Factors Affecting Isoflavone Content in Soybean Seeds: Changes in Isoflavones, Saponins, and Composition of Fatty Acids at Different Temperatures during Seed Development

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Factors affecting the isoflavone contents of soybean seeds were studied. Isoflavone contents of seeds of varieties grown at different locations, on different planting dates, and under different temperatures during seed development were determined by HPLC analysis. Fatty acid composition and contents of DDMP-conjugated saponin were also analyzed. The isoflavone content, together with the ratio of linoleic plus linolenic acid to total fatty acid, significantly decreased in the seeds harvested after growth at a high temperature for all soybean varieties tested. A general decrease was observed for all isoflavones, rather than a decrease restricted to a single molecular species. Of the total seed isoflavones, 80–90% were located in cotyledons, with the remainder in the hypocotyls. The hypocotyls had a higher concentration of isoflavones on a weight basis compared with cotyledons. While the isoflavone content of cotyledons exhibited large changes in response to high temperature during seed development, the isoflavone content remained high in the hypocotyls. As previously reported for other saponins, the DDMP-conjugated saponin content of seeds remained stable in response to elevated temperatures during seed development. These studies provide a basis for attempts to improve seed quality by the reduction of isoflavone content.

Keywords: *Isoflavone; genistein; glycitein; daidzein; saponin; soyasaponin; fatty acid; soybean; Glycine max*

INTRODUCTION

Although soybeans are an important food source, undesirable flavors and objectionable bitter and astringent tastes can be associated with soy products. Many attempts to improve the unfavorable characteristics of soybean seeds by genetic means have been made. Although the elimination of lipoxygenases from seeds was successfully achieved (Kitamura et al., 1983, 1985; Kitamura, 1984; Davies and Nielsen, 1986; Hajika et al., 1991) and contributes to the improvement of the bean flavor of soybean products (Matoba et al., 1985; Davies et al., 1987; Kitamura, 1993), factors that impart bitter and astringent flavors have remained. These undesirable characteristics are considered to be due to saponins (Iijima et al., 1987; Kitagawa et al., 1988; Taniyama et al., 1988; Okubo et al., 1992), phenolic acids (Arai et al., 1966), oxidized phospholipids (Sessa et al., 1976), oxidized fatty acids (Usuki and Kaneda, 1980), and isoflavones (Huang et al., 1981; Matsuura et al., 1989; Kudou et al., 1991; Okubo et al., 1992).

The group A saponins have been demonstrated to be responsible for an undesirable bitter and astringent taste (Iijima et al., 1987; Kitagawa et al., 1988; Taniyama et al., 1988; Okubo et al., 1992). At the same time, however, 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP)-conjugated saponins (Kudou et al., 1992, 1993) and their degradation products, or subgroup B and E saponins (Fenwick et al., 1991), have health benefits such as inhibition of the infectivity of the AIDS virus (HIV) (Nakashima et al., 1989) and inhibition of the activation of the Epstein–Barr virus early antigen (Konoshima and Kozuka, 1991). The reduction of saponins possessing undesirable characteristics by genetic means, together with an increase of the other saponins with health benefits, is considered to be of considerable importance. In this regard, the content of group A saponins is reported to depend more closely on genetic characteristics than on environmental effects (Shiraiwa et al., 1991; Tsukamoto et al., 1994). Therefore, the identification of a group of mutants deficient in group A saponins (Shiraiwa et al., 1990; Tsukamoto et al., 1992, 1993a,b, 1994) would contribute to the improvement of soybean-based foods.

Chemical structures, taste characteristics, and localization of isoflavones in seed have been well studied (Kudou et al., 1991). Many recent papers recognize the potential benefit of isoflavone components for reducing the risk of various cancers (Akiyama et al., 1987; Graber et al., 1992; Pagliacci et al., 1993; Mousavi and Adlercreutz, 1993; Fotsis et al., 1993; Mccabe and Orrenius, 1993; Jing et al. 1993) and for related pharmaceutical roles (Takano et al., 1993; Sargeant et al., 1993; Smith et al., 1993; Keung and Vallee, 1993). Therefore, as with the case of the saponins, enhancement of beneficial isoflavones, together with the reduction or elimination of other members of this group of compounds, could also contribute to improvement of soybean-based foods.

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In addition to potential medicinal applications, isoflavones are reported to perform a number of important physiological functions involved in the growth and development of soybeans. For example, soybean isoflavones induce *nod* genes in *Bradyrizobium japonicum* (Cho and Harper, 1991; Kape et al., 1991; Smit et al., 1992), and they are associated with the response of soybeans to infection by *Phytophthora megasperma* (Graham et al., 1990). Becuase of these physiological responses, it has been assumed that isoflavone content might be difficult to control by genetic means. However, certain soybean cultivars have recently been identified that have remarkably low isoflavone contents in seeds (Kitamura et al., 1991). Despite a low isoflavone content, these seeds germinated and grew without adverse visible effect to important agronomic characteristics such as plant stature and seed yield. Although the low seed isoflavone content of these cultivars was found to be stable over the 2-year period of examination when grown under standard culture conditions, they produced seeds with nearly normal isoflavone contents when seed fill occurred under late culture conditions in Japan (Kitamura et al., 1991). Growth under late culture conditions in Japan causes the plants to be exposed to lower temperatures during seed fill. This result suggests that while isoflavone accumulation in seeds is affected by environmental factors, it may also be manipulated by genetic methods. Although it is known that seed isoflavone content responds strongly to environmental changes (Eldridge and Kowlek, 1983), a systematic evaluation of this response has not been reported.

