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Full Length Research Paper

Characterization of a novel *curled-cotyledons* mutant in soybean [*Glycine max* (L.) Merr.]

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Cotyledons that affect the plant development are important part of soybean. We describe a recessive soybean mutant, designated as *curled-cotyledons* mutant which is derived from sodiumazide (NaN_3) and ^{60}Coγ ray mutagenized seeds of the 'Nannong 94-16' cultivar. The *curled-cotyledons* mutant has defective morphology in cotyledons development, compared to the wild-type plants. Additionally, it also has other aberrant agronomic character, such as longer growth period, and smaller plants. In the mutant, the embryo sac becomes smaller and bulbous, and ultrastructure of developing cotyledons exhibits larger vacuole, some organelles degradation, and membranous multilamellar appear at different stages. Protein and amino acid contents in seeds of mutant are higher than those of the wild type, especially methionine and cysteine. These results suggest that the *curled-cotyledons* mutant is a novel cotyledon development mutant, which could serve as a basic material to study seed composition and cotyledon development in soybean.

Key words: Soybean, mutant, *curled-cotyledons,* genes.

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.], is a representative dicotyledonous plant; its embryogenesis begins from the zygote division, through cotyledon development stage, and ends with a mature embryo. In soybean, cotyledons are the first aerial organ to differentiate and supply nutrition for plant growth. The development of cotyledon development is a complicated process which involves cell divisions, gene expression, and hormone regulation (Hughes and Galau, 1989; Mendoza et al., 2008).

With the explosion in the use of cotyledon mutants as tools for gene cloning, many genetic pathways have been well elucidated (Chandler, 2008; Liu et al., 1999; Madishetty et al., 2006; Meinke et al., 1994; Ogas et al., 1999; Piskurewicz et al., 2008). In *Arabidopsis thaliana*, after Bāumlein et al. (1994) first reported the regulate factor, (*FUS3*) gene, which affected cotyledon development during late embryogenesis, and Harada (2001) summarized the role of *leafy cotyledon* (*LEC*) genes in cotyledon development process, Braybrook and Harada (2008) demonstrated that the *LEC* genes affected the regulation networks of seed development. Qin and Zhao (2007) found that Arabinogalactan proteins (AGPs) played a part in embryo development, cotyledon formation and seedling morphology in *Nicotiana tabacum*. Chandler (2008) gave a detailed exposition in cotyledon organogenesis and a summary of the regulation of cotyledon development. The researchers above chose gain-of-function or loss-of function mutations, including insertional mutagenesis and RNA suppression, which have been widely used in plants to dissect seed development.

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Abbreviations: DAF, Days after flowering; **NaN_3,** sodiumazide; **EMS,** chemical mutagen.

Due to the complexity of genome database and the inefficiency of genetic transformation developing in soybean, chemical mutagenesis and ionizing radiation are used commonly which can be applied to most species, even to those that have complex genetic background (Senoo et al., 2000).

The combination of chemical mutagenesis and ionizing radiation with screening for induced changes in target genes of interest is a powerful technique for obtaining an allelic series that can be used to study gene function or crop improvement. With the availability of upgraded genomic database resources and sophisticated techniques, research on soybean cotyledon development becomes exciting.

In this paper, we present the characterization of a new mutation which is cotyledon defective. Based on the *curled-cotyledons* mutants treated by compound mutagenesis (NaN_3 and $^{60}Co\gamma$), we found that the mutant has higher protein and oil content, and has altered seed ultrastructure. The purpose of this experiment is to evaluate the features of soybean *curled-cotyledons* mutant.

MATERIALS AND METHODS

Mutagenesis and mutant screening

Mutants were generated in soybean cultivar 'Nannong 94-16' using NaN_3 and $^{60}Co\gamma$ as the mutagen on about 3,000 dry seeds, which were provided by National Center for Soybean Improvement in Nanjing Agricultural University (Nanjing, China). Three thousand dry seeds of the soybean cv 'Nannong 94-16' were treated with 2×10^3 mol/L NaN_3 and 350 Gy $^{60}Co\gamma$. M_1 seed was planted and propagated by single-seed descent. The seeds were grown in the experimental station in Nanjing, China. Only seedlings that showed inherited curled cotyledons were selected for further study. Since seeds of M_1 generation in 2003, we had collected M_8 generation seeds, including adding generation propagation in the winter of 2005 at Hainan, China.

Paraffin section

For paraffin section, the wild type and the mutant seeds of various developmental stages were fixed in formalin-acetic-alcohol (FAA) (glacial acetic acid/ ethanol, 3:1) for 10 to 12 h at 4°C, respectively in 2008 and 2009. Then, followed by dehydration and infiltration, the samples were embedded with paraffin. Samples were sectioned at 5 to 8 mm and stained with 1% safranin solution.

