Plant Biotechnology 30, 335–345 (2013)
DOI: 10.5511/plantbiotechnology.13.0314a

Original Paper

Characterization of hybrids between wild and genetically modified glyphosate-tolerant soybeans

Akihiro Kubo*, Mitsuko Aono, Nobuyoshi Nakajima, Toru Nishizawa, Masanori Tamaoki, Hikaru Saji

Center for Environmental Biology and Ecosystem Studies, National Institute for Environmental Studies, Tsukuba, Ibaraki 305-8506, Japan
* E-mail: kub@nies.go.jp Tel & Fax: +81-29-850-2391

Received August 17, 2012; accepted March 14, 2013 (Edited by Y. Ozeki)

Abstract With the first approval of a genetically modified (GM) soybean for uncontained use in Japan, information about hybrids of wild and GM or non-GM soybeans has become increasingly important. Therefore, we generated and characterized various hybrids between wild and GM or non-GM soybeans in a containment greenhouse, and investigated the inheritance of the *cp4 epsps* transgene and its effects on the hybrids. The hybrids inherited the *cp4 epsps* gene in a Mendelian fashion. The gene was stably expressed and produced functional protein, conferring glyphosate tolerance to the hybrids. We also examined the germination and survival rates of the second filial (F_2) seeds and those of hybrids backcrossed twice ($BC2F_2$) after a 3-month treatment at 4°C. Hybrids displayed similar germination characteristics as wild soybean after cold treatment. The majority of the $BC2F_2$ seeds survived as dormant seeds. There was no effect of the *cp4 epsps* gene on the survival rate. Furthermore, we examined the morphology, anthesis, and fecundity of the hybrids. On the whole, the F_1, F_2, and F_3 hybrids exhibited morphology, anthesis, and fecundity phenotypes that were intermediate between wild and cultivated soybeans; those of $BC2F_2$ hybrids were similar to those of wild soybean. Comparisons between GM and the corresponding non-GM hybrids did not reveal significant differences in fecundity. We suggest that hybrids containing half of their genes from wild soybean and half from cultivated soybean display fitness that are intermediate between wild and cultivated soybeans, whereas $BC2F_2$ hybrids have fitness similar to that of wild soybean. We also suggest that, despite being stably inherited, the *cp4 epsps* gene does not affect fitness in the absence of glyphosate treatment.

Key words: *cp4 epsps*, fitness, herbicide tolerance, introgression, wild soybean.

The total cultivation area of genetically modified (GM) crops is increasing worldwide. In 2011, 29 countries planted 160 million hectares of GM crops, occupying about 11% of cropland in the world (James 2011). The dominant GM crop is the herbicide-tolerant soybean, which occupied 75.4 million hectares in 2011 (James 2011). The potential environmental effects of GM crops include invasiveness, weediness, toxicity, and risks to biodiversity (Dale et al. 2002), which include transgene introgression from GM crops to their wild relatives (Warwick et al. 2009). Wild soybean (*Glycine soja* Siebold & Zucc.) is cross-compatible with cultivated soybean (*Glycine max* (L.) Merr.) and grows spontaneously in East Asia, where no GM soybean is yet commercially cultivated. In Japan, however, many GM soybean seeds are imported for oil extraction and other uses, mainly from the United States, where 94% of the soybean crop area in 2011 was planted with GM soybean according to the United States Department of Agriculture (http://usda01.library.cornell.edu/usda/current/Acre/Acre-06-30-2011.pdf). The Ministry of Agriculture, Forestry, and Fisheries of Japan identified a few escaped GM soybean plants near some Japanese import ports in fiscal 2009, 2010, and 2011 (http://www.maff.go.jp/j/syouan/nouan/carta/c_data/index.html). GM soybean varieties imported into Japan are approved for Type 1 Use, which is uncontained use of living modified organisms in Japan under the Cartagena Protocol domestic law of Japan (http://www.bch.biodic.go.jp/english/e_index.html). Some varieties are approved for cultivation in Japan. The first GM soybean approved for Type 1 Use in Japan was an herbicide glyphosate-tolerant soybean variety (*cp4 epsps*, *Glycine max* (L.) Merr.; 40-3-2, OECD UI:MON-Ø4Ø32-6), which harbors the *cp4 epsps* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase isolated from *Agrobacterium* sp. strain CP4 to confer glyphosate tolerance. Upon its approval for Type 1 Use in Japan in 2005, the Committee for Discussing Biological Diversity

Abbreviations: CP4 EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase isolated from *Agrobacterium* sp. strain CP4; GM, genetically modified; OECD, Organization for Economic Co-operation and Development; PCR, polymerase chain reaction; QTL, quantitative trait locus.

This article can be found at http://www.jspcmb.jp/
Published online September 5, 2013

Risk Assessment requested the collection of the following information in order to enrich the available scientific evidence: (i) the crossing rate between *Glycine max* and *Glycine soja* in the natural environment, (ii) the fitness of the progeny of the hybrid obtained from a cross of *Glycine max* and *Glycine soja*, (iii) the natural crossing rate between this recombinant soybean and *Glycine soja*, and the fitness conferred by the *cp4 epsps* transgene in the hybrid progeny, (iv) the geographical genetic variation of *Glycine soja*, and (v) a model concerning the behavior of the transgene based on (i) through (iv) (http://www.bch.biodic.go.jp/download/en_lmo/Soybean40_3_2enRi.pdf). Hybridization frequencies through pollen flow from cultivated soybean to wild soybean have been evaluated experimentally in fields. Nakayama and Yamaguchi (2002) reported a mean hybridization rate of 0.73% between a local soybean cultivar, Tambaguro, and a wild soybean accession from Kyoto Prefecture, Japan. Mizuguti et al. (2009, 2010) reported hybridization rates ranging from 0 to 0.097% between GM soybean cultivars derived from the approved GM soybean (line 40-3-2) and a wild soybean population in Tsukuba, Japan. Kuroda et al. (2010) found morphological intermediates between wild and cultivated soybeans in their natural habitats in Japan. Because there is a possibility that hybrids between wild and GM soybeans arise in open fields, the characterization of such hybrids will provide valuable scientific information for assessing the environmental risk of GM soybeans in East Asia.

In this study, we made various hybrids between wild soybean accessions and GM soybean cultivars derived from the line 40-3-2 or their parental non-GM cultivars. We characterized these hybrids in a containment greenhouse and investigated the inheritance and the effects of the *cp4 epsps* gene.

Materials and methods

Plant materials

Seeds of GM soybean cultivars, A3244RR and A3525RR, and their respective parental non-GM cultivars, A3244 and A3525, were provided by the Monsanto Company (St. Louis, Missouri, USA). A3244RR and A3525RR are homozygous for the *cp4 epsps* transgene and are descendants of herbicide glyphosate-tolerant soybean (*cp4 epsps*, *Glycine max* (L.) Merr.; 40-3-2, OECD UI:MON-Ø4Ø32-6) developed by the Monsanto Company. The 40-3-2 GM soybean line contains one copy of the intact *cp4 epsps* gene and two inactive fragments of the gene, which are stably inherited together (http://www.bch.biodic.go.jp/download/en_lmo/Soybean40_3_2enRi.pdf). Seeds of wild soybean accessions, Nasu-5 and JP110755, were obtained from the National Institute of Agrobiological Sciences (NIAS) Genebank (Tsukuba, Ibaraki, Japan). Nasu-5 and JP110755 originated in Aomori and Hiroshima Prefectures, Japan, respectively. Inbred lines of these accessions (inbred Nasu-5 and inbred JP110755) were developed and provided by Dr. Kaga (NIAS).

Plant cultivation and production of hybrids

Cultivation in this study was performed in a containment greenhouse at our institute (Tsukuba, Ibaraki, Japan). The containment greenhouse was set at 25°C with a relative humidity of 70% under natural light. In 2005, eight types of first filial generation (F_1) hybrids were produced by artificially pollinating wild soybean accessions (Nasu-5 and JP110755) with GM (A3244RR and A3525RR) and non-GM (A3244 and A3525) cultivars. To break the seed dormancy of wild soybean, partial seed coat removal was done by scraping a small portion of the seed coat distal to the hilum with a pair of nippers prior to sowing. The parental plants were grown in Wagner pots (1/10,000 a) with mixed soil containing Akadama (small), vermiculite, and Supersoil No. 1 (Mitsui Toatsu Fertilizers, Tokyo, Japan) in a ratio of 2 : 1 : 1. They were fertilized with Hyponex 6-10-5 (Hyponex Japan, Osaka, Japan) diluted 1 : 2,000 twice weekly beginning four weeks after germination. The day length was adjusted to 15 h with supplementary metal halide lamps. Vines were allowed to twine around three stakes on each of the growing pots. The development of flower buds of wild soybean was induced by short-day treatment of 8 h per day using boxes to cover the plants. All eight types of F_1 hybrids were sown along with their parental lines on May 22, 2006 to examine their characteristics and produce second filial generation (F_2) seeds by self-pollination. To break dormancy, F_1 hybrid seeds were treated similarly to wild soybeans before sowing, and were assessed for the CP4 EPSPS protein (described later). Cultivation was performed as mentioned above except that different pots (18 cm in diameter and 15 cm in height) were used and that supplementary lighting was terminated on July 7 to induce flower initiation under natural light. Because F_1 seedlings do not have vines at early developmental stages, seedlings with vines from self-pollinated seeds were eliminated. The positions of all pots in the greenhouse were changed twice per week. In order to examine characteristics of the F_2 plants and produce third filial generation F_3 seeds by self-pollination, (Nasu-5×A3244) F_2 and (Nasu-5×A3244RR) F_2 hybrids were sown along with A3244 and A3244RR in the containment greenhouse with supplementary lighting on May 25, 2007. Additional comparisons of (Nasu-5×A3244) F_2 hybrids with Nasu-5 and A3244 were performed in another controlled greenhouse without supplementary light. The seeds sown in 2007 had been collected from all the parental plants grown in 2006. The seeds were sown in small pots (11 cm in diameter and 7 cm in height), and seedlings were transplanted to Wagner pots (1/10,000 a) 36 days after sowing. Other growth conditions were the same as those mentioned above. On May 23, 2008, the (Nasu-5×A3244RR) F_3 hybrid was sown along with Nasu-5 and A3244RR in the containment greenhouse to examine their characteristics. Seeds harvested in 2007 from

the most fertile parent of each line were used for sowing. The seed pool of the selected F_3 hybrid included both GM and non-GM seeds, as determined by segregation of the *cp4 epsps* gene. Growth conditions were the same as those used for the parental F_2 hybrid except that transplantation was performed 47 days after sowing and that supplementary lighting was terminated on September 25. F_2 and F_3 seeds had a soft seed coat and germinated without the need for a treatment to break dormancy. In 2007, only two kinds of first filial generation hybrids backcrossed once ($BC1F_1$), containing the *cp4 epsps* gene, were successfully produced by artificial pollination from (JP110755×A3244RR) F_1 or (JP110755×A3525RR) F_1 to JP110755 by the methods described for the production of the F_1 hybrid. The short-day treatment was applied to all the parental lines to induce flower buds. In 2008, using the methods described for the production of the $BC1F_1$ hybrid, two $BC1F_1$ hybrid lines obtained in 2007 were backcrossed to JP110755 to produce first filial generation hybrids backcrossed twice ($BC2F_1$), which also contained the *cp4 epsps* gene. On June 5, 2009, (JP110755×A3525RR) $BC2F_1$ seeds were sown along with JP110755 and A3525RR in Wagner pots in the containment greenhouse to examine the characteristics of $BC2F_1$ plants and produce seeds of second filial generation hybrids backcrossed twice ($BC2F_2$) by self-pollination. All seeds of (JP110755×A3525RR) $BC2F_1$ produced in 2008 were sown, and wild soybean seeds produced by self-pollination on seed parents of the $BC2F_1$ hybrid were used for sowing. Growth conditions were the same as those used for the F_3 hybrid except that supplementary lighting was not applied. Additionally, two and three long-lived plants of the wild soybean and hybrid, respectively, were allowed to die by withdrawing water after February 3, 2010. On May 28, 2010, (JP110755×A3525RR) $BC2F_2$ seeds were sown along with JP110755 and A3525RR seeds in the containment greenhouse to examine characteristics of the $BC2F_2$ plants. Seeds harvested in 2009 from the most fertile parent of each line were used for sowing. Growth conditions were the same as those used for the $BC2F_1$ hybrid except that mixed soil of Akadama (medium), vermiculite, and Kureha soil (Kureha, Tokyo, Japan) in a ratio of 2:1:1 was used and that watering was continued until the plant died. All backcrossed lines ($BC1F_1$, $BC2F_1$, and $BC2F_2$) were examined for the presence of the CP4 EPSPS protein before sowing (method described in the next section). This treatment broke seed dormancy. In 2009, four kinds of F_1 hybrids were produced by artificial pollination of inbred Nasu-5 and inbred JP110755 with A3244RR and A3244 pollen using the methods described for the F_1 hybrid production in 2005 except that supplementary lighting was not applied. The inbred wild soybean lines used were seventh generation plants from seeds in the NIAS Genebank. On June 4, 2010, these four kinds of F_1 hybrids and their parental lines were sown after breaking seed dormancy in order to examine their characteristics. Growth conditions were the same as those used for the $BC2F_2$ hybrid.

