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Full Length Research Paper

Evaluation of genetic diversity in self-incompatible broccoli DH lines assessed by SRAP markers

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In this article, we investigated self-compatibility index (SCI) in broccoli double haploid (DH) lines, and the relationship and genetic diversity of 15 self-incompatible (SI) broccoli DH lines were analyzed by sequence related amplified polymorphism (SRAP). 11 primer combinations selected from 88 primer pairs revealed a total number of 129 unambiguous bands, 61 of which were polymorphic with a polymorphism frequency of 47.3%. Analyzed by NTSYS software, the genetic similarity coefficient of the 15 broccoli resources ranged from 0.76 to 0.98. Based on the coefficient value of 0.79, these broccoli DH lines were clustered into three multiple-member groups by unweighted pair group method with arithmetic mean (UPGMA) analysis, which provided molecular reference for parent selection in broccoli breeding.

Key words: *Brassica oleracea* L. var. *italica*, self-compatibility index, double haploid, genetic diversity, sequence related amplified polymorphism (SRAP).

INTRODUCTION.

Broccoli (*Brassica oleracea* L. var. *italica*) is a traditional European crop, and now has become widespread in Asian in recent decades (Branca, 2008). In broccoli, F_1 hybrids have advantages especially in uniform maturity, high total yield, better curd/head quality, and resistance to diseases and unfavourable weather conditions (Branca, 2008). For producing hybrid seeds of cabbage, cauliflower, broccoli, Brussels sprout and kale, a self-incompatibility (SI) character is utilized which is controlled by the *S*-locus (King, 2003). For establishing the homozygous SI parental lines, it usually needs years to get inbred line, survey and choose for several generations. Microspores culture could fleetly get homozygous lines-double haploid) (DH) lines and surveying self-compatibility indexes (SCI) of DH lines could quicken the getting of homozygous SI lines. Broccoli belongs to *Brassicaceae*. In the *Brassicaceae*, a conserved sporophytic self-incompatibility (SSI) system is present, and detailed genetic studies have resulted in the identification of highly polymorphic *S* genes that confer this trait.

The SSI system has been best characterized in the genus *Brassica*, and is primarily controlled by a receptor–ligand system encoded in two tightly linked and multi-allelic genes: the S receptor kinase (SRK), and the small cysteine-rich secreted protein, SP11/SCR (Samuel et al., 2008). SRK is the sole determinant of specificity in the stigma, and encodes a membrane-associated receptor protein kinase with extracellular, transmembrane and cytoplasmic kinase domains (Takasaki et al., 2000; Silva et al., 2001). SP11/SCR is the male determinant of S-locus specificity in the pollen side and encodes a low-molecular weight cysteine-rich protein which specifically expresses in the anther tissues (Shiba et al., 2001). The co-evolved SRK and SP11/SCR alleles constitute different S-haplotypes, and 'self' pollen rejection occurs

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Abbreviations: SCI, Self-compatibility index; **DH,** double haploid; **SRAP,** sequence related amplified polymorphism; **UPGMA,** unweighted pair group method with arithmetic mean; **SI,** self-incompatible; **SSI,** sporophytic self-incompatibility; **SRK,** S receptor kinase; **SP11/SCR,** small cysteine-rich secreted protein; **DNA,** deoxyribonucleic acid; **CTAB,** cetyl trimethylammonium bromide; **PCR,** polymerase chain reaction; **SC,** self-compatible; **ORPs,** open reading frames; **AFLP,** amplified fragment length polymorphism.

Table 1. SRAP primers.

Primer	Sequences (5'-3')	Primer	Sequences (5'-3')
me1	TGAGTCCAAACCGGATA	em1	GACTGCGTACGAATTAAT
me2	TGAGTCCAAACCGGAGC	em2	GACTGCGTACGAATTTGC
me3	TGAGTCCAAACCGGAAT	em3	GACTGCGTACGAATTGAC
me4	TGAGTCCAAACCGGACC	em4	GACTGCGTACGAATTTGA
me5	TGAGTCCAAACCGGAAG	em5	GACTGCGTACGAATTAAC
me6	TGAGTCCAAACCGGTAA	em6	GACTGCGTACGAATTGCA
me7	TGAGTCCAAACCGGTCC	em7	GACTGCGTACGAATTCAA
me8	TGAGTCCAAACCGGTGC	em8	GACTGCGTACGAATTCTG
		em9	GACTGCGTACGAATTCGA
		em10	GACTGCGTACGAATTCAG
		em11	GACTGCGTACGAATTCCA

when the S-haplotype of the pollen parent matches the pistil *S*-haplotype (Boyes and Nasrallah, 1993).

