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DEVELOPMENT OF SEQUENCE-TAGGED-SITE (STS) PCR MARKERS AND STUDY OF SIMPLE-SEQUENCE-REPEATS (SSR) FOR VARIETY TESTING IN OILSEED RAPE (*BRASSICA NAPUS*)

Document prepared by experts from France

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DEVELOPMENT OF SEQUENCE-TAGGED-SITE (STS) PCR MARKERS AND STUDY OF SIMPLE-SEQUENCE-REPEATS (SSR) FOR VARIETY TESTING IN OILSEED RAPE (*BRASSICA NAPUS*)

Yong Xiang Zhang[1], Stéphane Fouilloux[1] , Vincent Lonbard[1], Dominique Brunel[2], Régine Delourme[3], Françoise Blouet[4], And Mireille Bourgoin-Greneche[1].

I. INTRODUCTION

Oilseed rape (*Brassica napus*) is a very important oil seed production crop in France. As it has a great potential of non-food use, various variety improvement programs have been developed by the public and private breeders. This leads to an increasing number of varieties of oilseed rape in the national registration list and a great diversification of variety types (lines, hybrids, hybrid-line composites, hybrid-hybrid composites, *etc*...). This causes an insufficiency of traditionally used descriptors (morphologic characters and isozymes) to describe and differentiate correctly the new varieties. It is so necessary to develop new types of descriptors, in particular, DNA markers for helping variety testing in oilseed rape.

Among the available systems of molecular genetic markers, those which are reliable, easy-to-use, codominant and cost-effective are always desirable for Distinctiveness, Uniformity and Stability (DUS) testing in the context of plant variety protection. For this reason, simple-sequence repeats (SSRs) markers seem to be very attractive for the DUS testing ; however, the high investment required to develop SSRs is a limiting factor for many laboratories and species. In this paper, we explored the sequence-tagged-site (STS) (Tragoonrung *et al.*, 1992 and Talbert *et al.*, 1994), an alternate strategy to simple-sequence repeats (SSRs), for development of codominant PCR markers in oilseed rape. Furthermore, SSRs reported by different teams in *B. napus* (Lagercrantz *et al.*, 1993, Kresovich *et al.*, 1995 and Szewc-McFadden *et al.*, 1996) have also been studied ; the results will be reported as well.

II. Development of STS PCR markers

Twelve oilseed rape cultivars were studied (Table 1), 5 were spring type lines and 7 were winter type lines. They represented different genetic and geographical origins. Total DNA of each genotype was isolated from a pool of 10 young seedlings of 1-2 weeks age, growing in the green house, according to the CTAB protocol of Rogers et Bendich (1988), with small modifications. Sequence information was obtained from GENBANK. Eleven sets of consensus-primers were designed, with 'Oligo' software, from 11 published and known function genes of *A. thaliana* and *B. napus* (Table 2).

1: GEVES, labo. de biochimie, B.P.52, F-17700 Surgeres ; 2 : INRA, Station de Génétique et d'Amélioration des Plantes, route de St-Cyr, F-78026 Versailles Cedex. 3 : INRA, Station d'Amélioration des Plantes, B.P.29, F-35650 Le Rheu. 4 : GEVES La Minière, 78285 Guyancourt Cedex.

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1. Polymorphism of amplification

All the primer sets provided PCR products. At first, the PCR products have been resolved on 2% agarose gels and stained by BET : no length polymorphism was observed. So, the PCR products were then resolved on 5% polyacrymide gels. Length polymorphism was observed at least on 5 systems (AG, AP3, FAD7, LFY3 and ADH) among the 11 included in this study (Fig.1). These 5 sets of primers amplified 17 genes in *B. napus*, among which 10 showed polymorphism on the 12 genotypes used (Table 3). For each set of consensus-primer derived from one gene of *A. thaliana*, 2 to 4 fragments were in general amplified on oilseed rape genotypes. This can be explained by the amphidiploid origin of *B. napus*. However, there are some difficulties to interpret the results of GAPC, ABI3 and NIA1 and NIA2 because the resolution was not good enough and hence it needs to be improved. The number of profiles observed on the polymorphic systems varied from 2 to 5. The polymorphic genes presented generally 2 alleles across the 12 genotypes of oilseed rape. Out of the 10 informative genes, 4 were dominant.

2. Polymorphism of restriction of PCR products

Polymorphism of restriction has also been explored in order to increase the polymorphism level. PCR products of 6 sets of primers were assayed using 11 restriction enzymes of 4-, 5- and 6-base-cutters. All the digested products have been resolved on 2% agarose gels. Polymorphism of restriction was revealed on all these assayed loci (Table 3) by at least one enzyme. Two loci FAD3 and GAPC which had been monomorphic became polymorphic and their level of polymorphism was important, especially for locus GAPC. Eleven profiles were observed for GAPC on 12 genotypes. Anyhow, the profiles were more or less complex. It is difficult to do allelic analysis for this system. One could think that the level of polymorphism was not improved for loci AG, FAD7 and LYF3. It should be indicated that for a given locus, the information was usually redundant if polymorphism could be generated by several restriction enzymes ; this means that once polymorphism of restriction is revealed by one enzyme, there is no need to try other restriction enzymes.

