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Specific Inheritance of a Mutant Gene Controlling α, β Subunits-Null of β-Conglycinin in Soybean (*Glycine max* (L.) Merrill) and Observation of Chloroplast Ultrastructure of the Mutant

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Summary

In order to improve the nutritional value of soybean protein through the decrease of the β-conglycinin content or increase of the glycinin content, 3,000 dry seeds of a cultivar 'Wasesuzunari' were treated with 10 to 50 kR γ-rays. The 3,500 M2 seeds were observed for the analysis of the protein composition of the mutants by SDS-PAGE. As a result, a mutant trait characterized by the lack of both the α and β subunits of β-conglycinin and lethal chlorosis was detected. Investigation on the segregation of the mutant trait was carried out from the M3 to M8 generations and a peculiar segregation ratio (3 normal : 4 with half the amount : 1 null for the α, β subunits) was obtained from heterozygous individuals from the previous generations. Reciprocal hybridization experiments were conducted between the heterozygous genotype for the mutant trait and a normal cultivar 'Keburi'. As a result of this experiment, it was suggested that the mutant trait is controlled by a recessive gene (a), and that the pollens with the recessive gene are produced less than those with the dominant gene (A) as a : A ratio = 1 : 3. Ultrastructure of the chloroplasts in the null (aa) individuals was examined. Abnormalities were detected in the grana stacks and the thylakoid systems. The inheritance of the two traits (α and β subunits-null and lethal chlorosis) may be due to chromosomal deletion over a wide area including the loci controlling the α, β subunits of β-conglycinin and the chloroplast structure.

Key Words : Soybean (*Glycine max*), seed protein, γ-ray irradiation, protein subunit null mutant, lethal chlorosis, distorted segregation, chloroplast ultrastructure.

Introduction

Nutritional deficiency of soybean seed protein is characterized by a small amount of sulfur-containing amino acids such as methionine and cysteine (Derbyshire *et al.* 1976, Coates *et al.* 1985). The content of both essential amino acids is about half of or less (about 2 %) than that of animal proteins present in meat, egg or milk (about 4 %). The increase of the amount of sulfur-containing amino acids to a level equivalent to that of animal proteins by genetic methods is one of the major problems confronting soybean breeders (Kaizuma 1986).

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The two major components of soybean seed protein, glycinin (11S) and β-conglycinin (7S), exhibit differences in the amount of the sulfur-containing amino acids: in the former the content is about three to four times higher than in the latter (Thanh and Shibasaki 1977, Staswick *et al.* 1981). It is generally recognized that both protein components show a very strong negative correlation between their content (Kitamura and Kaizuma 1981, Ogawa *et al.* 1989, Mizuno *et al.* 1994, Kitamura 1995). This fact implies that the decrease of the content of β-conglycinin or increase of the content of glycinin results in the increase of the content of the amino acids in seed protein. Kitamura and Kaizuma (1981) identified two cultivars with a lower quantity of β-conglycinin subunit proteins: 'Keburi' as a null variant for the α' subunit of β-conglycinin protein and 'Mo-shidou Gong 503' as a variant with a low production of both α and β subunits of the protein. They confirmed that in these two variants the amount of sulfur-containing amino acids in seed protein is larger than that of ordinary cultivars. Ogawa *et al.* (1989) hybridized these two cultivars and obtained several strains with a combination of both characteristics, and also demonstrated that the amount of the sulfur-containing amino acids in seed protein increased. In order to further increase the amount of sulfur-containing amino acids, we attempted to induce the formation of a subunit-null mutant for α, β or both α and β subunits using γ-rays irradiation and we succeeded in producing a null mutant for both α and β subunits of β-conglycinin.

This mutant did not survive due to severe chlorosis in the case of the homozygous genotype. As a result, the amount of sulfur-containing amino acids in seed protein could not be determined. We analyzed the mode of inheritance of the null trait and the fertilization behaviour between the pollen grains and eggs harbouring the mutant gene. Furthermore, electron microscopic observation was carried out to analyse the progression of chlorosis in this mutant.