Electron microscopy

According to soybean seed development, eight days after flowering (DAF8) was at heart-cotyledon stage, 14 days after flowering (DAF14) was at late-cotyledon stage (Hymowitz and Singh, 1987). In 2008 and 2009, seeds of DAF8 and DAF14 were prepared and fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer; pH 7.2, for more than 4 h at 4°C, postfixed for 2 h in 1% osmic acid, and then sent to Nanjing Forestry University Electron Microscope Laboratory using standard preparation techniques, respectively. Later, the pictures were observed and recorded on a transmission electron microscope (75 kV, HITACHI, H-600).

Protein and oil content analysis

Dried mature seeds were collected from M_3 to M_8 generation. Expect M_4 generation, seeds of other generation were used for protein and oil examination. Seed protein and oil content were analyzed using FOSS-tecator INFRATEC 1255 Food and Feed Analyzer with three repeats each sample.

Amino acid analysis

In 2008, leaves and developing seeds of soybean cultivar 'Nannong 94-16' and mutants were collected used for amino acid content measurement during full flowering and seed developing stage. We sent leaves and seed powders to Physics and Chemistry Test Center of Jiangsu Province for amino acid analysis using A200 Amino Nova amino acid analyzer, with four times replications. Chinese National Standard (GB/T5009.124-2003) was used for the national detection criterion. T-test was used for the statistical analysis.

Genetic analysis

The field experiment was carried out at Jiangpu Trial Station of Nanjing Agricultural University. Segregation analysis was performed using F2 populations originating from the cross '*curled-cotyledons* mutant' × 'Nannong 94-16' in 2007 summer. Segregation in the F2 generation was analyzed with a χ^2 test for goodness of fit.

RESULTS

Phenotypes analysis of the *curled-cotyledons* mutant

We screened over 2,000 M_2 progeny of a NaN_3-60Coγ mutant population; the *curled-cotyledons* mutant was picked out. After continuous self-fertilization and back-cross, the *curled-cotyledons* mutant was found to be heritable, indicating that the mutant was homozygous. The cotyledons of the wild type are shown in Figure 1. Unlike wild type cotyledons, mutant cotyledons showed curled shape, from seedling to maturation phase (Figure 1D-1F). Comparison of the cotyledons of the wild type are shown in Figure 1; mutant showed curled cotyledons, from seedling to maturation phase (Figure 1D-1F).*Curled-cotyledons* mutant was embryo defective that affected cotyledons identity and it was initiated from morphogenesis phase and maintained throughout the whole seed development.

In order to better describe the phenotype of plants, we analyzed cotyledon color, hilum color, leaf shape, flower color, pubescence color, pod development, growth habit, and 100-seed weight of the wild type and the mutant, which were no obviously different. However, *curled-cotyledons* mutants were slightly later matured, about 8 to 10 days later. Moreover, the plant height of mutants was significantly lower than that of wild type (Table 1). Through several years of research results (from 2005 to

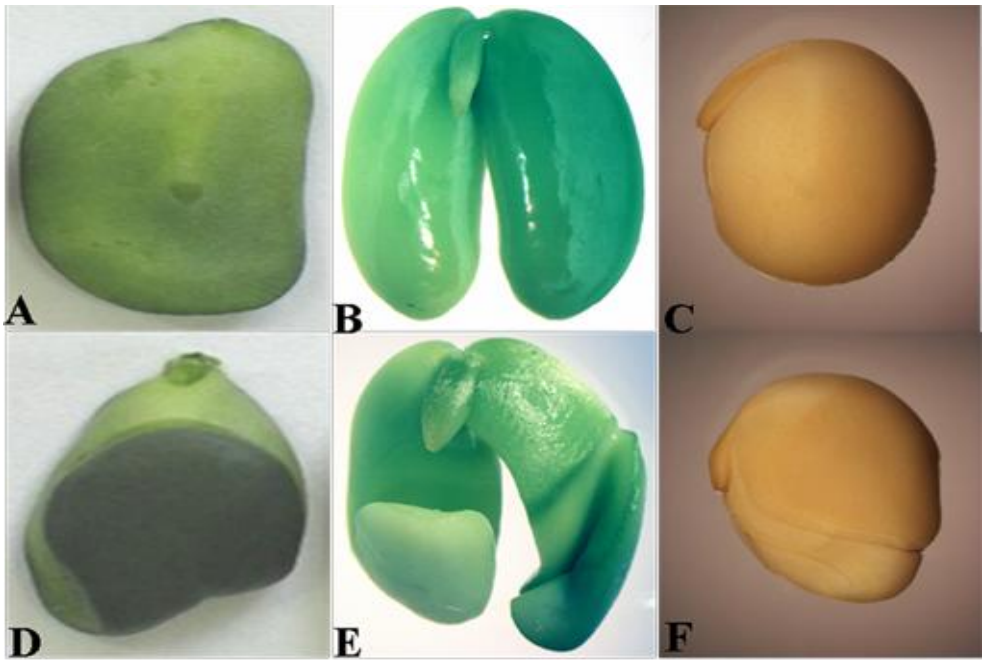

Figure 1. Morphological phenotype of cotyledons between the wild type and the mutant under dissecting microscope. A-C, the wild type cotyledon; D-F, the mutant cotyledon. A, normal cotyledon in seedling period, 10 days after germination; B, normal cotyledons at seed-filling stage, 30 days after flowering; C, normal mature cotyledons; D, curled cotyledons in seedling period, 10 days after germination; E, curled cotyledons at seed-filling stage, 30 days after flowering; F, curled mature cotyledons.

