Changes of Chemical Components During Seed Development in Black Soybean (*Glycine max* L.)

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ABSTRACT : Changes in the level of metabolites in leaves and pods were examined with respect to the seed chemical composition in black soybean. There was no further increase in pod length after 42 days after flowering (DAF). Pod weight, however, persistently increase until 73 DAF, thereafter the weight was slightly lowered. The seed storage protein, however, increased drastically as the increasing rate of pod weight was lessened at 61 DAF. The accumulation of seed storage proteins was occurred conspicuously as the increasing rate of pod weight was slowed down. The chlorophyll content both in leaves and pods was drastically decreased after 50 DAF. The beginning of drastic reduction in chlorophyll content was occurred concomitantly with the reduction of soluble protein content in leaves. The sugar content in leaves showed similar tendency with chlorophyll and soluble protein content. The starch level in leaves, however, showed different changing pattern during seed development. The starch content in leaves was increased persistently until 66 DAF, thereafter the content was decreased drastically to about 55% of maximal value at 66 DAF. Total phenolics content in leaves and the anthocyanins content in seeds were stable without noticeable increase until 66 DAF. The contents were increased dramatically after 66 DAF showing the synchronized pattern with the decrease in starch level in leaves. The levels of the selected metabolites in leaf and seed suggested that the accumulation of chemical components of black soybean seed is launched actively at 66 DAF. The profile of storage proteins was nearly completed at 61 DAF because there was no large difference in densitometric intensity among protein subunits after 61 DAF. In soybean, chemical maturation of seed begins around 61 to 66 DAF at which most metabolites in vegetative parts are decreased and remobilized into maturing seeds.

Keywords: black soybean, seed development, storage proteins, anthocyanins, remobilization

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S oybean [*Glycine max* (L.) Merrill] is one of the most important upland crops in Korea. Despite an increasing requirement for soybean production, only a small portion (105,024 tons) was produced domestically in Korea and a large portion of soybean (1.54 million tons) was imported in 2003. The demand for domestically produced soybeans, however, is increasing now due to the higher safeness and quality of soybean produced in domestic. The foods made from the soybean have been used for a long time throughout the world (Holt,1997; Kyoko,1998). In addition, especially, the seed of black *Glycine* spp. have been used as folk medicine in Korea. Of the supposed medicinal components in black soybean, the well-known chemical components are anthocyanins (Kolde,1997; Wang *et al.*, 1997), isoflavones such as phytoestrogens (Holt,1997; Kim,1996), oligosaccharides (Choi *et al.*,1995), and saponins (Kim *et al.*,1994; Oakenfull,1981; Kim, 2003) The pharmaceutical activities of anthocyanins have been mainly attributed to their antioxidant properties (Pool-Zobel *et al.*,1999; Goldbohm *et al.*,1998). In addition, anthocyanins in plant tissues are also highly related to many ecological functions.

The contents of major chemical constituents of seed are dependent on the growth stages of seed and the condition of source tissues. There are also many results about the relationship between growth stage and the concentration of chemical constituents in seed. Giaquinta *et al.* (1984) reported that sucrose content in leaves kept constantly in the course of seed development and then increased in the early ripening stage. Seong and Park (1993) reported that soluble sugar content in leaves increased by full seed stage and then decreased in *G. max*. The starch levels in leaves increased until the beginning of maturity. Pate *et al* (1977) reported that nitrogen in plant actively translocated through the vascular system, the xylem (11%) and phloem (89%). The protein content in leaves decreases according to the reproductive stage, the content in pod and seed, on the contrary, increases (Seong & Park,1993).

Soybean seed contains about 40% protein and 20% oil. The storage proteins consist of several kinds of proteins. The majority of chemical constituent is protein represented by

salt-soluble globulins which consist of 11S, 7S, and 2S fractions based on the sedimentation property. 11S and 7S globulins are referred to as glycinin and β-conglycinin, respectively. The percentage of these two proteins is about 70~80% of total seed protein. Glycinin (11S) and β-conglycinin (7S), the two main classes of multi-subunit seed storage proteins in *Glycine* spp., account for approximately 70 ~ 80% of total protein in a typical soybean seed (Hill *et al.*, 1974). Although β-conglycinin (7S) is abundant in seeds, the nutritional quality of β-conglycinin is poor due to the deficiency in sulfur-containing amino acids.