Although the interactions between SRK and SP11/ SCR has been well mapped out, still there are several questions to be solved, such as how temperature and humidity have impact on the SI, and how to overcome SI during reproduction of SI plants. Research on broccoli SI is seldom. In this article, we investigated SCI in broccoli DH lines, and evaluated genetic diversity of SI materials in broccoli by sequence related amplified polymorphism (SRAP).

MATERIALS AND METHODS

DH lines were planted in conservatory in 2008 autumn and SCI were determined in the next year spring when plants flowered.

Determination of self-compatibility index

Before buds anthesis, florets were protected from other pollens pollinated by bags. When most buds of the florets flowered, the bags were wiped off and flowers were pollinated with the pollen of the same plant and they were protected from other pollens for about 1 week after pollination. In every DH lines, 50 flowers were pollinated in early flower (in March). All other flowers or florets were discarded and the indication of plant name was written on a tag. SCI was determined again when plants were in the final-phase flower (in April).

DNA extraction

According to the result of determination of SCI, tender leaves of low SCI plants were taken for genomic deoxyribonucleic acid (DNA) isolation according to a cetyl trimethylammonium bromide (CTAB) procedure (Li and Quiros, 2001).

SRAP analysis

In this assay, exceptional SRAP primers (me6-me8, em7-em11) were designed except those mentioned in Li and Quiros' paper. All the primers were commercially synthesized (Sangon biological engineering technology and service Co. LTD., Shanghai). A total of 88 different combinations were employed using eight forward primers and 11 reverse primers (Table 1). Polymerase chain reaction (PCR) amplification was according to the procedure of Li and Quiros (2001).

Scoring and data analysis

The PCR products from SRAP analyses were scored qualitatively for presence or absence of bands. Only clear and apparently unambiguous bands were scored for SRAP. Genetic similarities between the SI DH lines were measured by the Dice similarity coefficient based on the proportion of shared alleles using 'simqual' sub-program of software NTSYS-PC version 1.8 (Exeter Software, Setauket, NY, U.S.A.) software package (Rohlf, 1993). The resultant distance matrix data was used to construct dendrograms by using the un-weighted pair-group method with an arithmetic average (UPGMA) subprogram of NTSYS-PC (Rohlf, 1993).

RESULTS

Self-compatibility indexes of broccoli DH lines tested

SCI of 124 broccoli DH lines were determined (Table 2). Self-compatible (SC) broccoli DH lines existed, but most of the broccoli DH lines were SI. Out of 124 broccoli DH lines, SCI of 15 lines were greater than 1, that is self-compatible, and 105 lines SI. b08266, b08267, b08268, b08269, four broccoli DH lines' SCI s were different hugely in the two SCIs tests. Out of 105 invariable (Tables 2 and 3).

Table 2. SCI test of broccoli DH plants (2009).