Table 1. Categorical description of 11 traits in mutant and wild type.

Trait	Mutant	Wild type
Seed color	Yellow	Yellow
Cotyledon color	Green	Green
Hilum color	Light brown	Light brown
Leaf shape	Elliptic	Elliptic
Flower color	Purple	Purple
Pubescence color	Gray	Gray
Pod development	Determinate	Determinate
Growth habit	Erect	Erect
100-seed weight (g)	29.78 ± 0.31	30.33 ± 0.50
Plant height (cm)	32.57 ± 0.64**	39.33 ± 0.57
Entire growth period (day)	104.13 ± 2.80**	94.38 ± 2.50

* Significant at $P < 0.05$; ** Significant at $P < 0.01$.

2010), it was confirmed that these different phenotypes were tightly linked to the cotyledon phenotype without separation.

Altered seed microstructure and ultrastructure in the *curled-cotyledons* mutant

To further characterize the *curled-cotyledons* mutant, we performed paraffin section for DAF8 and DAF14 stage, and found the space of cotyledon formation became narrow in the mutant (Figure 2). From Figure 2, it was seen that embryo sac looked like bulbous in the mutant, of which was oval in the wild type. The cell architecture of inner integument was more compact in the mutant, and easy to be stained.

There is also significant difference in cell ultrastructure between wild type and *curled-cotyledons* mutant.

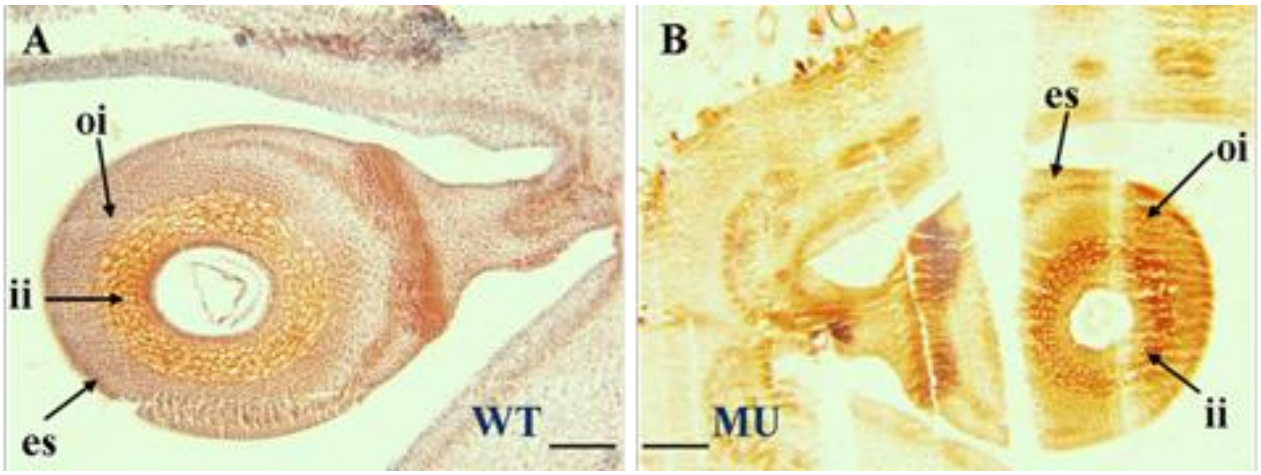

Figure 2. Light micrographs of the wild type and the mutant. ii, inner integument; oi, outer integument; es, embryo sac. Scale bars: 5 µm.

Compared to wild type cotyledons, membranous multilamellar was in larger vacuole while other organelles were unrecognizable in *curled-cotyledons* mutants at DAF8 and DAF14 stage (Figure 3).

At DAF8 stage, cotyledons started to develop in soybean, and were at heart stage. In wild type, the cell ultrastructure showed normal organelles (Figure 3A), while the larger vacuole like mature vacuolar structure, degradation of some organelles and appearance of membranous multilamellar were observed in *curled-cotyledons* mutant (Figure 3 B and C). At DAF14 stage, late cotyledon stage, the vacuole enlarged to reach the level of cell maturation in the wild type (Figure 3D). In mutants, membranous multilamellar was also recognizable in vacuole but not in wild type (Figure 3 E and F). No membranous multilamellar were ever observed in the wild type.

