© Indian Academy of Sciences

RESEARCH ARTICLE

AFLP studies on downy-mildew-resistant and downy-mildew-susceptible genotypes of opium poppy

MUKESH K. DUBEY[1,3], AJIT K. SHASANY[2*], OM P. DHAWAN[1], ASHUTOSH K. SHUKLA[2] and SUMAN P. S. KHANUJA[2,4]

[1]*Genetics and Plant Breeding Division,* [2]*Genetic Resources and Biotechnology Division, Central Institute of Medicinal and Aromatic Plants (CSIR), P.O. CIMAP, Lucknow 226 015, India*
[3]*Present address: Avesthagen Limited, Discoverer 9th Floor, International Technology Park, Whitefield Road, Bangalore 560 066, India*
[4]*Present address: C41-42, DS, Ramesh Nagar, New Delhi 110 015, India*

Abstract

Downy mildew (DM) caused by *Peronospora arborescens*, is a serious disease in opium poppy (*Papaver somniferum*), which has a world-wide spread. The establishment of DM-resistant cultivars appears to be a sustainable way to control the disease. In this paper, we present the results of a study aimed at the identification of amplified fragment length polymorphism (AFLP) markers for DM-resistance in opium poppy. Three opium poppy genotypes (inbred over about 10 years): Pps-1 (DM-resistant), Jawahar-16 (DM-susceptible) and H-9 (DM-susceptible) were crossed in a diallel manner and the F_1 progeny along with the parents were subjected to AFLP analysis of chloroplast (cp) and nuclear DNA with seven and nine *Eco*RI / *Mse*I primer combinations, respectively. cpDNA AFLP analysis identified 24 Pps-1 (DM-resistant)-specific unique fragments that were found to be maternally inherited in both the crosses, Pps-1 × Jawahar-16 and Pps-1 × H-9. In the case of nuclear DNA AFLP analysis, it was found that 17 fragments inherited from Pps-1 were common to the reciprocal crosses of both (i) Pps-1 and Jawahar-16 as well as (ii) Pps-1 and H-9. This is the first molecular investigation on the identification of polymorphism between DM-resistant and DM-susceptible opium poppy genotypes and development of DM-resistant opium poppy genotype-specific AFLP markers. These AFLP markers could be used in future genetic studies for analysis of linkage to the downy mildew resistance trait.

[Dubey M. K., Shasany A. K., Dhawan O. P., Shukla A. K. and Khanuja S. P. S. 2010 AFLP studies on downy mildew-resistant and -susceptible genotypes of opium poppy. *J. Genet.* **89**, xx–xx]

Introduction

Opium poppy (*Papaver somniferum*) is one of the most important crops due to the medicinal values of its alkaloids like morphine and codeine. Morphine is a preferred analgesic for cancer patients and codeine is a cough depressant (Kapoor 1995). There has been a constant increase in the global consumption of morphine in the last 10 years (International Narcotics Control Board 2009). To ensure a sufficient supply of opiates for legitimate medical needs, opium poppy is cultivated on a licit basis in a number of countries under the supervision of International Narcotics Control Board (INCB). Seeds obtained from the poppy capsules contain 48–52% oil, which have cholesterol lowering property (Singh *et al.* 1990). Seeds are edible and also used in confectionary etc. (Nergiz and Otles 1994). However, the poppy crop is seriously affected by downy mildew (DM) disease caused by the fungus, *Peronospora arborescens*. The pathogen attacks the plant at seedling stage and produces two kinds of symptoms, viz. systemic and topical. The plants with systemic infection die prematurely thereby causing severe loss in terms of seeds and alkaloids (Sattar *et al.* 1995). The biochemical mechanism of DM infection and the resistance response of the plant have been recently studied in detail (Dubey *et al.* 2010).

The disease can be controlled by means of fungicides, but such treatments are undesirable due to their cost and, particularly, their failure to achieve sustainable productivity. Identi-

*For correspondence. E-mail: akshasany@yahoo.com.

Keywords. AFLP; disease resistance; DNA markers; downy mildew; *Papaver somniferum*; *Peronospora arborescens*.

fication of stable resistance sources and development of DM-resistant cultivars thus appears to be the best eco-compatible way to control the disease. Few distinct sources of resistance to DM in opium poppy have been identified (Dubey 2008; Dubey *et al.* 2009a; Singh *et al.* 2003). Inheritance studies for DM resistance have shown recessive nature of the gene involved, and cytoplasmic control of the disease has also been reported (Dhawan *et al.* 1998; Dubey *et al.* 2009a,b). DM spreads rapidly under humid conditions by secondary infection through conidia produced on systemically infected plants (Sattar *et al.* 1995). Therefore, evaluation of breeding population for DM is difficult when weather conditions do not favour strong fungal growth and source of secondary infection is unavailable. Polymorphism analysis as well as development of molecular markers could be helpful in identification of the genome segments associated with DM resistance, which may in turn help in selecting DM-resistant genotypes without the uncertainties involved due to environmental interactions and screening errors.

There are several different DNA analysis procedures that have been used to develop molecular markers. Each procedure has its own requirements, sensitivity, and reliability. AFLP is one of the most reliable DNA fingerprinting techniques, which combines assay flexibility with high degree of sensitivity and reproducibility (Vos *et al.* 1995). The degree of polymorphism detected per reaction is much higher than other DNA fingerprinting techniques (Lin *et al.* 1996). AFLP has been extensively used for developing polymorphic markers associated with disease resistance in different crops like *Hordeum* (Altinkut *et al.* 2003), *Lycopersicon* (Giovanni *et al.* 2004), *Triticum* (Najimi *et al.* 2002), *Malus* (James *et al.* 2004), *Zea mays* (Agrama *et al.* 2002), *Oryza* (Jain *et al.* 2004), *Brassica* (Farinho *et al.* 2000), *Solanum* (Meksem *et al.* 1995), *Nicotiana* (Nishi *et al.* 2003), *Medicago* (Obert *et al.* 2000) etc. Although Saunders *et al.* (2001) have employed AFLP to analyse genetic diversity in opium poppy, there is no report yet on the identification and development of AFLP-based molecular markers for DM-resistance in the plant. Development of such molecular markers for DM-resistance in opium poppy would provide important information about the existing polymorphism that may be used in future genetic studies for linkage to DM-resistance trait.