Materials and Methods

Mutagen treatment and detection of seed protein mutants

A total of 3,000 dry seeds of a soybean cultivar, 'Wasesuzunari', were treated with 10 to 50 kR γ-rays (10 kR

intervals at a rate of 5 kR/h) at the Institute of Radiation Breeding, National Institute of Agrobiological Resources, in Japan. After irradiation, the seeds were sown in a greenhouse at the Faculty of Agriculture, Iwate University, Morioka in 1987. The seedlings in the first trifoliate stage were transplanted in the experimental field. At maturity 3,500 M 2 seeds were harvested from each of the 423 M 1 plants derived from the seeds treated with 40 and 50 kR γ-rays.

Detection of seed protein mutants was performed by SDS-PAGE for all of the harvested M2 seeds. For SDS-PAGE sample solution a small portion (5.0 mg) of partially cut off M2 seed was used. Preparation of sample solutions and detection of mutants by SDS-PAGE followed the method of Laemmli (1970). A variant with a very low production of α and β subunits of β-conglycinin was detected in a M2 seed. The M2 plant was grown and seven M3 seeds were collected. These seeds showed a distorted segregation for the mutational trait. In order to determine the mode of inheritance of the trait, the segregation ratio from M4 to M8 was determined, using individual seeds from the heterozygous phenotype seeds selected from the preceding generations.

Reciprocal hybridizations for the determination of gametophyte ratios between the mutant and normal genes

As described in the results, the mutant trait showed a peculiar distorted segregation for normal : intermediate : null production of α and β subunits of β-conglycinin. The frequency of mature gametophytes with the mutant or normal genes varied due to distorted segregation. Therefore, reciprocal hybridizations were conducted as shown below. Frequency of female gametes with the mutant or normal genes was investigated based on the segregation ratio of F_1 plant genotypes obtained from hybridizations between the heterozygous genotype (female) and 'Keburi' (male). Likewise, the frequency of male gametes harbouring the mutant or normal genes was analysed based on the segregation of F_1 plant genotypes obtained from the hybridization between 'Keburi' (female) and the heterozygous genotype (male).

In order to obtain hybridized F_1 seeds, two specific markers were utilized: normal or null α' subunit of β-conglycinin, and difference in electrophoretic mobility of Kunitz trypsin inhibitors, *Tia* or *Tib*. The cultivar 'Keburi' lacks the α' subunit and shows the *Tib* mobility. In contrast, the heterozygous type originating from 'Wasesuzunari' harbours the α' subunit and shows the *Tia* mobility. Both traits can be discriminated by SDS-PAGE and Davies system PAGE for a small portion of the hybridized seeds.

Electron microscopic observations of leaf cell organs

Soybean seeds with the three genotypes (normal, intermediate and null production of α and β subunits of β-conglycinin) harvested from an M7 heterozygous genotype plant were sown in the greenhouse in 1992. 'Wasesuzunari'(original cultivar) was used for comparison. Small leaf portions from the first trifoliate leaves of each genotype were cut at three growth stages corresponding to 15, 25 and 35 days after germination.

These leaf samples were fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.20) for 3 hours at 4°C, followed by postfixation in 1% osmium tetroxide in 0.14 M Veronal buffer (pH 7.25) for 2 hours. Then the fixed samples were dehydrated in a graded ethanol series and infiltrated with propylene oxide and then embedded in Epon-Araldite resin (Mollenhauer 1964). The samples cut with glass knives on a Porter Blum MTI ultramicrotome were stained with 1% uranyl acetate and 2.5% lead citrate. At least two preparations were made for electron microscopy from each of the three genotypes and a cultivar 'Wasesuzunari' at the three growth stages listed above. Hitachi H-800 transmission electron microscope was used for observation at 8000 to 15000 X.

Numbers of chloroplasts and mitochondria per cell were determined in at least 50 cells in both palisade and spongy tissues. Numbers of starch deposits and osmiophilic bodies per chloroplast were counted in 10 chloroplasts (11 X 15 cm photographs). Changes in the chloroplast ultrastructure during chlorosis were also observed.

