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대청에서 AFLP를 이용한 종자순도검사와 평가

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Seed Purity Test and Evaluation in *Isatis tinctoria* var. *yezoensis* (Ohwi) Ohwi Using AFLP Markers

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ABSTRACT : *Isatis tinctoria* var. *yezoensis* (Ohwi) Ohwi (Cruciferae) is one of major natural dyeing crops in the world and also have used as a medicinal plant in Korea. We evaluated seed purity in F_1-hybrid accessions using amplified fragment length polymorphism (AFLP) markers. One hundred sixty seeds from the male and female harvests were subsequently screened for seed purity with ten primers. The 13 accession-specific bands and many variable AFLP bands scored for accessions. Especially, E-AAC/M-CAA and E-AAG/M-CAT were presented clear hybrid bands for F_1 hybrids. F_1 hybrids maintained higher average level of genetic diversity compared with their correspondent parents. Self-inbred seeds from the female and male harvests were revealed 8.0% and 5.0%, respectively. The AFLP may lead to a better insight in to the hybrid seed purity test in *I. tinctoria* var. *yezoensis*.

Key Words **:** AFLP, F_1-hybrid, Genetic Diversity, Seed Purity

INTRODUCTION

Isatis tinctoria var. *yezoensis* (Ohwi) Ohwi (Cruciferae) is one of major natural dyeing crops in the world and listed a dye plant as symbols of stamps in Korea. Indigo played an important role in many countries' economics because natural blue dyes are rare (Choi *et al*., 2008). The plants also have used as a medicinal crop in Korea.

I. tinctoria var. *yezoensis* has been established itself as a persist member of coastal communities in several places in eastern North Korea and it have been cultivated as horticulture and a indigo blue dye in the South Korea. Spread of native habitats may generate interesting information for the prediction of invasion success, yet few such studies do not have been conducted in coastal species. Early introduced accession mostly occurs in the south part of Korea and flowers in May. Whereas, recently introduced accession flowers in June. In addition, morphological differences of two types or accessions were found in leaves as ecotype. Many people enjoy the yellow flower of this species like *Brassica campestris* L. ssp. *napus* var. *nippo-oleifera* Makino.

Production and distribution of high-quality seeds are fundamental to modern agricultural systems (Baxter and Copeland, 2008). The majority of annual crops is established each season from seeds, and seed quality can have a major impact on potential crop yield. Seeds carry the genetic traits incorporated by years of breeding and selection to create varieties that are adapted to specific production environments.

DNA molecular marker technologies were provided powerful tools for variety identification and seed quality control in various crops with the advantages of time-saving, less labor-consumption, and more efficiency (Paran *et al*., 1995). Allozyme exhibit Mendelian inheritance and is a co-

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dominant maker. Because of these prosperities allozymes are in many cases still a good choice in terms of the information they provide and cost-effectiveness. However this procedure may be limited by environmental conditions and tissue type, and may require selection of a suitable isozyme for purity test (Liu *et al*., 2007). Other methods, such as random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP), have also been used for the detection of contamination in hybrid seed lots (Livneh *et al*., 1990; Matsuura *et al*., 1994; Mongkolporn *et al*., 2004). Amplified fragment length polymorphism (AFLP) is one of the polymerase chain reaction (PCR) and the restriction enzyme digestion based genetic markers for rapid screening of genetic diversity. AFLP markers have been found to be effective in analyses of genetic variation below the species level, particularly in investigations of low level taxa and the differentiation of subpopulations (Paul *et al*., 1997; Kardolus *et al*., 1998). This study was to establish whether AFLP analysis could be use for hybrid seed purity testing of *I. tinctoria* var. *yezoensis*.

MATERIALS AND METHODS

1. Plant materials and hybridization

Two accessions and their hybrids were provided for this study. Early flowering plants (May) selected seeds from *I. tinctoria* var. *yezoensis* Ohwi cv. MAY (thereafter MAY) and late flowering plants (June), *I. tinctoria* var. *yezoensis* cv. JUNE (thereafter JUNE) were sown into an outdoor plot and 80 seedling plants from each origin (total 160) were randomly sampled. Because we can not test each and every seeds harvested from a field, testing a representative seeds that are drawn from a population (seed lot) is an acceptable procedure in seed testing.

Allozyme analysis was conducted to estimate seed pure lines according to the methods of Soltis *et al*. (1983). Isocitrate dehydrogenase (IDH) was showed a good result.