The present study reports the identification of nuclear and chloroplast DNA-based DM resistance-specific markers through AFLP analysis of specific DM-resistant and DM-susceptible inbred genotypes and their F_1 hybrids from reciprocal crosses to discriminate between the gene pool of DM-resistant and DM-susceptible genotypes and to study the inheritance pattern of the specific markers.

Material and methods

Plant material and disease evaluation

The plant material consisted of three opium poppy germplasm accessions viz. Pps-1 (DM-resistant), Jawahar-16 (DM-susceptible) and H-9 (DM-susceptible). The genotype Pps-1 is an inbred line with fringed leaf margins and partially petaloid sepals (Dhawan *et al.* 2007). On the other hand, Jawahar-16 is a released variety from Madhya Pradesh, India, having entire leaf margins and fringed petal margins, which has been selfed by us for the past 11 generations and could now be considered as an inbred line. H-9 is an inbred line with yellowish green leaves (suspected chlorophyll variant) with broad leaf margins. These genotypes are maintained in the National Gene Bank for Medicinal and Aromatic Plants at the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India. Disease reactions were scored by calculating the disease severity index (DSI) as described by us earlier (Dubey 2008; Dubey *et al.* 2009a,b). The three accessions along with the F_1 hybrids (DM-resistant × DM-susceptible; including respective reciprocal crosses) were used for AFLP analysis. For nuclear DNA AFLP analysis, six plants selected from the parental lines were analysed for differences in AFLP profiles and found to be similar. Hence, one randomly selected plant from each of the three parental lines were used for crossing. Similarly, exactly similar AFLP profiles were obtained, when 20 F_1 plants from each cross were analysed. Therefore, the AFLP profile of a F_1 plant was compared with parents for analysis of the inheritance pattern. For the cpDNA AFLP experiment, bulked populations of parents as well as F_1's (more than 500 seedlings for each) constituted the material for cpDNA isolation.

DNA isolation and AFLP analysis

Total cellular genomic DNA was isolated and purified from young leaf material of individual genotypes and hybrids following the modified CTAB extraction procedure (Khanuja *et al.* 1999).

Chloroplast DNA (cpDNA) was isolated from 5 g material of 7–10 day-old seedlings using the protocol described by Triboush *et al.* (1998) with some modifications (Dubey 2008). For chloroplast isolation, the seedlings were kept in dark for 48 h prior to harvesting (about 5 g) to reduce the starch content, rinsed, cooled to 0°C and ground in a cold mortar with 20 mL of STE buffer (400 mM sucrose, 50 mM Tris pH 7.8, 20 mM EDTA-Na_2, 0.2% bovine serum albumin, 0.2% β-mercaptoethanol). The last two components of the buffer were added just before the start of the experiment. All the chloroplast isolation steps were performed on ice. The homogenate was filtered through dense (50 μM) nylon mesh and the extract was centrifuged at 1000 rpm (200 g) for 20 min to remove the cellular debris and nuclei. The supernatant was then re-centrifuged at 4000 rpm (3700 g) for 20 min to pellet the chloroplasts. The chloroplast pellet was re-suspended in STE buffer using a soft paint brush and intact chloroplasts were re-pelleted by centrifugation at 4000 rpm (3700 g) for 20 min. Due to differential centrifugation force required for pelleting different cytoplasmic organelles, the chloroplast preparation was free of mitochondria that require a centrifugation at 12000 rpm (18000 g) for pelleting.

To avoid any nuclear DNA from contaminating the cpDNA, a DNase treatment step was applied. The isolated chloroplasts were homogenized in a minimal volume (not more than 0.2 mL) of ST buffer (400 mM sucrose, 50 mM Tris pH 7.8, 0.1% bovine serum albumin). DNase-1 (125 μg) was freshly dissolved in 0.2 mL of ST buffer with 0.02 M $MgCl_2$ and added to the chloroplast suspension (the final volume was then adjusted to 0.5 mL to get a DNase concentration of 250 μg/mL). Incubation with DNase was carried out at 37°C for 20 min. The reaction was stopped by adding EDTA-Na_2 to a final concentration of 0.2 M. DNase wash-off was performed in a special NETF buffer (1.25 M NaCl, 50 mM EDTA, 50 mM Tris pH 8.0, 50 mM NaF). Chloroplasts were pelleted by centrifugation at 4000 rpm (3700 g) for 20 min. For cpDNA isolation the chloroplast pellet was resuspended in TEN buffer (100 mM Tris pH 7.2, 50 mM EDTA, 100 mM NaCl and 0.2% β-mercaptoethanol) and lysed in 1% SDS at 60°C for 30–60 min. Extraction with phenol, phenol:chloroform (1:1) and chloroform:isoamyl alcohol (24:1) in that order was used for removal of proteins. The cpDNA in the supernatant was precipitated with 1/10 volume of 5 M ammonium acetate and 1 volume of isopropanol at −20°C for 2–3 h. The DNA pellet was washed repeatedly with ethanol (70%, 96%), air-dried, and redissolved upto 20 μL TE buffer (1 mM Na_2-EDTA pH 8.0, 10 mM Tris-HCl pH 8.0). Additional cleaning of cpDNA was carried out by potassium acetate-SDS precipitation as per the modified method of Wilson and Chourey (1984). After complete dissolution of cpDNA in TE buffer it was heated briefly with TEN buffer and SDS and finally potassium acetate was added. The mixture was shaken for few minutes (until the precipitate was dissolved) and then frozen for 30 min. The mixture was centrifuged and the supernatant was collected carefully avoiding the transfer of pellet particles. The supernatant was poured into ammonium acetate and isopropanol, mixed and incubated at −20°C for 30 min. The precipitated cpDNA was pelleted and washed repeatedly with ethanol (70%, 96%), dried at room temperature, and redissolved in TE buffer.

