Evaluation of Genetic Purity of F₁ Hybrid Seeds in Cabbage with RAPD, ISSR, SRAP, and SSR Markers

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Abstract. Four molecular marker systems—RAPD (random amplified polymorphic DNA), ISSR (intersimple sequence repeat), SRAP (sequence-related amplified polymorphism), and SSR (simple sequence repeat)—were used to evaluate seed genetic purity of a hybrid cabbage cultivar 'Zaoxia 16'. Genetic relationships of the F₁ hybrids and their parents were analyzed with 157 RAPD primers, 54 ISSR primers, 84 SRAP primer combinations, and 44 SSR primers. Three RAPD primers (NAURP2006, NAURP2020, and NAURP2031), two ISSR primers (NAUISR1058 and NAUISR1062), one SRAP primer combination (NAUSR04/NAURS05), and two SSR primers (NAUSSR1011 and NAUSSR1031), which produced male and female parent-specific markers simultaneously, were selected for testing the genetic purity of the F₁ seeds. A total of 210 'Zaoxia 16' hybrid individuals were investigated with these eight selected primers. Of these, 12 appeared to be false hybrids. Nine of the 12 putative false hybrids, confirmed with all eight primers, exhibited similar banding patterns to the female parent, suggesting that they could be derived from selfing of the female parent. The results were in accordance with those from field evaluations. This study showed that RAPD, ISSR, SRAP, and SSR markers are highly efficient and reproducible for genetic purity testing of cabbage commercial hybrid seeds.

Cabbage (*Brassica oleracea* var. *capitata*, cc, 2n = 2x = 18) belongs to the Brassicaceae family and is one of the most important vegetables in the world due to its wide

adaptation, high yield, long shelf time, and high economic significance. Cabbage has prominent heterosis and F₁ hybrid seeds are widely used in commercial production (Fang et al., 2000). Generally, F₁ hybrid seeds in *Brassica* vegetables are produced using established self-incompatible or male-sterile systems. However, hybrid seeds are often contaminated with seeds from selfing of female parents or outcrossing with other cabbage cultivars because of weakening of self-incompatibility or restoration of pollen fertility in male-sterile lines (Crockett et al., 2000). Low genetic purity would cause seed suppliers a great loss from the planters' claim and could make it easy for a competitor to steal the inbred parent of a hybrid. Therefore, it is critical for seed suppliers to control seed genetic purity before marketing.

The purity of F₁ hybrid seeds is traditionally assessed in a field grow-out trial (GOT), but these trials are time-consuming and labor-intensive and require large plots of land (Ballester and de Vicente, 1998; Dongre and Parkhi, 2005). Furthermore, morphological differences between true and false hybrids of cabbage are not always apparent and cannot be recognized easily, especially when parents are genetically similar, causing potential inaccuracy. Isozyme analysis has also been used in cabbage purity testing (Arus et al., 1985); however, this method may be limited by environmental conditions and tissue type and may require selection of a suitable isozyme (Crockett et al., 2000; Liu et al., 2004).

Because F₁ hybrids contain DNA from both parents, identification of male and female parent-specific markers will allow differentiation of true hybrids from selfed parental lines and outcrossed lines. Molecular markers, such as RAPD, ISSR, SSR, SRAP, AFLP (amplified fragment length polymorphism), and RFLP (restriction fragment length polymorphism) have been used in cultivar fingerprinting, seed purity testing, and germplasm identification for many species (Crockett et al., 2000; Dongre and Parkhi, 2005; Hu and Quiros, 1991; Li and Quiros, 2001; Liu et al., 2004; Nandakumar et al., 2004; Paran et al., 1995). Unlike radioactive chemicals involved RFLP and patent-protected AFLP, several other molecular markers, including RAPD, ISSR, SRAP, and SSR, could be effectively used for seed genetic purity testing and variety identification. Reports using RAPD markers are still limited, and no report has been published with ISSR, SSR, or SRAP markers to test cabbage seed purity (Crockett et al., 2000). In this study, four molecular marker systems—RAPD, ISSR, SRAP, and SSR—were used for testing seed purity of an elite commercial cabbage F₁ hybrid, 'Zaoxia 16'. An efficient and precise method was established for rapid and reliable genetic purity testing of commercial hybrid cabbage seeds.