The objective of this study was to know the changes in leaf starch and sugar contents and seed storage proteins during reproductive growth in black soybean and to verify the relationship between the protein content and the level of sugar and starch in leaves during seed maturation. The results may provide the basic information for the production of high-quality grain in black soybean

MATERIALS AND METHODS

Plant materials

The seeds of black soybean (land race) were provided from Seedbank for Wild Herbaceous Species, Korea University, Seoul, Korea. The seeds of black soybean were sown on sandy loam soil on May 25 and were harvested on October 16 (full ripe). The emergence date was June 5 and the beginning bloom was August 9. The sampling dates were 41 DAF (Sep 18), 46 DAF (Sep. 23), 53 DAF (Sep. 30), 62 DAF (Oct. 9), and 69 DAF (Oct. 16). Each sample was stored at -70°C until used. All data reported throughout this paper are the average of three replicates.

Analysis of leaf metabolites

Soluble sugar was extracted by incubating 100 mg of fully expanded leaf sample with 10 ml of 95% ethanol overnight. After filtering the residue was used for starch analysis. Soluble sugar and starch were determined with the anthrone method (Yoshida *et al.*,1976) Soluble protein concentration was determined spectrometrically according to Bradford (1976) Chlorophyll content determination was carried out according to Arnon (1949). Total phenolics concentration was measured with Folin-Ciocalteu method as described by Waterman and Mole (1994). Tannic acid (Sigma chemicals) was used for a reference curve. In brief, approximately 50 mg of powder was extracted in 5 ml of 50% v/v methanol in water for 1 h at 40°C with gentle. After centrifugation, the clear supernatant was used for the determination of total phenolics.

Anthocyanin analysis

Anthocyanins were extracted from 20 mg of ground seed with 1 ml of methanol containing 1% HCl in the dark at 4 °C for 24 hrs. The extract was centrifuged at 12,000 g for 20 min, and then stored at -4 °C until analysis. The filtrate was diluted properly with extracting solvent. Appropriate amount of filtrate was used to determine anthocyanin concentration using spectrophotometer at 530 and 650 nm Anthocyanin content was calculated as cyanidin-3-glucoside using an extinction coefficient of 34,300 (Siegelman & Hendricks, 1958).

Electrophoresis of seed storage proteins

Freshly collected seeds were dried and ground with mortar and pestle. Twenty mg of powder was defatted twice with cold-hexane, and protein was extracted with 1.0 ml of protein extraction buffer [0.05 M Tris-HCl, pH 8 0; 0.2% SDS, 0.2 M β-mercaptoethanol and 5 M urea] by shaking vigorously for 30 min The mixture samples were placed in boiling water bath for 10 min and centrifuged to remove debris at 12,000 g for 15 min. An aliquot (0.4 ml) of supernatant was mixed with 0.1 ml sample buffer [60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 5% mercaptoethanol and 1% bromphenol]. The samples were subjected to 12 5% SDS polyacrylamide gel electrophoresis (Laemmli,1970). After electrophoresis the separated protein bands were visualized by Coomassie brilliant blue R-250 solution. The separated protein bands were stored as digital image for analyzing relative amount of protein fraction. Relative amount of each fraction was determined using an image analysis software (Kodak D1, Kodak, USA).