Accessions of MAY and JUNE were self-pollinated with same types for three years and finally pure lines (F_4) were gotten. Male parent (M, *I. tinctoria var. yezoensis* cv. JUNE, F_4) was used the pure line for IDH-AA and female parent (F, *I. tinctoria* var. *yezoensis* cv. MAY, F_4) was used the pure line for IDH-BB. We ascertained that F_1 is all type AB and F_2 was the truth of 1 : 2 : 1 using chi-square and significant tests. We reused the ascertained F_1 to get F_2 and to determine hybrid seed purity.

All plants which were shown the selected phenetics were performed AFLP analysis and labelled phenetics. Before the buds are swollen to bursting, their stamens were removed from buds and the buds were cover with a paper bag to avoid contamination. Plants were out-crossed between the labelled female and male parents. One maternal plant has many flowers and can be crossed with other plants of same accession. 100 full F_1 hybrids of MAY x JUNE and 160 their correspondent parents were performed AFLP analysis.

In addition, each parent and hybrids were used to validate the homogeneity of the inbred lines.

2. AFLP analysis

DNA was extracted using the plant DNA Zol Kit (Life Technologies Inc., Grand Island, New York, USA) according to the manufacturer's protocol. The DNA concentration of each sample was determined spectrometrically and was electrophoresed on a 1.5% agarose gel to confirm quality.

AFLP analysis was carried out with AFLP-TM Analysis System I kit (Life Technologies Inc.) according to the manufacturer's instruction with minor modifications. Genomic DNA (125 ng) was double digested with 1.25 units of each *Eco*RI and *Mse*I restriction enzymes at 37℃ for three hours. Specific adapters were ligated to the restriction fragments with one unit of T_4 DNA ligase in 10 mM Tris-HCl (pH 7.5), 10 mM DTT, 50 mM KCl and 50 mM K-acetate, for two hours at 20. Pre-amplification was done using pre-amp primer mix with diluted template DNA.

Selective amplification was performed in a 20 $\mu\ell$ final volume containing 5 $\mu\ell$ of preamplification products with primers sets having ten combinations of *Eco*RI/*Mse*I primers, E-AAC/M-CAA, E-AAC/M-CAG, E-AAC/M-CAT, E-AAC/M-CTA, E-AAC/M-CTC, E-AAG/M-CAA, E-AAG/M-CAG, E-AAG/M-CAC, E-AAG/M-CAT and E-AAG/M-CTC. Gel electrophoresis was performed following the protocol of the AFLP™ Analysis System I (Promega, Madison, WI, USA). The staining, developing, and fixing of AFLP bands were done using the Silver Sequence DNA Sequencing™ system's protocol (Promega).

The amplification products were separated by electrophoresis on SDS-PAGE gels and stained with silver staining.

3. Data analysis

All AFLP bands were scored by eye and only unam-

biguously scored bands were used in the analyses. For the AFLP resolved in more than one zone of activity, the most anodally migrating band was designated as '1', and other subsequent fragments were sequentially numbered. Homogeneity of variance among varieties was tested by Bartlett's statistics.

The degree of polymorphism was quantified using Shannon's index of phenotypic diversity (Bowman *et al.*, 1971):

$$H_O = -\Sigma pi \log pi$$

where pi is the frequency of a particular phenotype I.

H_O can be calculated and compared for different varieties. Let

$$H_{VAR} = 1 / n\Sigma H_O$$

be the average diversity over the n different varieties and let

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

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

RESULTS

A total of ten primer combinations were surveyed for this analysis using 260 different individuals (80 for MAY, 80 for JUNE, and 100 for F_1 hybrids from their parents underdevelopment and decayed seeds). These primers were found to be suitable for this investigation based on the criteria outlined above. The remaining bands were noninformation across all individuals surveyed.

The ten primers resulted in two accessions- and their hybrid-specific bands. The 51 polymorphic and 92 monomorphic bands were scored for all individuals across accessions. These data were used to calculate the frequency of each locus among the individuals per accession. The 12 accession-specific bands were used to test seed purity.