Quality test of the *curled-cotyledons* mutant

Compared with the wild type, the protein content was significantly higher in the *curled-cotyledons* mutant from 2005 to 2009. A quantitative measurement of the protein content of the seeds showed that the mutant displayed increased protein content (Figure 4A). The protein content ranged from 48 to 51 % in *curled-cotyledons* mutant, the highest was even up to 51.64%, while it ranged from 44 to 46 % in wile type. The oil content was significantly lower in the mutant compared with the wild type (Figure 4B). The average content of oil was about 17.8% in wild type plants, whereas it was about 16.6% in the mutant. These results may be expected as previous researches reported that the protein content was negatively correlated with the oil content.

We also tested the amino acid content of *curled-cotyledons* mutants. 17 kinds of amino acids contents in the mutant's seeds varied from the wild type (Table 2). The results indicate that the mutant possessed higher not only free amino acid but also hydrolytic amino acid content in mature seeds. Among the 17 kinds of free amino acids, except for tyrosine, the ratio was below 1; others were all above 1. Histidine content varied most significantly, and the ratio reached 6.7. With respect to hydrolytic amino acids, methionine content was of maximum variation. Cysteine content in mutants was also increased. Other amino acids contents of mutant plants were also higher than that of wild type plants, and the ratios were all above 1 (Figure 5).

Genetic analysis of the *curled-cotyledons* locus

To understand the molecular mechanism of the *curled-cotyledons* mutant, we performed hybridization by crossing the mutant with the wild type. All 5 F1 plants had the normal phenotype, indicative of a recessive mutant phenotype. In the F2 generation, the progenies of hybridization produced normal and cotyledons curled plants in a ratio of ≈15:1 (Table 3). These results suggest that the presence of two recessive interacting genes mutation based on nuclear inheritance were responsible for the *curled-cotyledons* phenotype.

DISCUSSION

We isolated a cotyledon defective mutant in soybean with high content of protein and amino acid. It provides a new opportunity to study the genetic mechanism and gene expression in cotyledon development. Here, we discuss the results of morphological and genetic analysis of the

