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Determination of Seed Purity in Radish (*Raphanus sativus* L.) Using Allozyme

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Radish (*Raphanus sativus* L.) is one of very important crop plants in the world. It is very important to determine hybrid seed quality in the production of hybrid *Brassica* vegetable seeds to avoid unacceptable contamination with self-inbred (sib) seeds. The allozyme for evaluating seed purity in a commercial F_1-hybrid radish cultivar is demonstrated. Three hundred sixty seeds from the male and female harvest were subsequently screened for seed purity using 27 isozyme loci. Especially, F1 hybrids of radish, *Per-1* (aa x bb), *Lap-1* (aa x bb), *Est-1* (aa x bb) were presented clear hybrid bands. *Est-1* locus revealed that 15 (8.3%) seeds from the female harvest and 26 (14.4%) seeds from the male harvest were sibs. It maintains higher than average level of genetic diversity compared with their correspondent parents. Shannon's index of phenotypic diversity (*I*) of hybrids was the highest of all accessions (*R. sativus* L. cv. Daepeng, *R. sativus* L. cv. Backza, and their hybrids). The allozyme may lead to a better insight into the hybrid seed purity.

Key words : Allozyme, radish, *Raphanus sativus*, seed purity

Introduction

To determine hybrid seed quality, companies need quality controls. These controls are convinced to verify that the designed cross has been occurred, the number of self or sib-pollination between plants of the female parents meets the necessary purity required by law, and the product has an adequate quality (vigor and viability) [2]. The grow-out trial has been used as a common method for seed purity analysis by commercial seed enterprises [2]. The trial includes the growing of a representative number of hybrid seeds for phenotypic identification based on morphological markers that can be easily affected by environmental conditions. This type of trial is time consuming, space demanding, usually performed off-season, and often not allows the unequivocal identification of genotypes [5].

Isozymes, being nearly direct gene-products are excellent, reliable, easily obtained single markers [19]. In addition, their expression is dominant, which makes it unnecessary to carry out genetic crosses beyond the F_1 generation; as such, they have been, and still are, extremely useful in taxonomic and genetic studies [19].

Other methods, such as random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP), have also been used for the deletion of contamination in hybrid seed lots [1,14,17]. Use of isozyme variation represents only a small proportion of the total genetic variation [3]. Therefore, allozyme analysis can be limited by the number of detectable loci, or lack variable loci, when compared to other molecular markers [13,22,23].

The cultivated radish (*Raphanus sativus* L.) is a fast-growing annual species that grows in habitats distributed in six continents [18]. The somatic chromosome number of this species is 2n=18 [12]. *Raphanus sativus* is self-incompatible and therefore requires insect pollination for successful reproduction [18].

The cultivated radish is known as processing very high genetic diversity [7,10]. However, the majority of genetic variation of radish resided within populations. It is general knowledge that commercial accessions, particularly hybrid accessions of vegetable crops, are based on increased narrow variation at the genetic level [2].

In consequence, it is often difficult in discriminating between elite lines and their hybrids. On this point, the present study was conducted to find whether allozyme analysis could be use for hybrid seed purity testing of a commercial radish cultivar or not.

Materials and Methods

Plant materials and hybridization

Two accessions (*Raphanus sativus* L. cv. Daepeng and *R. sativus* L. cv. Backza) and their hybrid were provided for

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this study (Germplsam Institute, Kyoto University, Japan). Randomly selected 200 seeds from each variety were sown into an outdoor plot. When the seedlings were three weeks old, 128 plants from each variety or accession were randomly sampled and their leaves were used for molecular analysis.

Allozyme analysis and phenetical labelling were performed for all plants. Plants were selected based on the phenetical labeling and found three types to be useful in determining the seed purity of hybrids. Before the buds are swollen to bursting, their stamen were removed from buds and covered with a paper bag to avoid contamination. Plants were crossed and the female and male parents were labelled. F_1 hybrids of radish, *Per-1* (aa x bb), *Lap-1* (aa x bb), *Est-1* (aa x bb), and their correspondent parents were analyzed in this study. Each parent and hybrids were used to validate the homogeneity of the inbred lines.

Isozyme analysis

The leaf samples were collected from plant materials described above for DNA analysis. The procedures for the homogenization of tissues, starch gel electrophoresis, and enzyme assays followed the methods of Soltis et al. (1983)[21]. Young leaves were homogenized in Tris-HCl grinding buffer with PVP (pH 8.0) as described in Soltis et al. [21]. Electrophoresis was performed using 11.0% starch gels, and 12 enzymes were assayed. Acid phosphatase (ACP), glutamate oxaloacetate transaminase (GOT), and leucine aminopeptidase (LAP) were resolved on system 9 of Soltis et al. (1983). Esterase (EST) and peroxidase (PER) were resolved on system of morpholine-citrate buffer (pH 6.1). Isocitrate dehydrogenase (IDH), malic enzyme (ME), malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (PGD), phosphoglucose isomerase (PGI), phosphoglucomutase (PGM), and shikimate dehydrogenase (SKD) were resolved on system 10 of Soltis et al. [21].