We found many phenetic bands for determining the seed purity of two accession (MAY and JUNE) and their hybrids. Eight fragments (E-AAC/M-CAA-03, E-AAC/M-CAG-06, E-AAC/M-CAG-22, E-AAC/M-CAT-11, E-AAG/M-CAA-10, E-AAG/M-CTA-22, E-AAG/M-CAT-06, and E-AAG/M-CAT-11) were specific for MAY (Table 1). Five fragments (E-AAC/M-CAA-04, E-AAC/M-CAG-11, E-AAC/M-CAG-18, E-AAC/M-CAT-15, and E-AAG/M-CAT-12) were specific for JUNE. For example, MAY (female parent) generated the E-AAC/M-CAA-03 band which was absent in JUNE (male parent) (Fig. 1). JUNE (female parent) had E-AAC/M-CAA-04 band with same primer which was absent in MAY (male parent). All hybrids of MAY x JUNE had both bands, E-AAC/M-CAA-03 (from female parent) and E-AAC/M-CAA-04 (from male parent). E-AAG/M-CAT-11 and E-AAG/M-CAT-12 showed same trends. E-AAG/M-CAT-11(a)

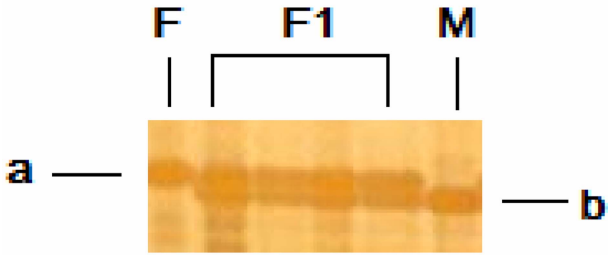

Fig. 1. DNA amplification patterns of E-AAC/M-CAA-03 (a) and E-AAC/M-CAA-04 (b) in *Isatis tinctoria* var. *yezoensis*.
M; Male parent type (*I. tinctoria* var. *yezoensis* cv. JUNE).
F; Female parent type (*I. tinctoria* var. *yezoensis* cv. MAY).
F_1; hybrid type
a; Specific band to female parent.
b; Specific band to male parent.

Table 1. List of primers selected from screening based on polymorphic fragments amplified in *Isatis tinctoria* var. *yezoensis*.

No. of primer	No. of fragments	Accession-specific fragments	
		MAY	JUNE
E-AAC/M-CAA	18	E-AAC/M-CAA-03	E-AAC/M-CAA-04
E-AAC/M-CAG	25	E-AAC/M-CAG-06, 22	E-AAC/M-CAG-11, 18
E-AAC/M-CAT	20	E-AAC/M-CAT-11	E-AAC/M-CAT-15
E-AAG/M-CAA	32	E-AAG/M-CAA-10	–
E-AAG/M-CTA	26	E-AAG/M-CTA-22	–
E-AAG/M-CAT	25	E-AAG/M-CAT-06, -11	E-AAG/M-CAT-12
Total	143	8	5

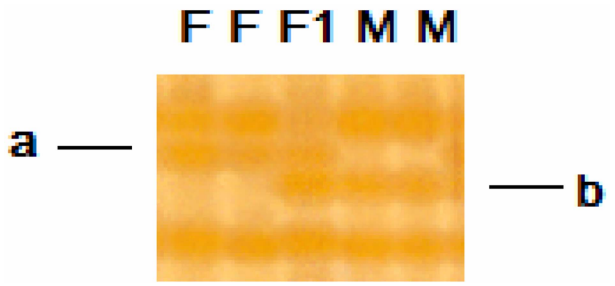

Fig. 2. DNA amplification patterns of E-AAG/M-CAT-11 (a) and E-AAC/M-CAA-12 (b) in *Isatis tinctoria* var. *yezoensis*.
M, F, and F_1 are the same as Fig. 1.
a; Specific band to female parent.
b; Specific band to male parent.

Table 2. Comparison of the number of sibs detected in two *Isatis tinctoria* var. *yezoensis* accessions using AFLP markers.

Parent	No. of sibs	% sibs
MAY (female)	8	8.0
JUNE (male)	5	5.0

(female parent) and E-AAG/M-CAT-12(b) (male parent) were also found for F_1 hybrids of MAY x JUNE (Fig. 2). However, although some bands such as E-AAC/M-CAG-06, E-AAC/M-CAG-22, E-AAC/M-CAT-11 were accession-specific to MAY, they were not contributed to test seed purity. Thus only two primers (E-AAC/M-CAA and E-AAG/M-CAT) were good to solve the hybrid purity test.

AFLP amplification of DNA extracted from germinated individuals from the female harvest reveal that 8 of 100 seeds were contaminated (8.0%) (Table 2). AFLP analysis of hybrid seeds from the male harvest revealed 5 of the 100 seeds were contaminated (5.0%).

The levels of variability of the hybrids were examined because they were not subject to any of the selection pressures of hybridization. Hybrids had high genetic diversity compared with their correspondent parents (Table 3). An assessment of the proportion of diversity present within species, $(H_{SP} - H_{VAR})/H_{SP}$, indicated that about 2.8% of the total genetic diversity was among accessions. Thus, the majority of genetic variation (97.2%) resided within accessions. Shannon's index of phenotypic diversity (H_O) of hybrids was the highest in all accessions (MAY, JUNE, and their hybrids). Although the mean H_E value for the female parent was slightly greater than for the male parent, it was not significance ($U < 0.05$; one-tailed Mann-Whitney test).