AFLP analysis was performed using the AFLP analysis kit (Applied Biosystems, Foster City, USA) following the manufacturer's guidelines and the protocols described by Shasany *et al.* (2005). Initially, the explorer gel for all 64 primer combinations (eight florescent labelled *Eco*RI primers and eight unlabelled *Mse*I primers) with nuclear and cpDNA of Pps-1 was run to determine the most responsive primer combinations for the poppy genome. The nine primer combinations: *Eco*RI-CAT/*Mse*I-ACA, *Eco*RI-CTG/*Mse*I-AAC, *Eco*RI-CAT/*Mse*I-ACT, *Eco*RI-CTC/*Mse*I-ACA, *Eco*RI-CTG/*Mse*I-AGC, *Eco*RI-CAC/*Mse*I-ACG, *Eco*RI-CTC/*Mse*I-AGC, *Eco*RI-CTC/*Mse*I-AGG and *Eco*RI-CAC/*Mse*I-AGG, generating the maximum number of fragments for nuclear DNA, were selected from the explorer gel. Similarly, seven primer combinations: *Eco*RI-CAA/*Mse*I-ACT, *Eco*RI-CTT/*Mse*I-ACG, *Eco*RI-CTC/*Mse*I-AGC, *Eco*RI-CAC/*Mse*I-ACT, *Eco*RI-CAC/*Mse*I-ACA, *Eco*RI-CAC/*Mse*I-AAG and *Eco*RI-CAG/*Mse*I-ACT, generating the maximum number of fragments with the cpDNA were selected. Further, all the accessions were subjected to selective amplifications with these selected primer combinations. The AFLP profiles were analysed using GeneScan analysis software version 3.1 (Applied Biosystems, Foster City, USA). For quantification of similarity, pairwise comparison of banding pattern were made by calculating indices of similarity using the similarity coefficient methods of Nei and Li (1979).

Results

The present study describes the detection of inherited DNA markers in reciprocal F_1 hybrids (Pps-1 × Jawahar-16, DSI (disease severity indices) = 63.27; Jawahar-16 × Pps-1, DSI = 69.99; Pps-1 × H-9, DSI = 56.99; H-9 × Pps-1, DSI = 66.57) between the DM-resistant (Pps-1, DSI = 8.33) and susceptible (Jawahar-16, DSI = 88.87; and H-9, DSI = 72.21) genotypes through AFLP. A total of 64 (8 *Eco*RI × 8 *Mse*I) primer combinations were used with both nuclear and cpDNA samples of Pps-1 to identify the best primer combinations that generated maximum number of fragments. From the 64 combinations, nine and seven primer combinations were selected for selective amplification of nuclear and chloroplast DNA, respectively.

AFLP analysis of chloroplast DNA

A total of 734 fragments were obtained from these three selected genotypes with the seven primer combinations ranging in size from 45 to 500 bp. The AFLP analysis of individual genotypes with seven primer combinations resulted in the amplification of 211 fragments for Pps-1, 258 fragments for Jawahar-16 and 265 for H-9. Of the 211 fragments obtained for Pps-1, 167 (79.15%) were found to be monomorphic, and 20 (9.48%) were polymorphic whereas 24 (11.37%) were unique. Of the 258 fragments generated in the case of Jawahar-16, 167 DNA fragments were monomorphic (64.73%), 71 (27.52%) were polymorphic and only 20 (7.75%) DNA fragments were found to be unique. In the case of H-9, 265 fragments were generated, of which 167 (63.02%) fragments were observed to be monophorphic whereas, 77 (29.06%) and 21 (7.92%) fragments were found to be polymorphic and unique, respectively. The size of unique fragments generated for the selected genotypes with different primer combinations are given in table 1.

To study the inheritance pattern of unique cpDNA fragments obtained from the DM resistant (Pps-1) and susceptible (Jawahar-16 and H-9) genotypes of opium poppy, AFLP analysis of F_1 progeny obtained from the reciprocal crosses (Pps-1 × Jawahar-16, Jawahar-16 × Pps-1; Pps-1 × H-9, H-9 × Pps-1 and Jawahar-16 × H-9, H-9 × Jawahar-16) were carried out with their parents (Pps-1, Jawahar-16 and H-9) using the same primer combinations, which were used for parental

cpDNA analysis. A total of 1386 fragments were generated from six crosses (three forward and three reciprocals) with the selected seven primer combinations, of which, 792 (57.14%) fragments were monomorphic, 589 (42.50%) were polymorphic and only five (0.36%) (3 for Pps-1 × Jawahar-16 and 2 for Jawahar-16 × Pps-1) were found to be unique. Of the 1386 fragments, 207 fragments were generated for the cross Pps-1 × Jawahar-16, 252 for Jawahar-16 × Pps-1, 201 for Pps-1 × H-9, 252 for H-9 × Pps-1, 233 for Jawahar-16 × H-9 and 241 fragments for H-9 × Jawahar-16.

The average similarity indices calculated showed that the similarity between the two susceptible genotypes (H-9 and Jawahar-16) was higher (75%) than that between the resistant (Pps-1) and susceptible genotypes (62% and 56% with H-9 and Jawahar-16, respectively) (table 2). This implies that susceptible genotypes, Jawahar-16 and H-9, were closer to each other than to the resistant genotype, Pps-1. The high level of polymorphism found with AFLP markers enabled an estimation of genetic variability in cpDNA of resistant and susceptible genotypes of opium poppy. The hybrids of resistant and susceptible genotypes, in which Pps-1 was selected as a female parent and Jawahar-16 as a male parent, showed 93% similarity to the female parent (Pps-1) and only 55% to the male parent (Jawahar-16). Similarly, the F_1 hybrids of the reciprocal cross in which Pps-1 was selected as male parent and Jawahar-16 as the female parent showed 93% similarity to the female parent (Jawahar-16) and only 55% similarity to the male parent (Pps-1). The results obtained from the reciprocal crosses of Pps-1 and H-9, were on similar lines (table 2). These results clearly indicate the predominance of maternal inheritance of cpDNA in opium poppy. However, the similarity index of the maternal parent with it's cognate F_1 hybrid is ~0.9 rather than the theoretically expected 1.0 and the reason for this may be either paternal inheritance of some cpDNA as observed in case of some plants (Shore and Triassi 1998) or possibly due to amplification of some contaminating nuclear DNA (even though DNase treatment was given to the chloroplast preparation). The identification of polymorphism and specific AFLP markers on the organelle (chloroplast) genome of the DM-resistant genotype would provide a tool for future genetic studies aimed at elucidating the linkage to the DM resistance trait in opium poppy.