Materials and Methods

'Zaoxia 16', an F₁ hybrid bred by the Shanghai Academy of Agricultural Science (SAAS), China, is characterized by its early maturity and heat resistance and is widely grown in Southern China. The two parental lines were grown in the vegetable breeding station at SAAS. Young leaves were collected for genomic DNA isolation. Genomic DNA was isolated and purified according to the CTAB method described elsewhere (Liu et al., 2003).

RAPD-PCR and ISSR-PCR was performed in a PTC-100 thermocycler (MJ Research, Waltham, Mass.) according to the protocols of Williams et al. (1990) and Zietkiewicz et al. (1994), respectively. The amplification products were separated on an agarose gel and photographed under ultraviolet light.

SRAP-PCR amplification and product analysis were based on the reported methods

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(Bassam et al., 1991; Li and Quiros, 2001), except the amplicons were separated by non-denaturing acrylamide gels and detected by silver staining (Bassam et al., 1991).

The SSR primer synthesis and SSR-PCR reactions were performed according to reported procedures (Lowe et al., 2004). The PCR products were detected with an 8% non-denatured polyacrylamide gel and then silver stained (Bassam et al., 1991).

All primer sequences will be provided upon request and purpose.

Commercial seeds of F₁ hybrid 'Zaoxia 16' were grown for GOTs at the Jiangpu Horticultural Crop Breeding Station at Nanjing Agriculture University (Nanjing, China) in Aug. 2005. Hybrid seeds of 'Zaoxia 16' are produced by intercrossing self-incompatible lines (Ren et al., 2004). Two hundred ten individuals were randomly selected and numbered in the field plots, and young leaves from the individuals were collected for genomic DNA isolation. At seedling and heading stages, purity evaluation was conducted on the basis of morphological traits including height, leaf shape and color, thickness of wax powder layer on leaves, head shape, heat tolerance at the seedling stage, and cold tolerance at the heading stage.

Results and Discussion

Between 'Zaoxia 16' and its parents, 126 out of 157 RAPD primers screened produced 347 polymorphic bands, in which 49 and 44 primers produced female and male parent-specific bands, respectively. Three primers, NAURP2006, NAURP2020, and NAURP2031, which produced one female parent-specific (FPS) and one male parent-specific (MPS) markers, were identified (Fig. 1). Further tests on all 210 'Zaoxia 16' hybrids grown in the field showed that 200 individuals produced both FPS and MPS markers using the primer NAURP2006. The remaining 10 individuals exhibited either MPS or FPS markers (Fig. 1, Table 1). Therefore, hybrid purity was 95.2% (200 out of 210) (Table 1). When screened with the other two RAPD primers (NAURP2020 and NAURP2031), 12 individuals were identified with only MPS or FPS markers. Therefore, the hybrid purity was calculated to be 94.3% (198 out of 210) (Table 1).

As shown in Table 1, 12 of the 210 individuals did not show FPS-MPS marker patterns, but 9 individuals (28, 32, 36, 74, 80, 81, 82, 193, and 208) produced only identical FPS markers, suggesting that these individuals might be false hybrids derived from selfing of the female parents. The remaining three individuals (29, 62, and 68) gave different results with different primers. In individual 29, both MPS marker NAURP2006₈₇₅ (875 bp) and FPS marker NAURP2006₁₆₀₀ (1600 bp) were amplified with primer NAURP2006, but only the MPS markers, NAURP2020₁₅₀₀ and NAURP2031₅₅₀ were amplified with primers NAURP2020 and NAURP2031, respec-

Fig. 1. RAPD analysis of 'Zaoxia 16' individuals and parents. F₁ hybrids were screened with the identified primers (a) NAURP2006, (b) NAURP2020, and (c) NAURP2031: lane 1, female parent; lane 2, male parent; lanes 3–16, individuals 62–75; lane M, DL 2000 DNA ladder (Takara Bio, Japan). Arrows indicate male parent- and female parent-specific markers.

Table 1. Genetic purity of 210 hybrid 'Zaoxia 16' individuals determined by identified molecular markers and field GOTs.