An assessment of the proportion of diversity present within varieties, H_{VAR}/H_{SP}, indicated that about 9.8% the total genetic diversity was among varieties. Thus, about 90.2% of genetic variation resided within varieties (Table 3).

Table 3. Partitioning of the genetic diversity into within and among varieties of *Isatis tinctoria* var. *yezoensis*.

Accession	H_{VAR}	H_{SP}	H_{VAR}/H_{SP}	$(H_{SP}-H_{VAR})/H_P$
MAY (female)	2.072	2.089	0.992	0.008
JUNE (male)	2.152	2.214	0.972	0.028
F_1	2.241	2.338	0.960	0.040

DISCUSSION

In today's market of quality conscious seed suppliers and buyers, there is a need to know what information is generated by various purity tests (Macko and Grzebelus, 2008). This document explains the value of each test, and the table below compares the most common exams used in seed testing. This information will help customers to select the proper test, or combination of tests, based on their needs. Morphological characteristics such as seed coat, seed size, shape, and hilum color can be used to differentiate between closely related cultivars. DNA fingerprint are the tests used to distinguish among cultivars and assure their genetic integrity.

Genetic test in genus *Brassica* has been investigated in several studies using isozyme and molecular markers (Arus *et al*., 1985; Crockett *et al*., 2000). Especially, RAPD markers for evaluating seed purity in a commercial F_1-hybrid cabbage (*Brassica oleracea* var. *capitata*) cultivar were demonstrated (Crockett *et al*., 2000). The 30 hybrids and their parents were evaluated for *Brassica napus* L. using isozymes and RAPD for 2 years (Yu *et al*., 2005).

In order to test determine hybrid seed purity, we conducted AFLP analyses. A large amount of molecular variation in *I. tinctoria* var. *yezoensis* was detected in molecular marker systems.??? The level of genetic variation of this species was high. For example, polymorphic values for AFLP were 66.7%. However, the majority of genetic diversity observed at the polymorphic loci in *I. tinctoria* var. *yezoensis* occurred within populations or varieties. In this study, AFLP variations were also maintained within accessions rather than among accessions (male, female, and hybrid lines), judging from the partitioning degree of the genetic diversity $[(H_{SP} - H_{VAR})/H_{SP}]$ (Table 3). Hence, the genetic similarity among the varieties is very high.

Fig. 1 showed a part of the results of typical AFLP using the selected primer E-AAC/M-CAA. The primer produced the female and male parent specific markers (~2200 bp) and was chosen to screen F_1 seeds from the female harvest. Use of primer E-AAC/M-CAG also resulted in amplification of the female and male specific bands (~800 bp), hence the primer was chosen the hybrid seeds from the male harvest.

We found AFLP markers useful in determining the seed purity of hybrids. The efficiency of AFLP marker for purity determination was about 2.8% (4/143 bands). The value was a little lower or similar to that found for tomato hybrids, another *Solanaceae* species, in which ten primers showed good polymorphism between parents out of 160 primers tested (Ballester and de Vicente, 1998). In carrot, among the 56 polymorphic *DcMaster* transposon markers, ten markers appeared to be potentially useful for hybrid seed purity testing (Paran *et al*., 1995).

The rate of contamination by plants is estimated by bulk PCR analysis (Komori and Nitta, 2004). When qualified seeds of MAY x JUNE and their accessions are used for hybrid seed production, it is likely that the seed purity of the resultant hybrid will be high enough for commercial use. Nevertheless, it is necessary to test the seed purity of the hybrid because seed contamination can be occur during the hybrid production process (Hashizume *et al*., 1993).

Why are different flowering periods of *I. tinctoria* var. *yezoensis* occurred within species or populations? It is not yet well understand component of studies on phenotypic evolution. Plant from nutrient-poor habitats tend to reduce the rate of nutrient cycling (Bertiller *et al*., 2002). In Korea, some species are shown in both inland and seashore areas (Kim *et al*., 2007). *I. tinctoria* var. *yezoensis* may be reflected ecological differences such as temperature and salt component between inland and seashore areas to contribute to population differentiation and effect on life cycling.

Conclusionally, we have shown in this report that AFLP analyses can be used as an useful method for seed purity testing of commercial accessions or accession and for detecting varieties in the *I. tinctoria* var. *yezoensis* tested.

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