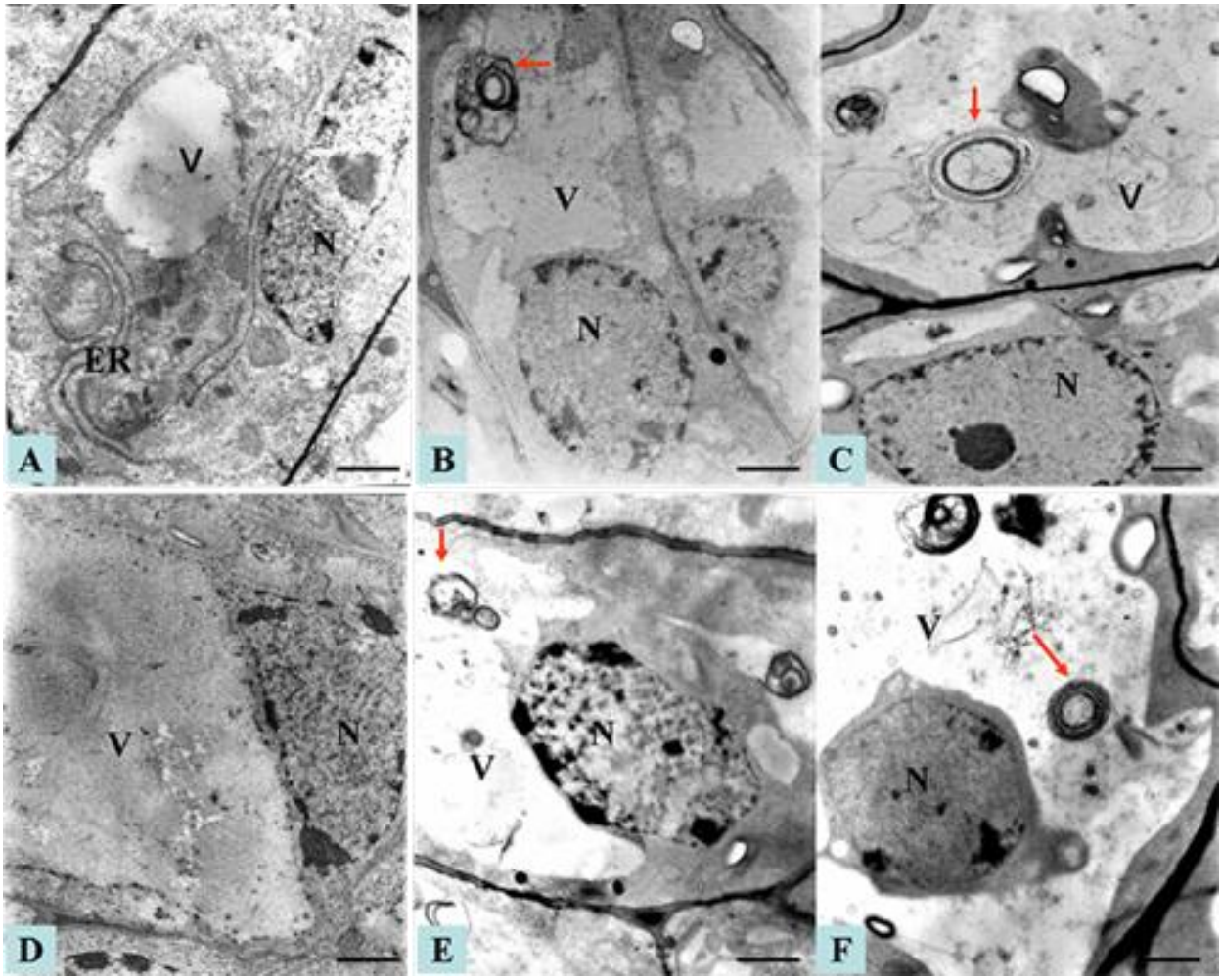

Figure 3. Cell ultrastructure of DAF8 and DAF14 developing cotyledons. A, Cell ultrastructure in the wild type at DAF8; B-C, cell ultrastructure in the mutant at DAF8; D, cell ultrastructure in the wild type at DAF14; E-F, cell ultrastructure in the mutant at DAF14. ER, endoplasmic reticulum; N, nuclear; V, vacuole. Red arrow points at multiple concentric membranes. Scale bars: 1 μm.

curled-cotyledons mutant. In addition to their abnormal cotyledon development, the mutant shows other closely related phenotypic traits, of which the most visible are stunted growth and late-maturing.

The combination of chemical mutagenesis with screening for induced changes in a gene target of interest is a powerful technique for obtaining an allelic series that can be used to study gene function or crop improvement. The *Etr* soybean mutant mutagenized with NMU has reduced ethylene sensitivity that can be beneficial against some pathogens (Hoffman et al., 1999). The *nts* soybean mutant mutagenized with chemical mutagen (EMS) is affected in a nodule-development regulatory gene (Carroll et al., 1985). The soybean cultivars to a EMS, physical mutagen (gamma rays) have been found to be more effective in inducing chlorophyll mutations compared to individual treatments of gamma rays and EMS in both the cultivars (Khan and Tyagi, 2009). The chlorophyll-deficient soybean mutant (*cd1*) treated with EMS, displayed yellow-green leaves from seedling stage V1 to fully flowering stage R2, abnormal chloroplasts, lower total chlorophyll content, lower plant height and lower seed yield compared to its wild-type control (Zhang et al., 2011). In our paper, curled-cotyledons mutant which is mutagenized by NaN_3 and $^{60}Co\gamma$ rays is embryo defective mutation that affects cotyledons identity in the morphogenesis phase. Except curled cotyledons trait and lower germination rate, seeds of homozygous mutants are the same as that of wild type. Although LEC genes are also necessary for normal progression through the morphogenesis phase of zygotic embryogenesis (Harada, 2001), cotyledon mutants induced by ectopic expression of *LEC1* and *LEC2* triggers somatic embryogenesis not zygotic embryogenesis (Gaj et al.,