Table 1. Size distribution of unique AFLP fragments generated from cpDNA of three selected opium poppy genotypes.

Primer combination (*Mse*I/*Eco*RI)	Genotypes Jawahar-16 (DM-susceptible)	Pps-1 (DM-resistant)	H-9 (DM-susceptible)
CAA/ACT	60, 63, 68, 99, 117, 184, 185, 194	66, 86, 109	369
CTT/ACG	59	230, 234	48, 113
CTC/AGC	68	62, 65, 91	
CAC/ACT	55, 281	63, 65, 94, 122, 130, 132, 149, 225, 229, 234	91, 163
CAC/ACA	87, 114, 147, 297	93, 196, 234	275
CAC/AAG	156	203, 287	62, 81
CAG/ACT	84, 369, 372	110	182, 252, 254, 280, 286, 295, 298, 306, 311, 319, 325, 328, 334

Figures indicate the size of AFLP fragments in bp.

Table 2. Similarity indices for the opium poppy parents and their reciprocal hybrids obtained through cpDNA AFLP analysis.

	Jawahar-16	Pps-1	H-9	Pps-1 × Jawahar-16	Jawahar-16 × Pps-1	Pps-1 × H-9	H-9 × Pps-1
Jawahar-16	1						
Pps-1	0.56	1					
H-9	0.75	0.62	1				
Pps-1 × Jawahar-16	0.55	0.93	0.60	1			
Jawahar-16 × Pps-1	0.93	0.55	0.74	0.56	1		
Pps-1 × H-9	0.55	0.92	0.60	0.90	0.56	1	
H-9 × Pps-1	0.70	0.61	0.92	0.59	0.72	0.62	1

Specific unique fragment profiles of parental genotypes (DM-resistant and DM-susceptible) were compared with those of their respective reciprocal F_1 hybrids. Most of the unique fragments were found to be inherited maternally and were present in their respective F_1 hybrids (table 3). Twenty-four Pps-1, (DM-resistant) specific unique fragments were found to be maternally inherited in both the crosses, Pps-1 × Jawahar-16 and Pps-1 × H-9, and these may serve as candidates for markers for DM resistance in opium poppy in future genetic studies (table 3). The results obtained in this study indicated that one could reveal hybridization patterns distinctly through AFLP analysis.

Table 3. Inheritance pattern of the chloroplast DNA marker fragments generated through AFLP analysis of Pps-1, Jawahar-16, H-9 and their reciprocal hybrids.

Fragment size (bp)*	Pps-1	Jawahar-16	H-9	Pps-1 × Jawahar-16	Jawahar-16 × Pps-1	Pps-1 × H-9	H-9 × Pps-1
DSI (mean)	8.33	88.87	72.21	63.27	69.99	56.99	66.57
*Mse*I CAA, *Eco*RI ACT							
60	−	+	−	−	+	−	−
63	−	+	−	−	+	−	−
66	**+**	**−**	**−**	**+**	**−**	**+**	**−**
68	−	+	−	−	+	−	−
86	**+**	**−**	**−**	**+**	**−**	**+**	**−**
109	**+**	**−**	**−**	**+**	**−**	**+**	**−**
117	−	+	−	−	+	−	−
184	−	+	−	−	+	−	−
185	−	+	−	−	+	−	−
194	−	+	−	−	+	−	−
369	−	−	+	−	−	−	+
*Mse*I CTT, *Eco*RI ACG							
48	−	−	+	−	−	−	+
59	−	+	−	−	+	−	−
113	−	−	+	−	−	−	+
230	**+**	**−**	**−**	**+**	**−**	**+**	**−**
234	**+**	**−**	**−**	**+**	**−**	**+**	**−**
*Mse*I CTC, *Eco*RI AGC							
62	**+**	**−**	**−**	**+**	**−**	**+**	**−**
65	**+**	**−**	**−**	**+**	**−**	**+**	**−**
68	−	+	−	−	+	−	−
91	**+**	**−**	**−**	**+**	**−**	**+**	**−**
*Mse*I CAC, *Eco*RI ACT							
55	−	+	−	−	+	−	−
63	**+**	**−**	**−**	**+**	**−**	**+**	**−**
65	**+**	**−**	**−**	**+**	**−**	**+**	**−**
91	−	−	+	−	−	−	+
94	**+**	**−**	**−**	**+**	**−**	**+**	**−**
122	**+**	**−**	**−**	**+**	**−**	**+**	**−**
130	**+**	**−**	**−**	**+**	**−**	**+**	**−**
132	**+**	**−**	**−**	**+**	**−**	**+**	**−**
149	**+**	**−**	**−**	**+**	**−**	**+**	**−**
163	−	−	+	−	−	−	+
225	**+**	**−**	**−**	**+**	**−**	**+**	**−**
229	**+**	**−**	**−**	**+**	**−**	**+**	**−**
234	**+**	**−**	**−**	**+**	**−**	**+**	**−**
281	−	+	−	−	+	−	−
*Mse*I CAC, *Eco*RI ACA							
87	−	+	−	−	+	−	−
93	**+**	**−**	**−**	**+**	**−**	**+**	**−**
114	−	+	−	−	+	−	−
147	−	+	−	−	+	−	−
196	**+**	**−**	**−**	**+**	**−**	**+**	**−**
234	**+**	**−**	**−**	**+**	**−**	**+**	**−**
275	−	−	+	−	−	−	+

Table 3 (*contd*)

Fragment size (bp)*	Pps-1	Jawahar-16	H-9	Pps-1 × Jawahar-16	Jawahar-16 × Pps-1	Pps-1 × H-9	H-9 × Pps-1
297	−	+	−	−	+	−	−
*Mse*I CAC, *Eco*RI AAG							
62	−	−	+	−	−	−	+
81	−	−	+	−	−	−	+
203	**+**	**−**	**−**	**+**	**−**	**+**	**−**
287	**+**	**−**	**−**	**+**	**−**	**+**	**−**
*Mse*I CAG, *Eco*RI ACT							
84	−	+	−	−	+	−	−
110	**+**	**−**	**−**	**+**	**−**	**+**	**−**
182	−	−	+	−	−	−	+
252	−	−	+	−	−	−	+
254	−	−	+	−	−	−	+
280	−	−	+	−	−	−	+
286	−	−	+	−	−	−	+
295	−	−	+	−	−	−	+
298	−	−	+	−	−	−	+
306	−	−	+	−	−	−	+
311	−	−	+	−	−	−	+
319	−	−	+	−	−	−	+
325	−	−	+	−	−	−	+
328	−	−	+	−	−	−	+
334	−	−	+	−	−	−	+

*Only those unique fragments of the parental genotypes that have been inherited in F_1 progenies have been listed in this table. cpDNA fragments common to both the crosses, Pps-1 × Jawahar-16 and Pps-1 × H-9, are indicated in bold. DSI, disease severity index.