Results

Detection and mode of inheritance of α and β subunits-null mutant gene

In the SDS-PAGE analyses of 3,500 M2 seeds harvested in 1987, we detected one mutant seed with a markedly decreased production of both the α and β subunits of β-conglycinin (lane 2 of Fig. 1). The seed was derived from the M1 seed treated with 40 kR γ-rays. The M2 plant derived from this seed was grown and 7 M3 seeds were harvested in 1988. SDS-PAGE of these M3 seeds showed a segregation of 2 normal and 5 intermediate for the production of the α and β subunits of β-conglycinin. Then we grew 5 M3 plants derived from the 5 M3 intermediate variant seeds and harvested 55 M4 seeds. They segregated in the ratio of 20 normal, 31 intermediate and 4 null for the production of both α and β subunits of β-conglycinin (Fig. 1).

We investigated the segregation ratio of normal vs. intermediate vs. null production of the subunits among the set seeds of the plants derived from the seeds with the intermediate production from M4 to M8 generations. The observed segregation frequencies showed obviously significant difference from 1 : 2 : 1 ratio for a single codominant gene trait as shown in Table 1. Then we tentatively assumed that 3 : 4 : 1 segregation ratio may be caused by the unequal formation of male or female gametophytes between the normal and mutant genes at the ratio of 3 : 1, respectively (Table 2), considering that the null production types showed extraordinarily decreased frequencies compared with the normal production types. As indicated by χ^2-test, the segregation

Table 1. Segregation frequencies of the SDS-PAGE banding patterns for the α, β subunits-null mutant gene from the M4 to M8 heterozygous seed generations

Grown seed generation	No. of seeds grown	Set seed generation	No. of set seeds analyzed	Charasteristic of α, β subunits			Expected ratios	
				Normal	intermediate	Null	1 : 2 : 1* 3 : 4 : 1* X^2	P
M_3	5	M_4	55	20	31	4		
				(13.7)	(27.5)	(13.7)	10.19	<0.01
				(20.6)	(27.5)	(6.8)	1.61*	>0.30*
M_4	13	M_5	172	65	87	20		
				(43.0)	(96.0)	(43.0)	24.39	<0.001
				(64.5)	(86.0)	(21.5)	0.12*	>0.90*
M_5	14	M_6	515	177	273	65		
				(128.7)	(257.5)	(128.7)	50.57	<0.001
				(193.1)	(257.5)	(64.4)	2.28*	>0.30*
M_6	13	M_7	381	141	198	42		
				(95.2)	(190.5)	(95.2)	52.02	<0.001
				(142.9)	(190.5)	(47.6)	0.98*	>0.50*
M_7	25	M_8	726	284	357	85		
				(181.5)	(363.0)	(181.5)	109.28	<0.001
				(272.3)	(363.0)	(90.7)	0.96*	>0.50*

Data in ( ) show the expected numbers for segregation ratios (1 : 2 : 1 and 3 : 4 : 1).
* : χ^2 and probability values for segregation ratio of 3 : 4 : 1.

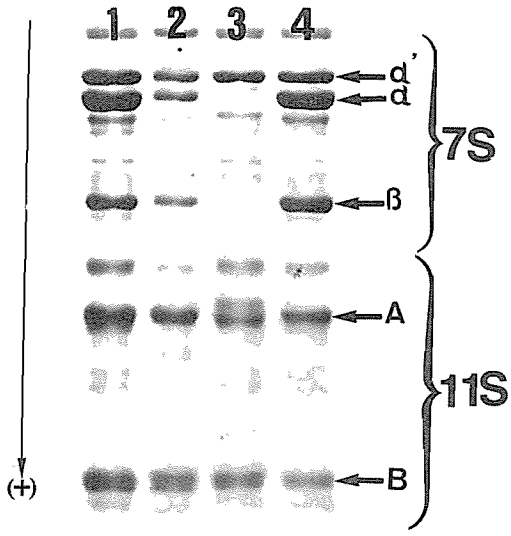

Fig. 1. SDS-slab PAGE of β-conglycinin (7S) and glycinin (11S) globulins from the original, Wasesuzunari and three genotypes for the null trait for α and β subunits of β-conglycinin. Soybean flour (5 mg) was dissolved in 0.5 ml of 50 mM Tris-HCL (pH 8.0) containing 0.2% SDS, 5 M urea and 0.1 M 2-mercaptoethanol. Protein samples (about 7 μl) were electrophoresed in 10% polyacrylamide separate gel with constant current of 15 mA for 4 hours. 1: Wasesuzunari; 2: intermediate type with α and β subunits (*Aa*); 3: α and β null type (*aa*); 4: α and β normal type (*AA*).