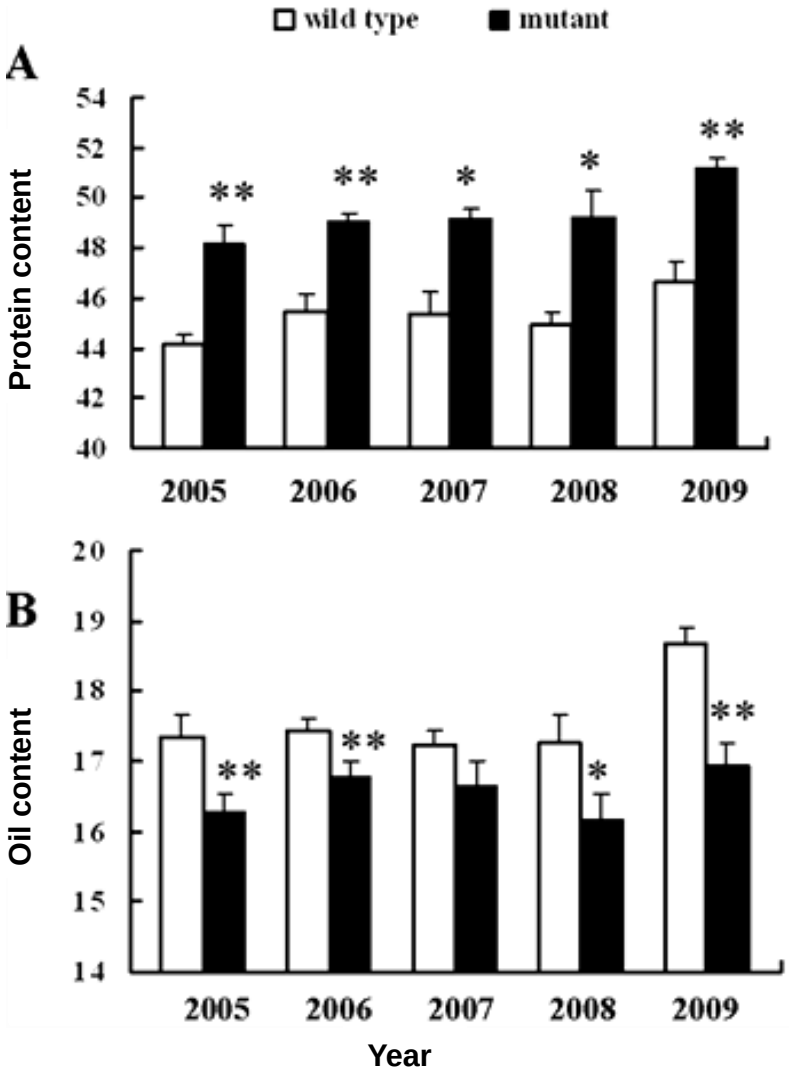

Figure 4. Protein and oil content between the wild type and the mutant from year 2005 to 2009. A. Protein content. B. Oil content. ***** Significant at $P < 0.05$; ****** significant at $P < 0.01$.

2005; Lotan et al., 1998; Stone et al., 2001). Our *curled-cotyledons* mutants belong to the latter.

As a consequence of curled cotyledons taking up smaller space, the space of embryo sac becomes smaller and bulbous may be due to curled cotyledons taking up smaller space. Ultrastructural changes of developing cotyledons in *curled-cotyledons* mutants also confirm that there are differences not only from phenotype but also to cell structure. Especially, membranous multilamellar appearance distinguishes mutants from wild types. Some researchers considered that membranous multilamellar developed from invagination of the tonoplast in cultured callus of *Stevia rebaudiana* (Shi et al., 1999), and other researchers showed that membranous multilamellar are involved in autophagy (Seglen et al., 1996; Benchimol, 1999). Verdus et al. (1993) reported that multilamellar structure of leaves treated by glycerol is associated with tannins which agreeds with the study of Ochs et al. (1994). In stress response, membranous multilamellar was found in ultrastructure cells of Onobrychis viciifolia (Han and Zhang, 2005). From ultrastructural change in our mutants, it is speculated that vacuole phagocytizes organelles in degradation form new multilayer body-membranous multilamellar.

In the *curled-cotyledons* mutant, protein and oil composition was changed; higher protein content and lower oil content. As the main component of protein, higher hydrolytic amino acids content gave reason to increase protein content in the mutant. Soybean protein is relatively rich in most of the essential amino acids, but

Table 2. 17 kinds of amino acid content between wild type and mutant at different stage.

