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Relationships between genome constitution and esterase composition in Brassica

Hiromi EGUCHI* and Tsuyoshi MATSUI*

Introduction

It is known that the change of gene can be studied through the differences in enzyme molecules synthesized in different genetic lines. In this point of view, one of the useful methods which will bring the information of phylogenetic importance in cultivated plants could be taken as comparative analysis of enzymes synthesized in different species and strains. ***Brassica*** species are regarded as distinct ones on the cytogenetical ground ; there exist 3 kinds of monogenomic group (*aa*, ***bb,*** and cc) and digenomic group ***(aabb, bbcc,*** and ***aacc),*** respectively (Morinaga, 1928; Morinaga and Fukushima, 1933; Manton, 1933 ; Sikka, 1940 ; Fukushima, 1945 ; Mizushima, 1952). Those plants contain relatively large amount of esterases in the plumules and young seedlings. Genetic control of esterase formation has been analyzed in ***Drosophila*** *melanogaster* (Ogita, 1962; Wright, 1963) and other animals.

Present paper deals with analyses of relationships between genome constitution and esterase composition in several ***Brassica*** species, employing the zymogram (Hunter and Marker-t, 1957).

Materials and methods

Brassica species used have been maintained in pedgree cultures in the Horticultural Laboratory of Kyushu University, and were listed in Table 1. Material seedlings were sampled 3 days after germination. Fifty

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Table 1. Material species and varieties in *Brassica.*

Species	Genome constitution	Chromosome number (n)	Horticultural variety
B. *pekinensis* Rupr.	***an***	***10***	Shimoyama-hakusai
B. *rapa* L.	*aa*	10	Shin-hakata-kabu
B. ***campestris*** L.	*aa*	***10***	Kanzaki-hanana
B. *chinensis* L.	*aa*	***10***	Seppaku-taisai
B. japonica Sieb.	*aa*	***10***	Okute-shiraguki-chisuji-kyona
B. *trilocularis* Hook	*a'a'*	10	
B. ***tournefortii*** Gouan	*TT*	*10*	——
B. nigra Koch	*bb*	*8*	California brown
B. oleracea L. var. *botrytis* D. C.	*cc*	9	Early snow ball
B. oleracea L. var. *capitata* D. C.	*cc*	*9*	Yoshin
B. oleracea L. var. *acephala* D. C.	*cc*	***9***	Kale
B. *juncea* Hemsel	*aabb*	*18*	Miike-takana
B. cernua Coss.	*aabb*	*18*	
B. *carinata* Braun	*bbcc*	17	Harron
B. napus L.	*aacc*	*19*	Rape

gm of the cotyledonary seedlings was frozen at -20°C and homogenized in 50 ml of *M/30* phosphate buffer of *p*H *7.0.* The homogenate was ultracentrifuged at *145,000 x g.* The supernatant was lyophilized and stored for the use to obtain esterase zymogram.

Agar-gel electrophoresis was employed for preparing the esterasc zymogram. Each gel medium for the electrophoresis was prepared with 0.7 gm of agar, and 2.0 gm of polyvinyl-pyrrolidonc (Ogita, 1962) in 100 ml of M/50 phosphate buffer of *p*H *7.0.* Agar-gel plates were made 2mm in thickness and supported by grass plate. The lyophilized extract was restored with deionized water, and a piece of filter paper (1.5 mm × 12.0 mm) was saturated with the extract and was placed on the agar-gel plate. The extract diffused from the filter paper into agar-gel. After 40 minutes, the filter paper was removed and the extract in agar -gel plate was exposed to the stabilized voltage of 20 V/cm at 0° C--5°C for 120 minutes. After the electrophoretic separation, 1 per cent solution of β-naphthyl acetate was sprayed on the surface of the agar-gel as the substrate of esterases. The agar-gel plate was incubated at 35°C for 30 minutes. The substrate, β-naphthyl acetate diffused into the agar-gel and was hydrolyzed by each esterase separated electrophoretically at individual location on an agar-gel plate. Naphthanil diazo blue B was used as the dye coupler.