RESULTS AND DISCUSSION

Time course of pod development

The changes of pod fresh weight and pod length showed different trends during seed development (Fig 1). As compared to the result of the change in pod length, the pod fresh weight was a more suitable trait to estimate the seed development. The maximum pod length was attained at 42 days after flowering (DAF). The fresh weight of pod, however, increased persistently until 73 DAF reflecting the threshold of physiological seed maturation commence after this time. This result suggested that the quantitative growth of seeds in black soybean was nearly stopped and the accumulation of specific component derived from assimilated material was launched at 73 DAF. Leaves and juvenile pods, typical source tissues supplying assimilates to sink (seeds), showed

a decrease in chlorophyll content. The content was drastically decreased after 50 DAF both in leaves and pods. Although there was a little differences in chlorophyll level in leaves and pods, the decreasing tendency and the time to
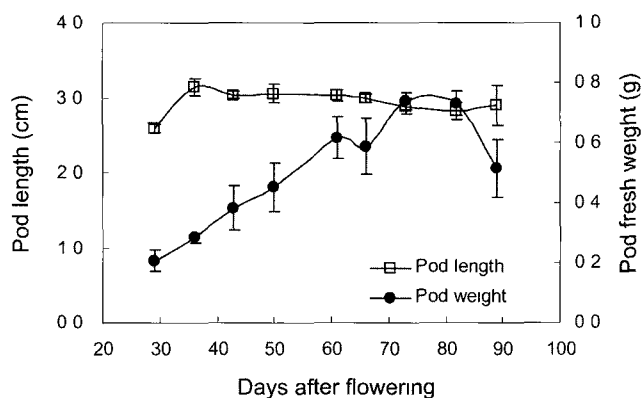

Fig. 1. Changes in pod length and fresh weight during seed development in soybean Pod fresh weight includes seeds in a pod Vertical bars indicate ±SD
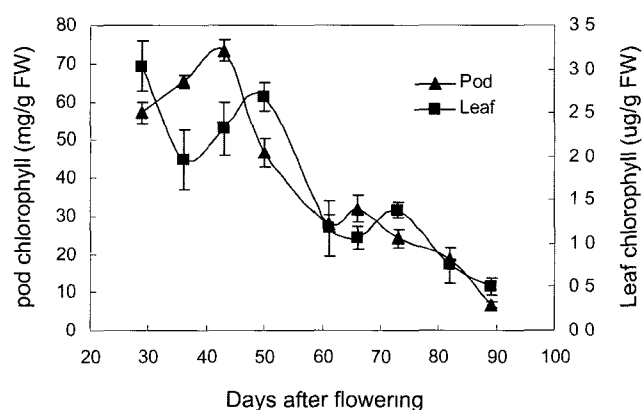

Fig. 2. Changes of chlorophyll content in leaves and pods during seed development in soybean The pod chlorophyll content was measured from the only maternal part do not including seeds. Vertical bars indicate ±SD

decrease was similar during seed development (Fig. 2).

The soluble protein contents in leaves and pods (Fig. 3) also showed similar pattern with chlorophyll. Soluble protein and chlorophyll content was highly correlated to the photosynthesis in leaf (Dreccer et al., 2000; Milroy & Bange, 2003). The result from chlorophyll determination suggests that primary metabolic activities including photosynthesis were lowered remarkably at 61 DAF in leaves. Most nitrogen exists as protein in leaves, which remobilized into seeds during seed development (Staswick, 1994; Rossato et al., 2001). Hence, the lowered level of proteins in leaves reflects the increased remobilization of nitrogen into seed, especially as storages protein (Table 1). The amount of soluble proteins which have roles related to basic metabolic activities in vegetative part of soybean also decreased from 50 DAF. The results imply that overall activity in leaves may persistent without deterioration until 50 DAF. The levels of soluble proteins of leaves were higher several times as compared to that of pod during seed development until 82 DAF, the difference was diminished from 82 DAF at which seeds were nearly matured and dried. Therefore, it was assumed that the metabolic activities which related to the accumulation of seed storage components were become more active from 82 DAF. Although the protein level in pods was lower than seeds, the substantial protein content in pod was not negligible suggesting that pod was an important tissue for supplying assimilates into seeds as a source tissue The photosynthetic activity of the leaves declines during senescence (Mondal et al.,1978), whereas the amount of N remobilizing from the leaves to the seed increase (Latimore et al.,1977). Additionally, Rossato et al. (2001) have reported that during flowering and pod development stages in Brassica napus, the capacity for N uptake significantly declines. Consequently, most nitrogen used for grain filling derives from mobilization of stored N in vegetative tissues.