III. Evaluation of SSRs markers for distinctiveness of varieties

A total of 21 SSR primer sets have been tested : 15 developed by Kresovich *et al* (1995) and Szewc-McFadden *et al.* (1996), 5 reported by Lagercrantz et al. (1993), and one developed by INRA Rennes. All the conditions of reaction and amplification used were the same as those described by Kresovich *et al* (1995). The prescreening of primers was performed on 4 genotypes (Yudal, Stellar, Drakkar and Darmor nain). Those which amplify a polymorphic locus have been assayed on the 12 genotypes (Fig. 2). All the results obtained are showed in table 4. Among the 21 sets of primers tested, amplification products have been produced on 18 sets ; however, polymorphism was observed only on 10 loci. The SSRs obtained on the 4 genotypes show 2 or 3 alleles (profiles) with a polymorphism information content (PIC) varying from 0.38 to 0.63. When amplification was performed on 12 genotypes, additional alleles have been detected only on 2 loci (B.n. 9A and X55). At present, only 6 of 10 polymorphic loci have been studied on the 12 genotypes.

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IV. Potential of SSRs and STS markers for variety testing in oilseed rape

The discriminate power of the STS markers and the SSRs markers is estimated by computing the PIC of each marker (or gene) using the results obtained on all the 12 genotypes. The value of PIC varied from 0.15 to 90 (table 5). To appreciate the real utility of these markers, the PIC was also computed only using the data obtained on the 9 European genotypes (table 5). By removing the 3 exotic genotypes (Stellar, Wesbrook and Yuddal), one SSR (B.n. 92A) and 5 STS became non-informative (table 5). This means that these 6 markers would not have utility in DUS testing of European varieties of oilseed rape. The value of PIC decreased more or less for the rest of loci. It should be indicated that polymorphic information produced by these 2 kinds of markers are not redundant. Compared to the results obtained by isozymes on the same genotypes, SSRs as well as STS are complementary in term of polymorphism.

Genetic distance between each pair of genotypes was estimated just by calculating the percentage of loci among the 18 polymorphic ones, which differed between the two genotypes (the distance is expressed between 0 to 1). The value of distance varied from 0.11 (2 differences) to 0.83 (15 differences) according to the pair of genotypes in comparison (table 6).

All the 12 genotypes could be differentiated from each other by these SSRs and STS markers even if the GAPC system is not used. So these DNA markers will be very useful to DUS testing of varieties in oilseed rape. The STS markers derived from consensus primers would be also potentially useful to other related *Brassica* species.

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Genotype	Origin	Type
Aligator (A)	Germany	Winter
Bristol (B)	France	Winter
Darmor nain (DN)	France	Winter
Falcon (F)	Germany	Winter
Goëland (G)	Germany	Winter
Link (L)	Germany	Winter
Vivol (V)	France	Winter
Drakkar (DK)	France	Spring
Jaguar (J)	Denmark	Spring
Stellar (S)	Canada	Spring
Wesbrook (W)	Australia	Spring
Yuddal (Y)	Korea	Spring

Table 1. list of genotypes used for development of STS and SSR markers.

Locus	Origin	Function
AG	*A. thaliana*	Agamous gene
AP3	*A. thaliana*	Homeotic flower gene
FAD7	*A. thaliana*	Linoleate desaturase
LFY3	*A. thaliana*	Floral meristem control gene
ADH	*A. thaliana*	Alcohol dehydrogenase
GAPC	*A. thaliana*	Glyceraldehyde 3P dehydrogenase C subunit
ABI3	*A. thaliana*	Abcissic acid insensitive gene
NIA1	*A. thaliana*	Nitrate reductase 1
NIA2	*A. thaliana*	Nitrate reductase 2
PHYA	*A. thaliana*	Phytochrome A
FAD3	*B. napus*	Linoleate desaturase

Table 2. Origin and function of loci studied.

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Locus	Number of genes		Polymorphism			
	amplified in oilseed	polymorphic rape	of number of profiles	amplification size of PCR products (bp)	of enzyme	restriction number of profiles
AG	4	3	5	550-670	Sau 3AI	3
AP3	4	3	4	600-650	Alu	3
FAD3	1	0	1	1900	Hae III	4
FAD7	2	1	2	900-1650	Taq 1	2
LFY3	4	2	3	700-800	Alu I	3
ADH	3	2	3	510-650	-	-
GAPC	> 4	?	1	600-1500	Taq I	11

Table 3. Different types of polymorphism observed on seven of 11 loci studied and on the 12 genotypes of oilseed rape.