Investigating the inheritance of the cp4 epsps *gene*

Sixty seeds each of (Nasu-5×A3525RR) F_2 and (Nasu-5×A3244RR) F_3 that was selected based on segregation of the *cp4 epsps* gene were sown. The 57 and 60 germinated seedlings, respectively, were grown in pots (11 cm in diameter and 7 cm in height) without transplantation. Other growth conditions were the same as those used for (Nasu-5×A3244RR) F_2 plants described above. In order to examine the presence or absence of the CP4 EPSPS protein in seedlings, the tip (approximately 1 mm) of the primary leaf was cut off and ground with a mortar and pestle in 3–4 ml of deionized water. The crude extracts were tested by an immunochromatographic lateral flow assay using Reveal for CP4 (Neogen, Lansing, Michigan, USA). The test strip was placed in the crude extract with the sample end down for approximately 5 min, and the presence of the CP4 EPSPS-specific line was examined. During cultivation of (Nasu-5×A3244RR) F_2 and (Nasu-5×A3244RR) F_3 plants (described above), the segregation rates of the *cp4 epsps* gene were also examined by immunochromatography of the primary leaves. The (JP110755×A3525RR) $BC2F_2$ seeds were also used to examine the segregation ratio of the *cp4 epsps* gene. In order to examine the presence or absence of CP4 EPSPS protein in seeds, a small portion of cotyledon surrounded by the seed coat away from the hilum was used instead of the primary leaf.

Detection of the *cp4 epsps* gene was performed by polymerase chain reaction (PCR). The true leaf was cut off, and the plant material was transferred onto a FTA PlantSaver Card or FTA Classic Card (Whatman, Clifton, New Jersey, USA) by pressing the leaf onto the card. After the card was dried, a disk of 2 mm in diameter was punched out of the card with the transferred plant material. The disk was put in a microtube, purified with FTA Purification Reagent (Whatman) according to the manufacturer's instructions, and subjected to PCR. PCR primers were EPSPS7 (5′-AAG AAC TCC GTG TTA AGG AAA GCG A-3′) and EPSPS8 (5′-AGC CTT AGT GTC GGA GAG TTC GAT-3′), which corresponded to sequences in the *cp4 epsps* gene. Blend Taq (Toyobo, Osaka, Japan) was used for PCR. Thermocycler conditions were: 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, and a final termination stage at 72°C for 10 min. PCR products were electrophoresed on 1.8% agarose gels and stained with ethidium bromide to visualize a 320-bp band corresponding to the *cp4 epsps* gene amplification product.

The seedlings were tested for glyphosate tolerance by wetting leaves sufficiently with a watering pot containing 41.0% isopropylammonium *N*-(phosphonomethyl) glycinate (Roundup; Nissan Chemical Industries, Tokyo, Japan) diluted 1:200 with deionized water 45 to 67 days after sowing in order to assess their tolerance approximately 1 week after treatment.

Investigation of cold tolerance of seeds

Intact seeds for (Nasu-5×A3525RR) F_2 and (Nasu-5×A3525) F_2 hybrids as well as Nasu-5, A3525RR, and A3525 were sown in pots (11 cm in diameter and 7 cm in height) containing

mixed soil of Akadama (small), vermiculite, and Supersoil No. 1 at a ratio of 2:1:1. They were watered sufficiently, covered with plastic bags to prevent desiccation, and kept in a dark room at 4°C for 3 months before examining germination rates in the containment greenhouse for 1 month. Growth conditions were the same as those used for (Nasu-5×A3525RR) F_2 (described above). The seeds that did not germinate were dug out, and the seeds that had not rotted were sown again after breaking seed dormancy (described above); the germination was then examined for 1 month. Detection of the GM hybrids containing the *cp4 epsps* gene was performed by immunochromatography using the primary leaf of the germinated seedlings.

Intact seeds for (JP110755×A3525RR) $BC2F_2$, A3525RR, and JP110755, which originated from the same grandmother plant as the $BC2F_2$ hybrid, were sown in pots (11 cm in diameter; 7 cm in height; 3 seeds/pot) containing mixed soil of Akadama (medium), vermiculite, and Kureha soil at a ratio of 2:1:1. They were examined for cold tolerance as described above.

Investigation of plant characteristics

Germination rates were determined 1 month after sowing. Test plants cultivated until maturity were randomly selected when not all of the seedlings could be maintained in the greenhouse due to space limitations. Plant height was measured between 1 and 2 months after sowing. The longest vine of the wild soybeans and hybrids was measured for plant height after straightening. To characterize the stems including the vines for extent of violet color, thickness, and length of hair, each plant was scored visually on a scale of 1 to 5. To evaluate leaf size, blade length of the central leaflet of the largest trifoliate leaf selected visually from each mature plant was measured. Basal diameter of the stem was measured twice at a right angle from the other measurement using electronic digital calipers to obtain the mean value for the plant. The dry weight of the final aboveground part was measured by removing it from the dead plant after harvesting the pods and drying it at 80°C for 2 days.

The flowering period of each plant was recorded as the period from opening of the first flower to wilting of the last flower.

The total weight of the seeds per plant was measured after the seeds were dried at room temperature. Seed weight per grain was calculated by dividing the total weight of the seeds for each plant by the total number of seeds for each plant.

Results

Production of hybrids between wild and cultivated soybeans

When GM soybean varieties are cultivated in fields of East Asia, pollen flow from GM soybeans to wild soybeans may generate hybrids in the natural habitats of wild soybeans. On the other hand, pollen flow from wild soybeans to GM soybeans may produce hybrid seeds in cropland, which could be ultimately harvested and consumed. Therefore, we made F_1 hybrids by hand-pollinating wild soybeans with the pollen of cultivated soybeans (Figure 1). Two GM cultivars (A3244RR and A3525RR) and their non-GM parental cultivars (A3244 and A3525, respectively) were used as pollen donors, and two wild soybeans, Nasu-5 and JP110755, were used as pollen recipients (Table 1). F_2 hybrids were obtained by self-pollination of the F_1 hybrids, and F_3 hybrids were produced by self-pollination of selected F_2 hybrids harboring the *cp4 epsps* gene (Figure 1). The F_1 hybrids were expected to contain 50% each of the wild and cultivated soybean-derived genes in the genome. The descendant F_2 and F_3 hybrids were also expected to contain approximately 50% each of the wild and cultivated soybean-derived genes (Figure 1). These hybrids may further cross with wild soybean growing in natural habitats, resulting in hybrids containing a higher proportion of genes derived from wild soybean. To characterize such hybrids, we generated $BC1F_1$ hybrids by artificially pollinating wild soybean with the pollen of the F_1 hybrid and $BC2F_1$ hybrids by artificially pollinating wild soybean with the pollen of the $BC1F_1$ hybrid (Figure 1). $BC1F_2$ and $BC2F_2$ hybrids were also produced by self-pollination of the $BC1F_1$ and $BC2F_1$ hybrids, respectively (Figure 1). The expected percentages of the wild soybean-derived genes in the genome were 75% for the $BC1F_1$ and $BC1F_2$ hybrids and 87.5% for the $BC2F_1$ and $BC2F_2$ hybrids (Figure 1). Because inbred wild soybean lines became available in 2009, F_1 hybrids between the inbred wild soybean and the GM or non-GM soybean were produced to further characterize F_1 hybrids. Table 1 shows the hybrids investigated in the present study.

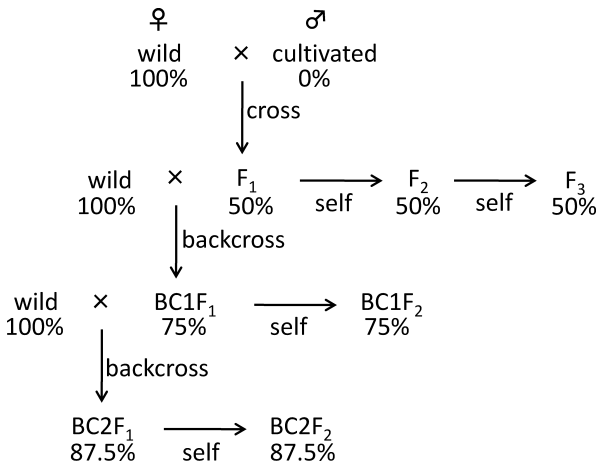


Figure 1. Production of hybrids between wild and cultivated soybeans. Expected percentages of the wild soybean-derived genes in the genome are shown.

Inheritance of the cp4 epsps *gene in hybrids of wild and GM soybeans*

In order to evaluate the stability and phenotype of the *cp4 epsps* gene in hybrids of wild and GM soybeans, seedlings of the segregating generation were analyzed using three different tests. First, the CP4 EPSPS protein in leaves was detected using immunochromatography, and the segregation ratio of CP4 EPSPS-positive and -negative phenotypes was tested statistically (Table 2). The results were consistent with dominant Mendelian inheritance, exhibiting a 3 : 1 segregation ratio (Table 2). Second, all CP4 EPSPS-negative plants, as well as some positive ones, were subjected to PCR for the *cp4 epsps* gene to search for the gene not expressed due to instability. However, there was no evidence for the presence of an unstable allele (Supplementary Table 1). Finally, the seedlings were subjected to the glyphosate tolerance test (Supplementary Table 1). All CP4 EPSPS-positive plants tested were glyphosate-tolerant, indicating that the enzymatic activity of the CP4 EPSPS protein was intact in all plants tested and that the glyphosate-tolerant phenotype is dominant in the hybrids.

Table 1. Hybrids investigated in the present study.

Hybrid	Number of test plants
(Nasu-5×A3244) F_1	6
(Nasu-5×A3244RR) F_1	6
(Nasu-5×A3525) F_1	6
(Nasu-5×A3525RR) F_1	2
(JP110755×A3244) F_1	6
(JP110755×A3244RR) F_1	2
(JP110755×A3525) F_1	6
(JP110755×A3525RR) F_1	1
(Nasu-5×A3244) F_2	15, 18
(Nasu-5×A3244RR) F_2	30, 30**
(Nasu-5×A3525) F_2	36*
(Nasu-5×A3525RR) F_2	60*, 57**
(Nasu-5×A3244RR) F_3	32, 60**, 100**
(JP110755×A3244RR) $BC1F_1$	5
(JP110755×A3525RR) $BC1F_1$	5
(JP110755×A3525RR) $BC2F_1$	5
(JP110755×A3525RR) $BC2F_2$	20, 120*, 66**
(inbred Nasu-5×A3244) F_1	10–52
(inbred Nasu-5×A3244RR) F_1	10–22
(inbred JP110755×A3244) F_1	10–133
(inbred JP110755×A3244RR) F_1	10–78

The name indicates: (seed parent of the cross×pollen parent of the cross) generation of the hybrid. Backcross (BC) was performed with the seed parent. *Plants tested for cold tolerance of seeds. **Plants tested for inheritance of the *cp4 epsps* gene.

Cold tolerance of the hybrid seeds

The germination rates of the test plants in this study were determined 1 month after sowing at 25°C (Supplementary Table 2). The germination rates of the hybrids and their parental wild and cultivated soybeans were similar in most cases, except that Nasu-5 showed a low germination rate when it was not treated to break seed dormancy (Supplementary Table 2).

In order to investigate the overwintering ability of sown seeds, F_2 and $BC2F_2$ hybrids were sown along with ancestral wild and cultivated soybeans, maintained at 4°C in the dark for 3 months, and cultivated at 25°C for 1 month to examine germination rates, and survival rates including dormant seeds (Figure 2). The germination rates of the cultivated soybeans were 0 (Figure 2A) or close to 0 (Figure 2B). Both the F_2 hybrids derived from GM or non-GM soybean and the $BC2F_2$ hybrids had germination rates comparable to their respective ancestral wild soybeans (Figure 2A, B). All ungerminated seeds of the cultivated soybeans and the F_2 hybrids had rotted, whereas almost all ungerminated seeds of the $BC2F_2$ hybrids and the wild soybeans had not rotted. A portion of the seedlings of the F_2 and $BC2F_2$ hybrids and JP110755 died after germination. The survival rates 1 month after cold treatment are shown in Figure 2C and 2D. The ungerminated normal-shaped seeds were evaluated for their ability to germinate after partial seed coat removal to break seed dormancy, and the germinating seeds were counted as dormant seeds. Survival rates of the F_2 hybrids were intermediate between their ancestral wild and cultivated soybeans (Figure 2C). On the other hand, the $BC2F_2$ hybrids showed a survival rate that did not significantly differ from that of JP110755 (Figure 2D).

The germination and survival rates of the F_2 hybrids derived from GM soybean did not significantly differ from those of the F_2 hybrids derived from non-GM soybean (Figures 2A, C). To further evaluate the effects of the *cp4 epsps* gene on the survival rate of the hybrids, the number of CP4 EPSPS-positive (+) and -negative (−) plants surviving after the cold treatment was examined in the hybrids of the segregating generation (Table 3).

Table 2. Segregation of CP4 EPSPS-positive (+) and -negative (−) phenotypes and the upper cumulative probability (*P*) of the chi-square test for Mendelian segregation.