The carbohydrates content of leaves, a typical source tis-

Table 1. Amount of each protein fraction separated by SDS-PAGE and quantified based on the densitometric intensity.

| Stage (DAF)	7S			11S		Total content*
	α'	α	β	Acidic	Basic	
42	47.0 (47 0)	53 0 (53.0)	0.0 (0 0)	0.0 (0 0)	0 0 (0.0)	0 3
50	12 3 (15 1)	37.4 (45 9)	2 5 (3.1)	19 1 (23.5)	10 1 (12 5)	6.1
61	11 8 (14.3)	20 8 (25.3)	4.7 (5 7)	29.9 (36 3)	15 2 (18.5)	21 1
66	10 4 (12 1)	17 8 (20 7)	5 0 (5 8)	31 0 (35 9)	22 0 (25 5)	22 1
73	7 0 (7.4)	17 9 (18 9)	4.8 (5 1)	33 9 (36 0)	30.7 (32.6)	31 9
82	8.5 (10 0)	12.2 (14 4)	7 5 (8 9)	33 1 (38 9)	23 6 (27 8)	31 6
89	10 0 (14 8)	8 4 (12 5)	7 7 (11 4)	21 5 (31 9)	19 9 (29 4)	38 0

Values in parenthesis are percentage.
*The content was calculated from the sum of detectable bands

sue assimilating carbohydrates, was analyzed (Fig. 4). The sugar content was decreased dramatically from 50 DAF The decrease in starch content, however, was observed 16 days later (66 DAF). The inconsistency of decreasing pattern between starch and sugar level may due to the late initiation of translocation of starch into seeds after 73 DAF. The pattern of changes in sugar content was nearly similar to the content of chlorophyll both in pod and leaf and soluble protein content in leaf. Because sugars are not representative storage materials, sugar level was linearly responded to the other physiological criteria such as chlorophyll, and soluble protein level. The onset of translocation of stored material from leaves (decrease in starch level) was begin at 66 DAF at which pod weight was maximal and the decreasing rate of pod and leaf chlorophyll and leaf soluble sugar content became slow-down. Mondal et al. (1978) reported an increase in leaf starch level in soybean during the seed filling period. On the other hand, Ciha & Brun (1978) reported no changes in starch levels in the leaves during reproductive growth. The starch levels declined from their peak values as
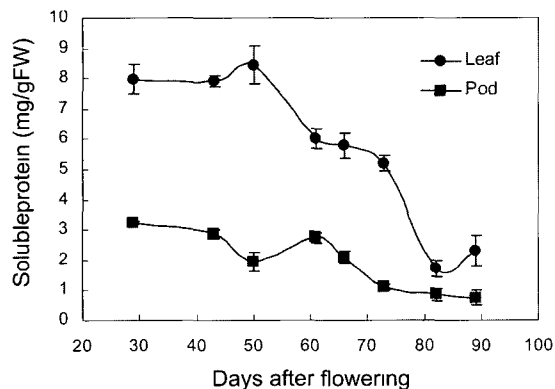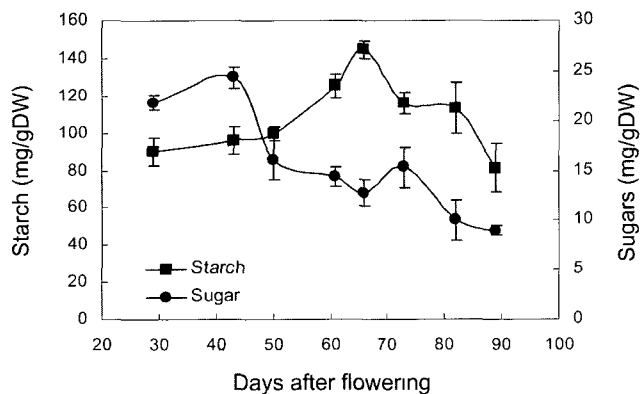
the plant approached maturity suggesting that some of the starch was redistributed into the seeds. Starch levels in leaves that were ready to be senescence were relatively high (about 8%). This result implies that photosynthate which will not attribute to seed yield still present in leaves after remobilization (Egli et al., 1980).

