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Application of Esterase and Peroxidase Zymograms to Breeding in *Brassica* with Reference to Nucleus Substitution

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Zymograms of leaf esterases and peroxidases were employed for the use of genetic examination in individual selection to promote nucleus substitution for breeding of new varieties, such as oleracea-cytoplasmic Brassica *campestris*. In successive strains derived from hybridization of *B. oleracea* x *B. campestris* and backcrosses with pollen of ssp. *pekinensis*, *two* of the esterases and one of the peroxidases were found to be specific to c genome. The pattern of the enzyme distribution was characterized by these enzymes, and there were two types of the plants in successive backcrossed strains; one had these enzymes specific to c genome, the other had none of them. Frequencies of the plants having the c genome-specific enzymes became lower according *to* a decrease in the number of chromosomes of c genome, in process of backcrosses with pollen of ssp. *pekinensis*. From this result, it was estimated that the chromosomes of c genome can be minimized by selecting the plant having none of the enzymes specific to c genome. and this method is useful for breeding of nucleus-substituted strains in *Brassica*.

INTRODUCTION

Nucleus substitution is known to be effective for breeding of new varieties, such as oleracea-cytoplasmic *Brassica campestris* which is used as a cultivar of Chinese cabbage with disease resistances (Nishi *et al.*, 1970). In procedure of the nucleus substitution, an adequate method of genetic examination for individual selection in successive backcrossed strains is needed. The genetic examinations have been performed by analyses of enzymes, since it was made clear that a gene is responsible for formation of an enzyme structure, and the zymogram (Hunter and Markert, 1957) has been used as a powerful tool in genetics (Ogita, 1962; Wright, 1963) and phylogenesis (Eguchi and Matsui, 1969a, b). Present paper deals with comparative analyses of leaf esterases and peroxidases in the zymogram, aiming at developing a new method of individual selection to promote the nucleus substitution in *Brassica*.

MATERIALS AND METHODS

Species and strains used in this experiment are listed in Table 1. *Brassica*

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napus used was derived from artificial synthesis by hybrid of *B. oleracea* var. *capitatax B. campestris* ssp. *pekinensis* and maintained in pedigree culture. Respective strains were obtained by backcrosses with pollen of ssp. *pekinensis* as described in Table 1, and their backcross generations were denoted as B_1, B_2 and B,. The chromosomes in the strains were observed in pollen mother cells. The strain in B_1 comprised c genome (9 of the chromosomes derived from *B. oleracea).* In B_2, the number of chromosomes of c genome was less than 9. The plants in B_3 were divided into two strains according to the number of chromosomes of c genome. Thus, four strains having the different number of chromosomes of c genome were obtained by means of cytological observation, and their compositions of genome and chromosomes were denoted as aac, $aa+\alpha$, $aa+\beta$ and $aa+\gamma$ in the respective strains, where $\alpha \leqq 9$, $\beta \leqq 5$ and $\gamma \leqq 1$ of the chromosomes of c genome.

Table 1. Explanation of material plants.

Species and strains in backcross generations	Crossing system	Genome	Number of plants used
B. canapestris ssp. *pekinensis*	—	aa	10
B. oleracea var. capitata		cc	10
B_1	*B. napus** × *B. campestris*	aac	10
B_2	B_1 × *B. campestris*	$aa+\alpha$**	11
B_3	B_2 × *B. canapestris*	$aa+\beta$**	4
		$aa+\gamma$ **	6

N.B. * Artificially synthesized by hybrid of *B. oleracea* var. *capitatax* ***B.*** *campestris* ssp. *pekinensis.*

** Chromosomes of c genome: $\alpha \leqq 9$, $\beta \leqq 5$ and $\gamma \leqq 1$.

Five grams of the 7th to 13th leaves of each plant was homogenized with 2.5 ml of M/30 potassium phosphate buffer of pH 7.0, and the homogenate was centrifuged at 150,000 × g for 2 hr to remove cell debris and particles. The supernatant was used as an enzyme solution for zymograms of esterases and peroxidases in agar-gel zone electrophoresis. The gel medium was prepared with 0.7 gm of agar and 4.0 gm of polyvinyl-pyrrolidone in 100 ml of M/30 potassium phosphate buffer of pH 7.0. The gel plate was made 1 mm thick and supported by a glass plate. A strip of filter paper (2x 12.5 mm) was saturated with the enzyme solution and was placed on the gel plate; the enzyme solution diffused from the filter paper into the agar gel at 2°C, and the filter paper was removed after 40min. A gel plate was exposed to stabilized voltage of 17 V/cm for 140min for esterase separation, and another plate was exposed to 18 V/cm for 240 min for peroxidase separation. For histochemical detection of esterases, 1 % solution of β-naphthyl acetate was sprayed on the surface of the agar gel as a substrate. After incubating the gel plate at 38°C for 40 min, naphthanyl diazo blue B was sprayed on the plate as a dye coupler. For histochemical detection of peroxidases, the gel plate was soaked at 25°C for 30 min in a solution containing 1 % hydrogen peroxide and 0.05 % o-dianisidine, which were used as substrate and as dye coupler, respectively. In respective zymograms, esterases and peroxidases were separated enough to examine ge-

netic variation.