kinds of amino acid	Full flowering stage		Seed-filling stage		Mature stage	
	WT	MU	WT	MU	WT	MU
Free amino acid						
Asp	0.252 ± 0.062	0.303 ± 0.009	0.346 ± 0.009	0.196 ± 0.019	0.041 ± 0.003	0.052 ± 0.001
Thr	0.346 ± 0.034	0.134 ± 0.007	0.061 ± 0.007	0.040 ± 0.019	0.161 ± 0.033	0.469 ± 0.027
Ser	0.226 ± 0.017	0.135 ± 0.005	0.134 ± 0.005	0.103 ± 0.006	0.011 ± 0.002	0.023 ± 0.007
Glu	0.227 ± 0.058	0.354 ± 0.019	0.039 ± 0.002	0.042 ± 0.003	0.032 ± 0.001	0.054 ± 0.01
Gly	0.025 ± 0.002	0.012 ± 0.001	0.025 ± 0.001	0.019 ± 0.002	0.005 ± 0.001	0.009 ± 0.001
Ala	0.376 ± 0.042	0.182 ± 0.006	0.118 ± 0.005	0.084 ± 0.001	0.029 ± 0.005	0.072 ± 0.015
Cys	ND	ND	ND	ND	0.009 ± 0.001	0.009 ± 0.001
Val	0.056 ± 0.004	0.038 ± 0.001	0.057 ± 0.003	0.057 ± 0.004	0.004 ± 0.001	0.007 ± 0.001
Met	0.003 ± 0.001	0.002 ± 0.001	0.005 ± 0.001	0.005 ± 0.001	0.014 ± 0.001	0.016 ± 0.003
Ile	0.028 ± 0.003	0.016 ± 0.001	0.023 ± 0.001	0.021 ± 0.001	0.005 ± 0.001	0.005 ± 0.002
Lue	0.039 ± 0.006	0.021 ± 0.002	0.047 ± 0.004	0.036 ± 0.002	0.007 ± 0.001	0.010 ± 0.003
Tyr	0.018 ± 0	0.017 ± 0.001	0.038 ± 0.003	0.027 ± 0.002	0.021 ± 0.003	0.019 ± 0.002
Phe	0.799 ± 0.079	0.423 ± 0.009	0.359 ± 0.011	0.146 ± 0.135	0.007 ± 0.005	0.013 ± 0.005
His	0.023 ± 0.001	0.016 ± 0.001	0.047 ± 0.004	0.036 ± 0.001	0.025 ± 0.005	0.165 ± 0.006
Lys	0.031 ± 0.001	0.022 ± 0.002	0.029 ± 0.007	0.018 ± 0.004	0.008 ± 0.001	0.013 ± 0.001
Arg	0.048 ± 0.009	0.012 ± 0.002	0.084 ± 0.010	0.081 ± 0.010	0.166 ± 0.037	0.628 ± 0.01
Pro	0.075 ± 0.007	0.061 ± 0.005	0.106 ± 0.026	0.086 ± 0.014	0.041 ± 0.002	0.051 ± 0.023
Total	2.569 ± 0.088	1.744 ± 0.048	1.517 ± 0.071	0.996 ± 0.107	0.107 ± 0.08	1.613 ± 0.064
Hydrolytic amino acid						
Asp	9.302 ± 0.195	7.778 ± 0.280	6.390 ± 0.242	5.184 ± 0.312	4.991 ± 0.376	6.568 ± 0.869
Thr	3.888 ± 0.110	3.170 ± 0.113	2.532 ± 0.139	2.061 ± 0.137	1.426 ± 0.043	1.880 ± 0.781
Ser	4.665 ± 0.061	3.832 ± 0.194	2.729 ± 0.124	2.070 ± 0.098	2.243 ± 0.238	2.412 ± 0.036
Glu	11.621 ± 0.271	9.533 ± 0.323	7.567 ± 0.388	6.158 ± 0.098	8.242 ± 0.382	9.421 ± 0.435
Gly	4.672 ± 0.104	3.829 ± 0.088	3.314 ± 0.145	2.756 ± 0.132	1.742 ± 0.057	1.873 ± 0.088
Ala	5.970 ± 0.124	4.779 ± 0.150	3.740 ± 0.074	3.177 ± 0.074	1.777 ± 0.119	1.822 ± 0.103
Cys	0.085 ± 0.007	0.066 ± 0.006	0.044 ± 0.010	0.022 ± 0.004	0.119 ± 0.090	0.151 ± 0.007
Val	5.372 ± 0.152	4.482 ± 0.056	3.760 ± 0.061	3.254 ± 0.079	2.097 ± 0.107	2.179 ± 0.153
Met	0.310 ± 0.039	0.711 ± 0.019	0.189 ± 0.046	0.567 ± 0.176	0.559 ± 0.094	0.922 ± 0.199
Ile	3.712 ± 0.096	3.137 ± 0.066	3.061 ± 0.064	2.692 ± 0.097	3.591 ± 0.037	3.740 ± 0.149
Lue	8.216 ± 0.138	6.750 ± 0.171	5.467 ± 0.263	4.704 ± 0.215	2.005 ± 0.079	2.145 ± 0.078
Tyr	3.053 ± 0.058	2.324 ± 0.068	2.094 ± 0.159	1.675 ± 0.173	1.556 ± 0.576	1.576 ± 0.497
Phe	6.204 ± 0.186	4.863 ± 0.129	3.643 ± 0.071	3.085 ± 0.051	1.917 ± 0.113	1.999 ± 0.084
His	2.150 ± 0.036	1.802 ± 0.055	1.519 ± 0.095	1.326 ± 0.086	1.331 ± 0.033	1.538 ± 0.070
Lys	5.627 ± 0.095	4.661 ± 0.139	4.365 ± 0.219	3.642 ± 0.129	2.402 ± 0.027	2.663 ± 0.039
Arg	4.629 ± 0.103	3.471 ± 0.087	3.686 ± 0.226	2.893 ± 0.302	3.212 ± 0.068	3.935 ± 0.113
Pro	3.964 ± 0.173	3.040 ± 0.157	2.904 ± 0.471	2.638 ± 0.204	1.636 ± 0.385	2.020 ± 0.382
Total	83.435 ± 1.872	68.225 ± 1.985	57.004 ± 0.704	47.903 ± 0.797	40.844 ± 0.846	46.843 ± 1.493

WT, wild type; MU, mutant; ND, no detected. At full flowering stage and seed-filling stage, leaves were detected. At mature stage, seeds were chosen; Error bars represent ± standard deviation (SD) of the mean. N=4.

the concentration of the sulfur containing amino acids methionine and cysteine is relatively low. The increasing sulfur amino acid content, methionine and cysteine, not only make up for the deficiency, but also provided a good way to improve soybean quality (Krishnan, 2005). Moreover, amino acids content is important to plant development. In developing soybean cotyledons, Arginine as a nitrogen-rich compound, plays an important role in soybean cotyledon development (Goldraij and Polacco, 1999, 2000; Polacco and Holland, 1993; Van Etten et al., 1967). Asparagine and histidine content change can also affect plant growth (Hernández-Sebastià et al., 2005;