Nuclear DNA AFLP analysis

The nuclear DNA of the DM-resistant (Pps-1) and susceptible (Jawahar-16 and H-9) poppy genotypes was also analysed through AFLP. A total of 904 fragments were generated from these three selected genotypes with nine primer combinations, of which 319 were generated in Jawahar-16, 305 in Pps-1 and 280 in H-9. In Jawahar-16, 229 (71.79%) fragments were monomorphic, 48 (15.04%) were polymorphic and 42 (13.17%) were found to be unique. In Pps-1, 229 (75.08%) fragments were monomorphic, 22 (7.22%) were polymorphic and 54 (17.70%) were unique, while in H-9, 229 (81.78%) fragments were monomorphic, 40 (14.29%) were polymorphic and 11 (3.93%) were found to be unique. The number and size distribution of the unique fragments generated with the different selected primer combinations for the three selected genotypes are given in table 4. Polymorphism obtained through AFLP analysis between resistant and susceptible genotypes would be helpful in differentiating specific (resistant/susceptible) cultivars at a very young plant age. The DM-resistant and DM-susceptible genotype-specific unique DNA fragments could be used to develop DM-resistance/susceptibility specific markers. On the basis of average similarity indices (table 5), it was found that both the susceptible genotypes (Jawahar-16 and H-9) were 81% similar while the DM-resistant genotype (Pps-1) showed only 67% and 69% similarity to Jawahar-16 and H-9, respectively.

AFLP analysis of F_1 hybrids obtained from the reciprocal crosses of DM-resistant and DM-susceptible parents (Pps-1 × Jawahar-16, Jawahar-16 × Pps-1, Pps-1 × H-9, H-9 × Pps-1 and Jawahar-16 × H-9 and H-9 × Jawahar-16), was carried out with the parents (Pps-1, Jawahar-16 and H-9) using the same primer combinations that were used for analysis of the parents. This study was carried out with the aim to detect the inheritance pattern of the marker DNA fragments, which were obtained in DM resistant (Pps-1) and susceptible (Jawahar-16 and H-9) genotypes, in their reciprocal hybrids. A total of 2185 fragments were generated from the six crosses (three forward and three reciprocals) with the selected nine primer combinations, of which 1572 (71.95%) were monomorphic and 613 (28.05%) were polymorphic. Of the 2185 fragments, 377 were generated for the cross Pps-1 × Jawahar-16, 382 for Jawahar-16 × Pps-1, 362 for Pps-1 × H-9, 322 for H-9 × Pps-1, 378 for Jawahar-16 × H-9 and 364 for H-9 × Jawahar-16.

Thirty-one of the 54 unique marker fragments generated in DM-resistant genotype Pps-1 and 26 of the 42 unique marker fragments generated in DM-susceptible genotype Jawahar-16 were found in the reciprocal hybrids of Pps-1 and Jawahar-16 (table 6). In the reciprocal hybrid of Pps-1 and H-9 genotypes, 18 marker fragments were found to be inherited

Table 4. Size distribution of unique AFLP fragments generated from the nuclear DNA of three selected opium poppy genotypes.

Primer combination (*Mse*I/*Eco*RI)	Genotypes		
	Jawahar-16 (DM-susceptible)	Pps-1 (DM-resistant)	H-9 (DM-susceptible)
CAT/ACA	229, 306	206, 268	–
CTC/AGG	72, 75, 95, 103, 169, 182, 207, 210, 218, 235, 261, 295, 312, 350, 408	127, 159, 174, 180, 209, 216, 242, 252, 259, 273, 285, 290, 311, 354, 355, 373, 375, 415, 422, 442, 486	–
CAT/ACT	55, 266	83, 92, 99, 112, 147, 181, 183, 249, 253, 264, 313, 343, 385, 401	–
CAC/AGG	–	68, 168	178, 205
CAC/ACG	87, 129, 132, 151, 170, 183, 187, 199, 204, 205, 217, 218, 337, 396	–	139, 213, 260, 394
CTC/AGC	219	70	–
CTG/AAC	249	62, 79, 136, 142, 153, 197, 211, 230	–
CTG/AGC	88, 122, 192, 230, 240, 272, 292	178, 200	114, 132, 146, 194, 233
CTC/ACA	–	61, 72, 156, 164	–

Figures indicate the size of AFLP fragments in bp.

Table 5. Similarity indices for three opium poppy genotypes, Jawahar-16, Pps-1 and H-9 obtained through nuclear DNA AFLP analysis.

Genotypes	Jawahar-16	Pps-1	H-9
Jawahar-16	1.00		
Pps-1	0.67	1.00	
H-9	0.81	0.69	1.00

from Pps-1 and eight fragments (out of 11) were found from the DM-susceptible genotype H-9 (table 6). Of the 31 fragments inherited from Pps-1 in the reciprocal crosses of Pps-1 and Jawahar-16 and the 18 fragments inherited from Pps-1 in the reciprocal crosses of Pps-1 and H-9, 17 fragments were found to be common, and these may be used as candidates for development of markers for DM-resistance in future genetic linkage studies (table 6). The results also indicated that a number of unique fragments present in Pps-1 were not inherited in its reciprocal F_1 hybrids with H-9 (for example 159-bp and 174-bp fragments generated with *Mse*I CTC/*Eco*RI AGG) and Jawahar-16 (for example 112-bp fragment generated with *Mse*I CAT/*Eco*RI ACT). The reasons for the same are unclear.