Results and discussion

Esterase patterns at different growth stages of B. rapa: Extracts originated from seeds, seedlings (at cotyledonary stage), and from leaves of *B. rapa*, respectively were used for zymogram as shown in Fig. 1. Five bands of esterases designated as A1∼A5 were found in the young seedlings and also in the leaves. Al band migrated slowly toward cathode, while other bands migrated towards anode. There were no differences in electrophoretic mobilities between the esterases of the young seedlings and those of the leaves. The intensity of the esterase bands of the young seedlings was in general, stronger than that of the leaves. On the other hand, Al, A2, A5 bands and an immobile band locating at the original point, were found in the seeds. The A2 band of the seeds was stronger, and the A5 band was weaker than those of the young seedlings and of the leaves. The pattern of seedling esterases was similar to that of leaf esterases, but was quite different from that of seed esterases. These facts suggest that the estcrase composition changes through the germination at quite fast tempo, then it become almost constant during the growing period of the seedlings until the growth stage of the 7th leaves.

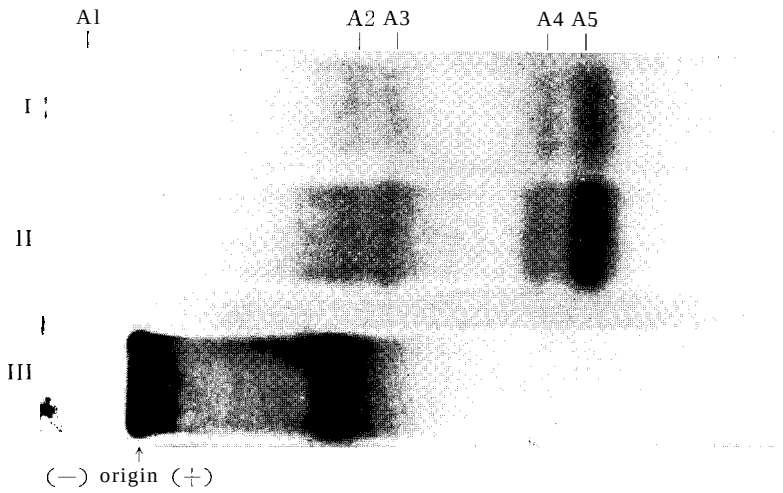

Fig. 1. Photograph of esterase zymogram showing the patterns of esterases occurred at various growing stages of ***B. rapa***. (I) leaves set at the 4-5th nodes of the 5-7 leaf plants. (II) cotyledonary seedlings collected 3 days after germination. (III) seeds.

The behaviors of esterases under the treatment with β-mercaptoethanol: Beta-mercaptoethanol (0.1 ml) was added to the extract (10 ml) of *B. rapa*

and of ***B. oleracea*** var. ***botrytis,*** and the treated extracts were used in zymograms. The treatment resulted in increasing the intensity of A2 and A5 bands in ***B. rapa,*** as well as C2 and C6 bands in ***B. oleracea.*** On the other hand, A3 band in ***B. rapa*** and C3 band in ***B. oleracea*** were scarcely detectable in the treated extract. The β-mercaptoethanol had no effect on Al and A4 band of ***B. rapa,*** and on Cl, C4, C5 and C7 bands of ***B. oleracea.*** All the bands in the treated extracts were not different from those untreated in their electrophoretic mobilities as shown in Fig. 2. The esterases of A2, A5, C2 and C6 bands were activated by the treatment with β-mercaptoethanol. In most cases of the following experiments, β-mercaptoethanol was added to the extract used for the zymograms.