Primer	Putative false-hybrid individuals*	No. of putative hybrids	Purity (%)
NAURP2006	28, 32, 36, 68 [▲] , 74, 80, 81, 82, 193, 208	200	95.2
NAURP2020	28, 29 [▲] , 32, 36, 62, 68, 74, 80, 81, 82, 193, 208	198	94.3
NAURP2031	28, 29 [▲] , 32, 36, 62, 68 [▲] , 74, 80, 81, 82, 193, 208	198	94.3
NAUISR1058	28, 32, 36, 62, 68 [▲] , 74, 80, 81, 82, 193, 208	199	94.8
NAUISR1062	28, 32, 36, 62, 68 [▲] , 74, 80, 81, 82, 193, 208	199	94.8
NAUSR04/RS05	28, 29 [▲] , 32, 36, 62, 74, 80, 81, 82, 193, 208	199	94.8
NAUSSR1011	28, 29 [▲] , 32, 36, 62, 68 [▲] , 74, 80, 81, 82, 193, 208	198	94.3
NAUSSR1031	28, 29 [▲] , 32, 36, 62, 74, 80, 81, 82, 193, 208	199	94.8
GOT	32, 36, 62, 68, 80, 82, 193	203	96.7

*[▲]Individuals with MPS markers; the rest are individuals with FPS markers.

tively. In individual 62, primer NAURP2006 generated NAURP2006₈₇₅ and NAURP2006₁₆₀₀ markers, which are MPS and FPS, respectively; but the other two primers only produced FPS markers NAURP2020₆₀₀ and NAURP2031₅₂₀. In individual 68, both MPS markers, NAURP2006₈₇₅ and NAURP2031₅₅₀, were identified, while only FPS marker NAURP2020₆₀₀ was amplified with primer NAURP2020.

Fifty-four ISSR primers were screened for 'Zaoxia 16' and its parents; 42 primers detected 112 polymorphic loci between the F₁

progeny and its parents, 14 primers produced only an FPS band, and 20 primers generated only an MPS band. Two primers (NAUISR1058 and NAUISR1062) amplified FPS and MPS markers in the F₁ hybrids simultaneously (Fig. 2). Primer NAUISR1058 produced an FPS marker (NAUISR1058₇₈₀) and an MPS marker (NAUISR1058₁₄₀₀), while primer NAUISR1062 produced an FPS marker (NAUISR1062₇₅₀) and an MPS marker (NAUISR1062₆₂₅) (Fig. 2). Using the two identified primers, the genotypes of 'Zaoxia 16' individuals were discriminated (Fig. 2).

However, 11 out of 210 individuals did not show the FPS-MPS pattern: 10 produced the FPS marker only, and one produced the MPS marker only (Table 1). Both MPS and FPS markers were detected from the other 199 individuals simultaneously. Thus, hybrid purity was 94.8%.

Eighty-four SRAP primers combinations were used to screen the F_1 hybrids of 'Zaoxia 16' and their parents: 11 primers produced either an FPS or an MPS band, and one primer, NAUSR04/NAURS05, was codominant in the F_1 and produced FPS marker NAUSR04/NAURS05₂₀₅ (205 bp) and MPS marker NAUSR04/NAURS05₂₃₀ (Fig. 3). Genotypic differences of the 210 individuals of 'Zaoxia 16' were identified with this NAUSR04/NAURS05 (Fig. 3). Eleven of 210 individuals did not generate codominant patterns: 10 individuals produced the FPS marker only, and one produced the MPS marker only (Table 1). Thus, hybrid purity was 94.8%.

Sixteen out of 44 SSR primers produced 47 polymorphic loci between the F_1 and its parents of 'Zaoxia 16'. Four SSR primers produced an FPS band, and three primers produced an MPS band. It was found that codominant primer NAUSSR1011 produced FPS marker NAUSSR1011₂₀₅ (205 bp) and

MPS marker NAUSSR1011₂₅₅ and that primer NAUSSR1031 produced FPS marker NAUSSR1031₁₂₀ and MPS marker NAUSSR1031₁₆₀ (Fig. 4). Two hundred ten 'Zaoxia 16' hybrids were screened with these two primers (Fig. 4). Primer NAUSSR1011 did not show the codominant pattern in 12 individuals, with 10 having the FPS marker only and two having the MPS marker only (Table 1). The amplification pattern of primer NAUSSR1031 showed that 11 individuals did not generate the codominant pattern (Table 1). Thus, hybrid purity was 94.8%. One out of 12 individuals (number 68) showed different results with these two primers. The expected codominant markers were amplified with primer NAUSSR1031, whereas only the MPS marker (NAUSSR1011₂₅₅) was amplified with primer NAUSSR1011.