Hybrid	CP4 (+)	CP4 (−)	*p*
(Nasu-5×A3244RR) F_2	21	9	0.53
(Nasu-5×A3525RR) F_2	43	14	0.94
(Nasu-5×A3244RR) F_3	39	21	0.074
(Nasu-5×A3244RR) F_3	79	21	0.36
(JP110755×A3525RR) $BC2F_2$	45	21	0.2

F_3 plants were obtained from a hemizygous F_2 parent.

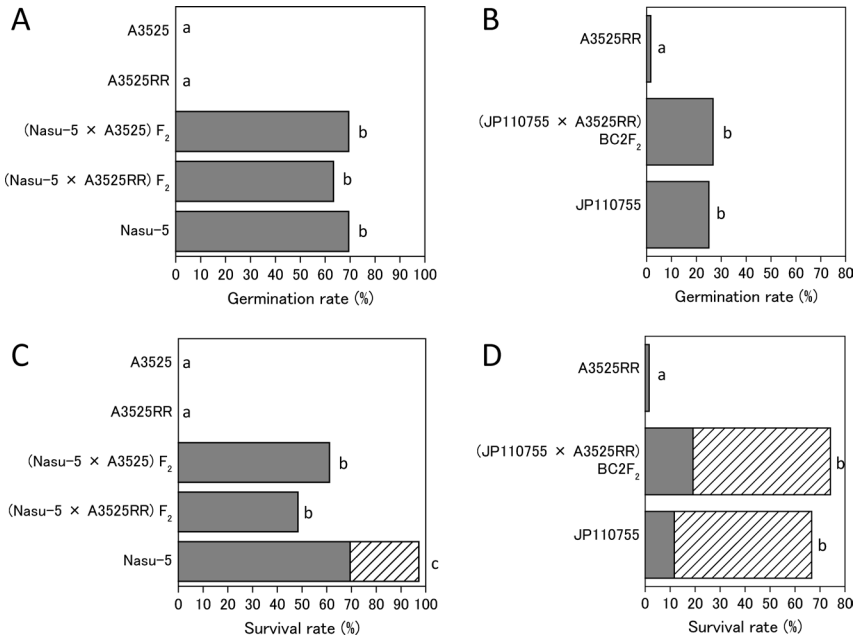


Figure 2. Germination and survival rates 1 month after cold treatment. F_2 (A, C) and $BC2F_2$ hybrids (B, D) were sown along with wild and cultivated soybeans from which they had been derived, maintained at 4°C in the dark for 3 months, and cultivated at 25°C for 1 month to examine germination rates (A, B) and survival rates including dormant seeds (hatched bars) (C, D). Identical letters to the right of the bars are representative of non-significant differences, assessed using Ryan's significance test for multiple comparison of proportions ($p<0.05$).

Table 3. Number of CP4 EPSPS-positive (+) and -negative (−) plants surviving after cold treatment and the upper cumulative probability (*P*) of the chi-square test for Mendelian segregation.

Hybrid	Surviving form	CP4 (+)	CP4 (−)	*p*
(Nasu-5×A3525RR) F_2	Seedling	22	7	0.91
(JP110755×A3525RR) $BC2F_2$	Seedling	16	7	0.55
(JP110755×A3525RR) $BC2F_2$	Dormant seed	39	10	0.46

No effect of the *cp4 epsps* gene on the survival rate of the hybrids was detected, since the surviving plants showed the expected Mendelian ratios (Table 3).

Morphology of the hybrid plants

We investigated the morphology of the hybrid plants. Wild soybean had vines, but cultivated soybean did not. All plants of F_1, $BC1F_1$, $BC2F_1$, and $BC2F_2$ hybrids had stereotropic vines, although young F_1 seedlings had no vines. Some plants of the F_2 and F_3 hybrids also had vines, and some of these emerged gradually during the growth of the plants. The plant height of the wild soybeans was larger than that of the cultivated soybeans between 1 and 2 months after sowing based on the longest vine of the wild soybeans, although the difference was not always significant (Supplementary Table 3). Plant height of the hybrids was either intermediate between that of wild and cultivated soybeans or was similar to that of the wild or cultivated soybean (Supplementary Table 3). Among corresponding hybrids with or without the *cp4 epsps* gene [i.e. (Nasu-5×A3244) F_1 vs. (Nasu-5×A3244RR) F_1, (inbred Nasu-5×A3244) F_1 vs. (inbred Nasu-5×A3244RR) F_1, (inbred JP110755×A3244) F_1 vs. (inbred JP110755×A3244RR) F_1, (Nasu-5×A3244) F_2 vs. (Nasu-5×A3244RR) F_2, (Nasu-5×A3244RR) F_2 (CP4−) vs. (Nasu-5×A3244RR) F_2 (CP4+), (Nasu-5×A3244RR) F_3 (CP4−) vs. (Nasu-5×A3244RR) F_3 (CP4+), and (JP110755×A3525RR) $BC2F_2$ (CP4−) vs. (JP110755×A3525RR) $BC2F_2$ (CP4+)], only (inbred JP110755×A3244RR) F_1 was significantly taller than (inbred JP110755×A3244) F_1 (Supplementary Table 3). Among the hybrids of the segregating generation, the Fisher's exact test revealed independence between the CP4 EPSPS protein and the presence of vines (Supplementary Table 4). Some characteristics of the stem including the vine (extent of violet color, thickness, and length of hair) were semi-quantitatively assessed among F_2 hybrids and their ancestral wild and cultivated soybeans. F_2 hybrids had intermediate stems between wild and cultivated soybeans, and the *cp4 epsps* gene had no effect on the stem morphology (Supplementary Table 5). The flowers and immature pods were similar among wild and cultivated soybeans and their hybrids. Wild and cultivated soybeans had cotyledons, primary leaves, and trifoliate true leaves. All the hybrids had the same kinds of leaves. The leaf size of the cultivated soybeans

was larger than that of the wild soybeans. The leaf size of the hybrids was intermediate between that of wild and cultivated soybeans or was similar to that of wild or cultivated soybean (Supplementary Table 6). Among six pairs of hybrids with or without the *cp4 epsps* gene, one pair showed a significant difference in leaf size: (Nasu-5×A3244) F_2 had larger leaves than (Nasu-5×A3244RR) F_2 (Supplementary Table 6).

The final basal diameter of the stem was measured after the plant died (Supplementary Table 7). The final basal diameter of the cultivated soybeans was larger than that of the wild soybeans. The final basal diameter of the hybrids was intermediate between that of wild and cultivated soybeans or was similar to that of wild or cultivated soybean, and there was no effect of the *cp4 epsps* gene on the basal diameter (Supplementary Table 7). The total stem length of each plant was measured after the plant died using F_1 hybrids and their parental wild and cultivated soybeans. The wild soybeans had longer stem lengths than the cultivated soybeans, and the F_1 hybrids had intermediate stem lengths between wild and cultivated soybeans. There was no significant effect of the *cp4 epsps* gene on the stem length (Supplementary Table 8). The dry weight of the final aboveground part was examined. The wild soybeans had a greater dry weight than the cultivated soybeans, and the hybrids were intermediate between wild and cultivated soybeans or were similar to wild soybean. There was no effect of the *cp4 epsps* gene (Supplementary Table 9). The colors of the mature pods of wild and cultivated soybeans were dark and light brown, respectively. (JP110755×A3525RR) $BC1F_1$ and $BC2F_1$ hybrids produced mature dark-brown pods. Each plant of (JP110755×A3525RR) $BC2F_2$ hybrids produced either dark-brown or light-brown pods (Supplementary Table 10). A Fisher's exact test showed independence between the CP4 EPSPS protein and the color of mature pods (Supplementary Table 10). The detached parts of the mature pod of the $BC2F_1$ and $BC2F_2$ hybrids exhibited a strong degree of twisting, similar to those of wild soybeans (data not shown).

The morphology of the $BC1F_1$ hybrids was similar to that of wild soybeans except that 2 out of the 5 (JP110755×A3525RR) $BC1F_1$ plants had wrinkled, small, pale green leaves, which were similar to the immature leaves of wild soybeans, and produced only a few seeds (data not shown). The morphologies of the $BC2F_1$ and $BC2F_2$ hybrids, which theoretically harbor 87.5% wild soybean-derived genes, were also similar to wild soybeans; there were no significant differences from JP110755, but there were some significant differences from A3525RR (Supplementary Tables 3, 6, 7, 9). In contrast, F_1, F_2, and F_3 hybrids, which theoretically harbor 50% each of wild and cultivated soybean-derived genes, showed some significant differences from wild and/or cultivated soybeans (Supplementary Tables 3, 5–7, 9). Soybean cultivars A3244, A3244RR, A3525, and A3525RR did not significantly differ from each other with respect to morphological traits investigated in this study, nor did wild soybean accessions Nasu-5 and JP110755 (Supplementary Tables 3, 5–9).

Comparison of the flowering periods

The flowering periods of the test plants were investigated (Supplementary Figure 1). The cultivated soybean cultivars had the earliest anthesis. JP110755 and the inbred JP110755 had the latest anthesis. Nasu-5 and inbred Nasu-5 had moderate anthesis. When seeds had been sown from May 22 to June 5, the anthesis of the cultivated soybeans partially overlapped with that of Nasu-5 and inbred Nasu-5, but never overlapped with that of JP110755 and inbred JP110755. The anthesis of F_1, F_2, and F_3 hybrids was intermediate between their parental or ancestral wild and cultivated soybeans. Long-day conditions prolonged the flowering periods. $BC2F_1$ and $BC2F_2$ hybrids had anthesis similar to that of their parental wild soybean, JP110755. There was no significant effect of the *cp4 epsps* gene on the flowering period (Supplementary Figure 1).

Fecundity of the hybrids

Since wild and cultivated soybeans are self-fertilizing plants, the test plants produced self-pollinated seeds without a pollinator in the containment greenhouse. Figure 3 shows the number of seeds produced per plant in seven experimental groups with different combinations of wild and cultivated soybeans and their hybrids. Fecundity varied among plants of the same line in different experimental groups, which was likely due to differences in growth conditions. Wild soybean plants produced more seeds than cultivated soybean plants, and there were no significant differences between wild soybean accessions or among cultivated soybean cultivars within each experimental group. The number of self-pollinated seeds of F_1, F_2, and F_3 hybrids was intermediate between that of wild and cultivated soybeans or was similar to that of wild or cultivated soybeans. The numbers of self-pollinated seeds of $BC2F_1$ and $BC2F_2$ hybrids were similar to those observed for wild soybean. There were no significant differences in the numbers of seeds between corresponding hybrids with or without the *cp4 epsps* gene (Figure 3). The number of pods per plant was counted in experimental groups 2, 6, and 7. The results were similar to those reported in Figure 3 (Supplementary Table 11). The number of seeds per pod ranged from 2.1 to 2.5 for all lines in experimental groups 2, 6, and 7 (Supplementary Table 12). The total weight of seeds per plant was examined in F_1 hybrids from inbred wild soybean, (JP110755×A3525RR) $BC2F_1$, and $BC2F_2$ hybrids (Supplementary Table 13). Despite the number

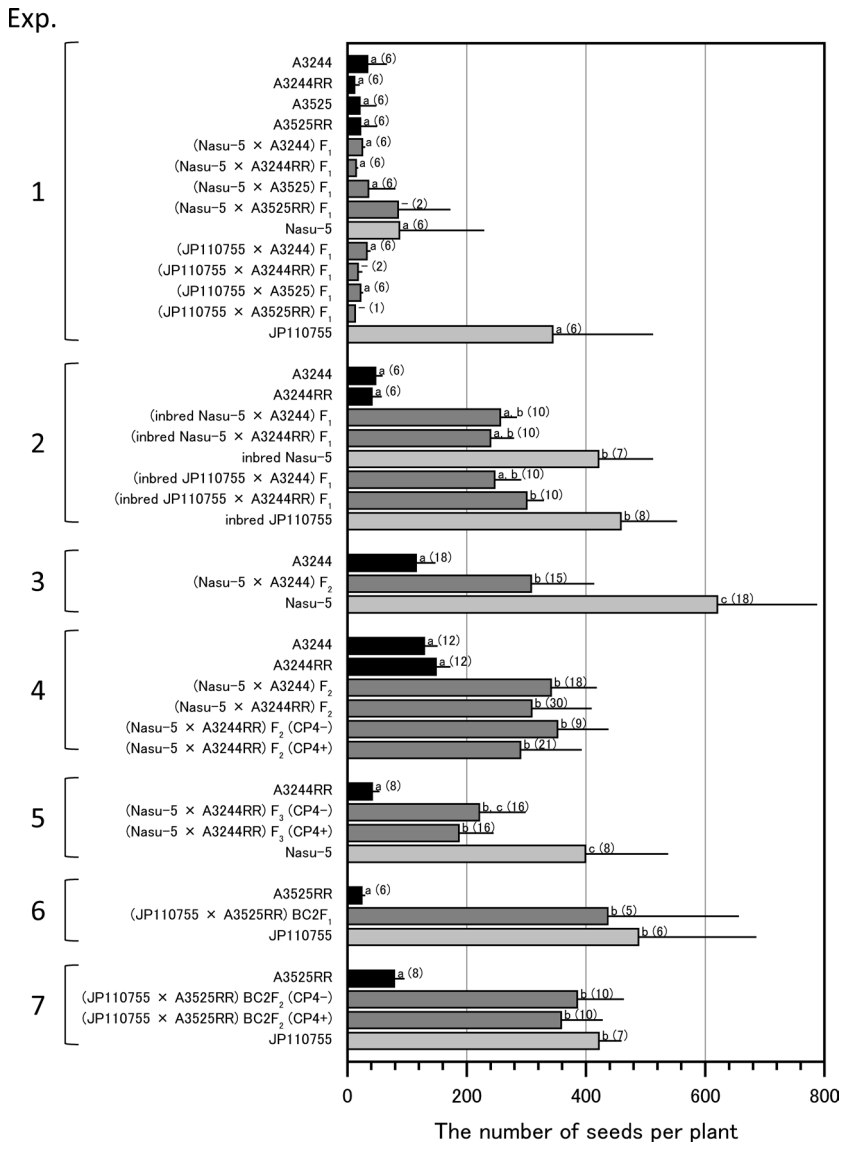


Figure 3. The number of seeds produced per plant. Experimental groups are shown on the left. Black (cultivated soybean), gray (hybrids), and light gray (wild soybean) bars represent the mean number of self-pollinated seeds per individual plant labeled on the left. Error bars represent standard deviations. Identical letters to the right of the bars are representative of non-significant within-group differences, determined by the Kruskal–Wallis test using Scheffé's comparison method ($p<0.05$; n is shown in parentheses after the letter). Data eliminated from statistical analyses due to small sample numbers are denoted by "–" to the right of the bars.