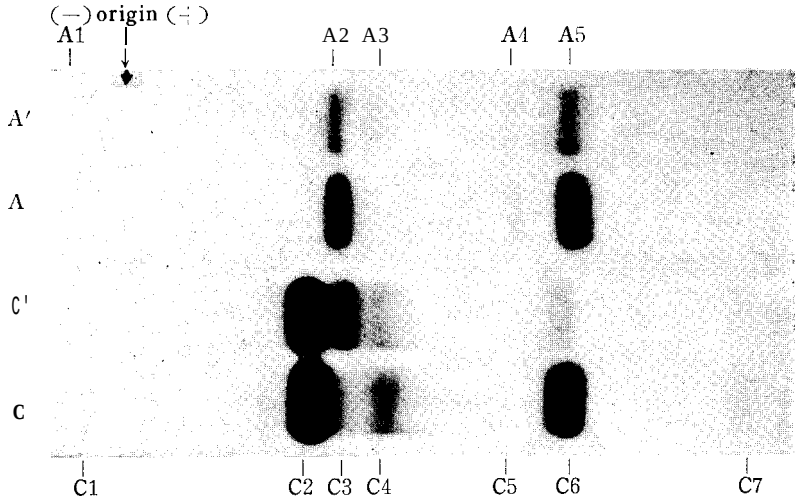

Fig. 2. Photograph of esterase zymogram showing comparison between the extract treated with β-mercaptoethanol and untreated extracts. (A') untreated extract of ***B. rapa.*** (A) treated extract of B. ***rapa.*** (C′) untreated extract of ***B. oleracea*** var. ***botrytis.*** ***(C)*** treated extract of ***B. oleracea*** var. ***botrytis.***

Relationships between genome constitution and esterase composition : Fig. 3 shows the patterns of esterases in the seedlings of the ***a*** genomic species and c genomic species. Al, A2, A3, A4 and A5 bands were generally found in the ***a*** genomic speces ; the Al band which was very weak, migrated slowly towards cathode, and the other bands migrated towards anode. The A4 band in ***B. chinensis*** was weaker than that in ***B. rapa*** and ***B. pekinensis.*** There were no differences in electrophoretic mobilities of esterases among those 3 different species having ***aa*** genome, i. e., ***B. pekinensis, B. chenensis,*** and ***B. rapa.*** But 7 bands designated as C1,

C2, C3, C4, C5, C6 and C7, were found in the c genomic species. The C3 band was not found in the extract treated with β-mercaptoethanol. The C5 and C7 bands were detected in var. ***botrytis*** only. All the bands of the c genomic species were different from those of the ***a*** genomic species in their electrophoretic mobilities. Thus, inter-genomic differences between ***a*** and ***c*** genomic species could be observed in differential electrophoretic mobilities of their esterases. While the intra-genomic differences could be found in the differential intensity of certain bands. The differences among 3 species having the ***aa*** genome, i. e., ***B. pekinensis, B. chinensis*** and ***B. rapa***, were taken to be on the same level as intra-specific differences among 3 varieties of ***B. oleracea*** having cc genome, i. e., ***B. oleracea*** var. ***botrytis,*** var. ***capitata,*** and var. ***acephala.*** From this view-point, the degree of the differentiation existing among 3 different species having ***aa*** genome, are regarded

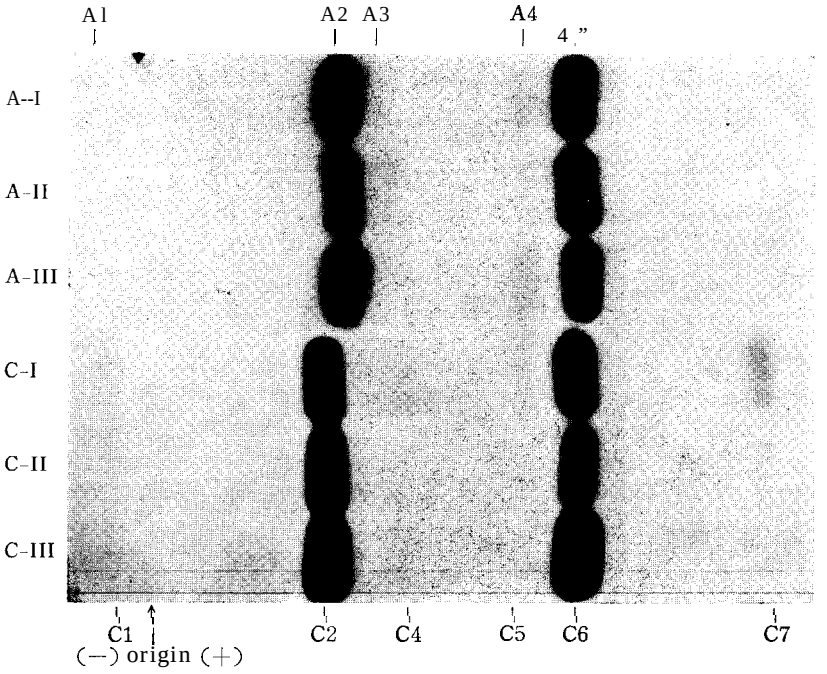

Fig. 3. Photograph of esterase zymograms derived from cotyledonary seedlings of ***Brassica,*** showing comparison between different monogenomic species (***aa*** and cc genomes). (A-l) ***B. pekinensis (aagenome).*** (A-II) ***B. chinensis*** (***aa*** genome). (A-III) ***B. rapa*** (***aa*** genome). (C-l) ***B. oleracea*** var. ***botrytis (cc genome).*** (C-II) ***B. oleracea*** var. ***capitata (cc*** genome). (C-III) ***B. oleracea*** var. ***acephala (cc*** genome).

as that being on a level with intra-specific differences.

