Molecular marker-assisted selection for development of yellow seeded Pol cms restorer lines in *Brassica napus* L.

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Abstract

The primary objective of this work was to transfer the partial-dominant yellow-seeded gene derived from a stable and pure yellow-seeded *Brassica napus* DH line No.2127-17 to Hui5148-2 to breed homozygous Polima cms restorer lines. To achieve this goal, the procedure of molecular marker-assisted selection (MAS) combined with phenotypic selection was performed in a modified recurrent backcrossing program. One dominant SCAR marker SCS1130 and one co-dominant CAPS marker SCA1 which closely flanked to the yellow-seeded gene and identified in our previous study were carried out in each segregating progenies to select the individuals carrying yellow-seeded gene. Simultaneously, a total of 88 RAPD primers and 60 AFLP primer pairs assays were used to analyze the background with a two-step selected approach, to identify the yellow-seeded plants genetically closer to the recurrent parent Hui5148-2 in BC_1F_1 and BC_2F_1, respectively. BC_2F_1 plants were finally self-pollinated to produce BC_2F_2. As a result, 5 superior introgression lines were identified for homozygous yellow-seeded restorer lines using SCA1 marker and further confirmed by phenotypic assessment in $BC_2F_{2:3}$. Background analysis subsequently demonstrated the 5 lines with 0.0157-0.0364 relative genetics distance to Hui5148-2. Further quality analysis and field trails consequently showed that they resulted in enhancing oil content and improving quality with desirable agronomic traits, which would offer the elite restorers in yellow-seeded hybrids breeding project.

Key words: Backcross, marker-assisted selection (MAS), phenotypic selection, restorer lines, yellow-seeded *Brassica napus* L.

Introduction

The genus *Brassica* provides a few important oilseed crop species, of which *Brassica napus* is the most economically important worldwide. The primary objectives in *Brassica napus* breeding are to improve the oil quality characteristic and increase the oil yield per unit area, which is determined by the seed yield potential and oil content in the seeds of a particular variety. A common strategy to enhance the yield potential is to develop hybrid varieties *i.e.* through the utilization of heterosis which has made prominent contributions to rapeseed production, whereas Polima cms is currently the most important hybrid system used for hybrid breeding in China. However, the approach to enhancing seed oil content is increasing the oil content in the embryos or reducing the hull proportion of the seeds. Compared with black seeds, yellow seeds of *Brassica* have significantly thinner seed coat, thereby leading to a lower hull proportion and, consequently, higher oil content, while some other advantages of yellow seeds include clearer oil, higher protein and lower fiber contents of the meal, giving rise to good quality and feeding value for livestock (Tang et al. 1997). Thus, the development of yellow-seeded Pol restorer lines to breed yellow-seeded hybrid cultivars can simultaneously improve the rapeseed oil quality characters and increase the oil yield. It has been considered as an ideal and effective breeding approach for canola breeders and there was a concerted effort to implement this goal.

The most critical step for breeding such a line is to transfer yellow-seeded gene to a desirable genetic background and then select the progenies possessing homozygous genotype. But only limited success has thus far been achieved using the conventionally breeding, which is partly due to the lack of elite and stable yellow-seeded germplasm or the complex inheritance and the environmental effect on yellow-seeded trait in *Brassica napus*. Moreover, the procedure via traditional breeding would be a laborious and time-consuming process. Fortunately, a yellow-seeded DH line No.2127-17 that produced stable and pure yellow seeds has been recently developed in our laboratory, which has been shown to be an elite interesting gene source due to the monogenic control and partial dominance of the trait (Liu et al. 2005). In addition, the use of genetic markers tightly associated with yellow-seeded gene can efficiently trace it at any stage of growth regardless of environmental conditions in a segregating population. At the same time, molecular fingerprints can help the selection of individuals that were genetically closer to the recurrent parent for expediting the recovery of the recurrent parent in a backcross breeding program (Francia et al. 2005; Joseph et al. 2004; Oliveira et al. 2005).

The primary objective of the study reported here was to transfer the yellow-seeded gene from No.2127-17 to Hui5148-2 using a combination of conventional phenotypic selection and assays of RAPD, AFLP, SCAR and CAPS markers identified in No.2127-17 (Liu et al. 2005, 2006), to develop stable and homozygous yellow- seeded Pol cms restorer lines.