Locus	Origin	Range of product sizes (pb)	Polymorphism on 4 genotypes		Polymorphism on 12 genotypes	
			number of allels	PIC*	number of allels	PIC
B.n. 9A	Kresovich et al. (1995)	180-260	3	0.63	5	0.67
B.n. 12A	Szewc-McFadden et al. (1996)	250-325	3	0.63	3	0.42
B.n. 26A	Szewc-McFadden et al. (1996)	90-120	2	0.38	2	0.38
B.n. 92A	Szewc-McFadden et al. (1996)	110-145	2	0.38	2	0.15
MB 2	Lagercrantz et al. (1993)	130	2	0.38	2	0.28
X55	INRA Rennes	180-200	2	0.38	3	0.57
B.n. 35D	Szewc-McFadden et al. (1996)	210-270	2	0.38	in progress	
B.n. 40C1	Szewc-McFadden et al. (1996)	130-150	2	0.50	in progress	
B.n. 59A1	Szewc-McFadden et al. (1996)	425-480	2	0.38	in progress	
MB 5	Lagercrantz et al. (1993)	90	2	0.38	in progress	
B.n.6A	Kresovich et al. (1995)	92-96	monomorphic			
B.n. 25C2	Szewc-McFadden et al. (1996)	130-175	monomorphic			
B.n. 38D	Szewc-McFadden et al. (1996)	150-200	monomorphic			
B.n. 72A	Szewc-McFadden et al. (1996)	240-300	monomorphic			
B.n. 80/3	Szewc-McFadden et al. (1996)	100-145	monomorphic			
MB 1	Lagercrantz et al. (1993)	160	monomorphic			
MB 3	Lagercrantz et al. (1993)	80	monomorphic			
MB 4	Lagercrantz et al. (1993)	50	monomorphic			
B.n. 19A	Szewc-McFadden et al. (1996)	175-225	no amplification			
B.n. 68/1	Szewc-McFadden et al. (1996)	200-400	no amplification			
B.n. 83B1	Szewc-McFadden et al. (1996)	190-240	no amplification			

* : PIC = polymorphism information content.

Table 4. Polymorphism of SSRs observed on the 12 genotypes of oilseed rape.

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Locus	Type	On 12 genotypes		On 9 European genotypes	
		number of alleles	PIC	number of alleles	PIC
B.n. 92A	SSR	2	0.15	1	0
B.n. 26A	SSR	2	0.38	2	0.35
B.n. 12A	SSR	2	0.42	2	0.35
B.n. 9A	SSR	5	0.67	4	0.62
MB 2	SSR	2	0.28	2	0.20
X55	SSR	3	0.57	3	0.49
FAD7-g3	STS/PCR	2	0.28	1	0
ADH-g3	STS/PCR	3	0.29	1	0
LFY3-g3	STS/PCR	2	0.40	2	0.49
LFY3-g1	STS/PCR	2	0.15	1	0
AP3-g4	STS/PCR	2	0.15	1	0
AP3-g3	STS/PCR	2	0.50	2	0.49
AP3-g1	STS/PCR	2	0.38	2	0.20
AG-g3	STS/PCR	2	0.50	2	0.44
AG-g2	STS/PCR	2	0.44	2	0.44
AG-g1	STS/PCR	2	0.15	1	0
FAD3	STS/HaeIII	4	0.64	2	0.49
GAPC	STS/TaqI	11	0.90	8	0.86

Table 5. Level of polymorphism of SSRs and STS and their potential for discrimination of European varieties.

	Stellar	Yudal	Drakkar	Darmor nain	Vivol	Link	Jaguar	Aligator	Bristol	Falcon	Wesbrook
Yudal	0,83										
Drakkar	0,39	0,61									
Darmor nain	0,56	0,44	0,33								
Vivol	0,50	0,61	0,33	0,39							
Link	0,50	0,61	0,22	0,39	0,17						
Jaguar	0,50	0,56	0,33	0,33	0,44	0,28					
Aligator	0,56	0,56	0,28	0,33	0,11	0,11	0,39				
Bristol	0,61	0,56	0,33	0,39	0,28	0,28	0,39	0,22			
Falcon	0,56	0,61	0,33	0,39	0,17	0,17	0,39	0,11	0,22		
Wesbrook	0,66	0,61	0,56	0,61	0,61	0,61	0,50	0,56	0,61	0,56	
Goeland	0,66	0,39	0,44	0,17	0,50	0,33	0,33	0,44	0,39	0,50	0,66

Table 6. Genetic distance between the 12 oilseed rape genotypes estimated using 18 DNA markers (6 SSRs and 12 STS)