Fig. 4 shows the relationship in esterase composition between an amphidiploid species, ***B. napus*** (aacc genome) and the basic monogenomic species, ***B. pekinensis (au*** genome) and ***B. oleracea*** var. ***acephala (cc*** genome). An array of Al, C2, A2, C4, C6 and A5 bands were found in ***B. napus***. The C6 and A5 bands in ***B. napus*** were superimposed on each other, according to their close migration. The array of esterase bands in the amphidiploid species, ***B. napus***, was found to be composed of the bands contained in the two basic monogenomic species.

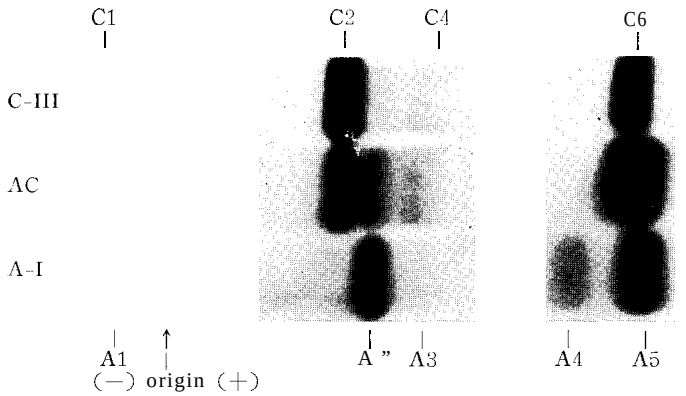

Fig. 4. Photograph of esterase zymogram derived from cotyledonary seedlings of ***Brassica,*** showing comparison between an amphidiploid species and its basic monogenomic species. (C-III) ***B. oleracea*** var. ***acephala (cc genome).*** (AC) ***B. napus*** (***aacc*** genome). (A-I) ***B. pekinensis (au*** genome).

On the other hand, ***b*** genomic species, ***B. nigra (bb*** genomc) appeared to have 7 bands of esterases, designated as B1, B2, B3, B4, B5, ***B6*** and ***B7,*** as shown in Fig. 5. The very weak B1 band migrated slowly towards cathode, while others towards anode. The B3 band which is closely adjacent to the position of the A3 band of a genomic species and the C4 band of c genomic forms, but the other 6 bands were distinctly different in their electrophoretic mobilities from those of other monogenomic species.

In an amphidiploid species, ***B. juncea (aabb*** genome), Al, A2, B4 and A5 bands were found. The B4 band of ***B. juncea*** was stronger than that of ***B. nigra.*** The A2 and A5 bands of ***B. juncea*** were weaker than those of the a genomic forms. The A4 band was not clear in ***B. juncea,*** because the A4 band appeared to be superimposed on the strong B4 band. In another amphidiploid species having ***aabb*** genome, ***B. cernua,*** Al, A2, X, A4 and A5 bands were found. The band designated as X could not be detected in either of the a genomic species or the ***b*** genomic species

as shown in Fig. 5. The A2 and A4 bands of ***B. cernua*** were weaker than those of the ***a*** genomic species

In ***B. carinata,*** an amphidiploid species having *bbcc* genome, Cl, C2, C4, X, C6 and C7 bands were found. This Cl band was very weak as quite like that of the c genomic species, and the C2, C4, C6 and C7 bands were definitely weaker than those of the c genomic species. It is strinkingly interesting that ***B. cernua (aabb*** genome) and B. ***carinata (bbcc*** genome) have the X band in common and have none of the bands which were present in ***B. nigra (bbb*** genome). The X band will be taken as the characteristic one of ***b*** genome. However, ***B. juncea (aabb*** genome) and ***B. nigra***