To analyze more precisely environmental effects on seed development, we studied the influence of soybean variety, location, and seeding time on the amount on isoflavone accumulation. Also measured during these studies were fatty acid composition (Collins and Howell, 1957; Howell and Collins, 1957; Chapman et al., 1976; Wolf et al., 1982) and saponin content (Shiraiwa et al., 1991; Tsukamoto et al., 1994). Finally, the effect of the temperature during seed development was examined using temperature-controlled growth cabinets to identify the factors affecting the isoflavone accumulation. The data obtained indicate that elevated growth temperature plays an important role in decreasing isoflavone content in seed cotyledons but has a less marked effect on the isoflavone accumulation in hypocotyls.

MATERIALS AND METHODS

Sources and Cultivation of Soybeans. Four soybean cultivars with low isoflavone content (Koganedaizu, Higomusume, Shirosaya 1, and Kairyoshirome) (Kitamura et al., 1991) and three controls with normal content (Suzuyutaka, Fukuyutaka, and Lee) were used in the field studies conducted in 1991. Soybean seeds for component analysis were obtained from the plants sown on April 19, May 22, June 27, and July 11 in the field of Kyushu National Agricultural Experiment Station, Kumamoto, latitude 33° N (Kyushu) or from the plants sown on May 7 and 28, June 10, and July 10 in the field of the National Agriculture Research Center, Tsukuba, latitude 36° N (Tsukuba).

Three cultivars, Koganedaizu, Higomusume, and Suzuyutaka, were grown at Tsukuba in temperature-controlled, sunlit Koitotron type S-152A growth chambers (Koito Industries Ltd., Yokohama, Japan). Seeds were sown on July 1, 1992, in pots filled with 5.6 kg of air-dried light-colored Andosol and chemical fertilizer [40 g of compound fertilizer ($N-P_2O_5-K_2O$ = 3:10:10), 15 g of $CaCO_3$, 30 g of fused magnesium phosphate]. They were initially grown outdoors under ambient conditions. When the plants reached the R5 stage of development, they were transferred into the growth chambers and remained there until the beginning of maturity (R7 stage). Each stage was identified according to the guidelines of Fehr et al. (1971). After reaching the R7 stage, the plants were transferred outdoors, and at physiological maturity the seeds were harvested for component analysis. Only the temperature was controlled in the growth cabinets, not the humidity and light intensity. Sufficient water was provided to avoid water-deficit stress.

Analysis of Isoflavone Content by High-Performance Liquid Chromatography (HPLC). The quantitative analysis of isoflavone glycosides was done according to the methods of Kudou (Kudou et al., 1991) and Kitamura (Kitamura et al., 1991), with some modifications. Either whole soybean seeds or seed hypocotyls (plumule plus radicle) were analyzed independently. For this purpose, whole seeds were ground into a fine powder with a mill (Retsch, Ultracentrifugal mill, 0.5-mm mesh screen). One hundred milligrams of the milled sample was extracted with 0.5 mL of 70% aqueous ethanol containing 0.1% acetic acid for 24 h at room temperature in screw-capped test tubes. Seed hypocotyls were collected from lyophilized seeds. They were ground into a powder, and then 25 mg of the dry sample was extracted in the same manner as for whole seeds. After centrifugation of the extract, a 20-μL aliquot of the supernatant was used directly for the HPLC analysis. Analysis of isoflavones from whole seeds was performed on ODS commercially packed columns (Tosoh Corp., Tokyo; TSKgel ODS-80 i.d. 4.6 × 250 mm), while hypocotyls were analyzed with a CAPCELL PAK, C_{18} SG120 column (Shiseido, Tokyo, i.d. 4.6 × 250 mm). A 0.1% acidic acid solvent system that contained a linear gradient of acetonitrile that ran from 15% to 40% during 25 min was used for the whole seed sample analysis. For hypocotyls, a linear gradient from 15% to 35% over a time period of 50 min was used. The solvent flow rate was 1.0 mL/min, and the UV absorption was measured at 260 nm. Purified soybean isoflavones obtained by Kudou's method (Kudou et al., 1991) were used as standards. Isoflavone contents were calculated as milligrams per 100 g of dry matter. Moisture content (percent) of whole seed powders was obtained after drying to constant weight at 105 °C for 24 h.

Analysis of DDMP-Conjugated Saponin Contents by HPLC. Three kinds of DDMP-conjugated saponins (soyasaponins αg, βg, and βa) were analyzed quantitatively by HPLC as described by Kudou et al. (1992, 1993). Whole soybean seeds were ground into a powder with a mill; 100 mg of the milled sample was extracted the same as for isoflavone analysis. A 20-μL aliquot of the supernatant was used directly for the HPLC analysis. Separations were performed on an ODS commercially packed column (Tosoh; TSKgel ODS-80 i.d. 4.6 × 250 mm). Saponins were subjected to isoclatic analysis using an acetonitrile–water–acetic acid [420:579:1 (v/v)] solvent system. The solvent flow rate was 1.0 mL/min, and the UV absorption was measured at 205 and 292 nm. Purified soybean saponins obtained by Kudou's methods (Kudou et al., 1992, 1993) were used as standards. DDMP-conjugated saponin contents were calculated as milligrams per 100 g of dry matter. Moisture content (percent) of whole seed powders was obtained after drying to constant weight at 105 °C for 24 h.

