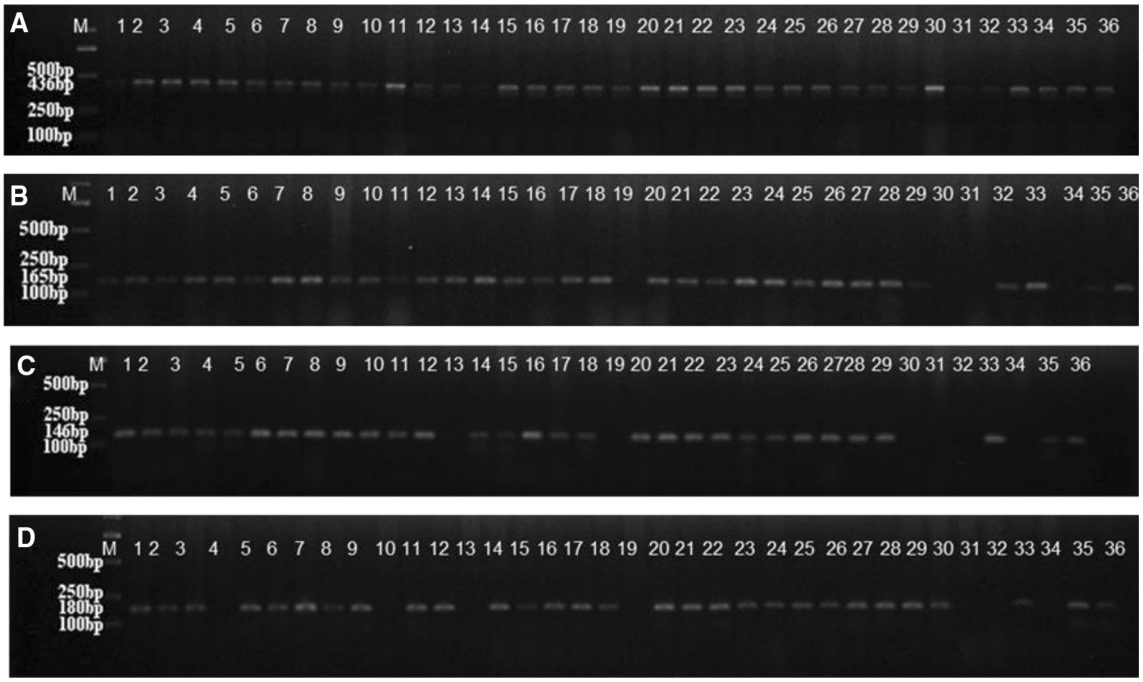

Fig. 3 PCR detection of glyphosate resistance gene. **a** PCR results for lectin. **b** PCR results for CaMV35S. **c** PCR results for *CP4 EPSPS*. **d** PCR results for the NOS terminator

Table 3 The protein and oil of parents and offspring

No.	Protein (%)	Oil (%)	Protein and oil (%)	No.	Protein (%)	Oil (%)	Protein and oil (%)
F_7-1	41.37	19.97	61.34	F_7-19	41.23	20.87	62.1
F_7-2	41.5	21.37	62.87	F_7-20	40.17	21.2	61.37
F_7-3	**42.43**	**22.57**	**65**	**F_7-21**	**43.57**	**21.83**	**65.4**
F_7-4	38.97	21.3	60.27	F_7-22	39.43	20.93	60.36
F_7-5	42.67	20.63	63.3	F_7-23	39.93	21.93	61.86
F_7-6	41.33	21.77	63.1	F_7-24	39.47	21.77	61.24
F_7-7	41.27	21.3	62.57	F_7-25	40.53	21.13	61.66
F_7-8	42.03	21.47	63.5	F_7-26	41.67	20.73	62.4
F_7-9	41.7	21.27	62.97	F_7-27	41.43	20.67	62.1
F_7-10	41.93	21.37	63.3	F_7-28	42.2	20.37	62.57
F_7-11	41.83	21.1	62.93	F_7-29	42.37	20.07	62.44
F_7-12	38.1	20.07	58.17	F_7-30	40.27	21.77	62.04
F_7-13	39.5	21.83	61.33	F_7-31	40.27	21.63	61.9
F_7-14	40.27	21.37	61.64	F_7-32	41.27	21.07	62.34
F_7-15	39.03	22.13	61.16	P_m-33	40.5	23.47	63.97
F_7-16	**42.17**	**22.37**	**64.54**	P_f-34	43.83	19.43	63.26
F_7-17	40.1	22.43	62.53	F_7-35	44.1	19.23	63.33
F_7-18	40.37	22.07	62.44	F_7-36	40.4	21	61.4

Parents in italics. Off spring in bold are have higher total % protein and oil than either parent. The P_m-33 is male parent RR1. The P_f-34 is female parent Jinda 73

(Saibi et al. 2015; Xun et al. 2015). These factors can reflect the influence of glyphosate on photosynthesis and plant resistance to stress.