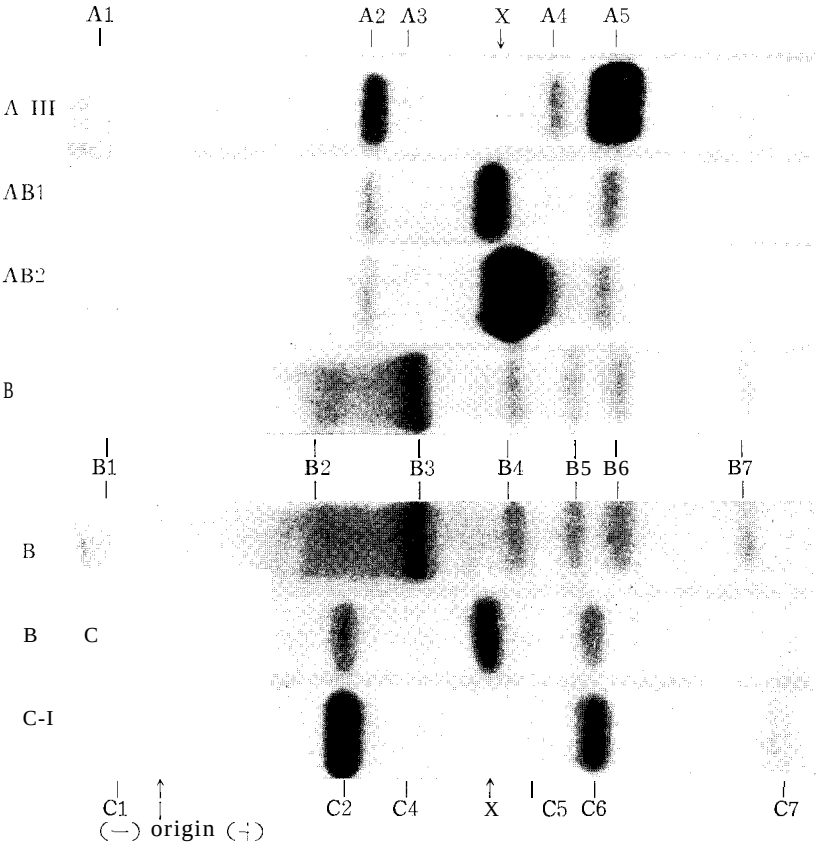

Fig. 5. Photograph of esterase zymograms derived from cotyledonary seedlings of *Brassica,* showing comparisons between different kinds of basic monogenomic species and the amphidiploid species. (A-III) ***B.*** *rapa* (*aa* genome). (AB1) ***B. cernua*** (*aabb* genome). (AB2) ***B. juncea*** (*aabb* genome). (B) ***B.*** nigra (*bb* genome). (BC) ***B. carinata (bbcc*** genome). (C-I) B. ***oleracea*** var. ***botrytis (cc*** genome).

do not contain the X band. While, ***B. juncea*** and ***B. cernua*** have the A2 and A5 bands in common. It could not be considered that the same gene mutation responsible for the formation of the esterase had occurred in both of ***B. carinata (bbcc*** gemone) and ***B. cernua (aabb*** genome) after spontaneous syntheses of these amphidiloid species and resulted in the production of the X band in both of those different amphidiploid species. It might be well conceivable that both of ***B. cernua*** and ***B. carinata*** had been spontaneously synthesyzed with the similar ***b*** genomic species having the X band, while ***B. juncea*** and ***B. cernua*** had been naturally synthesyzed independently with the different ***b*** genomic species and a similar ***a*** genomic species. From these considerations, it may be conceivable that the ***b*** genome of the amphidiploid species ***B. cernua*** and ***B. carinata,*** will be more or less different from the ***b*** genome of the present ***b*** genomic species, ***B. nigra.*** Fukushima ***et al. (1968)*** performed serological analyses of leaf proteins in ***Brassica*** species and reported that the ***b*** genome of ***B, carinata (bbcc*** genome) appears to be different from that of ***B. nigra (bb*** genome) in their precipitin patterns. The results from the present experiments agree well with the serological evidence, and clearly point out that there exists certain genome-specific differences in the esterase patterns among the monogenomic species, having ***aa, bb,*** or cc genomes. It is also apparent that the esterase patterns of amphidiploid species are generally composed of the bands which are present in the basic monogenomic species except the X band. For example, ***B. napus (aacc*** genome) is quitely intermediate in its esterase pattern between the ***a*** genomic species and the c genomic species The similar situation in the amphidiploid species was also observed in ***B. juncea (aabb*** genome), of which esterase pattern is composed of the bands of the ***a*** genomic and the ***b*** genomic species On the other hand, ***B. cernua (aabb*** genome) has some of the bands of the ***a*** genomic spcies, but has none of the bands of ***b*** genomic species, ***B. nigra.*** While ***B. carinata*** has some of the bands of the c genomic species, but has none of ***b*** genomic species, ***B. nigra.*** As mentioned above, the X band is present in ***B. carinata*** and ***B. cernua.*** In view of these facts, ***B. cernua*** seems to be situated much closer to the ***a*** genomic species than to the ***b*** genomic species ***B. nigra.*** But ***B. carinata*** seems to be closer to the c genomic species than to the ***b*** genomic species. Furthermore, there exists a closer relationship between ***B. cernua*** and ***B. carinata*** than between either of these amphidiploid species and the ***b*** genomic species ***B. nigra.*** The esterase bands detected in the present experiments were listed in Table 2.