RESULTS AND DISCUSSION

Esterase pattern

Figure 1 shows a zymogram of esterases in each of the plants of respective species and strains. In *B. oleracea*, **8** bands of esterases were found, which were designated as *EI, EII, EIII, EIV, Ec1*, EL?, *Ec3* and *Ec4*, corresponding to an array of the bands from cathode to anode. In *B. campestris*, *8* bands designated as *EI, EII, EIII, EIV, Ea1, Ea2, Ea3* and *Ea4* were found. The esterase bands of *EI, EII, EIII* and *EIV* were very slight and were found to be non-specific to species. The other bands of *Ecs* in *B. oleracea* were different in migration rate from the bands of *Eas* in *B. campestris*, and the bands of *Ecs* and *Eas* were clearly specific to respective species. In the plants in B_1 (*aac* genomes), esterases of *EI, EII, EIII, EIV, Ec1, Ea1, Ea2, Ea3* and *Ec4 were* found as listed in Table 2. Thus, in the digenomic plants having *aac* genomes, the esterase pattern consisted of the bands found in respective ancestor species of *B. oleracea* and *B. campestris*. In backcrossed strains in B_2 and B_3, there were two types of the plants; one had *Ec1* and/or *Ec4*, the other had none of these bands specific to c genome. Thus, genetic variation in the esterase

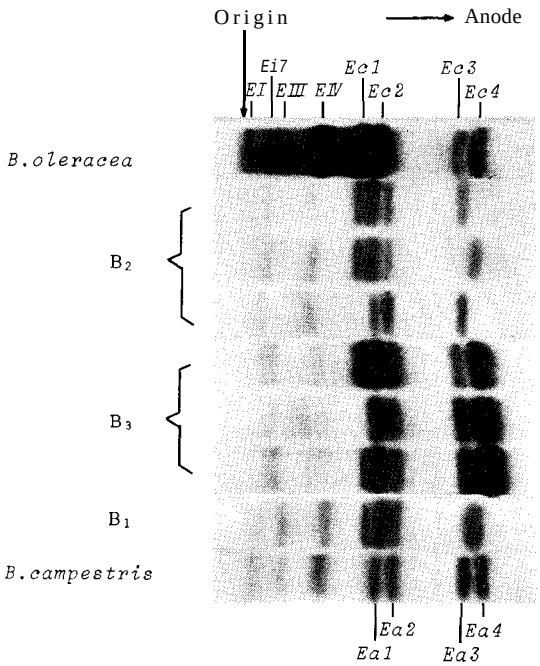

Fig. 1. Photograph of a zymogram of leaf esterases in respective monogenomic species of *aa* and cc, and successive strains derived from interspecific hybridization and backcrosses with pollen of ssp. *pekinensis*.

Table 2. Enzyme compositions in mono- and digenomic species.

Plants	Genome	Enzyme	Designated bands in zymogram
B. campestris ssp. *pekinensis*	*aa*	Esterase Peroxidase	*EZ EZZ EZZZ EZV Ea1 Ea2 Ea3 Ea4* *Pa1 Pa2 Pa3 PI PII PZZZ Pa4*
B_1	*aac*	Esterase Peroxidase	*EZ EZZ EZZZ EZV Ec1 Ea1 Ea2 Ea3 Ec4* *Pa1 Pc2 Pa2 Pc3 PI PZZZ*
B. oleracea var. capitata	*cc*	Esterase Peroxidase	*EI EZZ EZZZ EZV Ec1 Ec2 Ec3 Ec4* *Pc1 Pc2 PC3 PI PII PIII Pc4*