frequencies statistically fitted to 3 normal vs. 4 intermediate vs. 1 null production at higher probabilities than 0.30 (Table 1). On the other hand, we confirmed that there was no segregation in the progeny seed populations derived from the normal plants.

However, all the plants grown from the null type seeds developed lethal chlorosis soon after germination, and did not set seeds of the next generation, although

Table 2. Hypothesis for 3 *AA* (normal) : 4 *Aa* (intermediate) : 1 *aa* (null) segregation ratio of the α and β subunits-null mutant gene of β-conglycinin in the heterozygous plants

Formation of gametes in parents	Female or male	
Male or female :	3/4 *A* (Dominant)	1/4 *a* (Recessive)
1/2 *A* (Dominant)	3/8 *AA* (normal)	1/8 *Aa* (intermediate)
1/2 *a* (Recessive)	3/8 *Aa* (intermediate)	1/8 *aa* (null)

mature null type seeds were obtained in the plants derived from the seeds belonging to the intermediate type. To account for such a distorted segregation ratio, we assumed that gametophyte formation was irregular as indicated in Table 2.

Irregular gametophyte formation detected by analysis of reciprocal crosses

In order to determine the frequencies of male and female gametes bearing the mutant and normal alleles in the heterozygous plants we conducted several reciprocal hybridizations between the heterozygous individuals and 'Keburi'. Since 'Keburi' produces only the normal allele gametes for the trait in both sexual gametes, the number of F_1 plants inheriting the mutant allele depends on the ratio of the male and female gametes harbouring the normal and mutant alleles in the heterozygous parents. In hybridization in which the heterozygous individuals were used as a mother parent, the number of F_1 hybrid plants carrying a mutant allele depended on the ratio of female normal and mutant gametes in the mother parent. In the reciprocal hybridization of 'Keburi' X heterozygous individuals, the number of F_1 hybrid plants carrying the mutant allele depended on the male normal and

Table 3. Unequal formation of male gametophytes demonstrated y reciprocal hybridization between *AA* (Keburi) and *Aa* (heterozygous individuals)

Reciprocal Combinations	Number of F_1 plants tested	No. F_1 Genotypes with		Expected ratio	X^2	P
		AA	*Aa*			
AA (♀)×*Aa* (♂)	31	22 (23.25)	9 (7.75)	3 : 1	0.269	>0.50
Aa (♀)×*AA* (♂)	38	20 (19.00)	18 (19.00)	1 : 1	0.105	>0.70

Data in ( ) show the expected numbers for ratio.

mutant gamete ratio. The results of reciprocal hybridization analysis are shown in Table 3.

The F_2 seed populations from each of the 31 selfed F_1 plants derived from the hybridization of 'Keburi' as female and the heterozygous individuals as male were analyzed by SDS-PAGE to determine whether the distortion of the segregation ratio was controlled by the male gametophyte. As expected, nine F_1 plants containing F_2 segregating seeds for the α and β-subunits-null trait were transmitted with the mutant allele through the pollen gametes. A good fit to a 3 : 1 ratio for F_1 homozygous, normal and heterozygous plant number, respectively, was obtained. However, 18 of the 38 F_1 plants from the heterozygous individuals X 'Keburi' were found to harbour the mutant gene in heterozygous condition with a good fit to a 1 : 1 ratio.

These findings implied that the production of male gametes with the mutant gene was lower than that of gametes with the normal gene, though the production of female gametes with the mutant gene and of female gametes with the normal gene was identical.