In most cases, the intensities of the bands present in the amphidiploid species are different from those of the corresponding bands present in the basic species having monogenome in spite of their same electrophoretic mobilities. For example, the B4 band of ***B. juncea*** was

Table 2-a. Esterase composition in cotyledonary seedlings of ***Brassica*** species.

Species	Genome constitution	Esterase bands detected
B. pekinensis	***aa***	Al, **A2**, A3, A4, A5
B. rapa	***aa***	Al, A2, A3, A4, A5
B. chinensis	***aa***	Al, A2, A3, **A**4, A5
B. nigra	***bb***	B1, B2, B3, B4, ***B5, B6, B7***
B. oleracea var. ***botrytis***	***cc***	***Cl, C2, C4,*** C5, ***C6,*** *C*7
〃 var. ***capitata***	***cc***	***Cl, C2, C4, C6***
〃 var. ***acephala***	cc	***Cl,*** **C2**, ***C4, C6***
B. juncea	***aabb***	Al, ***A2, B5, A5***
B. cernua	***aabb***	Al, ***A2,*** **X**, A4, ***A5***
B. carinata	***bbcc***	***Cl, C2, X, C6. C7***
B. napus	***aacc***	Al, ***C2, A2, C4*** (=A3?), ***C6, A5***

N. B. Each of the extracts was treated with β-mercaptoethanol (1.0%).
Italic types show the bands with stronger intensity.
Bold-faces show the bands with the strongest intensity.

Table 2-b. Esterase composition in leaves obtained from the seedlings of various ***Brassica*** species having 10 pairs of chromosomes in somatic.

Species	Genome	Esterase bands detected
B. pekinensis	***aa***	Al, A2, A3, A4, A5
B. rapa	***aa***	Al, A2, A3, A4, A5
B. campestris	***aa***	Al, A2, A3, A4, A5
B. chinensis	***aa***	Al, A2, A3, A4, A5
B. japonica	***aa***	Al, A2, A3, A4, A5
B. trilocularis	***a′a′***	Al, A2, A3, A4, A5
B. tournefortii	***T T***	T1, **T2**

N. B. Italic types show the bands with stronger intensity.
Bold-faces show the bands with the strongest intensity.

stronger than that of ***B. nigra***, while the A2 and A5 bands of ***B. juncea*** were weaker than those of ***B. rapa***. Moreover, the C2 and C6 bands of ***B. carinata*** were weaker than those of ***B. oleracea*** as shown in Fig. 5. Wright (1963) reported a quantitative correlation between the intensity of an esterase band and the gene dosage responsible for its production in ***Drosophila melanogaster***; each of the gene dose could produce approximately equal amount of enzyme activity, while a band produced by one dose of the gene, is about half as intense as that produced by two

doses of the gene. In view of this evidence, it might be estimated that the dose of the gene responsible for the production of the esterase in an amphidiploid population would not be equal to that in the population of basic monogenomic species, composing such amphidiploid species ; for example, ***B. juncea*** population would possess a larger amount of genes responsible for the production of the esterase of B4 band than ***B. nigra*** population. These changes of the gene dosage might have occurred through evolutionary processes in nature and breeding in artificial force.