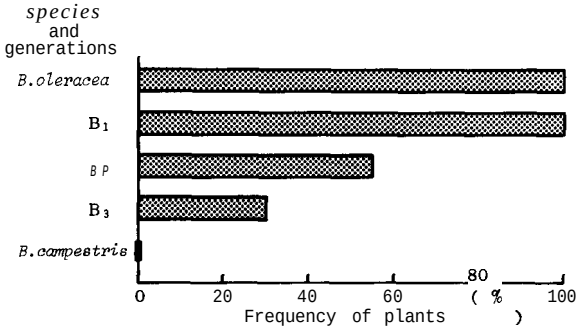

Fig. 2. Frequencies of plants having *Ec1* and/or *Ec4* on backcross generations.

pattern was characterized by *Ec1* and *Ec4*, and these bands **were** used as an index for the examination of genetic variation. Figure 2 shows frequencies of the plants having *Ec1* and/or *Ec4* on generations. These esterase bands

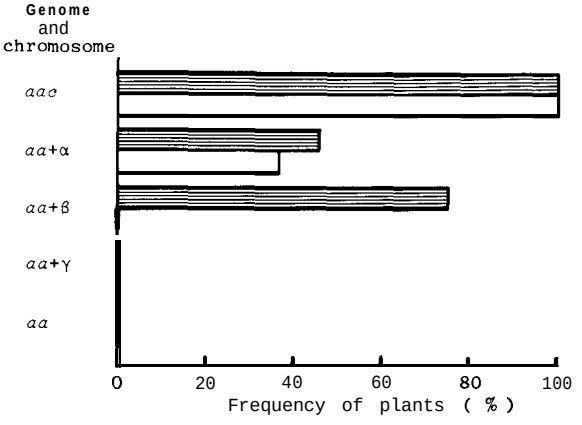

Fig. 3. Frequencies of plants having *Ec1* (▤) and *Ec4* (□) on the number of chromosomes of *c* genome, where α, β and γ are the number of chromosomes of c genome: $\alpha \leq 9, \beta \leq 5$ and $\gamma \leq 1$.

were found in all plants in B_1. These bands were found in 55 % of the plants in B_2 and 30 % in B_3. Thus, the frequency of the plants having these esterases decreased in backcrosses with pollen of ssp. *pekinensis*.

Figure 3 shows frequencies of the plants having *Ec1* and those having *Ec4* in respective strains on the number of chromosomes derived from c genome. As mentioned above, in digenomic strain (*aac*) having 9 chromosomes of c genome, *Ec1* and *Ec4* were found in all plants. In the strain ($aa+\alpha$) in which the number of chromosomes of c genome was less than 9, *Ec1* was found in 46 %, and *Ec4* was found in 36 % of the plants. In the strain ($aa+\beta$) having 5 chromosomes of c genome at its maximum, the percentage of the plants having *Ec1* was 75 %, and that of the plants having *Ec4* became 0 %. In the strain ($aa+\gamma$) having one or none of the chromosomes of c genome, these esterases were not found. Thus, the frequency of the plants having *Ec1* and/or *Ec4* became lower according to the decrease in the number of chromosomes of c genome derived from ***B.*** *oleracea*.

Peroxidase pattern

Figure 4 shows a zymogram of peroxidases in each of the plants. Peroxidase pattern in ***B.*** *oleracea* was composed of 7 bands which were designated as ***Pc1***, *Pc2*, *Pc3*, ***PI***, *PII*, *PIII* and *Pc4*, corresponding to an array of the bands

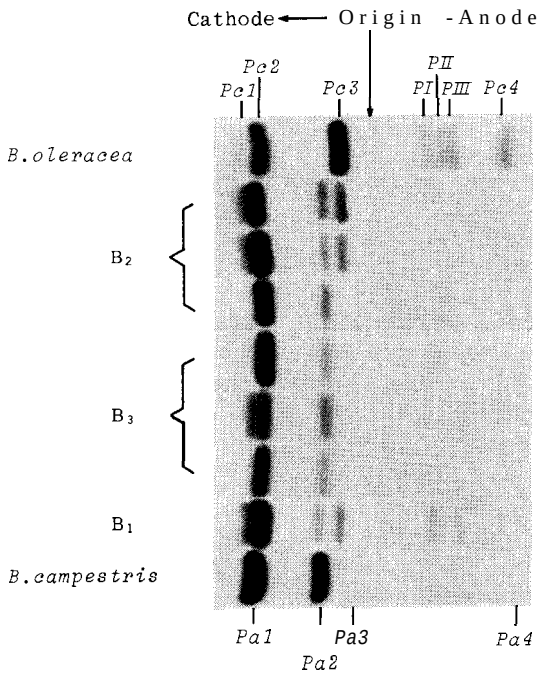

Fig. 4. Photograph of a zymogram of leaf peroxidases in respective monogenomic species of *aa* and cc, and successive strains derived from inter-specific hybridization and backcrosses with pollen of ssp. *pekinensis*.