After spraying glyphosate, Jinda 73 chlorophyll content declined significantly, whereas soluble sugar, MDA, relative conductivity, and proline content increased significantly. The changes of Jinda 73 soybeans are consistent with the plant stress. A previous study has also shown that increased glyphosate rate and late applications produced decreased leaf area and consequently decreased photosynthetic rates and shoot biomass (Zobiole et al. 2010). After treating with glyphosate, RR1 chlorophyll content began to decline, but recovered to a level similar to the untreated control RR1 plants by day 30. This would indicate that there is an effect of glyphosate treatment on plants, even those that are tolerant. Moreover, there was no significant change in soluble sugar, MDA, relative conductivity or proline after treating RR1 plants with glyphosate. Therefore, these results indicate that Jinda 73 has poor glyphosate resistance and RR1 has good glyphosate resistance.

Glyphosate is an inhibitor that targets EPSPS in plant chloroplasts. Glyphosate competes with PEP to form a stable EPSPS-S3P-glyphosate complex, resulting in the loss of EPSPS activity (Schonbrunn et al. 2001). The loss of EPSPS activity causes rapid accumulation of shikimic acid in the tissues, and then the aromatic amino acids which are necessary for protein synthesis are severely hampered resulting in inhibition of plant growth. Glyphosate has a significant effect on physiological and biochemical processes and it causes the degradation of chlorophyll (Gomes et al. 2014; Ozturk et al. 2008; Vivancos et al. 2011). In this study, MDA, soluble sugars, and relative conductivity were also affected by glyphosate treatment. As we have shown, chlorophyll content was reduced in both glyphosate resistant and glyphosate tolerant soybeans. Considering the results we show here that resemble oxidative stress, it is probable that glyphosate has several secondary or indirect effects on plant physiology (Gomes et al. 2014). As a result of oxidative stress, glyphosate damages the plant, and the plant produces more soluble sugars and proline in response to the damage. Glyphosate destroys chlorophyll which affects the photosynthetic rate and reduces metabolism. Eventually glyphosate causes plant death. Further studies are clearly needed to develop glyphosate resistant plants that are drought resistant, high protein and nutritious.

Among the 34 offspring, hybrid strains, 29 strains have glyphosate resistance and PCR analysis confirmed that these strains have imported the *CP4 EPSPS* gene. To select for high quality glyphosate-resistant soybeans, we selected three strains that had high concentrations of protein and oil. The contents of total protein and oil for sample F_7-3, F_7-16

and F_7-21 are higher than that of the parents. The concentration of glyphosate in the seeds was from 0.05 to 1.2 mg/kg and the concentration of AMPA was from 0.07 to 1.5 mg/kg. The results were consistent with a previous study (Arregui et al. 2004) and they found that glyphosate residues ranged from 0.1 to 1.8 mg kg^{-1} in grains. According to USDA federal regulations, the tolerance of glyphosate for soybean is 20 mg/kg (40CFR180.364). The content of glyphosate in our soybeans meets the standard. The high quality glyphosate resistant strains F_7-3, F_7-16 and F_7-21 can be used to cultivate seeds for the future and hopefully be improved upon to reduce plant stress symptoms of chlorophyll reduction (not tested in offspring).

Conclusion

We produced three glyphosate resistant soybean strains (F_7-3, F_7-16 and F_7-21) with higher total protein and oil content than their parent strains, Jinda 73 and RR1. Since glyphosate induced degradation has been documented even in glyphosate resistant plants, future research should be directed towards identifying the breakdown products of glyphosate, including AMPA, and elucidating the effects of these on chlorophyll biosynthesis in glyphosate resistant plants to understand the effects.

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Author's contributions Wei-yu Li and Gui-quan Li designed the study and prepared the manuscript. Ping Lu, Hao Xie, Jing-xuan Wang, Dong-yu Guo and Xing-yu Liang conducted the experimental work.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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