Such differences in the intensity of esterase bands were also observed among different species having *aa* genome. Fig. 6 illustrates comparative zymograms of leaf esterases present in different species having 10 pairs of chromosomes in somatic. The Al, A2, A3, A4 and A5 bands were generally found in the leaves of those species. These esterase patterns were similar to those in the seedlings of the a genomic species

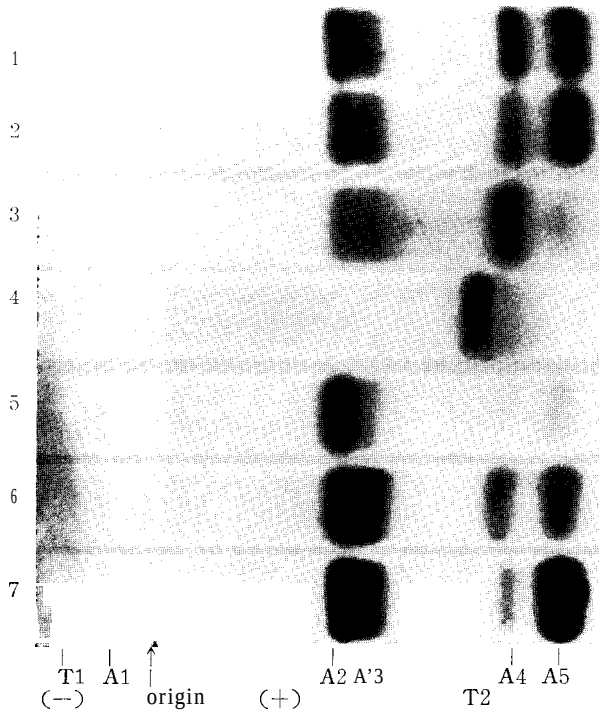

Fig. 6. Photograph of esterase zymograms derived from foliage leaves, showing the comparison among different ***Brassica*** species having 10 pairs of chromosomes in somatic. (Each extract used was not treated with β-mercaptoethanol.) (1) ***B. pekinensis (au*** genome). (2) ***B.*** *rapa*(*aa* genome). (3) ***B.*** *campestris* ***(au*** genome). (4) ***B. tourneforfii*** (*TT* genome). (5) ***B.*** *trilocularis* (*a'a'* genome). (6) ***B. juponicu (au*** genome). (7) ***B. chinensis (au*** genome).

shown in the above Figs. There were scarcely any appreciable differences in electrophoretic mobilities among different a genomic species, notwithstanding distinct differences among those species were found in intensities of the corresponding bands; the A4 band of *B. chinensis* was somewhat weaker, and the A5 band of ***B. campestris*** was much weaker than those of other ***a*** genomic species. Moreover, significant differences in the intensity were observed in the weaker bands, such as A3, A4 and A5 bands of ***B. trilocularis;*** especially the A4 band of ***B. trilocularis*** were so weak that it was scarcely detectable. These facts suggest that there would be significant differences in dose of genes responsible for the esterase production: A population of ***B. campestrissccms*** to possess small doses of the gene responsible for the production of A5 band esterase, furthermore, that of ***B. trilocularis seems*** to have smaller doses of genes reponsible for the production of A3, A4 and A5 band esterases. In view of these facts, it would be conceivable that ***B.*** *trilocularis* seems to be more different from the other species having *aa* genomes.

On the other hand, ***B. tournefortii*** showed a specific array of at least two bands, designated as T1, T2, and anindistinct band which is faster towards anode in its migration than the T2 band, not being discriminated according to its faint appearance. The T1 band showed remarkably weaker intensity and migrated slowly towards cathode and was somewhat faster in its migration than the Al band of the other species. The T2 band, which migrated towards anode, was slower in its migration than the A4 band of the other species. In view of this fact that the esterase pattern of ***B. tournefortii*** was different in electrophoretic mobilities from those of the other species having 10 pairs of chromosomes in somatic, it could be estimated that ***B. tournefortii*** would be different from the other species having *aa* genome on a quite level with an inter-genomic difference.