Changes of total phenolics in leaves and anthocyanins in seeds

Total phenolics content in leaf is related to the senescence (Saleem et al., 2001). Leaf phenolics content was persistently increased from 66 DAF (Fig. 5). This changing pattern suggested that leaf senescence and translocation and remobilization into seeds were accelerated after 63 DAF. As compared to the accumulating pattern of phenolics in leaves, anthocyanins were more dramatically accumulated in seeds. The accumulation of both phenolics in leaves and anthocyanins in seeds began simultaneously at 66 DAF at which the level of soluble proteins in pod the starch in leaves was decreased. Because anthocyanins are included to the phenolics, the levels of phenolic compounds are increased fast as the activity of vegetative organs decrease during seed development. It was assumed that the metabolic activity for accumulating seed storage components may maintains until the physiological maturity, although the level of chlorophyll, sugars, and protein in leaves was lowered from 66 DAF in soybean. Because the accumuation of anthocyanins in vegetative tissues is highly responsive to the stress (winkel-shirley, 2002), the anthocyanins level, however, may nat be responsive strictly to the seed development.

The accumulation mechanism of anthocyanins in seed, however, is still unclear. In black soybean, anthocyanin accumulation was commenced after full-growth of the pods (73 DAF). Therefore, it was deduced that anthocyanin biosynthesis in seeds was turned on when seed-oriented translo-

Fig. 3. Changes of soluble protein content in leaves and pods during seed development in soybean. The pod protein content was measured from the only maternal part do not including seeds. Vertical bars indicate ±SD.
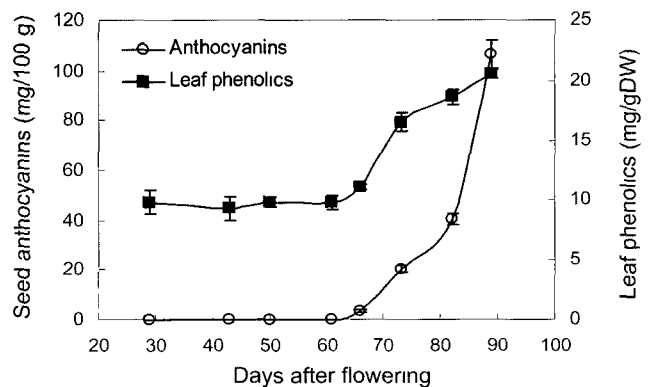

Fig. 4. Changes of starch and sugar protein content in leaves during seed development in soybean Vertical bars indicate ±SD

Fig. 5. Changes of in anthocyanins level in seeds and total phenolics in leaves during seed development in soybean Vertical bars indicate ±SD

cation and remobilization were finished. Because anthocyanin biosynthesis is triggered by stresses (Winkel-Shirlet, 2002), it is likely that artificial treatment of adverse condition to black soybean after quantitative maturation of seed may increases the anthocyanin content. In addition, anthocyanins are known as a functional component (Kolde, 1997; Wang *et al.*, 1997; Pool-Zobel *et al.*, 1999). If the black soybean is grown under adverse environment by which anthocyanins synthesis can be stimulated after 73 DAF, the seeds having higher level of anthocyanins can be produced without reduction in grain yield. In recent the demand for black soybean is increasing due to the increased demand for well-being. Besides the genetic improvement of anthocyanins level by breeding, the effort with controlling cultural practice to increase the level in seed should be conducted.