Discussion

Recently, genetic study of DM resistance in opium poppy revealed an involvement of cytoplasmic genes in addition to nuclear control (Dubey 2008; Dubey *et al.* 2009a,b). Nucleotide variations have been identified in the chloroplast encoded *rpoC1* gene in DM-resistant genotype (Pps-1) of opium poppy, which indicates a possible role of RNA polymerase β' subunit in resistance to *P. arborescens* (Dubey *et al.* 2009b). However the involvement of other genes in the development of DM-resistance in opium poppy could not be ruled out. Therefore, in the present study, AFLP analyses of chloroplast (cp) (as cytoplasmic factor) and nuclear DNA of DM-resistant (Pps-1), and DM-susceptible (Jawahar-16 and H-9) genotypes along with their normal and reciprocal hybrids were carried out in order to identify DM resistance-specific DNA markers based on the differences found in the AFLP banding pattern of DM-resistant and DM-susceptible genotypes. The highly stable DM-resistant line Pps-1 can be considered a potential germplasm donor and molecular marker technology may prove to be very useful for identifying DM-resistance specific DNA fragments in opium poppy. The parental origin of DNA markers as well as their allelic state was directly inferred from the presence of the DNA fragment in one parent, its absence in other and its inheritance in the F_1 progeny.

The advantage of AFLP analysis of the genotypes of a plant species with a narrow genetic base such as poppy is that a higher level of polymorphism could be detected with

Table 6. Co-inheritance pattern of the nuclear DNA marker fragments generated through AFLP analysis of reciprocal F_1 hybrids of Pps-1, Jawahar-16 and H-9.

Fragment size (bp)*	Pps-1	Jawahar-16	H-9	Pps-1 × Jawahar-16	Jawahar-16 × Pps-1	Pps-1 × H-9	H-9 × Pps-1
DSI (mean)	8.33	88.87	72.21	63.27	69.99	56.99	66.57
*Mse*I CTC, *Eco*RI AGG							
127	**+**	**−**	**−**	**+**	**+**	**+**	**+**
159	+	−	−	+	+	−	−
174	+	−	−	+	+	−	−
180	**+**	**−**	**−**	**+**	**+**	**+**	**+**
209	+	−	−	+	+	−	−
216	**+**	**−**	**−**	**+**	**+**	**+**	**+**
242	+	−	−	+	+	−	−
252	+	−	−	+	+	−	−
259	+	−	−	+	+	−	−
285	+	−	−	+	+	−	−
290	+	−	−	+	+	−	−
311	**+**	**−**	**−**	**+**	**+**	**+**	**+**
354	**+**	**−**	**−**	**+**	**+**	**+**	**+**
355	+	−	−	+	+	−	−
373	**+**	**−**	**−**	**+**	**+**	**+**	**+**
375	**+**	**−**	**−**	**+**	**+**	**+**	**+**
415	**+**	**−**	**−**	**+**	**+**	**+**	**+**
442	**+**	**−**	**−**	**+**	**+**	**+**	**+**
486	+	−	−	+	+	−	−
72	−	+	−	+	+	−	−
95	−	+	−	+	+	−	−
103	−	+	−	+	+	−	−
182	−	+	−	+	+	−	−
207	−	+	−	+	+	−	−
210	−	+	−	+	+	−	−
218	−	+	−	+	+	−	−
235	−	+	−	+	+	−	−
261	−	+	−	+	+	−	−
295	−	+	−	+	+	−	−
312	−	+	−	+	+	−	−
350	−	+	−	+	+	−	−
408	−	+	−	+	+	−	−
*Mse*I CAT, *Eco*RI ACT							
92	**+**	**−**	**−**	**+**	**+**	**+**	**+**
99	**+**	**−**	**−**	**+**	**+**	**+**	**+**
112	+	−	−	−	−	+	+
183	**+**	**−**	**−**	**+**	**+**	**+**	**+**
55	−	+	−	+	+	−	−
*Mse*I CAC, *Eco*RI AGG							
68	+	−	−	+	+	−	−
178	−	−	+	−	−	+	+
205	−	−	+	−	−	+	+
*Mse*I CAC, *Eco*RI ACG							
132	−	+	−	+	+	−	−
139	−	−	+	−	−	+	+
151	−	+	−	+	+	−	−
205	−	+	−	+	+	−	−
213	−	−	+	−	−	+	+
218	−	+	−	+	+	−	−
*Mse*I CTC, *Eco*RI AGC							

Table 6 (*contd*)

Fragment size (bp)*	Pps-1	Jawahar-16	H-9	Pps-1 × Jawahar-16	Jawahar-16 × Pps-1	Pps-1 × H-9	H-9 × Pps-1
219	−	+	−	+	+	−	−
*Mse*I CTG, *Eco*RI AAC							
79	**+**	**−**	**−**	**+**	**+**	**+**	**+**
197	**+**	**−**	**−**	**+**	**+**	**+**	**+**
211	+	−	−	+	+	−	−
230	+	−	−	+	+	−	−
*Mse*I CTG, *Eco*RI AGC							
88	−	+	−	+	+	−	−
114	−	−	+	−	−	+	+
122	−	+	−	+	+	−	−
132	−	−	+	−	−	+	+
146	−	−	+	−	−	+	+
192	−	+	−	+	+	−	−
230	−	+	−	+	+	−	−
233	−	−	+	−	−	+	+
240	−	+	−	+	+	−	−
272	−	+	−	+	+	−	−
292	−	+	−	+	+	−	−
*Mse*I CTC, *Eco*RI ACA							
61	**+**	**−**	**−**	**+**	**+**	**+**	**+**
72	+	−	−	+	+	−	−
156	**+**	**−**	**−**	**+**	**+**	**+**	**+**
164	**+**	**−**	**−**	**+**	**+**	**+**	**+**

*Only those unique fragments of the parental genotypes that have been inherited in F_1 progenies have been listed in this table. Nuclear DNA fragments common to both the crosses, Pps-1 x Jawahar-16 and Pps-1 x H-9, are indicated in bold. DSI, disease severity index.

fewer primer combinations, as evident in the present study. Our results are in line with the earlier reports that majority of flowering plants exhibit maternal mode of inheritance of chloroplast DNA (cpDNA) (Corriveau and Coleman 1988; Mogensen 1996). However, numerous exceptions have also been documented (Corriveau and Coleman 1988; Boblenz *et al.* 1990; Shore and Triassi 1998). In contrast, cpDNA has been reported to be paternally inherited in most of the gymnosperms (Mogensen 1996). However, maternal inheritance of the chloroplast genome has also been demonstrated in ferns (Guillon and Raquin 2000) and in red algae (Zuccarello *et al.* 1999). Variations at the cpDNA level have been used earlier to clarify the systematics of plants (Llaca *et al.* 1994). The chloroplast genome is well characterized and structurally vary stable (Clegg and Zurawski 1992), rarely recombines and has a slow structural evolutionary change (Palmer and Delwiche 1998). The specific AFLP markers detected in cpDNA of DM-resistant genotype would be stable and could be useful for genetic studies for DM-resistance.