from cathode to anode. In *B. campestris*, 7 bands designated as *Pal, Pa2, Pa3, PI, PII, PIII* and *Pa4* were found. The intensities of *PI, PII* and *PIII* were very slight and not species-specific. There were distinct differences in migration rate between *Pc* bands and *Pa* bands. The plants in B, comprised the peroxidases of ***Pal,*** *Pc2, Pa2, Pc3,* ***PI*** and *PIII* bands, as listed in Table 2. Thus, in the digenomic plants (*aac*) in B_1, peroxidase pattern consisted of the bands found in respective ancestor species of *B. oleracea* and *B. campestris*. However, the migration rates of *Pcl, Pal* and *Pc2* were close to each other, and it was difficult to use those bands as an index for the examination of genetic variation. In B_2 and B_3, some of the plants had *Pc3*, and others had not this enzyme. The genetic variation in peroxidase pattern was characterized clearly by *Pc3*. So, *Pc3* was used as an index for the examination of genetic variation. Figure 5 shows frequencies of the plants having *Pc3* on generations. The *Pc3* was found in all plants in B_1 as mentioned above and found *in* 55 % of the plants in B_2. However, *Pc3* was not found in B_3. Thus, the frequency of the plants having *Pc3* decreased in the process of backcrosses with pollen of ssp. *pekinensis*.

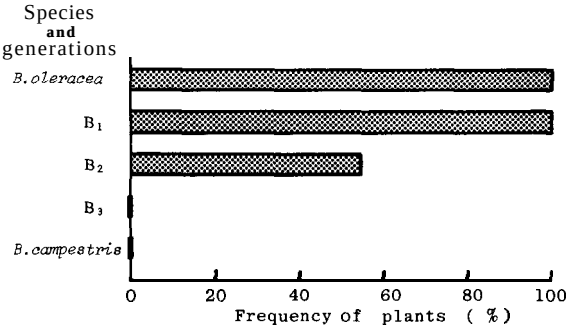

Fig. 5. Frequencies of plants having *Pc3* on backcross generations.

Figure 6 shows frequencies of the plants having *Pc3* in respective strains on the number of chromosomes derived from c genome. In digenomic strain (*aac*), *Pc3* was found in all plants as mentioned above. On the other hand, *Pc3* was found in 55 % of the plants of strain ($aa+\alpha$) having less than 9 chromosomes of c genome. In the strain ($aa+\beta$) having less than 5 chromosomes of c genome and the strain ($aa+\gamma$) having one or none of the chromosomes of c genome, *Pc3* was not found. Thus, the frequency of the plants having *Pc3* became lower according to the decrease in the number of chromosomes of *c* genome in the process of backcrosses with pollen of ssp. *pekinensis*, as well as the genetic variation examinkd in esterases.

Conclusion

In the successive strains derived from hybridization of *B. oleracea* var. *capitata* x *B. campestris* ssp. *pekinensis* and backcrosses with pollen of ssp. *pekinensis*, the number of chromosomes of c genome derived from *B. oleracea* varied

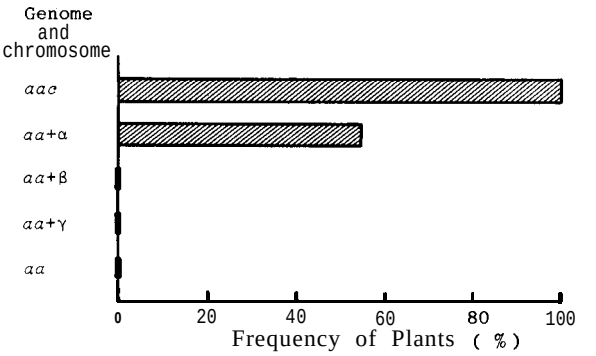

Fig. 6. Frequencies of plants having *Pc3* on the number of chromosomes of c genome, where α, β and γ are the number of chromosomes of c genome: $\alpha \leqq 9, \beta \leqq 5$, and $\gamma \leqq 1$.

with the respective generations (Iwasa, 1963). For promoting nucleus substitution, these chromosomes of c genome should be removed by individual selection in successive strains. Variations in enzyme pattern in the backcrossed strains related to the number of chromosomes of c genome, and the frequency of the plants having the enzymes specific to c genome became lower in proportion to the decrease in the number of these chromosomes. From this result, it was estimated that the chromosomes of c genome can be minimized by selecting the plant having none of the enzymes specific to c genome, and this method is useful for breeding of nucleus-substituted strains in *Brassica*.

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