Abnormality of the chloroplast ultrastructure

The ultrastructure of the chloroplasts was different between the null mutant genotype and the normal green plants. As shown in Fig. 2, the chloroplasts of the normal and heterozygous plants showed normal ultrastructural characteristics similar to those of the original cultivar at the three growth stages. The grana and stroma lamellar systems were well developed (Fig. 2A, B and C). However, in the null mutant yellow plants the ultrastructure of the chloroplasts was abnormal with disorientation, swelling and disruption of grana. The grana and thylakoid membranes of the chloroplasts in the null mutant genotype showed an abnormal development characterized by stacking in irregular and looser forms compared with the other genotypes (Fig. 2 D).

The relative size and frequency of the chloroplasts per cell and starch deposits per chloroplast did not vary significantly among the three genotypes and the original cultivar in both the palisade and spongy tissues at 15 and 25 day growth stages. However, there was an increase in the relative numbers of mitochondria and osmiophilic bodies in the tissues of the yellow plants (Table 4). The abnormalities in the chloroplast ultrastructure which were already present at the 15 day seedling stage (Fig. 3A) when chlorosis started to appear with very small yellow spots all over the surface of the first trifoliate leaf were enhanced at the 25 day stage when chlorosis progressed leading to the formation of uniformly yellow leaves (Fig. 3 B). The chloroplasts and other cell organelles by 35 days underwent complete degeneration in the leaf tissues (Fig. 3C). At this stage seedling growth was markedly delayed and the plant eventually died at 45 days after germination.

Discussion

Detection and mode of inheritance of the α and β subunits null mutant

The seeds with intermediate contents of α and β subunits of β-conglycinin in every generation were grown to mature plants and the set seeds of the next generation were harvested and subjected to SDS-PAGE analysis. As shown in Table 1, the segregation ratio of the normal vs. intermediate vs. null production of the α and β subunits of β-conglycinin was 3 : 4 : 1, respectively from the M 4 to M 8 generations. The set seeds of the normal type plant showed a uniform and normal production of the α and β subunits of β-conglycinin and did not segregate for that trait.

Interestingly, the α and β subunits-null trait was inherited together throughout the generations tested. There were always three kinds of phenotypes and phenotypes with the α-subunit-null trait only or the β-subunit-null trait only were not observed. This mutant trait was considered to be caused by a single gene mutation. Therefore, we used the gene symbol "*a*", for the mutated gene ("*A*", for the normal or wild gene).

However, if this designation is correct, it is necessary to explain why the peculiar segregation ratio of 3 normal vs. 4 intermediate vs. 1 null occurred instead of the 1 : 2 : 1 ratio, respectively. It was suggested that this ratio could be described to the unequal formation of male gametophytes (=pollen) in a heterozygous (*Aa*) plant.

It is well known that any decrease in the content of β-conglycinin subunits (α', α and/or β) is related to the decrease of the conversion of β-conglycinin to glycinin, and results in the increase of the amount of sulfur-con-