The inferences in the inheritance pattern of the AFLP fragments were made on the basis of DSI. For example, in the case of the cross between Pps-1 and Jawahar-16, the DSI was 63.27 whereas it was 69.99 in the reciprocal cross, which is significantly higher at 1% and 5% levels (Dubey 2008; Dubey *et al.* 2009a). The difference indicates the increment due to chloroplast genome. Similar was the case for the cross between Pps-1 and H-9.

When the DSI of the cross between Pps-1 and Jawahar-16 (63.27) was compared with that of the parents (8.33 and 88.87, respectively), the tendency of F_1 was found to lie towards the DM-susceptible parent, which was found to increase in the reciprocal cross (69.99). If the increase is assumed to be due to the maternal inheritance, still the F_1 are more DM-susceptible than the DM-resistant parent (Pps-1) and less DM-susceptible than the DM-susceptible parent (Jawahar-16). This formed the basis for scoring the AFLP markers for nuclear genome. Similar interaction was observed in the crosses between Pps-1 and H-9. The normal and reciprocal crosses of both the DM-susceptible genotypes (Jawahar-16 and H-9) have similar DSI (77.77) (Dubey 2008; Dubey *et al.* 2009a) with non-significant difference, which indicates the role of nuclear and cytoplasm interaction in DM resistance mechanism. These considerations formed the basis of scoring the AFLP markers.

A large number of studies have used DNA markers as tools to identify genes, QTLs, or to introduce new charac-

ters in elite germplasm lines (Rommens and Kishore 2000). This is the first molecular investigation on the identification of polymorphism and AFLP markers in DM-resistant and DM-susceptible genotypes of opium poppy and will form the basis for future development of linkage-based DM-resistance-specific markers. Such markers could enable the opium poppy breeders to tag and follow the inheritance of specific chromosome segments that are linked to DM-resistance traits from natural lines into improved cultivars. The markers could also be useful for defining DM-resistant genotypes while at an early seedling stage without uncertainties due to environmental interactions for disease development and predicting morphological parameters of adult plants. The identification of DM-resistance-specific nuclear and chloroplast DNA-based AFLP markers would also be useful for the genetic analysis of poppy crop; especially for the identification of disease resistance genes, construction of genetic linkage and quantitative loci maps, and for marker-assisted selection.

Acknowledgements

This work was supported by the financial assistance provided by the Council of Scientific and Industrial Research (CSIR), Government of India, New Delhi (in the form of Senior Research Fellowship 2-56/2002(I)EU.II to M. K. Dubey and funding in the Eleventh Five Year Plan CSIR Network Project NWP08).

References

Agrama H. A., Houssin S. F. and Tarek M. A. 2002 Cloning of AFLP markers linked to resistance to *Peronosclerospora sorghi* in maize. *Mol. Genet. Genomics* **267**, 814–819.

Altinkut A., Kazan K. and Gozukirmizi N. 2003 AFLP marker linked to water-stress-tolerant bulks in barley (*Hordeum vulgare* L.). *Genet. Mol. Biol*. **26**, 77–82.

Boblenz K., Nothnagel T. and Metzlaff M. 1990 Paternal inheritance of plastids in the genus *Daucus*. *Mol. Gen. Genet*. **220**, 489–491.

Clegg M. T. and Zurawski G. 1992 Chloroplast DNA and the study of plant phylogeny: present status and future prospects. In *Molecular systematics of plants* (ed. P. S. Soltis, D. E. Soltis and J. J. Doyle), pp. 1–13. Chapman and Hall, New York, USA.

Corriveau J. L. and Coleman A. W. 1988 Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 angiosperm species. *Am. J. Bot*. **75**, 1443–1458.

Dhawan O. P., Sharma J. R., Sattar A., Dwivedi S. and Lal R. K. 1998 Inheritance of downy mildew resistance in opium poppy: evidence of cytoplasmic control and paternal / biparental transmission. *J. Med. Aromat. Plant Sci*. **20**, 57–64.

Dhawan O. P., Dubey M. K. and Khanuja S. P. S. 2007 Detection of a true breeding homeotic gene mutant Pps-1 with partially petaloid sepals in opium poppy (*Papaver somniferum* L.) and its genetic behavior. *J. Hered*. **98**, 373–377.

Dubey M. K. 2008 Developing DNA markers for straw alkaloid production and disease resistance in opium poppy (Papaver somniferum L). Ph.D. thesis, University of Lucknow, Lucknow, India.

Dubey M. K., Dhawan O. P. and Khanuja S. P. S. 2009a Downy mildew resistance in opium poppy: resistance sources, inheritance pattern, genetic variability and strategies for crop improvement. *Euphytica* **165**, 177–188.

Dubey M. K., Shasany A. K., Dhawan O. P., Shukla A. K. and Khanuja S. P. S. 2009b Genetic variation revealed in the chloroplast-encoded RNA polymerase β' subunit of downy mildew-resistant genotype of opium poppy. *J. Hered*. **100**, 76–85.

Dubey M. K., Shasany A. K., Dhawan O. P., Shukla A. K. and Khanuja S. P. S. 2010 Lipid peroxidation and antioxidant activities involved in resistance response against downy mildew in opium poppy. *J. Phytopathol*. **158**, 88–92.

Farinho M. J., Coelho P. S., Monteiro A. A. and Leitão J. M. 2000 RAPD and AFLP markers linked to *Peronospora parasitica* resistance genes in broccoli. In *Proceedings of the 3rd ISHS International Symposium on Brassicas* (ed. G. J. King), pp. **75**. Horticulture Research International, 5–9 September 2000, Wellesbourne, Warwick, UK.