Materials and methods

Plant Materials

The recurrent parental line Hui5148-2 was an elite black-seeded Polima cms restorer line of hybrid *Brassica napus* and its hybrid with 1141A has been registered as Huaza No.7, which had been widely grown in china. The yellow-seeded donor parental line No.2127-17 was a pure and stable DH line without restorer ability based on broad testcross, while it has some undesirable agronomic traits, such as low yield, high amounts of erucic acid in the oil, high level of glucosinolates in the seed meal, which limit it to be used as immediate gene resource. In addition, an elite Polima cms line 1141A, extensively used in hybrid rapeseed production in China, was chosen as donor of sterility cytoplasm and was hybridized with Hui5148-2 and the selected lines. They were all obtained from Huazhong Agricultural University

DNA isolation, molecular markers analysis and seed colour classification

Total DNA was extracted from fresh leaves of seedlings in different generation and quantified according to Liu et al. (2006). The RAPD, AFLP, SCAR and CAPS analysis were carried out as followed by Liu et al. (2005 and 2006, respectively). While colour of self-pollinated seeds harvested from plants in different generations was analyzed using a method described by Liu et al. (2005).

MAS selection scheme

In an effort to breed stable and homozygous yellow-seeded Polima cms restorer lines, a modified MAS backcross breeding scheme was developed (Figure 1). In this scheme, molecular marker assay combined with phenotypic evaluation was adopted in each breeding generation. The progenies of each backcross were inspected with two markers, SCS1130 and SCA1, to identify if the yellow-seeded gene was present, followed by selection of homozygous and heterozygous plants in the later BC_2F_2 progenies using co-dominant marker, SCA1. In order to rapidly eliminate the genetic background of the No.2127-17 and efficiently identify individuals that have the least GD between Hui5148-2, the RAPD or AFLP analysis were designed to perform in the yellow-seeded lines (based on the marker genotype) using two-step approach, namely, firstly selected limited makers to analyze the progenies and to eliminate about half the individuals, then, followed an another increasing number of makers to analyze the remaining plants. As a result, three phenotypically superior individuals with least GD were selected to backcross with Hui5148-2 in BC_1F_1 and self-pollinate in BC_2F_1. Meanwhile, self-pollination was made to produce the F_2 in each progeny which was used for further seed quality and colour analysis to verify the results of molecular marker selection.

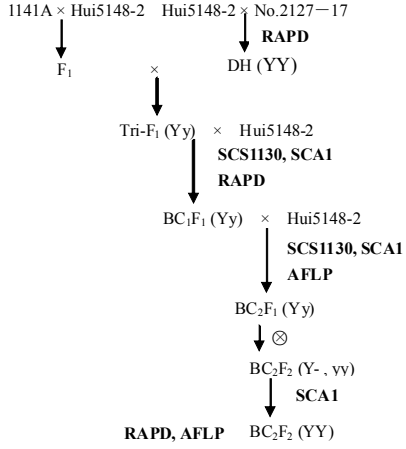

Figure 1 The modified molecular marker- assisted backcross breeding scheme for Hui5148-2 with yellow-seeded gene

Genetic distances (GDs) analysis

Only strong and distinguishable DNA bands in RAPD and AFLP analysis were considered. Presence of the band was recorded as 1, whereas absence of the band was recorded as 0. Polymorphic data were used to estimate GD. Genetic similarity (GS) was analyzed using the equation (Nei and Li, 1979): $GS = (2N_{ij})/(N_i+N_j)$, where N_{ij} is the number of fragments shared between populations i and j, N_i and N_j are the number of scored fragments of population i and j, respectively. GD was then calculated as GD=-ln(GS). UPGMA (Unweighted pair group arithmetric average) method was use in the cluster analysis.

Assessment of agronomic performance and analysis of seed quality

During the whole growth period, several types of agronomic traits were recorded, namely, vigour, uniformity, configuration of plant and leaf, flowering time, disease resistance, yield per plant, etc. Quality analysis of seeds from selected plants was done by using Near Infrared Reflectance Spectroscopy (NIRS) methods described by Gan et al. (2003) in terms of oil, glucosinolate, erucic, olieic and protein content.

Results

Background analysis in DH population

No.2127-17 was used as male donor and crossed with Hui5148-2 to obtain the yellow-seeded DH using microspore culture procedure. All DH individuals continued to grow until maturity and were harvested for colour and quality analysis. Out of 65 yellow-seeded DH lines, 12 with better quality were selected to identify selections with the least GD between Hui5148-2 based on RAPD analysis. By screening a total of 810 decamer primers, 240 primers revealed polymorphisms between parents, from which 142 primers were used for evaluation of their genetic background. Totally 830 bands were obtained with 314 polymorphic bands (2.2 bands per primer). Cluster analysis demonstrated that DH_{146} and DH_{21} were two accessions with the least GD (0.4329, 0.5022, respectively), consistent with phenotypic assessment in field. As a result, the two tri-crosses were backcrossed with Hui5148-2 to obtain BC_1F_1 populations, which were, for convenience, designated as $BC_{1\text{-}21}F_1$ and $BC_{1\text{-}146}F_1$, respectively, and selected for further analysis.