Fatty Acid Composition Determination by Gas Chromatography (GC). Fatty acid methyl esters (FAME) were prepared directly from whole seed powder. A 20-mg aliquot of the milled soybean seeds was dispersed in 0.8 mL of methanol that contained 12.5% acetyl chloride. The extractions at 80 °C for 3 h. Water (0.8 mL) and *n*-hexane (0.8 mL) were then added to the screw-cap vials and mixed vigorously. After centrifugation, a 5-μL aliquot of the upper layer was used directly for gas chromatography. A Shimadzu GC-6A apparatus (Shimadzu Corp., Kyoto, Japan) fitted with a flame ionization detector was used for this purpose. A commercially packed column [GL Science Inc., Tokyo; Chromosorb W(AW) 100/120 mesh coated with SP 2310 + SP 2300 (3+2%) packed in a glass column (i.d. 2.6 mm × 2 m)] was used for FAME separation. Nitrogen at 50 mL/min was used as the carrier gas. The temperature of the injector and detector was maintained at 230 °C, and the column temperature was kept

Table 1. Climatic Data at Kyushu and Tsukuba for the Period April–November 1991[a]

	Kyushu (latitude 33° N)					Tsukuba (latitude 36° N)				
	temperatures			sunshine		temperatures			sunshine	
month	mean	max	min	duration	precipitation	mean	max	min	duration	precipitation
April	15.4	21.0	9.8	83.2	123.0	13.6	18.6	9.3	136.6	91.5
May	18.3	22.6	14.0	126.2	247.6	17.0	22.1	12.2	166.0	60.5
June	23.6	26.4	20.7	97.1	506.5	22.1	26.3	18.6	75.8	118.5
July	27.2	30.4	24.0	147.8	327.5	24.6	29.3	21.1	77.3	127.0
Aug	27.2	31.4	22.9	202.1	128.0	23.8	28.2	20.7	109.7	184.0
Sept	24.7	29.5	19.8	209.3	104.5	22.3	26.2	19.4	87.9	419.0
Oct	17.3	22.9	11.8	123.4	63.0	16.6	20.1	13.3	75.7	457.5
Nov	10.8	18.1	3.5	185.8	72.5	10.2	15.5	5.5	148.1	89.0

[a] Values are expressed in °C (mean of mean, maximum, and minimum temperatures of a day for the month), h (sum of sunshine duration for the month), and mm (sum of precipitation for the month).

Table 2. Maturity Group, Harvest Date, 100-Seed Weight, and Moisture and Hypocotyl Contents of Soybeans Grown at Kyushu and Tsukuba at Different Sowing Dates in 1991[a]

variety name	maturity group	sowing date	harvest date	100-seed weight	moisture content	hypocotyl content
			Kyushu (33° N)			
Higomusume	I	April 19	Aug 14	17.4	13.6	2.4
		May 22	Aug 30	14.4	13.6	2.6
		July 11	Sept 25	14.2	13.6	2.8
Kairyoshirome	I	April 19	Aug 20	17.2	13.6	2.1
		May 22	Aug 31	13.7	13.4	2.1
		July 11	Sept 24	16.9	13.5	1.9
Shirosaya 1	I	April 19	Aug 21	16.0	13.6	2.6
		May 22	Aug 27	12.3	13.6	3.1
		July 11	Sept 25	14.9	13.5	2.7
Koganedaizu	II	April 19	Aug 21	21.9	13.5	2.3
		May 22	Sept 5	20.9	13.5	2.0
		July 11	Sept 30	13.7	13.5	3.2
Suzuyutaka	IV	April 19	Sept 9	17.0	13.5	2.5
		May 22	Sept 9	18.6	13.6	2.4
		June 27	Oct 2	21.4	13.5	2.5
Fukuyutaka	VI	June 27	Oct 28	25.5	13.6	2.1
		July 11	Oct 27	26.0	13.6	2.2
Lee	VI	April 19	Sept 9	13.2	13.6	2.8
		May 22	Sept 9	8.5	13.5	3.1
		July 11	Oct 25	14.9	13.5	2.6
			Tsukuba (36° N)			
Higomusume	I	May 7	Aug 24	19.0	13.5	2.3
		June 10	Sept 12	18.2	13.5	2.1
Shirosaya 1	I	May 7	Aug 24	19.0	13.4	2.3
		June 10	Sept 12	18.9	13.4	2.2
Koganedaizu	II	May 7	Aug 27	22.2	13.4	2.2
		May 28	Sept 17	23.3	13.3	2.3
		June 10	Sept 25	22.1	13.4	2.2
Suzuyutaka	IV	May 7	Oct 5	24.8	13.6	2.1
		July 10	Oct 16	25.5	13.5	2.0
Fukuyutaka	VI	May 7	Oct 31	33.0	13.7	1.9
		June 10	Nov 3	31.4	13.7	2.0
		July 10	Nov 5	31.9	13.6	2.0
Lee	VI	May 7	Oct 28	18.0	13.4	2.5
		June 10	Oct 31	17.8	13.4	2.7
		July 10	Nov 5	19.0	13.4	2.5

[a] Values are expressed in g (100-seed fresh weight) and in % (moisture and hypocotyl contents) and represent means, $n = 2$.

at 200 °C. Peaks were identified by using standard FAME (Sigma, St. Louis, MO), and the percentages of FAME were calculated from peak areas obtained by using an electronic integrator (Shimadzu C-R1A).

Statistical Analysis. Statistical differences between two means were examined by the *t*-test, using the program TMEAND (Wakimoto et al., 1984). Analysis of variance (ANOVA) was conducted by using the program AOV2 (Tanaka and Tarumi, 1986).

RESULTS AND DISCUSSION

Seven varieties, four low-isoflavone varieties (Higomusume, Kairyoshirome, Shirosaya 1, and Koganedaizu) and three ordinary varieties (Suzuyutaka, Fukuyutaka, and Lee), were grown at Kyushu (latitude 33° N) and Tsukuba (latitude 36° N) in 1991. Four sowing dates (April, May, June, and July) were used for each variety at each location so that advantage could be taken of naturally occurring differences in environmental conditions. Climatic data for the period April–November 1991 at Kyushu and Tsukuba are summarized in Table 1. Data showing maturity group, sowing and harvest dates, 100-seed weight, and moisture and hypocotyl contents of seeds harvested from these plants are shown in Table 2.