Data analysis

For the enzymes resolved in more than one zone of activity, the most anodally migrating isozyme was designated as '1', and other subsequent isozymes were sequentially numbered. The alleles of isozyme 1, 2, 3, and so on were designated sequentially as 'a', 'b', 'c', and so on, respectively.

A locus was considered polymorphic if two or more alleles were detected, and the frequency of the most common allele was less than 0.99. Several standard genetic parameters were estimated using a computer program developed by M.D. Loveless and A.F. Schnabel (2000) [6]. The percentage of polymorphic loci and gene diversity (H_E) were estimated from the data [9].

The degree of polymorphism was quantified using Shannon's index of phenotypic diversity [4]:

$$Ho = -\sum p_i \log p_i$$

where p_i is the frequency of a particular phenotype i [15].

H_O can be calculated and compared for different accessions. Let

$$H_{ACC} = 1/n\sum H_O$$

be the average diversity over the n different accessions and let

$$H_{SP} = -\sum p \log p$$

be the diversity of species calculated from the phenotypic frequencies p in all the accessions considered together. Then the proportion of diversity presented within accessions, H_{ACC}/H_{SP}, can compared with that of between accessions $(H_{SP} - H_{ACC})/H_{SP}$ $(=G_{ST})$.

An analysis of non-shared bands (NSB) was performed with all pairs of inbred lines [20].

Results

A total of 180 seeds from each parental variety were also tested to ensure homozygosity and reproducibility of polymorphic markers. A high level of genetic variation was found in the radish accessions. Fifteen of the 27 loci examined (59.3%) showed polymorphism in at least one variety, while the remaining twelve loci (*Acp-3*, *Acp-4*, *Est-3*, *Est-4*, *Est-5*, *Idh-1*, *Lap-2*, *Mdh-2*, *Mdh-3*, *Per-2*, *Per-3*, and *Pgm-1*) were monomorphic in all accessions.

I found many phenetic bands in determining the seed purity of two accessions (*R. sativus* L. cv. Daepeng and *R. sativus* L. cv. Backza) and their hybrids. Especially, F1 hybrids of radish, *Per-1* (aa x bb), *Lap-1* (aa x bb), *Est-1* (aa x bb) presented clear hybrid bands (Figs. 1, 2, and 3).

The hybrids maintained higher than average level of genetic diversity compared with their correspondent parents. Shannon's index of phenotypic diversity (I) of hybrids was highest of all accessions (*R. sativus* L. cv. Daepeng, *R. sativus* L. cv. Backza, and their hybrids). Although the mean H_E value for the female parent was slightly greater than

Fig. 1. Seed purity analysis using *Est-1* locus in *R. sativus*. MP: Male parent. FP: Female parent. F1: F_1 bybrid.

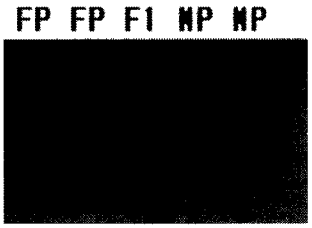

Fig. 2. Seed purity analysis using *Lap-1* locus in *R. sativus*. MP, FP, and F1 are the same as Fig. 1.

Fig. 3. Seed purity analysis using *Per-1* locus in *R. sativus*. MP, FP, and F1 are the same as Fig. 1.

that for the male parent, no statistical significance was found ($U<0.05$; one-tailed Mann-Whitney test). The *Est-1* locus showed two banded heterozygotes indicating that the allele is monomer. Three hundred sixty seeds from the male and female harvest were subsequently screened for seed purity using EST isozyme marker. Result from these experiments revealed that 15 (8.3%) seeds from the female harvest and 26 (14.4%) seeds from the male harvest were sibs. The isozyme survey generated values of genetic differentiation which were significantly different at the 5% level (Table 2).

In order to have a better understanding, an analysis of non-shared bands (NSB) was performed with data from those well resolved 15 loci for all pairs of inbred lines. This index gives a measure of the degree of dissimilarity between two genotypes (NSB=0:two genotypes are similar and NSB=1:two genotypes are different). As expected, the results of applying the NSB algorithm showed that the parents were very closely related (0.012).