Fukushima and Iwasa (1966) succeeded in discriminating the genomes of ***B.*** *trilocularis* and ***B.*** *tournefortii* **;** the genome of ***B. trilocularis*** has been designated as ***a',*** according to the evidence that the meiotic chromosome associations are shown as (9-10) II + (2-0) I in the F, hybrid raised by the cross between ***B. trilocularis*** and ***B. campestris.*** That is, the genome of ***B. trilocularis*** is more or less different from the original ***a*** genome to some extent. While the genome of ***B. tournefortii*** has been designated as ***T,*** according to the evidence that ***B. tournefortii*** is highly cross incompatible with several ***Brassica*** species having ***a, b*** or c genomes, and the meiotic chromosome associations are shown as (O-5) II + (20-10) I in the F, hybrid raised by cross between ***B, tournefortii*** and ***B. campestris,*** that is, ***B. tournefortii*** is composed of a certain genome differing from the a genome and others.

The results obtained through the present comparative analyses of leaf esterases of ***Brassica*** species having 10 pairs of chromosomes agree well with those cytogenetical evidences. That is, it could be estimated that ***B.*** *tournefortii* has completely different from the ***a*** genomic species in spite of their same numbers of chromosomes in somatic.

The comparative analyses of esterase compositions by means of zymogram were remarkably useful for the phylogenetic investigation of ***Brassica*** species, and could offer the following working hypotheses applicable for the phylogenetic studies of cultivated plants.

1) Inter-genomic difference : An array of esterase bands present in one species is different in electrophoretic mobilities of esterases.

2) Intra-genomic difference : Generally the esterase bands are different in intensities, and in some cases, a certain band is absent, though the other bands show the same electrophoretic mobilities.

3) Relationship in esterase composition between an amphidiploid species and their basic monogenomic species : The array of bands present in an amphidiploid form was mostly composed of some of the bands present in their basic monogenomic species. In some cases, the amphidiploid species does not contain the bands present in one of the basic species, but contains some of the bands present in the other one of the basic species and have the band which is not contained in either of the basic species.

The differences in esterase composition among various species of cultivated plants would show nothing but resuls of the change of several genes responsible for production of those esterases. However, the artificial selection for the esterase composition has not carried out ; the differences in esterase composition among the species would be brought by the indirect result of differentiations subsequent to natural selection over the long period of evolutionary history and artificial selection for the other characters. Consequently, the variation in esterase composition could reflect the fundamental difference in gene pool of the population,

Summary

Relationships between genome constitution and esterase composition in ***Brassica*** species, were analyzed by means of the zymogram, and the following results were obtained:

1) The differences among the ***a*** genomic species (*Brassica rapa* and others), the *b* genomic species ***(B. nigra),*** and the c genomic species ***(B.*** *oleracea*), could be observed in electrophoretic mobilities of their esterases.

2) Intra-genomic differences could be observed in differential intensities of some bands, and in some cases, observed in existence of some specific bands.

3) The array of the csterase bands in amphidiploid species of ***Brassica*** is composed of some of the bands present in the basic species having monogenome. Some of the amphidiploid species contain the bands present in one of their basic species, but have none of the bands present in the other one of their basic species, and further, they have a band which is not present in either one of the basic species ; ***B. juncea (aabb*** genome) has some of the bands present in the ***a*** genomic species and the ***b*** genomic species, while ***B. cernua (aabb*** genome) has some bands present in the ***a*** genomic species, but has none of the bands present in the ***b*** genomicspecies, and further, has one band designated as X, which is not contained in both of the basic species. Similar situation could be observed in ***B. carinata (bbcc*** genome), which has some of the bands present in the c genomic species and has the X band, but has none of the bands present in the ***b*** genomic species. These findings suggest that the ***b*** genome composing ***B. cernua (aabb*** genome) and ***B. carinata (bbcc*** genome) will be somewhat different from that of ***B. juncea (aabb*** genome) and ***B. nigra (bb*** genome).

4) In comparing the ***Brassica*** species having 10 pairs of chromosomes in somatic, it became apparent that ***B. tribocularis*** is distinctly different from the other species having ***aa*** genome in the intensities of some of the esterase bands, and ***B. tournefortii*** is much more different from the other species in electrophoretic mobilities of all the esterase bands. These findings agree well with the cytogenetic evidences proved by Fukushima and Iwasa (1966) who have assigned ***T*** genome to ***B. tournefortii*** and a' genome to ***B. tribocularis.***

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