DH line	SCI 1	SCI2	DH lines	SCI 1	SCI 2	DH lines	SCI 1	SCI 2
2151-3	4.153	4.363	2214-8	1.206	1.206	b08247	0.26	0.568
2201	0.063	0.121	2220-3	0	0.962	b08248	0.137	0.238
2201D1	6.463	6.982	2236-2	0	0	b08249	0	-
2201D2	0.933	0.821	2237-1	0	-	b08250	0	0.368
2203-1	0	0.325	2237-2	0.085	0	b08251	0.767	1.021
2204T	1.737	1.679	2239T	0	0.093	b08252	0.891	0.982
2205T	0.050	0.078	2241-3	0.024	0.368	b08253	0.071	0.282
2206-16	0	0.314	2242D1	0	0.326	b08254	0	0.291
2208-4	0	0.193	2243-5	0.172	0.031	b08256	1.686	1.922
2208-5	0	0.128	2244	0	0.561	b08257	0	0.016
2209-1	0.026	0.185	2245T2	0.046	0.096	b08258	0.902	1.215
2209-3	0.281	0.389	2246T	0	0.069	b08259	0.163	0.238
2209-4	0.023	0.153	2246T1	0.067	0.093	b08260	1.233	1.036
2209-8	0.022	0.182	2249T15	0.884	-	b08261	0.102	0.625
2209-13	0	0.073	2249-7	0.419	0.327	b08262	0	0.021
2209-16	0.545	0.328	2249-8	0.053	0.517	b08263	0.291	0.395
2209-17	0.039	0.187	2249-9	0.5	0.980	b08264	0.455	0.827
2209-18	0	0.165	2249C2	0.082	0.098	b08265	0.469	0.768
2209-19	0	0.132	2253-6	0.022	0.089	b08266	0	7.333
2209-21	0.105	0.795	2256T	-	2.048	b08267	0.021	3.846
2209-22	0	0.251	b08225	0	0	b08268	0	4.657
2209-26	0	0.194	b08227	0.351	0.533	b08269	0	7.062
2209-29	0.073	0.186	b08228	0.017	0.328	b08270	0.111	-
2209-30	1.509	1.752	b08229	-	0	b08271	0	0.322
2209-39	0.070	0.210	b08230	0	0.315	b08272	0.226	0.879
2209C4	0.585	0.671	b08231	0.186	0.685	b08273	2.125	2.675
2209T35	0	0	b08232	0	0.216	b08274	2.061	2.786
2209T36	0	0.055	b08233	0	-	b08275	0.309	0.785
2209T37	0	0.925	b08234	0	0.158	b08276	0.948	1.258
2209T50	0.030	0.710	b08235	0	-	b08277	0	0.051
2209D1	0.041	0.561	b08236	0	0.212	b08278	0	0.098
2209D2	0	0.398	b08237	0	0.125	b08279	1.804	1.672
2209D3	0.015	0.258	b08238	0.3	0.131	b08280	0	0.091
2209T1	0	0.026	b08239	0.049	0.145	b08281	0.069	0.162
2209T2	0	0.237	b08240	0.021	0.533	b08282	0.036	0.215
2209T3	0	0.094	b08241	0	0.516	b08284	0	0.319
2209T4	0	0.400	b08242	0.059	0.256	b08285	3.092	3.846
2214-1	0.870	0.658	b08243	0	0.312	b08286	0.266	0.266
2214-2	0.647	0.763	b08244	0.683	0.735	b08287	0.042	0.089
2214-3	1.146	1.753	b08245	0	-	b08292	0.16	0.343
2214-5	0.018	0.087	b08246	0	0.257	b08302	0.016	0.108
2214-7	0.175	0.352						

SCI 1, Self-compatibility index in early flower (in March); SCI 2, self-compatibility index in final-phase flower (in April).

Table 3. Source of self-incompatible broccoli DH lines.

Number	Name	Donor/generation	Origin (country)
01	2209-13	Li lv/ F_4	Japan
02	2209T1	Li lv / F_4	Japan
03	2209T37	Li lv / F_4	Japan
04	2214-5	No. 19 / F_3	China Taiwan
05	2246T	No.172 / F_1	Japan
06	2246T1	No.172 / F_1	Japan
07	2249C2	No.116 / F_1	Japan
08	b08287	Sheng lv/ F_1	Japan
09	2253-6	No.10/ F_1	Netherland
10	2237-2	Lv xiong 90 / F_1	Japan
11	b08225	Man tuo lv/ F_1	Netherland
12	2245T2	No.59/ F_3	Unknown
13	2239T	No.219/ F_2	Unknown
14	b08257	No.64/ F_1	Unknown
15	2236-2	No.64/ F_1	Unknown

SRAP analysis of 15 broccoli strongly self-incompatible DH lines

SRAP analysis revealed a large number of distinct, scorable fragments per primer pair and in total, 129 bands, both polymorphic and monomorphic were obtained using the 11 primer combinations in 15 broccoli strongly SI DH lines (Tables 3 and 4). The number of amplified fragments varied from eight to 17, with an average of 11.7±3.07 bands (electromorphs) per primer combination. Overall size of PCR amplified fragments using five primer sets ranged from 50 to 700 bp. Out of 129 bands, 61 bands were polymorphic and thus, the polymorphism percentage averaged to 47.3% across the 15 DH lines. Maximum number of polymorphic bands was obtained for em_1-me_1 (AAT-ATA) primer combination.