of produced seeds, the total weight of seeds per plant was not always larger in wild soybean than in cultivated soybean due to differences in seed weight per grain (Supplementary Table 14). Cultivated soybeans produced larger and heavier seeds than wild soybeans or hybrids. The F_1 hybrids produced seeds with intermediate weight between wild and cultivated soybeans, and $BC2F_1$ and $BC2F_2$ hybrids produced seeds similar in weight to wild soybeans (Supplementary Table 14). As a consequence of the number of seeds per plant (Figure 3) and seed weight per grain, F_1 hybrids produced more total weight in seeds per plant than wild or cultivated soybeans, and $BC2F_1$ and $BC2F_2$ hybrids produced a total weight in seeds per plant similar to wild soybeans (Supplementary Table 13). No effect on total weight of seeds per plant or seed weight per grain of the *cp4 epsps* gene was detected (Supplementary Tables 13, 14).

Morphology of the hybrid seeds

The morphology of the seeds was observed and photographed. The seed coat is a tissue originating from the seed parent. A3244, A3244RR, A3525, and A3525RR produced large, round, ocherous seeds, and Nasu-5 and JP110755 produced small, oval, dark-brown seeds (Supplementary Figure 2). F_1 seeds were similar to those of wild soybean, although they were somewhat warped (Supplementary Figure 2) and heavier (Supplementary Table 14). F_2 seeds showed intermediate

size, shape, and color between wild and cultivated soybeans (Supplementary Figure 2). F_3 and F_4 seed pools harvested from each parental plant showed color variation irrespective of the presence of the *cp4 epsps* gene (Supplementary Figures 3, 4). $BC1F_2$ seed pools harvested from each $BC1F_1$ plant had a color similar to either wild soybeans or F_2 hybrids (Supplementary Figure 5A). $BC2F_1$, $BC2F_2$, and $BC2F_3$ seeds were similar to wild soybean seeds (Supplementary Figures 5B−D, Supplementary Table 14).

Discussion

In this study, we made various hybrids between wild and GM or non-GM soybeans (Figure 1, Table 1) in order to characterize them and investigate the inheritance pattern of the *cp4 epsps* gene, as well as its effects. The genomes of F_1, F_2, and F_3 hybrids are expected to consist of 50% wild soybean-derived genes, whereas those of hybrids backcrossed to wild soybean are expected to contain 75% ($BC1F_1$, $BC1F_2$) or 87.5% ($BC2F_1$, $BC2F_2$) wild soybean-derived genes. If introgression of a transgene from GM soybeans to wild soybeans occurs, such backcrossed hybrids should appear. However, natural backcrossing rates seem to be low, because outcrossing rates of wild soybean populations are much lower than selfing rates (Fujita et al. 1997; Kiang et al. 1992; Kuroda et al. 2008). Kuroda et al. (2010) reported that introgression of cultivated soybean genes into wild soybeans appears to be rare based on the fate of intermediates between wild and cultivated soybeans in their natural habitats in Japan. Nevertheless, they found evidence suggesting that introgression of cultivated soybean genes into wild soybeans had occurred in the past (Kuroda et al. 2010).

It has been reported that the *cp4 epsps* gene is stably inherited and that the CP4 EPSPS protein is stably expressed in the progeny of the GM soybean line, 40-3-2 (http://www.bch.biodic.go.jp/download/en_lmo/Soybean40_3_2enRi.pdf). However, the stability of the *cp4 epsps* gene and the expression of the CP4 EPSPS protein in hybrids between wild and cultivated soybeans were previously unknown. Wild and cultivated soybeans have the same number of chromosomes ($2n=40$), are cross-compatible, and can produce fertile progeny (OECD 2000). Therefore, it seems that hybrids of wild and cultivated soybeans have stable genomes. However, gene silencing could occur in some transgenes (Kooter et al. 1999). This study investigated the segregation ratios of the *cp4 epsps* gene in F_2, F_3, and $BC2F_2$ hybrids to examine the stability of the gene. This gene was inherited in the hybrids in a Mendelian fashion (Table 2). Further investigation by *cp4 epsps* gene-specific PCR and glyphosate tolerance testing in seedlings (Supplementary Table 1) revealed that there was no evidence of inhibition of gene expression or protein function, indicating that this gene is expressed stably in the hybrids.

Wild soybeans disperse seeds in the fall, and the seeds germinate in the spring. In contrast, cultivated soybeans hardly overwinter. Therefore, the overwintering ability of the sown seeds seems important for survival in natural habitats. In this study, the germination and survival rates of F_2 and $BC2F_2$ seeds after a 3-month cold treatment at 4°C in the soil were examined to assess overwintering ability (Figure 2). The F_2 and $BC2F_2$ seeds germinated similarly to wild soybeans after cold treatment and most of the seedlings survived. The majority of the $BC2F_2$ seeds survived as dormant seeds, similar to the ancestral JP110755 seeds. In addition, the $BC2F_2$ seeds had seed coats similar to those of wild soybeans (Supplementary Figure 5C). The seed coat is thought to be a major determinant of dormancy (Moïse et al. 2005), and some loci controlling seed coat color have been shown to be associated with hard seededness in wild soybean (Sakamoto et al. 2004). Zhou et al. (2010) suggested that a major phenolic in the seed coat was functionally related to coat-imposed hard seededness in wild soybean. We cannot eliminate the possibility that hybrids of wild and cultivated soybeans have an overwintering ability, although environmental conditions in natural habitats of wild soybeans during the winter seem to be more severe than those in our experiments. Kitamoto et al. (2012) reported that F_2 hybrids between JP110755 (Hiroshima) and a cultivated soybean cultivar, Fukuyutaka, produced overwintered seeds with survival rates ranging from 0% to 90–100% based on field experiments using F_3 seeds buried in the soil from December 26 to March 16. In our study, the germination and survival rates of the F_2 hybrids derived from GM soybeans were not significantly different from those of the F_2 hybrids derived from non-GM soybeans (Figures 2A, C). Furthermore, no effects of the *cp4 epsps* gene on the survival rate of the segregating hybrids were detected (Table 3). In addition, there was no relationship between the *cp4 epsps* gene and the morphology of the hybrid seeds (Supplementary Figures 3, 4). These results are consistent with the fact that *cp4 epsps* is not functionally involved in cold tolerance or dormancy (OECD 1999).

We investigated the morphology of hybrid plants, specifically focusing on plant height, stem characteristics (extent of violet color, thickness, and length of hair), leaf size, final basal diameter of the stem, total stem length, and final dry weight of aboveground part (Supplementary Tables 3, 5–9). Plant morphology should influence plant fitness. Overall, F_1, F_2, and F_3 hybrids exhibited intermediate morphology between wild and cultivated soybeans, and backcrossed hybrids exhibited morphology similar to wild soybeans. The presence of the *cp4 epsps* gene did not significantly affect morphology in most cases, with the exceptions of plant height in (inbred JP110755×A3244RR) F_1 (Supplementary

Table 3) and leaf size in (Nasu-5×A3244RR) F_2 (Supplementary Table 6). However, the reasons for these morphological differences are not known. The *cp4 epsps* gene has nothing to do with morphology (OECD 1999). Despite a statistically significant difference in plant height and leaf size, *cp4 epsps* did not have a significant effect on seed production (Figure 3, Supplementary Tables 11, 13). Moreover, the CP4 EPSPS protein was independent of the presence of vines (Supplementary Table 4) and the color of the mature pod (Supplementary Table 10).

Two of the five plants of (JP110755×A3525RR) $BC1F_1$ hybrids had wrinkled, small, pale-green leaves similar to immature leaves of wild soybeans and produced only a few seeds. Two $BC2F_1$ seeds were obtained using pollen from one of the abnormal $BC1F_1$ plants, but they did not germinate (data not shown). The cause of this abnormality may be genetic instability of interspecific hybrids. However, the *cp4 epsps* gene is not likely to be the cause of this abnormality because three of the five plants of (JP110755×A3525RR) $BC1F_1$ hybrids were normal, harboring the *cp4 epsps* gene. Furthermore, the *cp4 epsps* gene encodes an enzyme to synthesize a common plant metabolite unrelated to morphology (OECD 1999). Even if such abnormal plants appear in natural habitats, their progeny would disappear, given that they produce only a few seeds.

The overlap of flowering periods is an important factor for hybridization in fields. Since periods of anthesis in F_1, F_2, and F_3 hybrids were intermediate between their parental or ancestral wild and cultivated soybeans (Supplementary Figure 1), these hybrids may further hybridize with wild and/or cultivated soybeans. On the other hand, anthesis in $BC2F_1$ and $BC2F_2$ hybrids was similar to that observed in their parental wild soybean accession, and therefore, they are able to backcross. We should note that anthesis of cultivated soybeans can change based on the sowing date and that unintended dispersal of GM soybean seeds can occur in all seasons during their transport. The *cp4 epsps* gene did not significantly affect the flowering period (Supplementary Figure 1).

The fitness of a population is defined as the number of offspring divided by the number of parents in the same life cycle stage in their natural habitat. Therefore, fecundity is the fundamental factor affecting fitness. On the whole, F_1, F_2, and F_3 hybrids exhibited intermediate fecundity between wild and cultivated soybeans, and backcrossed hybrids exhibited fecundity similar to that of wild soybeans (Figure 3). The difference in the number of seeds per plant most likely resulted from differences in the number of pods per plant rather than differences in the number of seeds per pod (Supplementary Tables 11, 12). Importantly, the *cp4 epsps* gene did not significantly affect the number of seeds per plant (Figure 3).

Based on the results from this study, we suggest that hybrids containing half of their genes derived from wild soybean and half from cultivated soybean have intermediate fitness between wild and cultivated soybeans, whereas hybrids backcrossed twice to wild soybean have fitness similar to that of wild soybean. Furthermore, we suggest that the *cp4 epsps* gene does not affect fitness in the absence of glyphosate treatment, although the gene is inherited stably in the hybrids. Kitamoto et al. (2012) presented a model to predict the frequency of integration of a neutral transgene from cultivated to wild soybeans by considering the linkage between a transgene and fitness-related quantitative trait loci (QTLs) for the number of produced seeds and winter survival. Based on our result that the effect of the *cp4 epsps* gene is neutral in the absence of glyphosate treatment, their model is applicable to the *cp4 epsps* gene. However, the *cp4 epsps* gene did not affect survival rate after cold treatment (Table 3) or the number of seeds produced (Figure 3) among segregating hybrids. This indicates that there was no linkage between the transgene and fitness-related QTLs for the number of produced seeds and winter survival, although the location of the *cp4 epsps* gene in the genome of the GM soybean line 40-3-2 is unknown.

Acknowledgements

We thank Dr. Kazuhito Matsuo of the National Institute for Agro-Environmental Sciences and Dr. Ryo Ohsawa of the University of Tsukuba for their advice. We thank Dr. Akito Kaga of NIAS for providing the inbred wild soybean seeds. We also acknowledge the Monsanto Company for providing the GM and non-GM soybean seeds and the NIAS Genebank for providing the seeds of the wild soybean accessions. This work was funded by the Ministry of the Environment of Japan and the project "Assurance of safe use of genetically modified organisms" from the Ministry of Agriculture, Forestry and Fisheries of Japan.