MAS analysis in BC_1F_1

Agronomic performance of the two BC_1F_1 families grown in the field were evaluated and quality analysis in the tri-F_2 was combined, one superior and most like Hui5148-2 family ($BC_{1\text{-}146}F_1$) with 353 plants along with the parental lines was consequently selected for MAS analysis. Genotyping of these plants using the two linked markers separately, SCS1130 or SCA1, confirmed that 169 plants possessed yellow-seeded characteristics (Fig.2A). However, when they were used in combination, 161 were proven to contain yellow- seeded traits (Table 1), which was further confirmed by seed color of the BC_1F_2 and BC_1F_0 (immediate F_1, the others are similar) plants. From the 161 plants, based on agronomic performance, 142 with vigorous growth and good resistance were identified for RAPD background analysis by 88 most polymorphic primers from the above-mentioned 142 ones, to select plants with the least GD between the Hui5148-2. Initially, 30 primers pre-screened all these 142 plants, and a total of 51 polymorphic bands (1.7 bands per primer) were detected (Fig.2B). After cluster analysis using the initial data, 46 plants were selected to create a sub-BC_1F_1 for further analysis. Subsequently, using the remaining 58 primers, 85 polymorphic bands had been amplified with average of 1.5 bands and the GD values among the 46 lines ranged from 0.2469 to 0.3815 (Table 2). Accordingly, three plants that showed the highest similarity to Hui5148-2 with the least GD were backcrossed to Hui5148-2 and self-pollinated to generate putative BC_2F_1 ($BC_{2\text{-}25}F_1$, $BC_{2\text{-}85}F_1$, $BC_{2\text{-}49}F_1$) and BC_1F_2 families, respectively.

Table 1 The selection results of SCS1130 and SCA1 markers in different generations

Generation	Markers Name	Homo-Y		Hetero- Yy			Homo- yy			Misclassi-fication	Double-crossover
		+	-	-	+	-	+	-	+	%	%
BC_1F_1	SCS1130	0	0	6	163	0	9	175	0	4.25	0.57
	SCA1	0	0	0	161	8	**2**	174	8	5.10	
BC_2F_1	SCS1130	0	0	5	100	0	8	110	0	5.83	0.45
	SCA1	0	0	0	98	7	**1**	112	5	5.83	
BC_2F_2	SCA1	9	0	0	22	1	0	15	2	6.12	

Table 2 The 10 individuals with the least GD in BC_1F_1 and BC_2F_1 populations

Order	BC_1F_1 population		BC_2F_1 population	
	Code	G D	Code	G D
1	**25**	**0.2469**	**278**	**0.0800**
2	**85**	**0.2534**	**248**	**0.0827**
3	**49**	**0.2624**	**243**	**0.1018**
4	35	0.2719	286	0.1054
5	68	0.2776	231	0.1119
6	147	0.2829	232	0.1231
7	69	0.2877	329	0.1244
8	127	0.2877	241	0.1263
9	6	0.2927	242	0.1263
10	11	0.2973	244	0.1335

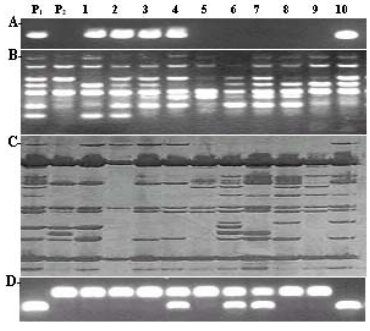

Figure 2 Amplification patterns in the partial individuals generated by SCS1130 (A) and RAPD S86 (B) in BC1F1, AFLP EA07MC12 (C) in BC2F1 and SCA1 (D) in BC2F2 progenies. lanes: P1 No.2127-17, P2 Hui5148-2, 1-10 individual plants.