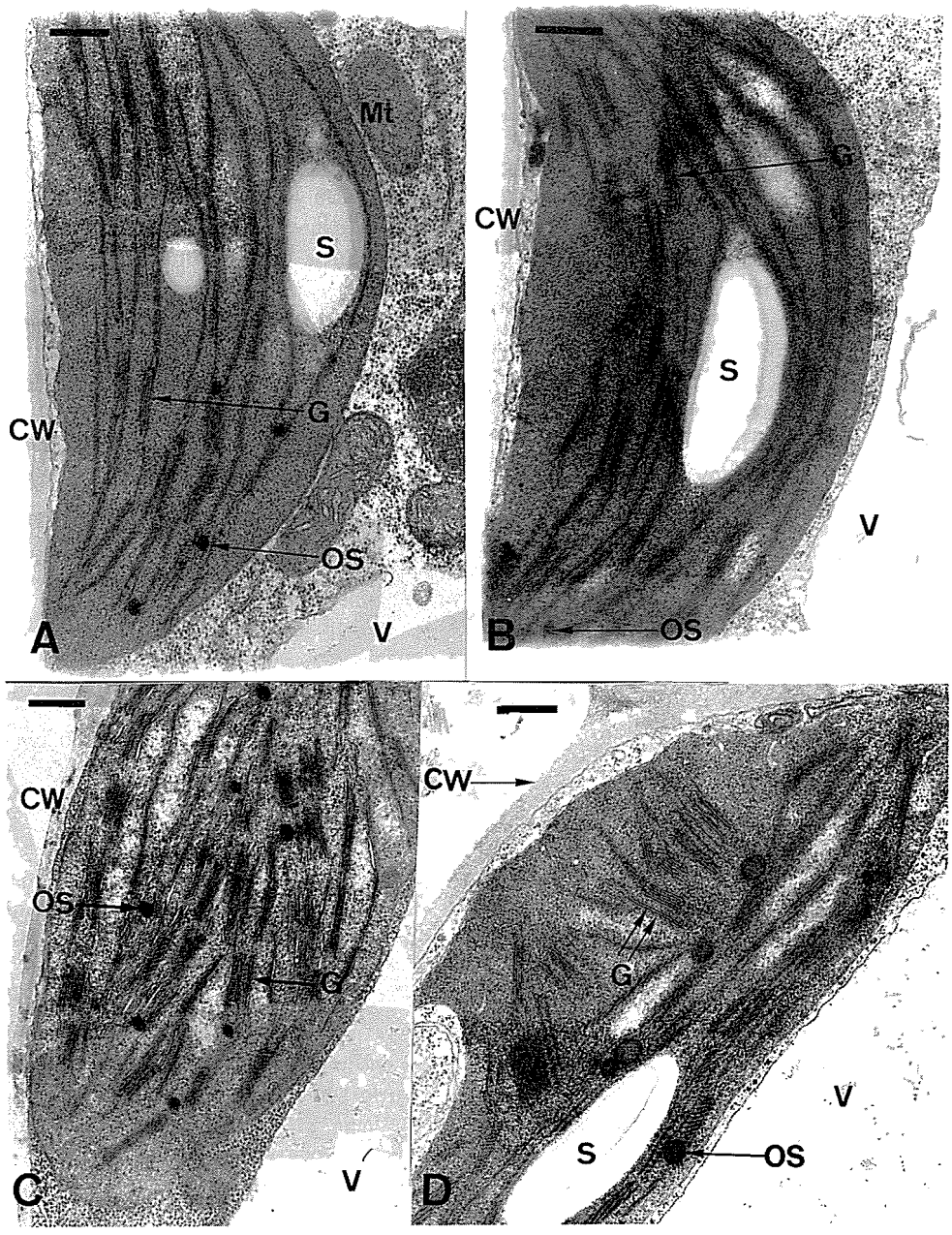

Fig. 2. Transmission electron microscopy of the green normal type (*AA*) and *aa* mutant chloroplasts grown at 25 days and heterozygous one (*Aa*) at 15 days. A. Normal chloroplast from leaf tissue of the original, Wasesuzunari, shows normal stacking of the lamellae into grana. Starch deposits accumulate. B. Chloroplast of homozygous plant (*AA*) showing normal chloroplast ultrastructure almost similar to that of Wasesuzunari. C. Normal chloroplast from the heterozygous plant (*Aa*). D. Chloroplast from null mutant (*aa*) showing some disorientation of grana stacks (⇄). S, starch deposit; G, grana; OS, osmiophilic body; Mt, mitochondria; CW, cell wall; V, vacuole. Bar represents 0.5 μm.

taining amino acids (Kitamura and Kaizuma 1981, Ogawa *et al.* 1989, Mizuno *et al.* 1994, Kitamura 1995).

This mutant gene could be an appropriate material for research on chromosomal changes related to the α and β subunits genes. Tsukada *et al.* (1986) revealed the presence of a strong linkage between the α and β subunits genes, which supports our findings that the α and β subunits-null characteristic corresponded to a single mutation gene. This suggests that a β-conglycinin null type could be induced by a slight mutation without concomitant chlorosis unlike in this study. Recently, Takahashi *et al.* (1994) have succeeded in inducing a null mutant for the α subunit only. They obtained a viable strain lacking α and α' subunits.