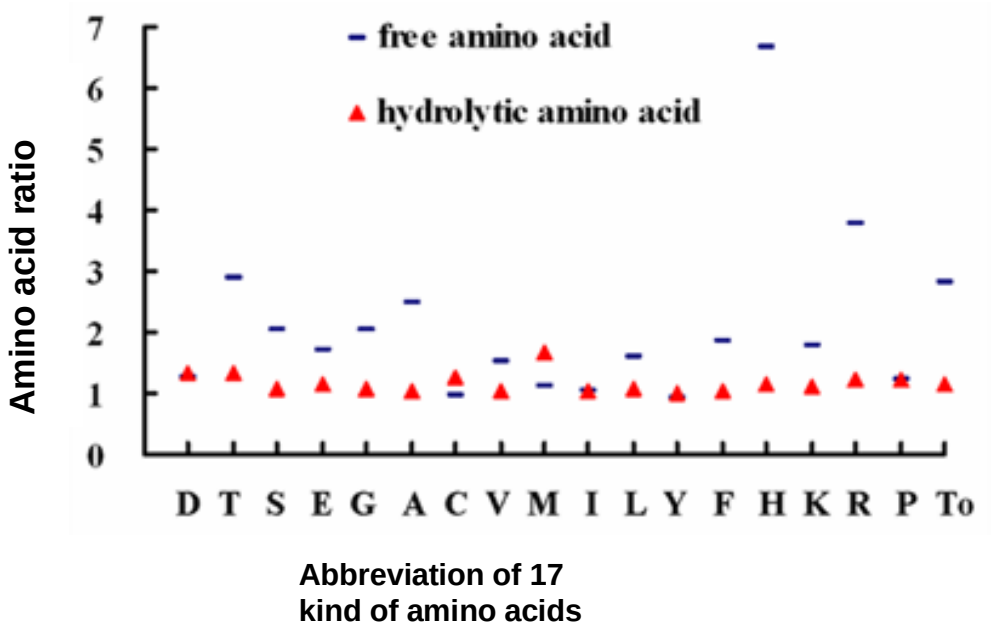

Figure 5. Compasiton of amino acids content between the wild type and the mutant. X-axis, the abbreviation of 17 kinds of amino acids; Y-axis, the ratio of the amino acids content between the mutant and the wild type. Ratio>1, mutant has higher content amino acid; ratio<1, wild type is higher in amino acid content. To, the total content of amino acids.

Table 3. Genetic analysis of the trait of curled cotyledon in F2 population.

Trait	Normal cotyledon	Curled cotyledon	χ^2
Phenotypic value	296	18	$\chi_c^2 = 0.0688 < \chi_{0.005,1}^2 = 3.84$
Theoretic value	294.375	19.625	
Segregation ratio	15 : 1		

F1 population was the cross of the curled cotyledon soybean × 'Nannong 94-16' cultivar. All the F1 plants were normal cotyledons. In F2 population, there were 296 plants with normal cotyledons, and 18 plants with curled cotyledons. The segregation ratio is 15:1.

Imamura et al., 2003).

The recent development of molecular maps has great promise for identifying genes associated with cotyledon development (Berrios et al., 2000; Botto et al., 2003; Conte et al., 2010; Ubayasena et al., 2010). In our research, we also try to determine the associations of simple sequence repeat (SSR) molecular markers with minor differences which could further serve to find candidate genes controlling *curled-cotyledons*. There are five polymorphic markers localized on D2 and G genetic linkages between the wild type and the *curled-cotyledons* mutant (unpublished). Panthee et al. (2006a) mapped molecular markers both on molecular linkage groups (MLG) G involved in determining methionine and cysteine concentration in soybean. In addition, a novel molecular marker (Satt570) associated with a protein quantitative trait locus (QTL) was also detected on MLG G in soybean (Panthee et al., 2005; Panthee et al., 2006b). It is interesting to note that molecular markers from the same genomic region on MLG G were detected to be associated with protein or methionine and cysteine. This is probably a genomic region involved in the amino acid production process. Besides the markers associated with protein and oil content, others may be involved in the cotyledon development. We also used the Affymetrix® GeneChip® Soybean Genome Array analysis, twice. In the GeneChip, there were 61,170 probe sets, 37,685 soybean transcripts; only 17 transcripts were common between the two Genechips (data not shown), of which there are some genes located on D2 and G linkages. The results confirm that the mutant character probably differ by only a limited number of genes. With the republish of

soybean genome sequence (Schmutz et al., 2010), these results are helpful for us to identify and validate candidate genes associated with cotyledon development in future. It is also very important to efficiently study and use genetic diversity resources in crop breeding and sustainable agriculture.

The new mutants generated in this study would provide additional intermediate for high protein content breeding and genetic material for studying the cotyledons development. This research lays a foundation for further study on the molecular mechanism of gene regulation cotyledon development in soybean. Further characterization of the functional genes is undergoing. We speculate that further genetic studies in the mutant will identify the majority of essential genes and define a regulatory network for cotyledon development.

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