In the GOT, seven of the 210 individual hybrids of 'Zaoxia 16' exhibited abnormal characteristics, including poor growth, reduced height, less upright and compact shape, light leaf color, and thicker layer of wax powder on the leaves. Compared with the true hybrids, these seven plants also showed reduced heat and cold tolerance. Thus, hybrid purity from the GOT was calculated to be 96.7%.

Fig. 2. ISSR analysis of 'Zaoxia 16' individuals and parents. F_1 hybrids were identified with primers (a) NAUISR1058 and (b) NAUISR1062: lane 1, female parent; lane 2, male parent; lanes 3–16, individuals 62–75; lane M, DL 2000 DNA ladder (Takara Bio, Japan). Arrows indicate male parent- and female parent-specific markers.

Fig. 3. SRAP analysis of 'Zaoxia 16' individuals using primer combination NAUSR04/NAURS05: lane 1, female parent; lane 2, male parent; lanes 3–16, individuals 62–75; lane M, 50-bp DNA ladder. Arrows indicate male parent- and female parent-specific markers.

Seed contamination is always a concern in hybrid seed production of cabbage. In this study, nine of the 210 F_1 hybrids generated the FPS markers only using the eight identified primers, indicating that these nine individuals were false hybrids that might be produced from selfing of the female parent. However, three individuals (29, 62, and 68) exhibited different patterns with different markers (Table 1). One possible reason for these discrepancies is that parental individuals are advanced inbred lines and may have residual amounts of heterozygosity that can only be detected at the molecular level (Nandakumar et al., 2004). Consequently, hybrid genotypes occasionally exhibit slight variation, which could account for the differences observed with these molecular markers. Furthermore, cabbage is a naturally cross-pollinating species. Parental individuals may not be genetically identical and could be heterozygous at one or more loci; consequently, few loci in some hybrids may be non-heterozygous. The trace amount of heterozygosity could be a likely cause of the occasional presence of only FPS or MPS markers in these hybrids with some primers, which could generate the FPS and MPS markers simultaneously. Therefore, it can be suggested that individuals 29 and 68 should be the true hybrids because they came from crosses between parents. In individual 62, primer NAURP2006 amplified both MPS and FPS markers; but when the other seven primers were used, only the FPS markers were amplified. This indicated that individual 62 was derived from a selfed female parent individual, which has a residual heterozygous locus detected by the primer NAURP2006. The combined results of the RAPD, ISSR, SRAP, and SSR marker analyses confirmed that 10 of the 210 F_1 plants were false hybrids. The overall genetic purity of this F_1 hybrid seed lot was 95.2%.

Results from marker analysis and GOT were not consistent for individuals 28, 68, 74, 81, and 208. The disparities between these two methods probably arise from the difficulty of visual analysis in the field, which could lead to potentially inaccurate identification of weak plants as false hybrids and of strong plants as true hybrids.

Although male or female parent-specific markers can be used to screen hybrid seeds (Ballester and de Vicente, 1998; Crockett et al., 2000; Dongre and Parkhi 2005), codominant markers are always preferred for assessment of hybrid seed purity. It is suggested that a single codominant marker is sufficient to distinguish false hybrids from real hybrids (Nandakumar et al., 2004; Yashitola et al., 2002). However, residual heterozygosity, detected only at the molecular level, occurs inevitably in many inbred cabbage lines; therefore, it is questionable to determine hybrid purity only using a single marker. This research showed that RAPD, ISSR, SRAP, and SSR markers are fast and effective, and results are generally consistent with morphological analyses in field plots. Despite the added cost, use of multiple

Fig. 4. SSR analysis of 'Zaoxia 16' individuals using primers (a) NAUSSR1011 and (b) NAUSSR1031: lane 1, female parent; lane 2, male parent; lanes 3–16, individuals 62–75; lane M, 50-bp DNA ladder. Arrows indicate male parent- and female parent-specific markers.

marker systems could result in more accurate and reliable assessment of hybrid seed purity of cabbage. Combination of effective markers identified in this study would be a good option for establishment of a seed quality control system to be applied for seed purity testing in commercial seed production of cabbage.