Genetic diversity analysis

The results obtained by the Dice coefficient show that the genetic similarity varied from 0.76 between DH line 2209T1 and 2249C2 (from varieties of different Japanese company) to 0.98 between DH line 2209T1 and 2209T37 (both from the same variety). No region-specific markers were found. The UPGMA analysis clustered 15 broccolis SI DH lines into three main large groups; cluster A comprising 10 DH lines, cluster B comprising three DH lines and cluster C comprising two DH lines from different country (Figure 1). The clustering of the DH lines based on genetic similarity did not in general reflect their geographic region of origin. Cluster A included three subgroups. Subgroup 1 comprised 2209-13, 2209-T1 and 2209T37; all from one variety of a Japanese company. Subgroup 2 comprised 2246T1, 2237-2, 2236-2, 2245T2, 2214-5 and 2253-6; the former two lines were both from Japan, mid two lines were of unknown origin, and the latter 2 lines were from China, Taiwan and Netherland respectively. The two unknown origin lines 2236-2 and 2245T2 had high genetic similarity with 2246T1 from Japan. Subgroup 3 only had 1 line (2246T) which had the same origin as 2246T1 in subgroup 2.

Cluster B comprised 2239T, b08257 and 2249C2; the former two lines had high genetic similarity (0.88) but were from two different varieties which both had unknown origin, and the last line was from Japan.

DISCUSSION

The result of SCI test revealed that most of the broccoli DH lines were SI, and a few were SC, which is consistent with the opinion of Branca (2008). Out of 124 broccoli DH lines, four DH lines' SCI were distinctly different and the other DH lines' SCI were not obviously different in the two SCIs tests, which demonstrated that the SI of some broccoli plants was affected by the environment such as temperature and humidity.

Broccoli's origin is Europe, and was introduced in China in the 1980s. In China, broccoli varieties in production are almost from foreign country such as Japan, Netherland and France. Broccoli resource is lean in China, so it is important to collect broccoli resource in order to research broccoli and breeding. During collection of broccoli resource, some broccoli materials were unknown. SRAP was developed by Li and Quiros (2001), which is aimed for the amplification of open reading frames (ORFs). The polymorphism fundamentally

Table 4. Data on SRAP fragments and polymorphism obtained using 11 primer combinations in 15 broccoli DH lines.

Primer combination	Total number of band	Number of polymorphic band	Number of monomorphic band	Polymorphism (%)
e_1m_1	14	10	4	71.4
e_1m_2	8	7	1	87.5
e_1m_3	11	8	2	72.7
e_2m_1	17	6	11	35.3
e_2m_4	9	7	2	77.8
e_2m_5	9	1	8	11.1
e_3m_5	11	4	7	36.4
e_3m_6	16	4	12	25
e_8m_8	14	3	11	21.4
e_9m_8	11	5	6	45.5
$e_{10}m_8$	9	6	3	66.7
Total	129	61	68	
Mean	11.7±3.07	5.5±2.50	6.2± 3.92	47.3