Unequal formation of male gametophyte demonstrated by reciprocal hybridization to heterozygous plants (Aa)

The distorted segregation ratio of the 3 normal (*AA*) : 4 intermediate (*Aa*) : 1 null (*aa*) for the α and β subunits of β-conglycinin was obtained based on SDS-PAGE analysis of the set seeds of the heterozygous (*Aa*) plants in M3 to M8 generations. We assumed that the

Table 4. Numerical differences in some traits of palisade tissues between genotypes and growth stages observed under the electron microscope

Genotypes	Mitochondria per cell		Osmiophilic bodies per chloroplast		Chloroplasts per cell		Starch deposits per chloroplast	
	15*	25*	15*	25*	15*	25*	15*	25*
Wasesuzunari (*AA*)	6.38 (0.42)	6.30 (0.92)	5.20 (0.77)	6.69 (0.84)	7.57 (0.43)	7.90 (0.78)	0.87 (0.02)	2.96 (0.04)
Normal (*AA*)	5.92 (0.54)	5.83 (0.64)	5.00 (0.68)	6.22 (0.42)	7.38 (0.63)	7.33 (0.48)	0.93 (0.03)	2.26 (0.34)
Intermediate (*Aa*)	5.98 (0.86)	5.42 (0.47)	5.42 (0.41)	6.33 (0.74)	7.60 (0.26)	7.92 (0.28)	0.91 (0.03)	2.69 (0.84)
Null (*aa*)	11.50 (0.97)	8.33 (0.64)	6.94 (0.85)	8.61 (0.87)	7.69 (0.63)	7.70 (0.79)	0.93 (0.08)	2.93 (0.38)

* : Days after germination.
All the data are expressed as mean values and standard deviations in ( ).