The isoflavones from whole seeds were isolated and identified. Daidzin, genistin, malonyldaidzin, and malonylgensitin were routinely detected, but other isoflavones (aglycons and acetyl forms) were present only in trace amounts. Although the amount of change in isoflavone content depended on the variety, total isofla-

Table 3. Isoflavone Content of Whole Seeds from Seven Varieties Grown at Kyushu and Tsukuba That Were Planted on Different Sowing Dates in 1991[a]

variety name	sowing date	daidzin	genistin	malonyldaidzin	malonylgenistin	total
			Kyushu			
Higomusume	April 19	4.2	0.7	13.6	9.7	28.2
	May 22	2.8	1.9	8.7	6.7	19.9
	July 11	9.9	11.7	37.5	48.6	107.6
Kairyoshirome	April 19	14.2	1.5	42.5	26.9	85.0
	May 22	10.1	7.4	25.3	20.7	63.4
	July 11	20.5	4.7	84.4	55.5	165.1
Shirosaya 1	April 19	11.5	4.3	33.9	22.6	72.2
	May 22	12.0	2.9	27.3	13.9	56.1
	July 11	16.2	8.9	79.8	57.7	162.6
Koganedaizu	April 19	5.5	0.7	21.1	17.9	45.2
	May 22	7.3	1.3	19.0	14.0	41.5
	July 11	21.0	6.9	97.4	88.5	213.7
Suzuyutaka	April 19	4.8	3.7	12.2	12.0	32.6
	May 22	4.9	1.6	19.5	13.6	39.5
	June 27	6.8	1.6	36.6	37.9	82.8
Fukuyutaka	June 27	5.0	7.3	18.9	40.8	72.0
	July 11	4.4	3.9	38.1	66.6	112.9
Lee	April 19	5.8	2.3	9.1	7.6	24.7
	May 22	7.5	nd	11.1	6.1	24.6
	July 11	9.4	5.7	58.9	68.4	142.3
			Tsukuba			
Higomusume	May 7	13.8	1.2	55.0	48.9	118.8
	June 10	6.5	0.7	36.3	35.6	79.0
Shirosaya 1	May 7	15.0	1.6	59.6	43.4	119.5
	June 10	10.9	1.6	44.0	34.7	91.1
Koganedaizu	May 7	18.7	nd	62.1	44.0	124.7
	May 28	12.9	nd	56.7	47.5	117.0
	June 10	15.1	nd	70.5	66.7	152.3
Suzuyutaka	May 7	17.3	4.1	76.1	89.4	186.8
	July 10	16.4	6.5	86.2	86.8	195.8
Fukuyutaka	May 7	14.3	1.8	73.4	124.8	214.2
	June 10	14.2	2.3	83.6	114.3	214.4
	July 10	13.0	1.7	81.1	107.5	203.2
Lee	May 7	23.3	10.7	143.9	145.8	323.6
	June 10	19.8	14.7	151.5	154.6	340.5
	July 10	19.6	14.9	162.8	153.7	351.0

[a] nd, not detected. Values are expressed in mg/100 g of dry seed and represent means, $n = 2$.

vone contents of almost all varieties sown in April and May and grown at Kyushu were much lower when compared to that in seeds from plants sown in June and July (Table 3). For example, the total isoflavone content of Lee sown July 11 was 5.8 times higher than that in plants sown May 22. Changes of isoflavone content in response to different sowing dates were observed not only in low-isoflavone varieties but also in ordinary ones when they were grown at Kyushu. The changes in the total isoflavone content were not restricted to one or two isoflavones, but rather a general increase of all components was found. Interestingly, no differences in isoflavone content as a function of sowing dates were detected in seeds from the plants grown at Tsukuba. This suggests that unknown climatic and environmental factors contribute to isoflavone contents in soybean seeds.

It was estimated that 10–20% (w/w) of the total isoflavones in the seeds were located in hypocotyls even though they account for only about 2% of total seed weight (Kudou et al., 1991). Reports about the physiological activities of isoflavones in soybean plants have been limited to the roots and hypocotyls of the plant, such as the induction of *nod* genes in *B. japonicum* (Cho and Harper, 1991; Kape et al., 1991; Smit et al., 1992) and the responses of soybean tissues to infection with *P. megasperma* (Graham et al., 1990). The concentration and distribution of these compounds in hypocotyls were next investigated.

Daidzin, glycitin, genistin, malonyldaidzin, malonylglycitin, and malonylgenistin were detected, but the other isoflavone components such as aglycons or acetyl forms were at the limits of detection with the method employed. In general, the isoflavone contents in the hypocotyls were much higher than in whole seeds and the changes in content were less pronounced (Table 4). These results suggest mechanisms exist that permit a higher isoflavone content to be maintained in hypocotyls than in cotyledons. The observation has important practical implications because it means that isoflavone content in cotyledons and hypocotyls can to a large extent be manipulated independently of one another.

Although there is a report that the contents of group A saponins (Shiraiwa et al., 1991) and subgroup B and E saponins (Tsukamoto et al., 1994) are not so influenced by environmental effects, the situation with DDMP-conjugated saponins remains to be investigated. Because DDMP-conjugated saponins are thought to be beneficial for human health, we considered it important to make this determination. Because soyasaponin αg is detected only in hypocotyls, soyasaponin βa is detected in cotyledons, and soyasaponin βg is detected in both parts (Kudou et al., 1993; Tsukamoto et al., 1993a), it was possible to distinguish among the various seed tissues by an analysis of whole seed powders. There was no difference among different sowing dates in Kyushu and Tsukuba (Table 5). The result suggested that like the other saponins tested previously, DDMP-conjugated saponin contents do not respond to environmental stress in the same manner as isoflavones.