Literature Cited

Arus, P., C.R. Shields, and T.J. Citon. 1985. Application of isozyme electrophoresis for purity testing and cultivar identification of *F₁* hybrids of *Brassica oleracea*. *Euphytica* 34:651–657.

Ballester, J. and M.C. de Vicente. 1998. Determination of *F₁* hybrid seed purity in pepper using PCR-based markers. *Euphytica* 103:223–226.

Bassam, B.J., G. Caetano-Anolles, and P.M. Gresshoff. 1991. Fast and sensitive silver stain-

ing of DNA in polyacrylamide gels. *Anal. Biochem.* 196:80–83.

Crockett, P.A., P.L. Bhalla, C.K. Lee, and M.B. Singh. 2000. RAPD analysis of seed purity in a commercial hybrid cabbage (*Brassica oleracea* var. *capitata*) cultivar. *Genome* 43:317–321.

Dongre, A. and V. Parkhi. 2005. Identification of cotton hybrid through the combination of PCR based RAPD, ISSR and microsatellite markers. *J. Plant Biochem. Biotechnol.* 14:53–55.

Fang, Z.Y., X.W. Wang, D.Y. Qu, and G.S. Liu. 2000. Hybrid seed production in cabbage, p. 109–129. In: Amarjit S. Basra (ed.). *Hybrid seed production in vegetables: rationale and methods in selected crops*. The Haworth Press, New York.

Hu, J. and C.F. Quiros. 1991. Identification of broccoli and cauliflower cultivars with RAPD markers. *Plant Cell Rept.* 10:505–511.

Li, G. and C.F. Quiros. 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR

reaction: its application to mapping and gene tagging in *Brassica*. *Theor. Appl. Genet.* 103: 455–461.

Liu, L.W., W.Z. Guo, X.F. Zhu, and T.Z. Zhang. 2003. Inheritance and fine mapping of fertility restoration for cytoplasmic male sterility in *Gossypium hirsutum* L. *Theor. Appl. Gene.* 106:461–469.

Liu, L.W., X.L. Hou, Y.Q. Gong, Y.M. Zhang, K.R. Wang, and J.F. Zheng. 2004. Application of molecular markers in variety identification and purity testing in vegetable crops. *Mol. Plant Breed.* 2:563–568.

Lowe, A.J., C. Moule, M. Trick, and K.J. Edwards. 2004. Efficient large-scale development of microsatellites for marker and mapping applications in *Brassica* crop species. *Theor. Appl. Genet.* 108:1103–1112.

Nandakumar, N., A.K. Singh, R.K. Sharma, T. Mohapatra, K.V. Prabhu, and F.U. Zaman. 2004. Molecular fingerprinting of hybrids and assessment of genetic purity of hybrid seeds in rice using microsatellite markers. *Euphytica* 136:257–264.

Paran, I., M. Horowitz, D. Zamir, and S. Wolf. 1995. Random amplified polymorphic DNA markers are useful for purity determination of tomato hybrids. *HortScience* 30:377.

Ren, Y.Y., J.X. Chen, and Y.M. Tong. 2004. Selection and breeding of a new heat tolerant cabbage 'Zaoxia 16'. *Acta Agr. Shanghai* 20:57–60.

Williams, J.G., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18:6531–6535.

Yashitola, J., T. Thirumurugan, R.M. Sundaram, M.K. Naseerullah, M.S. Ramesha, N.P. Sarma, and R.V. Stone. 2002. Assessment of purity of rice hybrids using microsatellite and STS markers. *Crop Sci.* 42:1369–1373.

Zietkiewicz, E., A. Rafalski, and D. Labuda. 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20: 176–183.