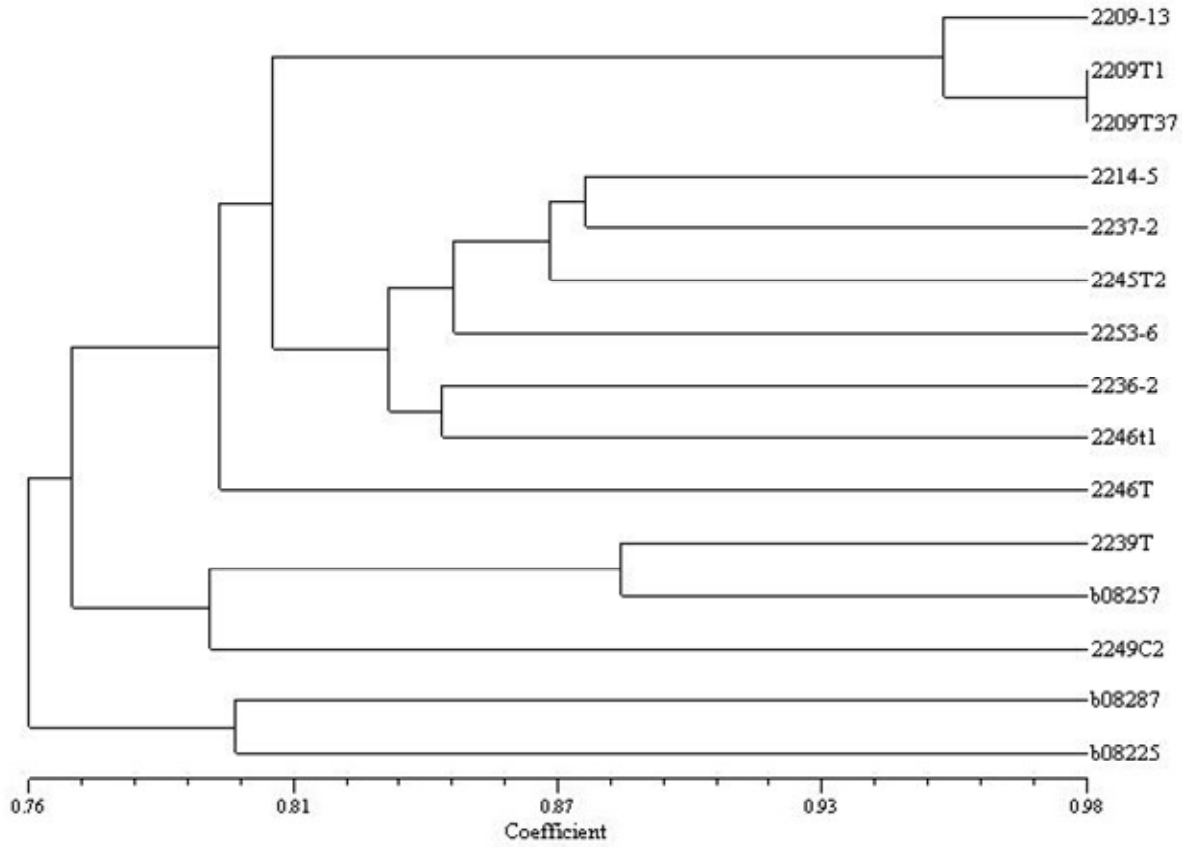

Figure 1. Dendrogram of the 15 broccoli self-incompatible DH lines based on SRAP bands using UPGMA cluster analysis.

originates from the variation of the length of introns, promoters and spacers, both among individuals and among species. In genetic diversity analysis, the information given by SRAP markers was more concordant to the morphological variability and to the evolutionary history of the morphotypes than that of the

amplified fragment length polymorphism (AFLP) markers (Ferriol et al., 2003). In the SRAP analysis, the number of amplified fragments varied from 8 to17, with an average of 11.7±3.07 bands per primer combination, and the polymorphism percentage averaged to 47.3% across all the varieties. SRAP analysis was successful in detecting genetic diversity and relationships between the broccoli SI DH lines. A low level of genetic diversity was found in the broccoli SI germplasm. In cluster analysis, 2209-13, 2209T1 and 2209T37, which were from Li lv F_4 inbred line, were highly similar (genetic similarity greater than 0.95). 2246T and 2246T1 from the same variety F_1 were less similar, and were clustered into two different groups. That is to say, genetic background of donor decides genetic relationship among donor's DH lines; the more miscellaneous genetic background of donor is, the more complex genetic relationship among donor's DH lines are. Contrarily, the more simplex genetic background of donor is, the lower genetic diversity among the donor's DH lines.

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