It has been reported that the fatty acid composition is markedly affected by the maximum temperature during seed development (Collins and Howell, 1957; Howell and Collins, 1957; Chapman et al., 1976; Wolf et al., 1982) and that the contents of linoleic and linolenic acids decrease while oleic acid increases as temperature increases. It has further been reported

Table 4. Isoflavone Content of Hypocotyls from Seeds of Seven Soybean Varieties Grown at Kyushu and Tsukuba in 1991[a]

variety name	sowing date	daidzin	glycitin	genistin	malonyldaidzin	malonylglycitin	malonylgenistin	total
				Kyushu				
Higomusume	April 19	84.0	58.3	51.3	180.4	104.1	170.0	648.0
	May 22	82.2	44.2	49.9	192.1	90.1	181.5	640.0
	July 11	75.9	58.7	65.1	242.1	143.5	206.7	792.0
Kairyoshirome	April 19	145.3	112.1	70.7	224.0	192.9	100.0	845.0
	May 22	126.9	87.6	66.7	248.7	202.9	107.8	840.6
	July 11	110.3	105.1	95.6	244.7	261.4	112.1	929.2
Shirosaya 1	April 19	137.5	122.2	72.1	213.5	269.2	138.4	952.9
	May 22	117.0	81.4	58.3	209.1	216.1	116.2	798.2
	July 11	138.2	125.9	85.1	317.7	332.2	181.9	1180.9
Koganedaizu	April 19	105.8	58.9	57.0	228.3	108.9	228.8	787.7
	May 22	98.3	56.0	68.6	211.9	128.3	208.1	781.3
	July 11	117.2	80.8	103.9	323.2	212.7	253.9	1091.8
Suzuyutaka	April 19	85.9	187.6	46.7	177.3	364.9	140.9	1003.2
	May 22	55.6	163.9	43.4	178.5	391.1	155.0	987.5
	June 27	53.0	185.2	51.0	152.5	414.4	152.6	1008.8
Fukuyutaka	June 27	62.6	50.4	67.5	248.1	152.4	286.6	867.4
	July 11	58.7	49.4	92.9	245.2	161.1	309.5	916.8
Lee	April 19	34.8	180.5	31.9	102.8	377.9	108.0	835.8
	May 22	16.5	94.1	21.5	61.2	249.0	82.3	524.7
	July 11	48.1	220.3	55.8	160.6	577.3	164.5	1226.5
				Tsukuba				
Higomusume	May 7	81.4	61.2	103.7	228.3	145.1	224.6	844.4
	June 10	113.4	81.8	136.6	286.6	190.4	226.6	1035.5
Shirosaya 1	May 7	96.7	78.9	101.3	247.7	185.9	197.3	907.6
	June 10	103.4	74.8	81.0	271.1	176.1	151.9	858.3
Koganedaizu	May 7	117.5	74.7	96.1	282.2	167.2	191.0	928.8
	May 28	121.9	78.1	101.5	335.2	197.7	204.2	1038.6
	June 10	121.6	74.4	111.2	324.5	171.6	201.0	1004.3
Suzuyutaka	May 7	58.6	216.1	95.3	278.4	557.9	146.4	1352.9
	July 10	128.6	225.0	132.2	288.9	567.6	257.2	1599.5
Fukuyutaka	May 7	51.7	64.6	82.6	201.7	152.2	304.2	857.0
	June 10	56.6	41.9	81.3	274.6	117.1	310.9	882.5
	July 10	71.1	47.6	82.9	346.9	125.2	296.5	970.2
Lee	May 7	40.5	212.4	65.0	157.3	491.0	181.8	1148.0
	June 10	61.9	234.1	83.0	263.5	556.3	193.2	1392.0
	July 10	51.4	220.6	59.8	228.5	547.2	175.5	1283.0

[a] Values are expressed in mg/100 g of dry seed and represent means, $n = 2$.

Table 5. DDMP-Conjugated Saponin Content of the Whole Seeds from Seven Soybean Varieties Planted on Different Sowing Dates Grown at Kyushu and Tsukuba in 1991[a]

variety name	sowing date	soyasaponin αg	soyasaponin βg	soyasaponin βa	total
		Kyushu			
Higomusume	April 19	6.9	76.4	63.7	146.9
	May 22	7.0	88.4	58.4	153.7
	July 11	12.0	122.9	104.5	239.4
Kairyoshirome	April 19	8.7	144.8	63.9	217.5
	May 22	11.8	166.2	56.3	234.4
	July 11	10.3	175.9	88.1	274.3
Shirosaya 1	April 19	8.5	109.2	68.6	186.3
	May 22	7.2	105.8	40.0	152.9
	July 11	9.0	132.6	95.8	237.4
Koganedaizu	April 19	9.6	118.6	68.5	196.7
	May 22	9.0	125.0	61.6	195.6
	July 11	14.0	146.5	113.9	274.4
Suzuyutaka	April 19	9.6	142.8	52.1	204.4
	May 22	11.6	167.4	62.3	241.2
	June 27	10.6	152.1	55.6	218.2
Fukuyutaka	June 27	12.4	135.7	46.5	194.6
	July 11	12.2	132.3	47.5	192.0
Lee	April 19	19.7	251.7	73.4	344.8
	May 22	18.3	244.9	57.2	320.4
	July 11	8.8	226.1	83.4	318.3
		Tsukuba			
Higomusume	May 7	11.7	118.8	117.9	248.4
	June 10	14.9	114.3	104.2	233.4
Shirosaya 1	May 7	11.2	97.7	95.9	204.9
	June 10	10.8	83.4	79.8	174.0
Koganedaizu	May 7	9.2	148.0	56.3	213.5
	May 28	8.2	124.4	74.6	207.2
	June 10	6.3	126.4	87.8	220.5
Suzuyutaka	May 7	10.9	125.4	55.2	191.5
	July 10	16.2	155.8	51.0	223.0
Fukuyutaka	May 7	17.6	127.5	50.2	195.6
	June 10	8.6	98.8	33.5	140.9
	July 10	13.3	114.3	32.2	159.7
Lee	May 7	15.9	189.8	70.7	276.4
	June 10	16.5	193.9	78.5	288.9
	July 10	13.9	171.2	68.2	253.3