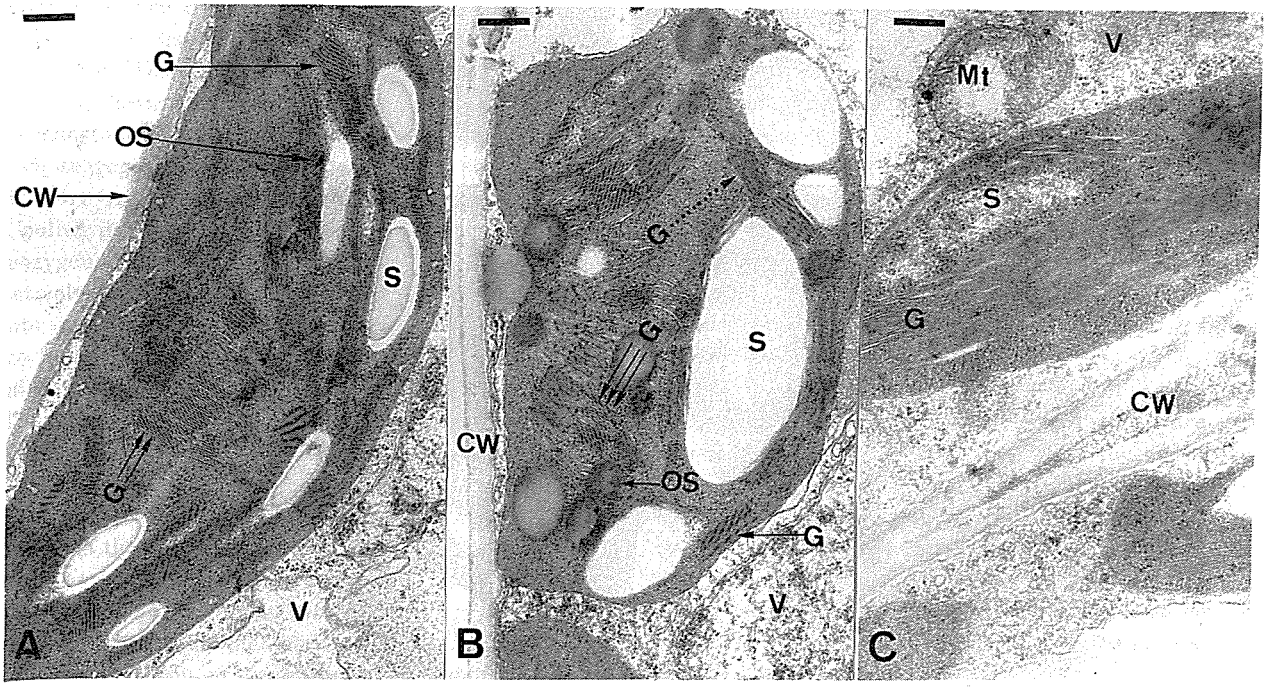

Fig. 3. Ultrastructure of *aa* mutant chloroplasts at three growth stages. A. Abnormal chloroplast at 15 days showed overlapping of several thylakoid membrances. Some grana stacks show disorientation, disruption and seem to be degraded. Starch deposits are initially developed. B. Chloroplast at 25 days showing severe degradation of thylakoid system and osmiophilic bodies. C. Chloroplast at 35 days with complete degradation of starch deposits, grana and osmiophilic bodies. ⟶ : normal grana, ⟹ : disoriented grana, ⇉ : swollen grana ⋯⋯> ; degraded grana. S, starch deposit; G, grana; OS, osmiophilic body; Mt, mitochondria; CW, cell wall; V, vacuole. Bar represents 0.5 μm.

unequal formation of the male or female gametophytes between "*a*" and "*A*" led to the distorted segregation ratio.

Then we determined experimentally which sex was responsible for the unequal formation. Reciprocal hybridizations between the heterozygous (*Aa*) and the normal (*AA*) plants were carried out and the F_1s were analysed for the determination of the ratio of homozygous (*AA*) vs. heterozygous(*Aa*) among the set seeds in F_1 plants by SDS-PAGE.

The distorted segregation ratio of 3 : 4 : 1 appeared when three-fourth *A* and one-fourth *a* gametophytes were formed in one sex and one-half *A* and one-half *a* gametophytes in the other sex (Table 2). As shown in Table 3, the male gametophytes in the heterozygous (*Aa*) were produced at the ratio of three-fourth *A* and one-fourth *a* because the F_1s segregated into 3 *AA* and 1 *Aa* only when the heterozygous (*Aa*) individuals were used as pollen parents.

The reason why the production of male gametophytes with "*a*" was lower remains unknown. Microscopic studies on pollen development in the heterozygous (*Aa*) are in progress. At least no abnormality was detected until tetrad formation (data not shown). Distorted segregation due to partial abortion of the male gametes has also been reported in several higher plants such as rice (Lin *et al.* 1992, Lin and Ikehashi 1993), maize (Sari Gorla and Rovida 1980), and barley (Zivy *et al.* 1992), suggest-

ing that some gametophyte loci were responsible for these phenomena.

Electron microscopic observations of chloroplast degradation

The α and β subunits-null seeds (*aa*) developed lethal chlorosis at the seedling stage soon after germination. Overall appearance was similar to that of other genotypes (*Aa* and *AA*). However, chlorosis began at first with the formation of small yellow spots scattered on the primary leaves. Then, the yellow spots rapidly enlarged and yellowing became more pronounced. The growth of the seedlings ceased about 2 weeks after germination and all of them eventually died about 4 to 5 weeks later.

Therefore, homozygous (*aa*) for the mutated gene could not be obtained as a plant, presumably due to the disorganization (= deletion) over a wide area of the chromosome segment with the α and β subunits genes. This chromosome segment may contain an important DNA sequence related to chloroplast development.

We compared the characteristics of the ultrastructure of the chloroplast and some other intracellular organs among the three genotypes (*aa, Aa* and *AA*) as shown in Table 4. The changes in the chloroplast ultrastructure involved mainly abnormalities of intrachloroplast organelles such as grana and lamellae. As shown in Fig. 3, swelling of the grana and disorderly stacking of the lamellae in the chloroplasts of the *aa* genotype started at the very early seedling stage, 15 days after germination. Such abnormal phenomena were not observed in the *Aa* and *AA* genotypes. Development of normal chloroplasts appeared to be strongly inhibited only in the *aa* genotypes and complete destruction of the chloroplasts occurred 25 days after germination.

As seen in Table 4, there were major differences in some characters until these seedling stages. However, the number of mitochondria per cell and the number of osmiophilic bodies per chloroplast increased significantly, presumably due to chloroplast destruction.

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