References

Dale PJ, Clarke B, Fontes EMG (2002) Potential for the environmental impact of transgenic crops. *Nat Biotechnol* 20: 567–574

Fujita R, Ohara M, Okazaki K, Shimamoto Y (1997) The extent of natural cross-pollination in wild soybean (*Glycine soja*). *J Hered* 88: 124–128

James C (2011) *Global Status of Commercialized Biotech/GM Crops: 2011. ISAAA Brief No. 43.* ISAAA, Ithaca

Kiang YT, Chiang YC, Kaizuma N (1992) Genetic diversity in natural populations of wild soybean in Iwate Prefecture, Japan. *J Hered* 83: 325–329

Kitamoto N, Kaga A, Kuroda Y, Ohsawa R (2012) A model to predict the frequency of integration of fitness-related QTLs from cultivated to wild soybean. *Transgenic Res* 21: 131–138

Kooter JM, Matzke MA, Meyer P (1999) Listening to the silent genes: transgene silencing, gene regulation and pathogen control. *Trends Plant Sci* 4: 340–347

Kuroda Y, Kaga A, Tomooka N, Vaughan DA (2008) Gene flow and genetic structure of wild soybean (*Glycine soja*) in Japan. *Crop*

Sci 48: 1071–1079
Kuroda Y, Kaga A, Tomooka N, Vaughan D (2010) The origin and fate of morphological intermediates between wild and cultivated soybeans in their natural habitats in Japan. *Mol Ecol* 19: 2346–2360
Mizuguti A, Ohigashi K, Yoshimura Y, Kaga A, Kuroda Y, Matsuo K (2010) Hybridization between GM soybean (*Glycine max* (L.) Merr.) and wild soybean (*Glycine soja* Sieb. et Zucc.) under field conditions in Japan. *Environ Biosafety Res* 9: 13–23
Mizuguti A, Yoshimura Y, Matsuo K (2009) Flowering phenologies and natural hybridization of genetically modified and wild soybeans under field conditions. *Weed Biol Manage* 9: 93–96
Moïse JA, Han S, Gudynaitę-Savitch L, Johnson DA, Miki BLA (2005) Seed coats: Structure, development, composition, and biotechnology. *In Vitro Cell Dev Biol Plant* 41: 620–644
Nakayama Y, Yamaguchi H (2002) Natural hybridization in wild soybean (*Glycine max* ssp. *soya*) by pollen flow from cultivated soybean (*Glycine max* ssp. *max*) in a designed population. *Weed Biol Manage* 2: 25–30
OECD (1999) Consensus document on general information concerning the genes and their enzymes that confer tolerance to glyphosate herbicide. http://www.oecd.org/dataoecd/17/11/46815618.pdf
OECD (2000) Consensus document on the biology of *Glycine max* (L.) Merr. (soybean). http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2000)9&doclanguage=en
Sakamoto S, Abe J, Kanazawa A, Shimamoto Y (2004) Marker-assisted analysis for soybean hard seededness with isozyme and simple sequence repeat loci. *Breed Sci* 54: 133–139
Warwick SI, Beckie HJ, Hall LM (2009) Gene flow, invasiveness, and ecological impact of genetically modified crops. *Ann NY Acad Sci* 1168: 72–99
Zhou S, Sekizaki H, Yang Z, Sawa S, Pan J (2010) Phenolics in the seed coat of wild soybean (*Glycine soja*) and their significance for seed hardness and seed germination. *J Agric Food Chem* 58: 10972–10978

Supplementary Table 1. Detection of the *cp4 epsps* gene and its expression using 3 different methods

Hybrid	Individual No.	CP4 EPSPS	*cp4 epsps*	Glyphosate tolerance
(Nasu-5 × A3525RR) F_2	1	+	+	+
	4–7, 9	+	nd	+
	10, 11	+	+	+
	12–16, 18–21, 23, 25, 27–30, 32–34, 36, 39, 41, 43–46, 49–54, 56, 58–60	+	nd	+
	2, 3, 8, 17, 22, 24, 26, 31, 35, 37, 42, 48, 55, 57	–	–	–
(Nasu-5 × A3244RR) F_3	1–3	+	+	+
	5, 9, 11, 12, 14–23, 26, 28, 29, 31, 32, 34–37, 40, 41, 43, 44, 46, 48–50, 53–55, 57, 59	+	nd	+
	4, 6–8, 10, 13, 24, 25, 27, 30, 33, 38, 39, 42, 45, 47, 51, 52, 56, 58, 60	–	–	–

nd, not determined

F_3 plants were obtained from a hemizygous F_2 parent.

Supplementary Table 2. Germination rates of the test plants 1 month after sowing at 25°C

Plants	Number of germinated/sown seeds	Germination rate (%)	Treatment to break seed dormancy	Sowing date
A3244	40/41	98	no	5/25/2007
A3244	12/12	100	no	6/4/2010
A3244RR	18/18	100	no	5/25/2007
A3244RR	15/15	100	no	5/23/2008
A3244RR	12/12	100	no	6/4/2010
A3525	23/36	64	no	11/30/2007
A3525	35/36	97	no	12/19/2008
A3525RR	35/36	97	no	11/30/2007
A3525RR	10/10	100	yes	6/5/2009
A3525RR	16/16	100	yes	5/28/2010
(inbred Nasu-5 × A3244) F_1	52/(52 + 2*)	≥ 96	yes	6/4/2010
(inbred Nasu-5 × A3244RR) F_1	23/23	100	yes	6/4/2010
(inbred JP110755 × A3244) F_1	133/133	100	yes	6/4/2010
(inbred JP110755 × A3244RR) F_1	78/(78 + 2*)	≥ 98	yes	6/4/2010
(Nasu-5 × A3244) F_2	33/60	55	no	5/25/2007
(Nasu-5 × A3244RR) F_2	30/36	83	no	5/25/2007
(Nasu-5 × A3525) F_2	35/36	97	no	8/10/2007
(Nasu-5 × A3525RR) F_2	57/60	95	no	1/12/2007
(Nasu-5 × A3244RR) F_3	60/60	100	yes#	1/25/2008
(Nasu-5 × A3244RR) F_3 (CP4−)	21/21	100	no	5/23/2008
(Nasu-5 × A3244RR) F_3 (CP4+)	79/79	100	no	5/23/2008
(JP110755 × A3244RR) BC1F_1	6/6	100	yes	7/18/2008 – 8/29/2008
(JP110755 × A3525RR) BC1F_1	5/6	83	yes	7/18/2008 – 8/29/2008
(JP110755 × A3525RR) BC2F_1	7/11	64	yes	6/5/2009
(JP110755 × A3525RR) BC2F_2 (CP4−)	19/20	95	yes	5/28/2010
(JP110755 × A3525RR) BC2F_2 (CP4+)	20/20	100	yes	5/28/2010
Nasu-5	30/30	100	yes	5/25/2007
Nasu-5	17/36	47	no	9/28/2007
Nasu-5	14/15	93	yes	5/23/2008
inbred Nasu-5	11/11	100	yes	6/4/2010
JP110755	16/17	94	yes	6/5/2009
JP110755	12/16	75	yes	5/28/2010
JP110755	19/30	63	yes	2/3/2012
JP110755	21/30	70	no	2/3/2012
inbred JP110755	12/12	100	yes	6/4/2010

*, ungerminated unidentified seeds

#, for 7 seeds similar to wild soybean

Supplementary Table 3. Plant height

Exp.	Plants	Days after sowing	Height ± SD (cm)	Number of plants	Statistical difference
1	A3244	52	36.4 ± 2.6	6	a
	A3244RR	52	33.3 ± 0.9	6	a
	A3525	52	37.7 ± 4.5	6	a
	A3525RR	52	37.2 ± 3.2	6	a
	(Nasu-5 × A3244) F_1	52	74.9 ± 8.7	6	a
	(Nasu-5 × A3244RR) F_1	52	65.2 ± 25.9	6	a
	(Nasu-5 × A3525) F_1	52	71.1 ± 9.5	6	a
	(Nasu-5 × A3525RR) F_1	52	61.3 ± 1.1	2	−
	Nasu-5	52	64.7 ± 8.7	6	a
	(JP110755 × A3244) F_1	52	43.7 ± 3.0	6	a
	(JP110755 × A3244RR) F_1	52	25.0 ± 10.1	2	−
	(JP110755 × A3525) F_1	52	36.3 ± 6.3	6	a
	(JP110755 × A3525RR) F_1	52	25.5	1	−
	JP110755	52	67.4 ± 9.0	6	a
2	A3244	42	19.9 ± 1.3	11	a, b, d
	A3244RR	42	17.6 ± 1.1	9	a, b, d
	(inbred Nasu-5 × A3244) F_1	42	28.5 ± 16.6	52	b, c
	(inbred Nasu-5 × A3244RR) F_1	42	28.0 ± 18.9	22	a, b, c
	inbred Nasu-5	42	77.0 ± 10.1	11	d
	(inbred JP110755 × A3244) F_1	42	14.5 ± 5.5	133	a
	(inbred JP110755 × A3244RR) F_1	42	20.0 ± 9.9	78	b
	inbred JP110755	42	55.2 ± 7.8	11	c, d
3	A3244	49	20.3 ± 2.7	18	a
	(Nasu-5 × A3244) F_2	49	25.2 ± 11.6	15	a
	Nasu-5	49	49.3 ± 16.7	18	b
4	A3244	49	19.0 ± 4.1	12	a
	A3244RR	49	19.4 ± 3.2	12	a
	(Nasu-5 × A3244) F_2	49	46.8 ± 31.1	18	a
	(Nasu-5 × A3244RR) F_2	49	26.3 ± 19.2	30	a
	(Nasu-5 × A3244RR) F_2 (CP4−)	49	28.3 ± 20.3	9	a
	(Nasu-5 × A3244RR) F_2 (CP4+)	49	25.4 ± 19.1	21	a
5	A3244RR	62	25.9 ± 2.6	8	a, b
	(Nasu-5 × A3244RR) F_3 (CP4−)	62	35.4 ± 20.7	16	a
	(Nasu-5 × A3244RR) F_3 (CP4+)	62	24.1 ± 11.2	16	a
	Nasu-5	62	60.6 ± 17.4	8	b

6	A3525RR	61	35.5 ± 3.7	6	a
	(JP110755 × A3525RR) $BC2F_1$	61	41.7 ± 22.3	5	a, b
	JP110755	61	62.4 ± 9.6	6	b
7	A3525RR	28	19.2 ± 1.8	8	a
	(JP110755 × A3525RR) $BC2F_2$ (CP4$^-$)	28	50.7 ± 16.5	10	b
	(JP110755 × A3525RR) $BC2F_2$ (CP4+)	28	52.4 ± 14.9	10	b
	JP110755	28	44.8 ± 9.5	5	a, b

Experimental groups are shown in the leftmost column for comparison of plant height (mean ± standard deviation). Identical letters in the rightmost column are representative of non-significant differences, determined by the Kruskal-Wallis test using Scheffé's comparison method ($p < 0.05$). Data eliminated from the statistical analyses due to small sample numbers are denoted by "–".

Supplementary Table 4. Test of independence between CP4 EPSPS protein and presence of vines by Fisher's exact test

Hybrid	Days after sowing	Number of plants with vines	Number of plants without vines	Two-tailed *P* value
(Nasu-5 × A3244) F_2	68	31	2	0.66
(Nasu-5 × A3244RR) F_2	68	27	3	
(Nasu-5 × A3244RR) F_2 (CP4−)	68	8	1	1.00
(Nasu-5 × A3244RR) F_2 (CP4+)	68	19	2	
(Nasu-5 × A3525RR) F_2 (CP4−)	45	17	26	0.11
(Nasu-5 × A3525RR) F_2 (CP4+)	45	2	12	
(Nasu-5 × A3244RR) F_3 (CP4−)	60	7	32	1.00
(Nasu-5 × A3244RR) F_3 (CP4+)	60	4	17	
(Nasu-5 × A3244RR) F_3 (CP4−)	42	5	16	0.32
(Nasu-5 × A3244RR) F_3 (CP4+)	42	11	68	

F_3 plants were obtained from a hemizygous F_2 parent.

Supplementary Table 5. Characteristics of stem including vine

Feature	Plants	Days after sowing	Score ± SD	Number of plants	Statistical difference
Extent of violet color					
	A3244	67	2.0 ± 0	30	a
	A3244RR	67	2.0 ± 0	12	a
	(Nasu-5 × A3244) F_2	67	3.2 ± 0.9	33	b
	(Nasu-5 × A3244RR) F_2	67	3.3 ± 0.9	30	b
	(Nasu-5 × A3244RR) F_2 (CP4$^-$)	67	3.2 ± 1.0	9	a, b
	(Nasu-5 × A3244RR) F_2 (CP4+)	67	3.3 ± 0.8	21	b
	Nasu-5	67	4.0 ± 0	18	b
Thickness					
	A3244	68	4.0 ± 0	30	a
	A3244RR	68	4.0 ± 0	12	a
	(Nasu-5 × A3244) F_2	68	3.0 ± 0.3	33	b
	(Nasu-5 × A3244RR) F_2	68	3.0 ± 0.3	30	b
	(Nasu-5 × A3244RR) F_2 (CP4$^-$)	68	3.0 ± 0	9	b, c
	(Nasu-5 × A3244RR) F_2 (CP4+)	68	3.0 ± 0.3	21	b
	Nasu-5	68	2.0 ± 0	18	c
Length of hair					
	A3244	68	4.0 ± 0	30	a
	A3244RR	68	4.0 ± 0	12	a, b
	(Nasu-5 × A3244) F_2	68	3.2 ± 0.6	33	c
	(Nasu-5 × A3244RR) F_2	68	3.1 ± 0.5	30	c
	(Nasu-5 × A3244RR) F_2 (CP4$^-$)	68	3.0 ± 0	9	b, c, d
	(Nasu-5 × A3244RR) F_2 (CP4+)	68	3.1 ± 0.6	21	c
	Nasu-5	68	2.0 ± 0	18	d

Stem features were evaluated by visually scoring each plant from 1 to 5. The mean scores with standard deviations are presented. Identical letters in the rightmost column are representative of non-significant differences, determined by the Kruskal-Wallis test using Scheffé's comparison method ($p < 0.05$).