[a] Values are expressed in mg/100 g of dry seed and represent means, $n = 2$.

Table 6. Relative Fatty Acid Composition and C18:2 + C18:3 to Total Fatty Acid Ratio of Whole Seeds from Seven Varieties Planted on Different Sowing Dates and Grown at Kyushu and Tsukuba in 1991[a]

variety name	sowing date	C16:0	C18:0	C18:1	C18:2	C18:3	(C18:2 + C18:3)/total FA
				Kyushu			
Higomusume	April 19	14.3	2.9	43.4	35.0	4.3	39.4
	May 22	14.9	3.4	37.4	39.0	4.9	44.1
	July 11	15.4	3.0	24.4	49.6	7.6	57.2
Kairyoshirome	April 19	11.8	2.3	52.0	29.0	4.6	33.7
	May 22	15.2	2.6	40.3	35.7	5.7	41.6
	July 11	14.2	2.5	26.4	48.6	8.0	56.7
Shirosaya 1	April 19	12.5	2.5	37.2	42.7	5.1	47.8
	May 22	14.1	2.8	38.2	39.8	5.3	45.0
	July 11	13.3	2.5	20.9	55.5	7.8	63.3
Koganedaizu	April 19	16.1	2.3	43.7	33.5	3.9	37.6
	May 22	16.5	2.6	41.8	34.2	4.7	38.9
	July 11	18.2	2.5	21.1	50.4	7.7	58.2
Suzuyutaka	April 19	14.7	2.7	30.3	45.8	6.7	52.4
	May 22	14.6	2.7	25.1	50.4	7.4	57.7
	June 27	14.3	2.9	21.7	52.7	8.4	61.1
Fukuyutaka	June 27	13.1	2.9	20.3	56.2	7.5	63.7
	July 11	13.4	2.8	18.2	57.7	8.0	65.7
Lee	April 19	15.6	2.9	23.5	51.8	6.3	58.1
	May 22	16.8	3.0	21.8	51.0	7.4	58.4
	July 11	13.0	3.0	19.8	55.7	8.5	64.2
				Tsukuba			
Higomusume	May 7	14.1	2.4	37.3	40.2	5.5	45.9
	June 10	14.5	2.7	35.7	41.0	5.7	46.9
Shirosaya 1	May 7	14.2	2.5	30.6	45.9	6.6	52.6
	June 10	14.4	2.8	30.9	44.2	7.4	51.8
Koganedaizu	May 7	12.9	2.8	47.8	31.9	4.6	36.5
	May 28	12.7	2.7	46.8	32.7	5.0	37.7
	June 10	13.2	2.3	40.1	38.9	5.5	44.4
Suzuyutaka	May 7	13.9	2.7	23.6	51.1	8.3	59.7
	July 10	13.6	3.2	17.8	54.2	11.0	65.3
Fukuyutaka	May 7	12.0	3.0	17.0	59.5	8.5	68.0
	June 10	11.7	2.5	20.5	57.3	7.9	65.2
	July 10	11.9	2.9	18.5	58.1	8.5	66.7
Lee	May 7	12.2	3.0	20.8	56.5	7.4	64.0
	June 10	11.7	2.7	23.4	54.4	7.7	62.2
	July 10	11.8	2.7	22.4	54.7	8.0	63.0

[a] Values are expressed in % and represent means, $n = 2$.

Table 7. Mean Values of Seed Component Analysis in Four Groups Composed of 70 Different Samples Belonging to Seven Soybean Varieties Based on the Difference in Locations and Harvest Periods in 1991[a]

location	harvest period	sample size	whole seed total isoflavone	hypocotyl total isoflavone	total DDMP–saponin	(C18:2 + C18:3)/total FA
Kyushu	before Sept 15	24	44.4 ± 20.5^{abc}	803.7 ± 148.8^{ab}	216.2 ± 61.8	46.1 ± 8.4^{ab}
	later than Sept 15	16	132.4 ± 45.4^{ad}	1001.7 ± 161.2^{a}	243.6 ± 42.6	61.2 ± 3.4^{ac}
Tsukuba	before Sept 15	10	107.6 ± 17.9^{be}	914.9 ± 103.9^{c}	214.9 ± 27.5	46.5 ± 5.8^{cd}
	later than Sept 15	20	229.9 ± 78.9^{cde}	1152.5 ± 246.9^{bc}	215.7 ± 46.9	59.2 ± 9.6^{bd}

[a] Values are expressed in mg/100 g of dry seed (isoflavone and saponin contents) and in % (fatty acid ratio). Means with the same letter in the same column are significantly different at 1% level by t-test.

that changes in other environmental factors such as photoperiod, light intensity and quality, and N, P, K, and S nutrition have little or no effect on the levels of these fatty acids. We therefore tested whether the ratio of linoleic and linolenic acids to total fatty acids changed in our plants in the same manner reported in the literature and at the same time changes were occurring in isoflavone content. The tendency of the changes of the ratio at different locations and sowing dates was very similar to those of the isoflavones (Table 6).