Table 1. Partitioning of the genetic diversity into within and among accessions of radish

Accession	H_{pop}	H_{sp}	H_{pop}/H_{sp}	$(H_{sp}-H_{pop})/H_{sp}$
Female	0.197	0.269	0.732	0.263
Male	0.186	0.244	0.762	0.238
F_1	0.221	0.295	0.747	0.251

Table 2. Comparison of the number of sibs detected in a commercial radish cultivar using isozyme markers

Gender	No. of seeds	No. of sibs	% sibs
Female	180	15	8.3
Male	180	26	14.4

Discussion

Isozyme analyses were applied in order to determine hybrid seed purity. A large amount of molecular variation in radish was detected in two marker systems. The level of genetic variation of radish was high. For example, polymorphic value for isozyme was 59.3%. According to a review of plant allozyme literature by Hamrick and Godt [9], the average percentage of polymorphic loci for annual species was 50.7% at the species level (reviewed for N=190 species) and 30.2% at the population level (N=187). However, the majority of genetic diversity at the polymorphic loci in *R. sativus* was observed within populations or accessions [10]. In this study, isozyme variations were maintained not only among accessions (male, female, and hybrid lines), but also within the accessions (Table 1). Hence, the genetic similarity among the accessions was very high. Thus, it was indicated that it was very hard to detect a unique molecular genotype which was specific for accession and botanical variety. Therefore, a number of molecular markers were necessary to produce reliable estimates of seed purity determination.

I found allozyme markers useful in determining the seed purity of hybrids. The efficiency to find an allozyme marker useful for purity determination was about 7.5%. The value is similar to that found for tomato hybrids, another *Salanaces* species, in which 13 primers showed good polymorphic between parents out of 160 primers tested [16].

In the present study, nearly all individuals of the same accession were grouped together in all dendrograms. Allozyme markers have shown valuable tool for accessing population genetic variation [13], and complete congruence has been found between gene diversity estimates derived

from isozyme data sets [11].

This index of non-shared bands (NSB) gives a measure of the degree of dissimilarity between two genotypes. The results of the NSB algorithm showed that the parents were very closely related (NSB=0.012).

I have shown in this report that isozyme analyse can be used as a method for seed purity testing in a commercial radish accession. Isozyme analysis was founded to be useful for detecting accessions in the radish cultivar tested. However, isozyme patterns have been shown to be limited as indicated by the failures to detect accessions in more closely related parental lines of watermelon, tomato, and broccoli [8]. Markers can be classified as either co-dominant (e.g. isozyme, microsatellite and most RFLP), meaning that banding patterns of homozygotes can be distinguished from the patterns of heterozygotes, or dominant (e.g. RAPD, AFLP), where banding patterns of heterozygotes are identical to the patterns of one of the homozygotes and thus homozygotes and heterozygotes can not be distinguished. If allozyme analyses might supplement with DNA markers for several crops, they lead to a better insight into the hybrid seed purity with efficacy in very reduced time compared to the classical *in vivo* methods.

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초록 : 알로자임에 의한 무 씨의 순수성 검증

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무(*Raphanus sativus* L.)는 세계적으로 중요한 작물 중의 하나이다. 십자화과 식물 종에서 종자 생산에서 원하지 않은 내교잡에 의한 종자 결실로 오염이 발생하므로 씨의 순수성 검증은 매우 중요하다. 재배종 진주 대평 무(*R. sativus* cv. Daepeng)와 백자 무(*R. sativus* cv. Backza)의 교잡 분석을 실시하였다. 알로자임으로 상업적으로 이용되는 잡종 제1세대(F_1) 무에 있어서 씨의 순수성을 평가하였다. 웅성과 자성 양친 360개체에 27개 대립유전자좌위를 조사하였다. 특히 *Per-1* (aa x bb), *Lap-1* (aa x bb), *Est-1* (aa x bb)에서 명확한 잡종 밴드를 나타내었다. *Est-1* 대립유전자좌위에서 자성 배우체로부터 기원된 것이 15개체(8.3%)가 발견되었고, 웅성 배우체로부터 기원된 것이 26개체(14.4%)가 발견되었다. 또한 다양도 측면에서 양친 계통에 비해 잡종 계통에서 높은 유전적 다양도를 유지하고 있었다. 샤논의 정보지수(Shannon's index)를 이용한 표현형 다양도는 교잡 계통이 가장 높았다. 알로자임에 의한 무 계통의 교잡에 의한 종자 생성에서 씨에 대한 순수성 검증이 효과적으로 탐지되어 육종 연구에 기여할 수 있을 것으로 판단된다.