Supplementary Table 6. Leaf size

Exp.	Plants	Days after sowing	Size ± SD (cm)	Number of plants	Statistical difference
2	A3244	96	11.0 ± 1.1	6	b, c
	A3244RR	96	10.2 ± 2.5	6	a, b, c
	(inbred Nasu-5 × A3244) F_1	103	11.7 ± 0.7	10	c
	(inbred Nasu-5 × A3244RR) F_1	103	11.1 ± 0.7	10	b, c
	inbred Nasu-5	117, 118	8.7 ± 0.7	7	a, b, c
	(inbred JP110755 × A3244) F_1	115	8.8 ± 0.6	10	a, b
	(inbred JP110755 × A3244RR) F_1	115	8.9 ± 0.6	10	a, b, c
	inbred JP110755	132	6.1 ± 0.3	8	a
3	A3244	125	12.7 ± 0.9	18	b
	(Nasu-5 × A3244) F_2	139	12.0 ± 1.3	15	b
	Nasu-5	181	8.7 ± 0.9	18	a
4	A3244	125	13.3 ± 1.0	12	a, c
	A3244RR	125	13.4 ± 0.7	12	c
	(Nasu-5 × A3244) F_2	140, 161	13.2 ± 1.4	18	c
	(Nasu-5 × A3244RR) F_2	140, 161	11.1 ± 2.1	30	b
	(Nasu-5 × A3244RR) F_2 (CP4−)	140, 161	11.0 ± 1.7	9	a, b, c
	(Nasu-5 × A3244RR) F_2 (CP4+)	140, 161	11.1 ± 2.3	21	a, b
5	A3244RR	108	9.5 ± 1.1	8	b
	(Nasu-5 × A3244RR) F_3 (CP4−)	150	9.1 ± 1.3	16	b
	(Nasu-5 × A3244RR) F_3 (CP4+)	150	9.1 ± 1.5	16	b
	Nasu-5	159	6.9 ± 0.7	8	a
6	A3525RR	104	6.9 ± 0.6	6	b
	(JP110755 × A3525RR) $BC2F_1$	129	5.3 ± 0.9	5	a
	JP110755	129	5.3 ± 0.8	6	a
7	A3525RR	101	8.1 ± 0.8	8	b
	(JP110755 × A3525RR) $BC2F_2$ (CP4−)	146	6.3 ± 0.8	10	a
	(JP110755 × A3525RR) $BC2F_2$ (CP4+)	146	6.7 ± 0.8	10	a, b
	JP110755	146	6.2 ± 0.3	5	a

Experimental groups are shown in the leftmost column for comparison of leaf size (mean ± standard deviation) represented by blade length of the central leaflet of the largest trifoliate leaf selected visually from each mature plant. Identical letters in the rightmost column are representative of non-significant differences, determined by the Kruskal-Wallis test using Scheffé's comparison method ($p < 0.05$).

Supplementary Table 7. The final basal diameter of stem

Exp.	Plants	Diameter ± SD (mm)	Number of plants	Statistical difference
1	A3244	3.8 ± 0.6	6	a
	A3244RR	3.3 ± 0.3	6	a
	A3525	3.3 ± 0.5	6	a
	A3525RR	3.6 ± 0.7	6	a
	(Nasu-5 × A3244) F_1	2.4 ± 0.1	6	a
	(Nasu-5 × A3244RR) F_1	2.5 ± 0.4	6	a
	(Nasu-5 × A3525) F_1	2.2 ± 0.3	6	a
	(Nasu-5 × A3525RR) F_1	2.9 ± 0.5	2	−
	Nasu-5	2.0 ± 0.4	6	a
	(JP110755 × A3244) F_1	2.9 ± 0.2	6	a
	(JP110755 × A3244RR) F_1	2.4 ± 0.1	2	−
	(JP110755 × A3525) F_1	2.4 ± 0.2	6	a
	(JP110755 × A3525RR) F_1	2.6	1	−
	JP110755	2.4 ± 0.5	6	a
2	A3244	4.4 ± 0.5	6	b, c
	A3244RR	4.6 ± 1.0	6	b, c
	(inbred Nasu-5 × A3244) F_1	3.4 ± 0.5	10	a, b
	(inbred Nasu-5 × A3244RR) F_1	3.3 ± 0.4	10	a, b
	inbred Nasu-5	2.3 ± 0.3	7	a
	(inbred JP110755 × A3244) F_1	4.8 ± 0.5	10	c
	(inbred JP110755 × A3244RR) F_1	4.5 ± 0.5	10	b, c
	inbred JP110755	4.0 ± 0.8	8	a, b, c
3	A3244	7.8 ± 1.2	18	c
	(Nasu-5 × A3244) F_2	4.5 ± 1.0	15	b
	Nasu-5	2.8 ± 0.4	18	a
4	A3244	8.9 ± 1.6	12	b
	A3244RR	10.5 ± 0.9	12	b
	(Nasu-5 × A3244) F_2	4.6 ± 0.8	18	a
	(Nasu-5 × A3244RR) F_2	5.0 ± 1.0	30	a
	(Nasu-5 × A3244RR) F_2 (CP4−)	4.8 ± 0.7	9	a
	(Nasu-5 × A3244RR) F_2 (CP4+)	5.1 ± 1.1	21	a
5	A3244RR	5.7 ± 0.7	8	c
	(Nasu-5 × A3244RR) F_3 (CP4−)	4.7 ± 0.8	16	b, c
	(Nasu-5 × A3244RR) F_3 (CP4+)	4.2 ± 0.7	16	b
	Nasu-5	2.2 ± 0.1	8	a

6	A3525RR	4.8 ± 0.1	2	−
	(JP110755 × A3525RR) $BC2F_1$	3.8 ± 0.5	5	a
	JP110755	4.1 ± 0.4	6	a
7	A3525RR	6.9 ± 0.8	8	b
	(JP110755 × A3525RR) $BC2F_2$ (CP4−)	4.7 ± 0.5	10	a
	(JP110755 × A3525RR) $BC2F_2$ (CP4+)	5.3 ± 0.4	10	a, b
	JP110755	4.4 ± 0.4	5	a

Experimental groups are shown in the leftmost column for comparison of basal diameter of stem (mean ± standard deviation). Identical letters in the rightmost column are representative of non-significant differences, determined by the Kruskal-Wallis test using Scheffé's comparison method ($p < 0.05$). Data eliminated from the statistical analyses due to small sample numbers are denoted by "–".

Supplementary Table 8. Total stem length including vines

Plants	Total stem length ± SD (cm)	Number of plants	Statistical difference
A3244	63.9 ± 27.6	6	a
A3244RR	40.7 ± 2.9	6	a
A3525	57.3 ± 39.2	6	a
A3525RR	60.6 ± 38.9	6	a
(Nasu-5 × A3244) F_1	289.3 ± 41.6	6	a, b
(Nasu-5 × A3244RR) F_1	207.2 ± 77.9	6	a, b
(Nasu-5 × A3525) F_1	326.7 ± 174.8	6	a, b
(Nasu-5 × A3525RR) F_1	705.0 ± 744.6	2	–
Nasu-5	1076.8 ± 1698.6	6	a, b
(JP110755 × A3244) F_1	258.3 ± 43.8	6	a, b
(JP110755 × A3244RR) F_1	121.8 ± 21.6	2	–
(JP110755 × A3525) F_1	210.8 ± 7.4	6	a, b
(JP110755 × A3525RR) F_1	150.0	1	–
JP110755	4160.3 ± 2403.0	6	b

Total stem length (mean ± standard deviation) of each plant was measured after the plant died. Identical letters in the rightmost column are representative of non-significant differences, determined by the Kruskal-Wallis test using Scheffé's comparison method ($p < 0.05$). Data eliminated from the statistical analysis due to small sample numbers are denoted by "–".

Supplementary Table 9. Dry weight of the final aboveground part

Exp.	Plants	Dry weight ± SD (g)	Number of plants	Statistical difference
2	A3244	2.0 ± 0.5	6	a, b
	A3244RR	1.7 ± 0.6	6	a
	(inbred Nasu-5 × A3244) F_1	5.2 ± 1.5	10	a, b, d
	(inbred Nasu-5 × A3244RR) F_1	4.8 ± 1.3	10	a, b, c
	inbred Nasu-5	9.3 ± 2.7	7	c, d
	(inbred JP110755 × A3244) F_1	6.3 ± 1.6	10	a, b, d
	(inbred JP110755 × A3244RR) F_1	7.3 ± 1.0	10	b, d
	inbred JP110755	12.3 ± 1.3	8	d
3	A3244	4.1 ± 1.6	18	a
	(Nasu-5 × A3244) F_2	12.3 ± 5.1	15	b
	Nasu-5	21.6 ± 9.3	18	b
4	A3244	5.7 ± 2.0	12	a
	A3244RR	9.2 ± 2.2	12	a, b
	(Nasu-5 × A3244) F_2	26.6 ± 11.3	18	c
	(Nasu-5 × A3244RR) F_2	23.6 ± 9.9	30	c
	(Nasu-5 × A3244RR) F_2 (CP4−)	27.4 ± 7.8	9	c
	(Nasu-5 × A3244RR) F_2 (CP4+)	22.0 ± 10.4	21	b, c
5	A3244RR	5.8 ± 2.4	8	a
	(Nasu-5 × A3244RR) F_3 (CP4−)	12.4 ± 6.0	16	b
	(Nasu-5 × A3244RR) F_3 (CP4+)	8.6 ± 5.9	16	a, b
	Nasu-5	8.1 ± 2.5	8	a, b
6	A3525RR	3.5 ± 1.5	2	–
	(JP110755 × A3525RR) $BC2F_1$	8.7 ± 3.9	5	a
	JP110755	9.8 ± 3.7	6	a
7	A3525RR	4.9 ± 1.6	8	a
	(JP110755 × A3525RR) $BC2F_2$ (CP4−)	18.5 ± 2.8	10	b
	(JP110755 × A3525RR) $BC2F_2$ (CP4+)	20.5 ± 2.2	10	b
	JP110755	19.5 ± 2.1	5	b

Experimental groups are shown in the leftmost column for comparison of dry weight of the aboveground part (mean ± standard deviation). Identical letters in the rightmost column are representative of non-significant differences, determined by the Kruskal-Wallis test using Scheffé's comparison method ($p < 0.05$). Data eliminated from the statistical analyses due to small sample numbers are denoted by "–".

Supplementary Table 10. Test of independence between the CP4 EPSPS protein and the color of mature pods using the Fisher's exact test

Hybrid	Number of plants with dark-brown pods	Number of plants with light-brown pods	Two-tailed *P* value
(JP110755 × A3525RR) $BC2F_2$ (CP4−)	8	2	1.00
(JP110755 × A3525RR) $BC2F_2$ (CP4+)	7	3	

Supplementary Table 11. Number of pods per plant

Exp.	Plants	Number of pods per plant ± SD	Number of plants	Statistical difference
2	A3244	20.5 ± 4.6	6	a
	A3244RR	18.8 ± 5.9	6	a
	(inbred Nasu-5 × A3244) F_1	103.4 ± 12.2	10	a, b
	(inbred Nasu-5 × A3244RR) F_1	98.7 ± 19.3	10	a, b
	inbred Nasu-5	182.7 ± 42.2	7	b
	(inbred JP110755 × A3244) F_1	103.9 ± 19.4	10	a, b
	(inbred JP110755 × A3244RR) F_1	131.0 ± 8.7	10	b
	inbred JP110755	205.9 ± 24.8	8	b
6	A3525RR	11.3 ± 1.2	6	a
	(JP110755 × A3525RR) $BC2F_1$	195.8 ± 99.4	5	b
	JP110755	217.7 ± 81.5	6	b
7	A3525RR	35.4 ± 5.8	8	a
	(JP110755 × A3525RR) $BC2F_2$ (CP4−)	166.9 ± 31.1	10	b
	(JP110755 × A3525RR) $BC2F_2$ (CP4+)	161.6 ± 31.0	10	b
	JP110755	192.0 ± 18.6	5	b

Experimental groups are shown in the leftmost column for comparison of the number of pods per plant (mean ± standard deviation). Identical letters in the rightmost column are representative of non-significant differences, determined by the Kruskal-Wallis test using Scheffé's comparison method ($p < 0.05$).