We divided the data, which included 70 different samples that consisted of duplicate determinations of 7 varieties collected in the field experiment of 1991, into four groups. The four groups related to plants harvested either before or after September 15 at each of the two growth locations. When harvest took place before September 15, the soybeans were likely to be exposed to high temperatures during seed development. Furthermore, as shown in Table 1, means of temperature at Kyushu (latitude 33° N) were much higher than those at Tsukuba (latitude 36° N). The isoflavone contents of the groups harvested before September 15 were significantly lower than those of the group harvested after September 15, even for the plants grown at Tsukuba (Table 7). The same tendency was observed for the fatty acid composition. Thus, temperature during seed development rather than sowing date seems to have a major influence on total seed isoflavone and unsaturated fatty acid content. Isoflavone content in hypocotyls was also affected by harvest dates, but the changes in the contents were less pronounced than those in whole seeds.

To further examine the effect of the temperature on the isoflavone content during seed development, we performed an experiment using temperature-controlled growth cabinets. Growth conditions, dates for flowering, seed development (R5 stage), beginning of maturity (R7), and harvest, 100-seed weight, and moisture and hypocotyl contents of the seeds are summarized in Table 8. Because soybeans grown at high temperature produced some flat and wrinkled seeds, these were omitted from analysis. The seeds used for the data listed in Table 9 were normal in color and shape, and all of the seeds ($n = 10$) germinated well in a germination test.

Table 8. Flowering, Developing Seed (R5), Maturity (R7), and Harvest Dates, 100-Seed Weight, and Moisture and Hypocotyl Contents of Soybeans Grown under Different Conditions in Growth Cabinets in 1992[a]

variety name	condition[b]	F[c]	R5[c]	R7[c]	H[c]	100-seed weight[d]	moisture content[d]	hypocotyl content[d]
Higomusume	high	Aug 10	Aug 29	Sept 21	Sept 28	9.6 ± 1.0[a]	13.3 ± 0.5	2.3 ± 0.2
	low	Aug 9	Aug 29	Sept 28	Oct 21	14.1 ± 1.9[a]	13.6 ± 0.0	2.2 ± 0.1
Koganedaizu	high	Aug 18	Aug 31	Oct 4	Oct 4	8.2 ± 0.4[a]	13.5 ± 0.1	2.6 ± 0.6
	low	Aug 18	Aug 29	Oct 19	Oct 23	14.7 ± 1.0[a]	13.5 ± 0.1	2.2 ± 0.1
Suzuyutaka	high	Aug 9	Aug 29	Oct 4	Oct 11	10.8 ± 1.1[a]	13.3 ± 0.3	2.3 ± 0.2
	low	Aug 9	Aug 29	Oct 26	Oct 27	22.2 ± 0.5[a]	13.6 ± 0.0	2.0 ± 0.0

[a] Values are expressed in g (100-seed fresh weight) and in % (moisture and hypocotyl weight) and represent means ± SD, $n = 3$. [b] Plants were sown on July 1 and grown outdoors except from R5 to R7 stages, during which temperature treatment was applied in growth cabinets. High, daytime (8:00 a.m. to 6:00 p.m., 10 h) 38 °C and nighttime 28 °C; low, daytime (5:00 a.m. to 7:00 p.m., 14 h) 25 °C and nighttime 10 °C. [c] Dates for F (flowering), R5, R7, and H (harvest) indicate mode of triplicate samples. [d] Means with the same letter in the same column in the same variety are significantly different at 1% level by t-test.

Table 9. Analysis of Seed Components of Soybeans Grown at Different Temperatures during Seed Development in Growth Cabinets[a]