Changes in storage protein fractions in seeds

SDS-PAGE profile of seed storage protein is presented in Fig. 6. Each subunit of 11S and 7S protein fractions was separated well enough to discriminate the subunits. The fraction of 11S protein was separated into three subunits, α', α, and β subunit, and 7S protein was separated clearly into two fraction, acidic and basic subunit. The accumulation of β subunit of 11S was initiated later than the other subunits of 11S and 7S protein fraction. There was no large difference in the protein profile of seeds sampled from 61 DAF to 89 DAF. From 61 DAF, accumulation pattern did not show large qualitative (banding pattern) difference. In addition, the A_5 subunit of 11S basic protein was clearly detectable at the late stage of seed development (89 DAF). Therefore, the occurrence of A_5 subunit can be used as a criterion for determining whether the seed is attained to maturity. Glycinin

Fig. 6. Electrophoretic profiles of seed storage protein from developing soybean seeds M standard markers, 1 29 DAF, 2 42 DAF, 3· 50 DAF, 4·61 DAF; 5·66 DAF, 6 73 DAF, 7 82 DAF, 8·89 DAF, respectively

(acidic and basic subunits) and β-conglycinin (α', α, and β subunits) are the major storage proteins in soybeans. Both proteins constitute 58~78% of the extractable protein (Cai & Chang, 1999). The relative composition of 7S proteins was higher in early stage from 29 DAF to 61 DAF (Table 1). The 11S protein fractions were accumulated later as compared to 7S proteins. The proportion of 7S α' fraction was decreased as seeds are matured On the other hands, the α' fraction was increased up to 7 7 %.

The overall profile of proteins fractions at 61 DAF was similar to that from harvest. The result showed that the onset of declining of starch level in leaves was consistent with the independent accumulation of protein fractions in soybean seeds during seed development. The 11S/7S ratio of soybean storage protein has been known as a factor influencing to the gel firmness of soybean curd (Kang *et al.*, 1991). The 11S/7S ratio after 61 DAF, however, was not highly variable as compared to the quantitative changes in protein fractions The result of Kim *et al.* (2004) showed no qualitative differences among different colored soybean varieties. Although the result presented here was from black (colored) soybean, the accumulating kinetics of seed storage proteins during seed development may be similar with that of other varieties. The ratio of 11S/7S proteins changed from 0.56 at 42 DAF to 1.58 at 89 DAF. As compared to the previous result of Taira (1972) and Murphy & Resurreccion (1984), the ratio of 1.58 at physiological maturity was slightly different. The ratio, however, is variable depending on the genetic and environmental differences (Murphy & Resurreccion, 1984).

In conclusion, the substantial accumulation of seed chemical components including proteins and anthocyanins were occurred after 61 DAF at which the increase in pod fresh weight was nearly stopped and chlorophyll and soluble protein content both in leaves and pods became to decrease. The SDS-PAGE protein profile at 61 DAF was completed without large difference with that at physiological maturity (89 DAF). There was a little fluctuation in relative content of protein subunits among seed developing period after 61 DAF. The results suggested that qualitative maturation of seed with respect to the chemical composition was completed at 61 DAF, although quantitative changes were persistent until harvest in black soybean.
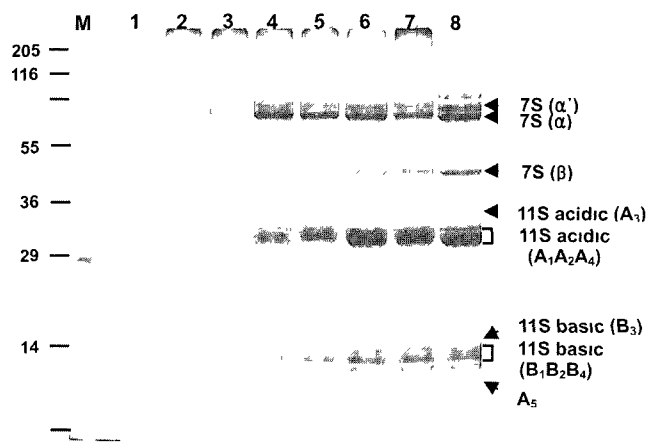
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