MAS analysis in BC_2F_1

Based on the BC_2F_1 field performance and quality analysis in BC_1F_2 and BC_2F_0 seeds, $BC_{2\text{-}85}F_1$ family composed of 223 individuals was further analyzed via SCS1130 and SCA1. As a result, 98 plants were identified to contain the yellow-seeded gene because of simultaneous detection of the two markers (Table 1). Because AFLP techniques usually exhibited higher multiplex ratio and high efficiency, the background of the selected lines was subsequently conducted in the same manner based on AFLP assays using 60 primer pairs showing high polymorphism between the parents selected from a set of 256 E+3/MC+2 and 256 E+3/MG+2 primer pairs. Out of the 98 plants, 94 were subsequently selected to pre-conduct with a total of 20 pairs. The results showed that 91 out of 1800 intense and reproducible bands could be scored as polymorphisms with a mean of 5.5 per primer pair (Fig.2C). As a result, 46 plants were identified to survey with the rest 40 pairs, 160 polymorphic bands (4.0 bands per pair) from total 3400 bands were detected. The cluster analysis demonstrated that the value of minimum GD was 0.08 (Table 2). Finally, 3 individuals with least GD were self-pollinated to produce BC_2F_2 ($BC_2F_{2\text{-}278}$, $BC_2F_{2\text{-}248}$, $BC_2F_{2\text{-}243}$) progenies, which was in good agreement with the morphological data.

Further analysis in BC_2F_2

For a successful backcross breeding program, it is necessary to identify the BC_2F_2 population genotype to obtain homozygous lines since they might be segregated for the yellow-seeded gene. For these purpose, co-dominant SCA1, which can simplify this process without an additional generation of progeny testing to make this distinction, was used. Based on the field appearance and the seed quality, out of the 3 BC_2F_2, $BC_2F_{2\text{-}248}$ containing 49 individuals was finally selected and subjected to SCA1 analysis (Fig.2D), which revealed that 9 plants were identified as homozygous dominant (YY), 23 heterozygous (Yy), and 17 homozygous recessive (yy) in a segregation of 1:2:1 on the basis of Chi-square analysis (χ^2=2.80) and was verified by the seed colour segregation in advanced $BC_2F_{2:3}$. At last, five elite and homozygous lines were further identified in this study. In order to estimate the percentage of the Hui5148-2 background in the 5 lines, RAPD and AFLP were used. The results showed that GD values to Hui5148-2 among the 5 lines ranged from 0.0157 to 0.0364, implying that the recovery of the Hui5148-2 alleles was very high.

Figure 3 The seed colour of two parents, one selected line and its hybrid.

Seed colour analysis of the 5 lines demonstrated that they ranged from rank 2 to 3 (Fig. 3). In addition, these 5 lines showed good oil quality, high oil content and the same agronomic performance as the original restorer lines, implying that they can be used for elite yellow-seeded hybrid breeding in the future.

Discussion

We primarily examined the combining ability of the 5 improved restorer lines and the agronomic performance of the hybrids derived from the crossing between 1141A and Hui5148-2 or the 5 lines. However, no significant difference was detected in yield, indicating that the combining ability of Hui5148-2 was well maintained in the 5 lines; while the agronomic performance among the hybrids was almost identical, for example, oil content and quality have been significantly improved compared with the original hybrid (data not shown), which may be due to the stringent phenotypic selection for Hui5148-2 plant type accompanied with quality evaluation at each stage of MAS breeding. Additionally, the 5 dominant yellow-seeded lines obtained herein have particular advantages in the combination with hybrid breeding. If they were crossed with black-seeded male sterile line (1141A), hybrids with black seeds could be produced that yield yellow commodity seeds for the crushing industry. Furthermore, they can also be directly developed into commercial varieties in yellow-seeded *Brassica napus* breeding programs. To our knowledge, this is the first report and a successful example of the development of elite yellow-seeded restorer lines by a combination of MAS and phenotypic selection in *Brassica napus*.

In the present study, high selection efficiencies and environmental limitation against the yellow-seeded gene derived from No.2127-17 using markers SCS1130 and SCA1 together were obtained. The use of these markers in a segregation population easily distinguished the yellow- and black-seeded plants, and also differentiated homozygous and heterozygous individuals, which suggested it can greatly narrow the size of breeding population for effective selection and short the time needed in genotype determination (Yang et al. 2003). Simultaneously, analysis of the progeny background by stepwise RAPD or AFLP is useful in rapidly determining the relative GD to Hui5148-2 which reduced the cost of MAS through the progressive reduction in the number of individuals subjected to final marker analysis (Oliveira et al. 2005), in line with previous work on rice (Joseph et al. 2004). However, since phenotypic selection is sometimes affected by experience at some rate, it is anticipated that an increased complement between molecular technologies and conventional breeding in the near future are needed for a more efficient improvement of the yellow-seeded *Brassica napus*. (Francia et al. 2005).

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