	Higomusume		Koganedaizu		Suzuyutaka	
component	high	low	high	low	high	low
isoflavones (whole seed)						
daidzin	0.6 ± 0.1	7.7 ± 1.7	0.9 ± 0.6	10.2 ± 1.8	1.5 ± 0.4	6.1 ± 2.2
genistin	1.6 ± 0.2	11.9 ± 2.7	1.1 ± 0.6	15.0 ± 1.5	1.9 ± 0.3	10.2 ± 3.8
malonyldaidzin	3.5 ± 1.7	47.5 ± 8.7	3.5 ± 0.5	69.0 ± 10.3	1.8 ± 0.0	60.2 ± 15.0
malonylgenistin	5.2 ± 2.0	81.1 ± 8.7	5.2 ± 1.4	100.7 ± 5.1	4.1 ± 0.3	80.3 ± 9.6
total[b]	10.9 ± 3.9[a]	148.3 ± 21.8[a]	10.7 ± 3.1[a]	194.9 ± 15.1[a]	9.4 ± 0.8[a]	156.8 ± 29.9[a]
isoflavones (hypocotyl)						
daidzin	17.1 ± 3.3	38.7 ± 3.4	14.5 ± 4.0	48.3 ± 13.0	16.6 ± 3.5	33.4 ± 4.1
glycitin	11.9 ± 0.8	35.4 ± 4.4	4.3 ± 1.8	39.8 ± 14.6	42.6 ± 11.1	162.5 ± 4.3
genistin	10.8 ± 3.0	28.8 ± 3.5	9.1 ± 0.9	45.9 ± 10.2	8.5 ± 2.5	32.8 ± 7.6
malonyldaidzin	100.4 ± 12.1	283.2 ± 6.4	84.3 ± 19.1	428.6 ± 69.5	79.9 ± 7.5	303.9 ± 6.4
malonylglycitin	47.0 ± 26.3	169.4 ± 13.1	13.2 ± 5.1	155.5 ± 31.2	145.8 ± 24.7	548.3 ± 7.1
malonylgenistin	36.2 ± 20.3	130.7 ± 10.1	10.2 ± 3.9	119.9 ± 24.1	112.5 ± 19.1	422.8 ± 5.4
total[b]	223.3 ± 61.9[a]	686.2 ± 21.2[a]	135.6 ± 33.1[a]	838.0 ± 161.4[a]	405.9 ± 64.6[a]	1503.7 ± 28.9[a]
DDMP–saponins						
soyasaponin αg	9.1 ± 0.3	7.7 ± 1.5	11.7 ± 1.0	9.9 ± 0.9	9.7 ± 1.2	7.9 ± 2.1
soyasaponin βg	122.6 ± 7.7	96.5 ± 7.9	165.6 ± 6.3	137.8 ± 11.5	149.5 ± 16.3	131.8 ± 10.4
soyasaponin βa	68.6 ± 6.1	92.8 ± 14.1	71.8 ± 3.1	89.3 ± 10.7	34.5 ± 3.8	59.6 ± 3.8
total[b]	200.3 ± 12.4	197.0 ± 23.4	249.1 ± 7.5	237.0 ± 23.0	193.7 ± 20.7	199.2 ± 15.0
fatty acids						
palmitic (C16:0)	13.8 ± 0.4	12.2 ± 0.2	13.2 ± 0.6	12.2 ± 0.2	15.3 ± 0.3	12.2 ± 0.3
stealic (C18:0)	2.8 ± 0.1	2.8 ± 0.2	2.8 ± 0.1	2.6 ± 0.2	2.7 ± 0.3	2.2 ± 0.1
oleic (C18:1)	41.9 ± 2.5	25.2 ± 0.5	41.2 ± 3.5	24.7 ± 1.9	27.7 ± 3.4	23.5 ± 1.7
linolic (C18:2)	36.9 ± 2.3	51.2 ± 0.1	37.8 ± 2.6	51.2 ± 1.1	47.9 ± 2.9	51.2 ± 1.0
linolenic (C18:3)	4.0 ± 0.1	8.1 ± 0.5	4.3 ± 0.6	8.9 ± 0.9	6.1 ± 0.6	10.6 ± 0.9
C18:2 + C18:3 ratio[b]	41.2 ± 2.3[a]	59.6 ± 0.6[a]	42.4 ± 3.1[a]	60.4 ± 1.9[a]	54.2 ± 3.3[a]	62.0 ± 1.9[a]

[a] Values are expressed in mg/100 g of dry seed (isoflavones and saponins) and in % (fatty acids) and represent means ± SD, $n = 3$. [b] Means with the same letter in the same row in the same variety are significantly different at 1% level by t-test.

Table 10. *F* Values and Levels of Significance of ANOVA of Seed Components of Three Cultivars Grown in Growth Cabinets at Different Temperatures during Seed Development[a]

source	df	whole seed total isoflavone	hypocotyl total isoflavone	total DDMP-saponin	(C18:2 + C18:3)/total FA
between varieties	2	2.20NS	51.62**	9.53**	12.99**
between temperatures	1	273.85**	281.20**	0.05NS	118.73**
variety × temperature	2	2.20NS	16.93**	0.71NS	6.55*

[a] NS, not significant; * and **, significant at 5 and 1%, respectively.

Seeds from plants grown at high temperatures were significantly lower in 100-seed weight than those of the soybeans grown at low temperature. Data from the analysis of the seed components of the soybeans grown in growth cabinets during the seed development are summarized in Table 9. Both the isoflavone content and the ratio of linoleic plus linolenic acids to total fatty acids were reduced significantly (1% level) at elevated temperatures. The difference in the isoflavone content was 14–16-fold in the whole seed, while it was 3–6-fold in the hypocotyl for the varieties studied. The DDMP-conjugated saponin contents were not significantly different between the two temperature conditions. Again, there was a general decrease in isoflavone content in response to high temperature rather than changes confined to certain compounds. The decrease of linoleic plus linolenic acid in response to elevated temperature was accompanied by an increase in oleic acid content. We also examined the effect of higher temperatures (daytime 38 °C and nighttime 33 °C) in the same experiment.

The results of ANOVA (Table 10) of the data obtained in this experiment suggested that (1) the isoflavone contents in the whole seed (hypocotyl part) depended on the temperature during seed development, (2) the isoflavone contents in the hypocotyl and C18:2 + C18:3 ratio of fatty acids depended largely on the temperature during seed development but also on the variety, and (3) the DDMP-conjugated saponin contents depended only on the variety. From these results, it is apparent that one of the factors affecting isoflavone content in soybean seeds is high temperature during seed develop-

ment. Higher temperatures result in lower isoflavone content. However, these results also suggested that there may exist a mechanism to maintain high isoflavone contents in seed hypocotyls.

Although the isoflavone contents in the hypocotyl remained high when the soybeans were grown outdoors, they decreased when the soybeans were grown in growth cabinets. It is possible that insects or microorganisms stimulate the production of isoflavones in seed hypocotyls. It was estimated that about 80–90% (w/w) of the isoflavone components in the ordinary soybean seeds were present in the cotyledons. Therefore, in using soybeans as a food material, emphasis should be placed on the isoflavone contents in the cotyledons.

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Registry No. Provided by the Authors: Daidzin, 552-66-9; glycitin, 40246-10-4; genistin, 529-59-9; malonyldaidzin, 124590-31-4; malonylglycitin, 137705-39-6; malonylgenistin, 51011-05-3; soyasaponin βg, 143519-54-4; C14:0, 544-63-8; C16:0, 57-10-3; C18:0, 57-11-4; C18:1, 112-80-1; C18:2, 60-33-3; C18:3, 463-40-1.

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