Giovanni C. D., Dell'Orco P., Bruno A., Ciccarese F., Lotti C. and Ricciardi L. 2004 Identification of PCR-based markers (RAPD, AFLP) linked to a novel powdery mildew resistance gene (*ol-2*) in tomato. *Plant Sci*. **166**, 41–48.

Guillon J.-M. and Raquin C. 2000 Maternal inheritance of chloroplasts in the horsetail *Equisetum variegatum* (Schleich.). *Curr. Genet*. **37**, 53–56.

International Narcotic Control Board 2009 Report of the International Narcotic Control Board for 2008. United Nations Publication, New York, USA (www.incb.org).

Jain A., Ariyadasa R., Kumar A., Srivastava M. N., Mohan M. and Nair S. 2004 Tagging and mapping of a rice gall midge resistance gene, *Gm8*, and development of SCARs for use in marker-aided selection and gene pyramiding. *Theor. Appl. Genet*. **109**, 1377–1384.

James C. M., Clarke J. B. and Evans K. M. 2004 Identification of molecular markers linked to the mildew resistance gene *Pl-d* in apple. *Theor. Appl. Genet*. **110**, 175–181.

Kapoor L. D. 1995 *Opium poppy - botany, chemistry and pharmacology*. The Haworth Press, Binghamton, NY, USA.

Khanuja S. P. S., Shasany A. K., Darokar M. P. and Kumar S. 1999 Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. *Plant Mol. Biol. Rep*. **17**, 1–7.

Lin J.-J., Kuo J., Ma J., Saunders J. A., Beard H. S., MacDonald M. H. *et al*. 1996 Identification of molecular markers in soybean comparing RFLP, RAPD and AFLP DNA mapping techniques. *Plant Mol. Biol. Rep*. **14**, 156–169.

Llaca V., Delgado Salinas A. and Gepts P. 1994 Chloroplast DNA as an evolutionary marker in the *Phaseolus vulgaris* complex. *Theor. Appl. Genet*. **88**, 646–652.

Meksem K., Leister D., Peleman J., Zabeau M., Salamini F. and Gebhardt C. 1995 A high-resolution map of the vicinity of the *R1* locus on chromosome V of potato based on RFLP and AFLP markers. *Mol. Gen. Genet*. **249**, 74–81.

Mogensen H. L. 1996 The hows and whys of cytoplasmic inheritance in seed plants. *Am. J. Bot*. **83**, 383–404.

Najimi B., Boukhatem N., El Jaafari S., Jlibene M., Paul R. and Jacquemin J.-M. 2002 Amplified fragment length polymorphism (AFLP) analysis of markers associated with *H5* and *H22* Hessian fly resistance genes in bread wheat. *Biotechnol. Agron. Soc. and Environ.* **6**, 79–85.

Nei M. and Li W. H. 1979 Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* **76**, 5269–5273.

Nergiz C. and Otles S. 1994 The proximate composition and some minor constituents of poppy seeds. *J. Sci. Food Agric*. **66**, 117–120.

Nishi T., Tajima T., Noguchi S., Ajisaka H. and Negishi H. 2003 Identification of DNA markers of tobacco linked to bacterial wilt resistance. *Theor. Appl. Genet*. **106**, 765–770.

Obert D. E., Skinner D. Z. and Stuteville D. L. 2000 Association of AFLP markers with downy mildew resistance in autotetraploid alfalfa. *Mol. Breed*. **6**, 287–294.

Palmer J. D. and Delwiche C. F. 1998 The origin and evolution of plastids and their genomes. In *Molecular systematics of plants II DNA sequencing* (ed. D. E. Soltis, P. S. Soltis and J. J. Doyle), pp. 375–409. Kluwer, Boston, USA.

Rommens C. M. and Kishore G. M. 2000 Exploiting the full potential of disease-resistance genes for agricultural use. *Curr. Opinion Biotechnol*. **11**, 120–125.

Sattar A., Samad A., Alam M., Zaim M., Dhawan O. P., Singh S. P., Bajpai S. and Lal R. K. 1995 Screening of opium poppy (*Papaver somniferum*) germplasm for disease resistance. *Curr. Res. Med. Aromat. Plant* **17**, 315–320.

Saunders J. A., Pedroni M. J., Penrose L. D. J. and Fist A. J. 2001 AFLP analysis of opium poppy. *Crop Sci*. **41**, 1596–1601.

Shasany A. K., Darokar M. P., Dhawan S., Gupta A. K., Gupta S., Shukla A. K. *et al*. 2005 Use of RAPD and AFLP markers to identify inter- and intraspecific hybrids of *Mentha. J. Hered*. **96**, 542–549.

Shore J. S. and Triassi M. 1998 Paternally biased cpDNA inheritance in *Turnera ulmifolia* (Turneraceae). *Am. J. Bot*. **85**, 328–332.

Singh S. P., Khanna K. R., Dixit B. S. and Srivastava S. N. 1990 Fatty acid composition of opium poppy (*Papaver somniferum* L.) seed oil. *Indian Agric. Sci*. **60**, 358–359.

Singh H. B., Singh A., Singh P., Srivastava S., Shukla S. and Singh S. P. 2003 Evaluation of germplasm of opium poppy (*Papaver somniferum*) for resistance to downy mildew (*Peronospora arborescens*). *Indian J. Agric. Sci*. **73**, 391–394.

Triboush S. O., Danilenko N. G. and Davydenko O. G. 1998 A method for isolation of chloroplast DNA and mitochondrial DNA from sunflower. *Plant Mol. Biol. Rep*. **16**, 183–189.

Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T., Hornes M. *et al*. 1995 AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res*. **23**, 4407–4414.

Wilson A. J. and Chourey P. S. 1984 A rapid inexpensive method for the isolation of restrictable mitochondrial DNA from various plant sources. *Plant Cell Rep*. **3**, 237–239.

Zuccarello G. C., West J. A., Kamiya M. and King R. J. 1999 A rapid method to score plastid haplotypes in red seaweeds and its use in determining parental inheritance of plastids in the red alga *Bostrychia* (Ceramiales). *Hydrobiologia* **401**, 207–214.

Received 30 March 2009, in final revised form 2 September 2009; accepted 22 September 2009
Published on the Web: 22 February 2010