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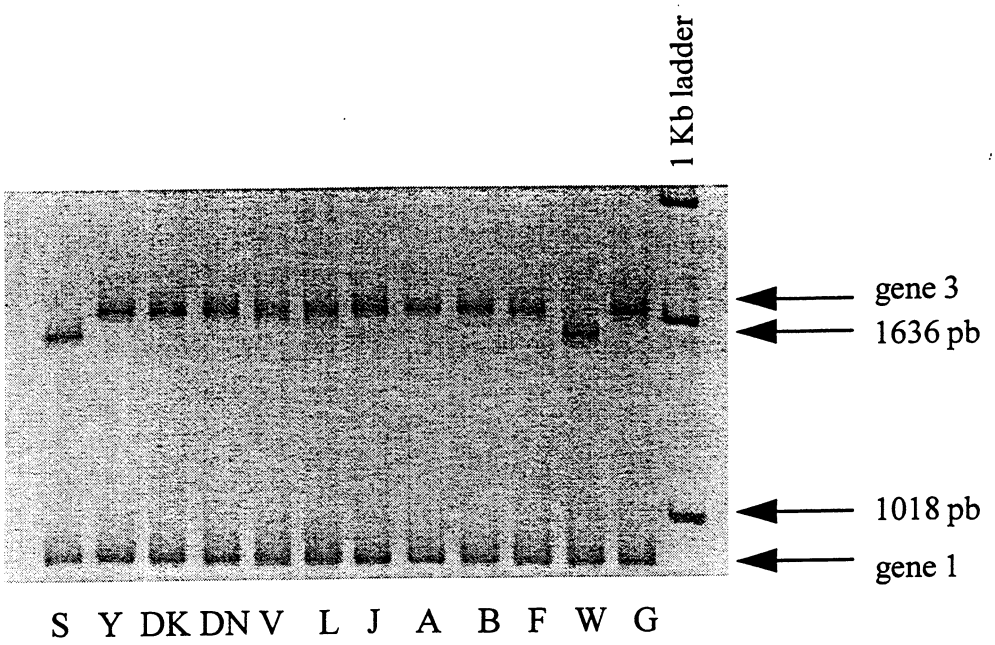

Fig.1 : Polymorphism of STS locus FAD7 on 12 genotypes of oilseed rape, resolved on 5% acrylamide gel with silver staining.

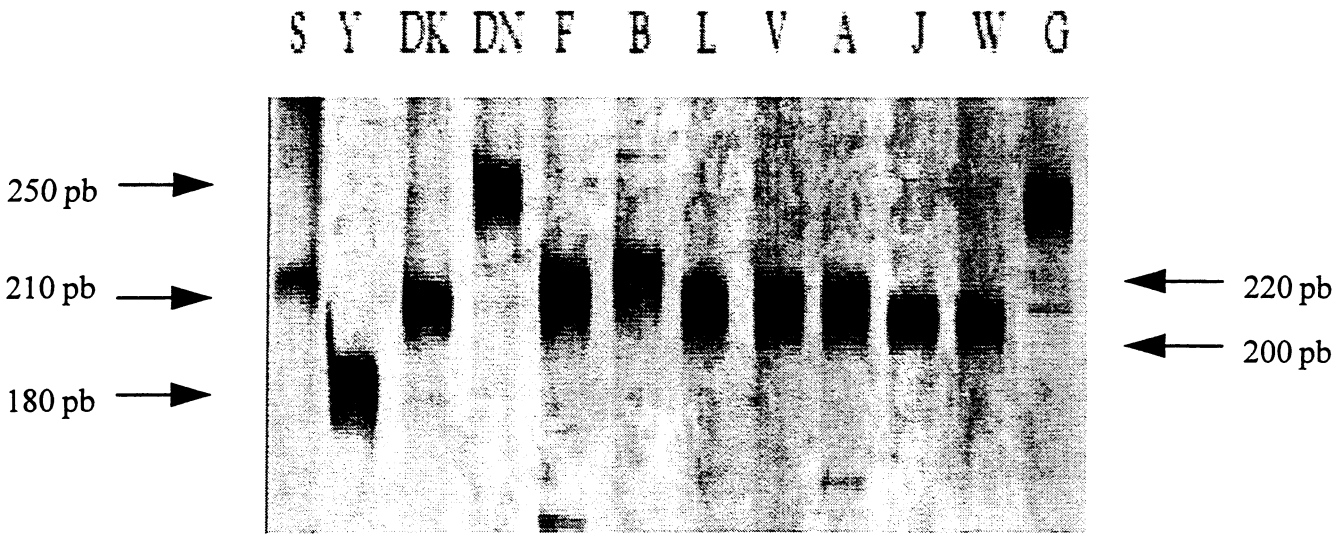

Fig.2 : Polymorphism of SSR locus Bn9A on 12 genotypes of oilseed rape, resolved on 10% acrylamide gel with silver staining.

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