Supplementary Table 12. Number of seeds per pod

Exp.	Plants	Number of seeds per pod ± SD	Number of plants	Statistical difference
2	A3244	2.3 ± 0.1	6	a, b
	A3244RR	2.1 ± 0.2	6	a
	(inbred Nasu-5 × A3244) F_1	2.5 ± 0.1	10	b
	(inbred Nasu-5 × A3244RR) F_1	2.4 ± 0.1	10	a, b
	inbred Nasu-5	2.3 ± 0.1	7	a, b
	(inbred JP110755 × A3244) F_1	2.4 ± 0.2	10	a, b
	(inbred JP110755 × A3244RR) F_1	2.3 ± 0.1	10	a, b
	inbred JP110755	2.2 ± 0.2	8	a, b
6	A3525RR	2.1 ± 0.2	6	a
	(JP110755 × A3525RR) $BC2F_1$	2.3 ± 0.1	5	a
	JP110755	2.2 ± 0.1	6	a
7	A3525RR	2.2 ± 0.2	8	a
	(JP110755 × A3525RR) $BC2F_2$ (CP4$^-$)	2.3 ± 0.1	10	a
	(JP110755 × A3525RR) $BC2F_2$ (CP4+)	2.2 ± 0.1	10	a
	JP110755	2.2 ± 0.1	5	a

Experimental groups are shown in the leftmost column for comparison of the number of seeds per pod (mean ± standard deviation). Identical letters in the rightmost column are representative of non-significant differences, determined by the Kruskal-Wallis test using Scheffé's comparison method ($p < 0.05$).

Supplementary Table 13. Total weight of seeds per plant

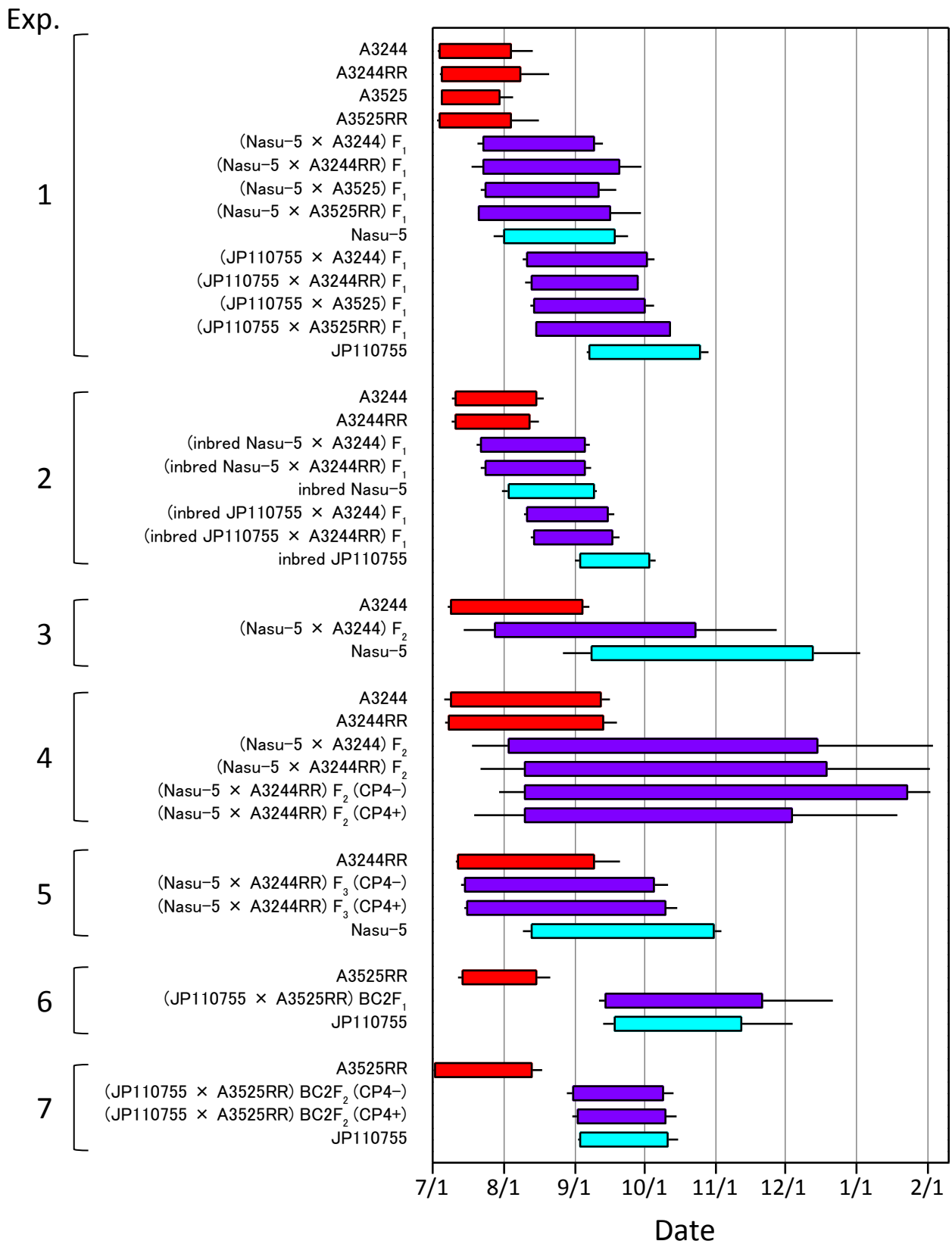
Exp.	Plants	Total weight of seeds per plant ± SD (g)	Number of plants	Statistical difference
2	A3244	9.7 ± 2.4	6	a, b
	A3244RR	7.6 ± 2.8	6	a
	(inbred Nasu-5 × A3244) F_1	16.8 ± 1.5	10	b, c
	(inbred Nasu-5 × A3244RR) F_1	14.5 ± 2.2	10	a, c
	inbred Nasu-5	10.3 ± 2.1	7	a, b
	(inbred JP110755 × A3244) F_1	15.0 ± 3.0	10	a, c
	(inbred JP110755 × A3244RR) F_1	18.2 ± 1.4	10	c, d
	inbred JP110755	11.1 ± 2.3	8	a, b
6	A3525RR	3.6 ± 1.4	6	a
	(JP110755 × A3525RR) $BC2F_1$	12.4 ± 7.6	5	a
	JP110755	11.5 ± 4.7	6	a
7	A3525RR	15.0 ± 3.2	8	b
	(JP110755 × A3525RR) $BC2F_2$ (CP4$^-$)	9.5 ± 2.6	10	a, b
	(JP110755 × A3525RR) $BC2F_2$ (CP4+)	8.0 ± 1.8	10	a
	JP110755	8.2 ± 1.1	5	a

Experimental groups are shown in the leftmost column for comparison of total weight of seeds per plant (mean ± standard deviation). Identical letters in the rightmost column are representative of non-significant differences, determined by the Kruskal-Wallis test using Scheffé's comparison method ($p < 0.05$).

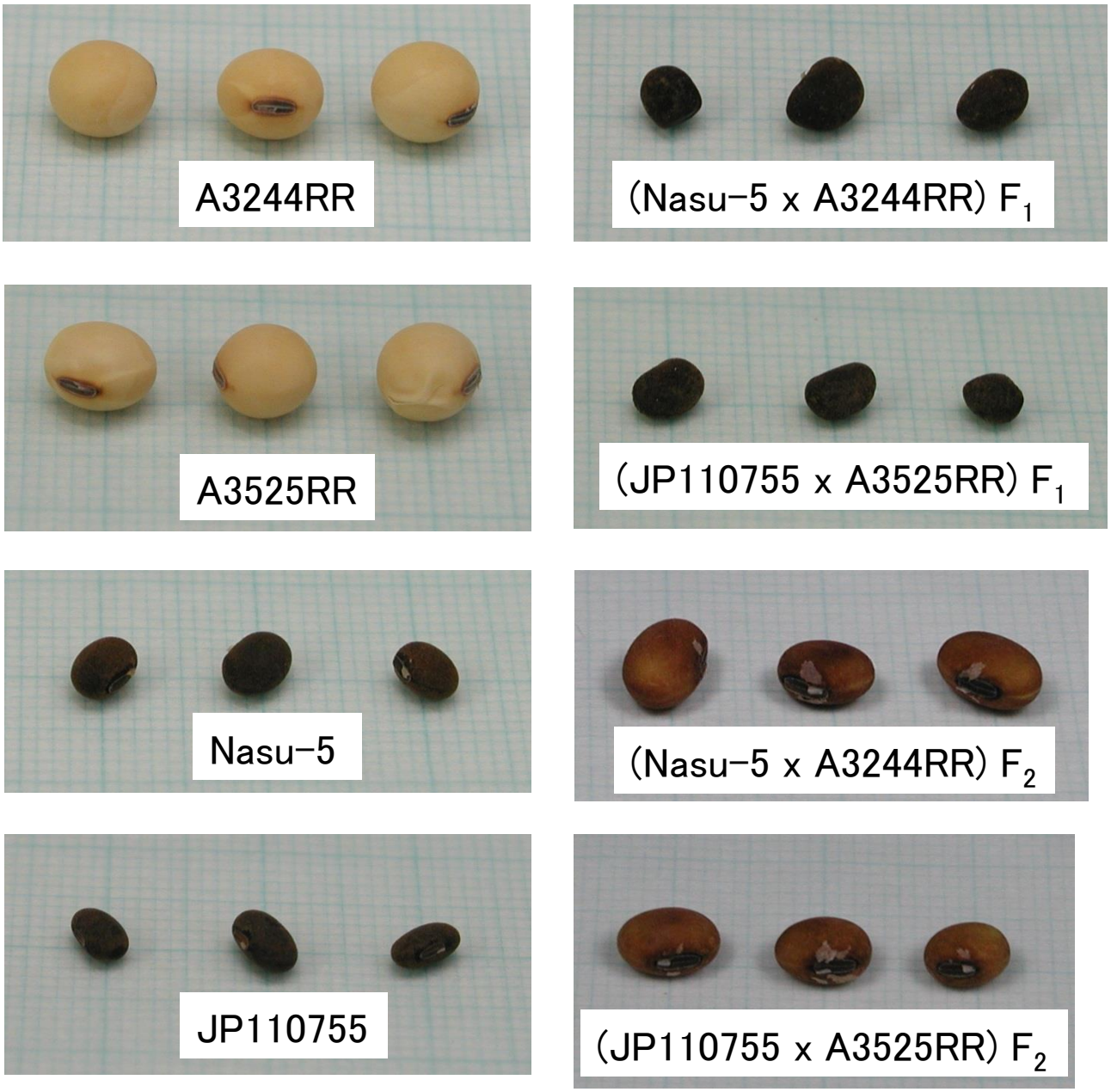
Supplementary Table 14. Seed weight per grain

Exp.	Parental plants	Seed weight per grain ± SD (mg)	Number of plants	Statistical difference
2	A3244	205.8 ± 14.3	6	b
	A3244RR	188.2 ± 10.7	6	b
	(inbred Nasu-5 × A3244) F_1	65.8 ± 6.3	10	b
	(inbred Nasu-5 × A3244RR) F_1	60.8 ± 3.9	10	a, b
	inbred Nasu-5	24.5 ± 0.8	7	a
	(inbred JP110755 × A3244) F_1	60.7 ± 3.0	10	a, b
	(inbred JP110755 × A3244RR) F_1	60.8 ± 3.3	10	a, b
	inbred JP110755	24.4 ± 2.9	8	a
6	A3525RR	147.3 ± 32.9	6	b
	(JP110755 × A3525RR) $BC2F_1$	26.4 ± 6.2	5	a, b
	JP110755	23.4 ± 1.4	6	a
7	A3525RR	191.2 ± 6.9	8	b
	(JP110755 × A3525RR) $BC2F_2$ (CP4−)	24.3 ± 2.7	10	a, b
	(JP110755 × A3525RR) $BC2F_2$ (CP4+)	22.1 ± 1.5	10	a
	JP110755	19.4 ± 0.8	5	a

Experimental groups are shown in the leftmost column for comparison of seed weight per grain (mean ± standard deviation). Identical letters in the rightmost column are representative of non-significant differences, determined by the Kruskal-Wallis test using Scheffé's comparison method ($p < 0.05$).



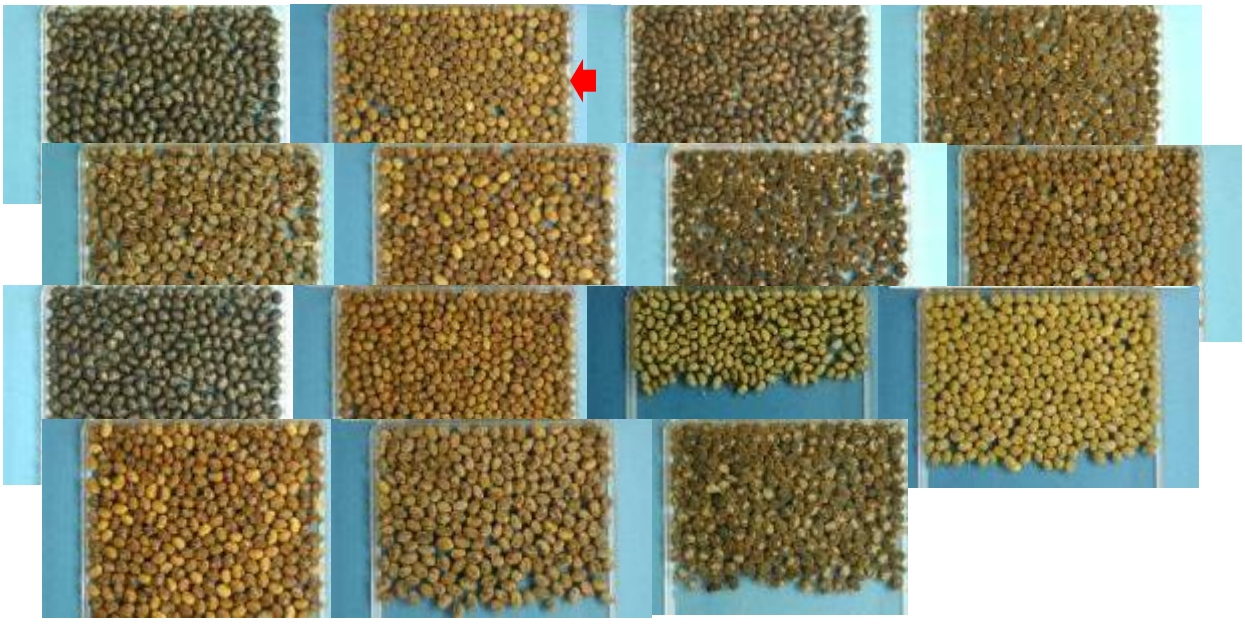
Supplementary Figure 1. Flowering periods of the test plants. Experimental groups are shown on the left. Seeds were sown on May 22, 2006 (Exp. 1), June 4, 2010 (Exp. 2), May 25, 2007 (Exp. 3, 4), May 23, 2008 (Exp. 5), June 5, 2009 (Exp. 6), and May 28, 2010 (Exp. 7). Supplementary lighting for a 15-h photoperiod was conducted for Exp. 1 (terminated on July 7), Exp. 4, and Exp. 5 (terminated on September 25). Red (cultivated soybean cultivars), violet (hybrids), and blue (wild soybean accessions) bars represent flowering periods from the mean starting to the mean ending dates in the plants labeled on the left. Error bars represent standard deviation.



Supplementary Figure 2. Examples of seeds of GM and wild soybeans and F_1 and F_2 hybrids. Generation names represent the seed itself not the parental plant. Seeds were placed on 1-mm graph paper.

From (Nasu-5 x A3244RR) F_2 *cp4 epsps* (−/−)

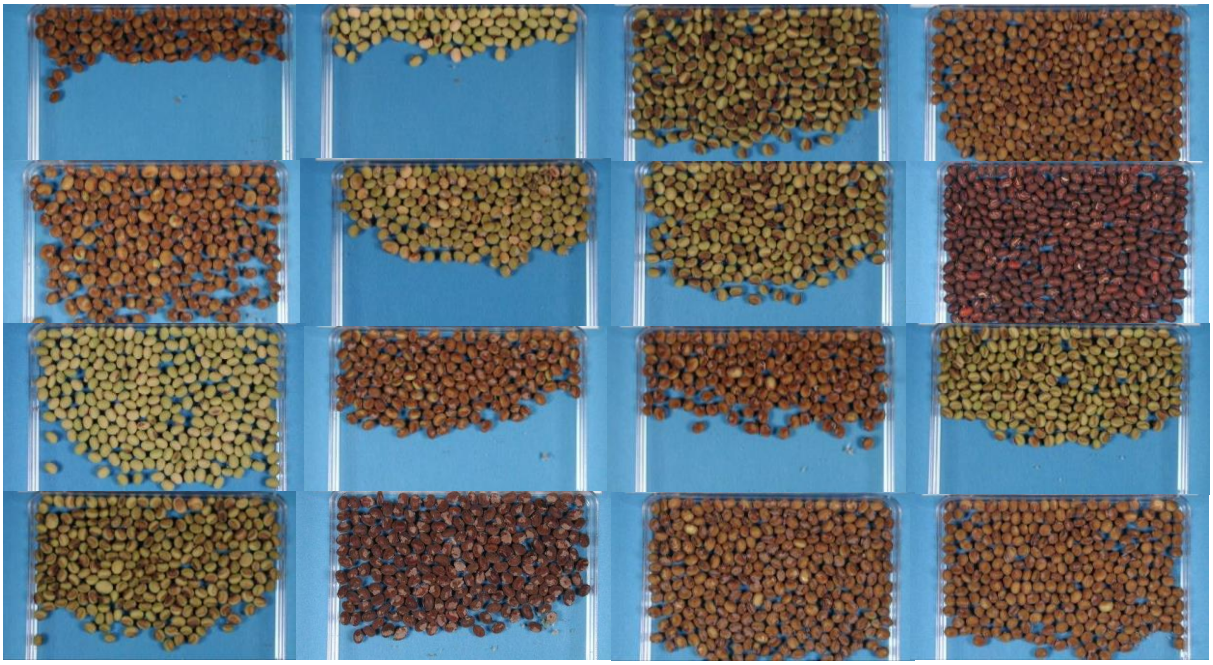
From (Nasu-5 x A3244RR) F_2 *cp4 epsps* (+/−)



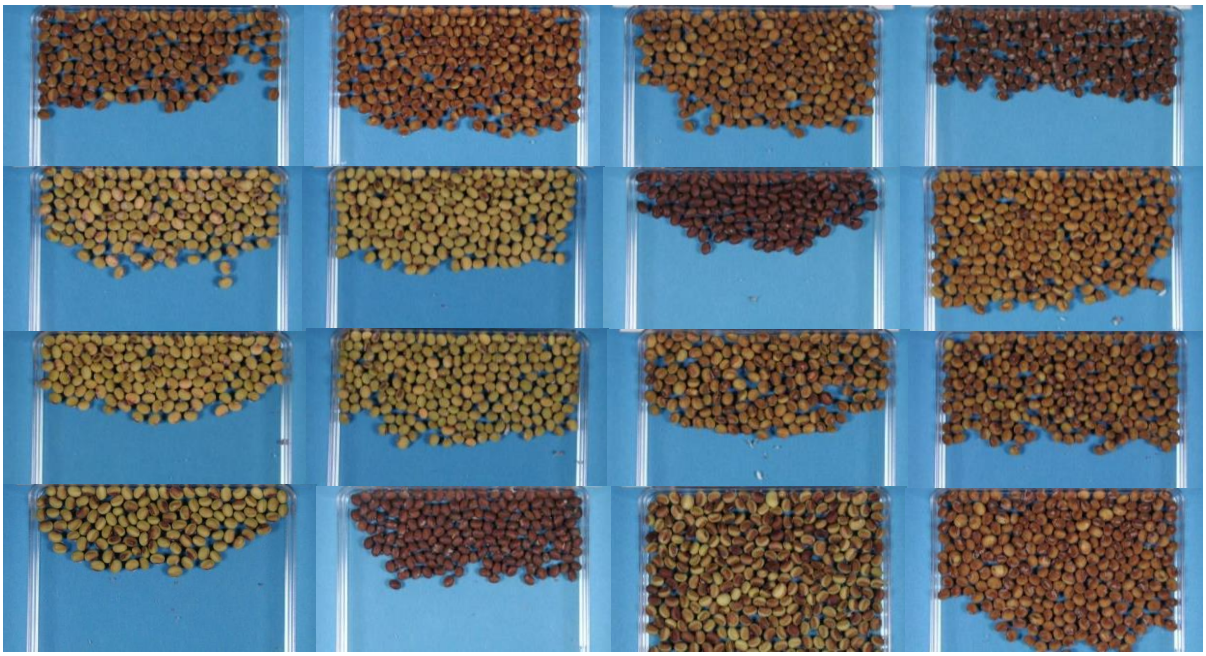
From (Nasu-5 x A3244RR) F_2 *cp4 epsps* (+/+)

Supplementary Figure 3. F_3 hybrid seeds. Pools of seeds harvested from each F_2 plant are shown under the *cp4 epsps* genotype of the F_2 plant. The dish on which the seeds were placed was 96 mm in inside width. The red arrow indicates a seed pool used to characterize F_3 hybrids.

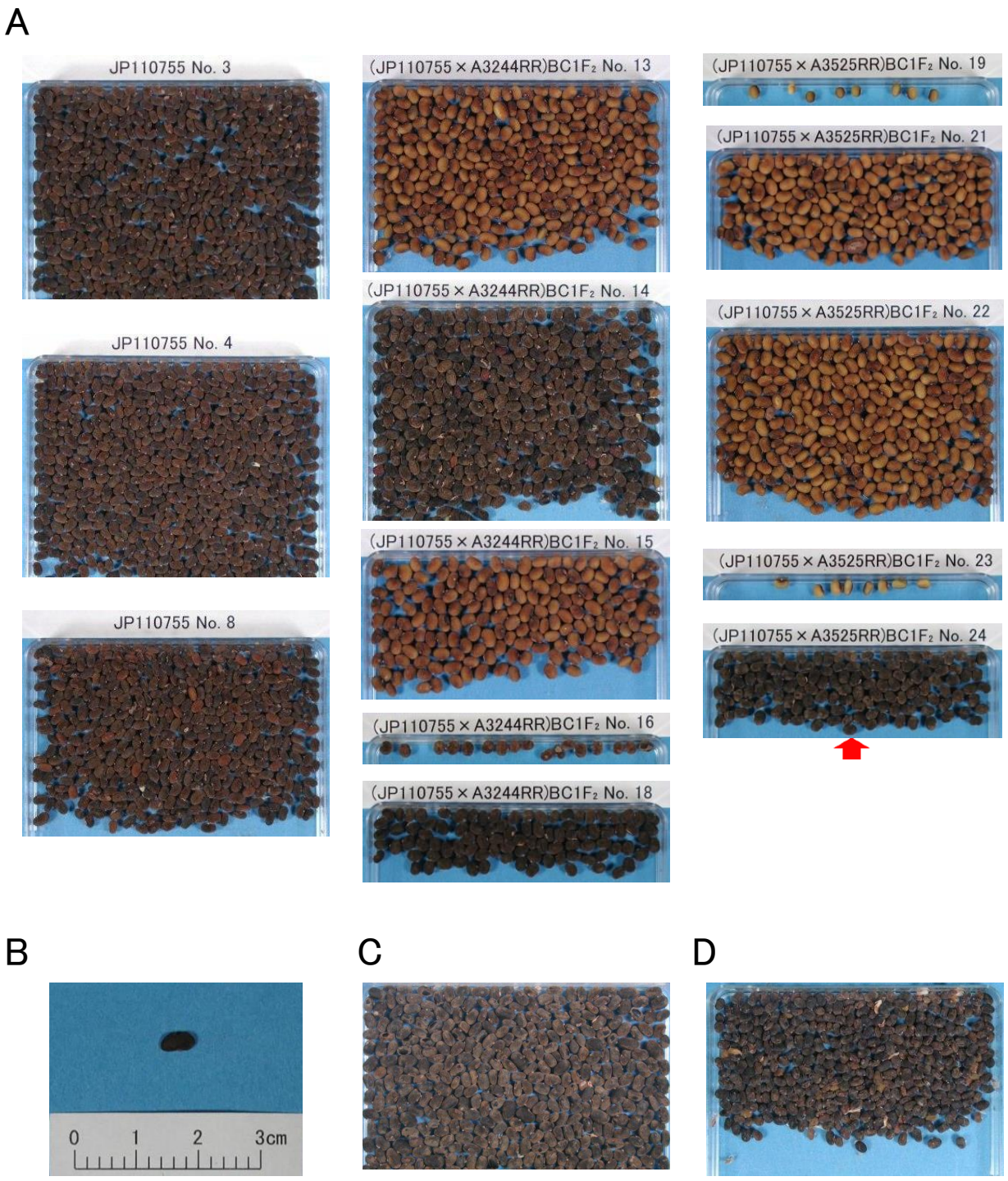
From (Nasu-5 x A3244RR) F_3 CP4 EPSPS (−)



From (Nasu-5 x A3244RR) F_3 CP4 EPSPS (+)



Supplementary Figure 4. F_4 hybrid seeds. Pools of seeds harvested from each F_3 plant are shown under the CP4 EPSPS phenotype of the F_3 plant. The dish on which the seeds were placed was 96 mm in inside width.



Supplementary Figure 5. $BC1F_2$, $BC2F_1$, $BC2F_2$, and $BC2F_3$ hybrid seeds. (A) JP110755 and $BC1F_2$ seeds. Seed color segregates in $BC1F_2$. Pools of seeds harvested from each parental plant are shown and are distinguished by their ID number. The red arrow indicates a seed pool harvested from a $BC1F_1$ plant that was an ancestor of the $BC2F_1$, $BC2F_2$ and $BC2F_3$ hybrids in this study. (B) A (JP110755 × A3525RR) $BC2F_1$ seed. (C) A pool of (JP110755 × A3525RR) $BC2F_2$ seeds. (D) A pool of (JP110755 × A3525RR) $BC2F_3$ seeds. The dish on which the seeds were placed was 96 mm in inside width.