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Genetic Variation Revealed in the Chloroplast-Encoded RNA Polymerase β′ Subunit of Downy Mildew–Resistant Genotype of Opium Poppy

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Abstract

Two accessions of opium poppy, Pps-1 (dark green leaves, highly resistant to downy mildew [DM]) and H-9 (yellowish green leaves, susceptible to DM), which originated from common progenitor SPS49 were selected, and their F_1 and F_2 progenies showed that leaf color trait was governed by single recessive nuclear gene, whereas DM resistance appeared to be the interaction between cytoplasmic and nuclear genes. Chloroplast DNA (cpDNA) analysis of these 2 accessions through arbitrarily-primed polymerase chain reaction generated a unique fragment in Pps-1. Subsequent sequence analysis upon cloning of this cpDNA fragment revealed its similarity with the plastid-encoded RNA polymerase β′ subunit (*rpoC1*). Full-length *rpoC1* DNA was therefore isolated from both the genotypes that was 2707 bp long with a 658-bp intron (436–1093) and a 2049-bp open reading frame encoding 682 amino acid long polypeptide. Comparative sequence analysis of the *rpoC1* gene from both the genotypes, revealed 4 single-nucleotide substitutions at 4 positions that caused 3 amino acid changes in the protein sequence—1) A to C transversion at position 825 (Glu275Asp), 2) A to G transition at position 1203 (Ile401Met), and 3) T to C transition at position 1422 and G to A transition at position 1423 both in same codon of the reading frame (Ala475Thr). This investigation is the first report indicating base substitution changes in the plastid-encoded *rpoC1* gene in DM-resistant genotypes of opium poppy. This finding may lead to implication of possible role of RNA polymerase β′ subunit in resistance to DM caused by *Peronospora arborescens*.

Key words: *opium poppy, downy mildew, disease resistance, RNA Polymerase β′ subunit, amplification refractory mutation system*

Opium poppy (*Papaver somniferum* L.) produces more than 80 alkaloids belonging to various tetrahydrobenzylisoquinoline-derived classes (Weid et al. 2004), several of which are pharmaceutically important, including the analgesic morphine, the antitussive codeine, the muscle relaxant papaverine, the antitumorigenic drug noscapine, and the antimicrobial agent sanguinarine (Facchini and Park 2003). The seeds obtained from the capsules of the plant are edible and used in confectionary (Nergiz and Ötles 1994). However, the crop suffers from several bacterial, fungal, and viral diseases, of which downy mildew (DM) disease caused by the fungus *Peronospora arborescens* (Berk) de Bary is the most destructive and widespread. It attacks the plants at seedling stage with systemic as well as topical symptoms. Most of the plants with systemic infection die prematurely, whereas those with topical infection survive with reduced growth, seed, and latex yield as compared with healthy plants.

Plants respond to pathogen infection via the *Avr/R* recognition system (Flor 1955; Dangl and Jones 2001). The recognition of pathogen-encoded Avr protein by the product of plant resistance (*R*) gene activates a signal transduction pathway leading to hypersensitive response (HR) and induced systemic acquired resistance (Ryals et al. 1996). However, successful pathogens have evolved specialized strategies to suppress plant defense responses and induce disease susceptibility in otherwise resistant hosts (Abramovitch et al. 2003). Recently, many fungal pathogenicity genes have been identified with roles in the formation of infection structure, cell wall degradation, overcoming or avoiding plant defense, responding to the host environment, production of toxins, and in signal

cascades. These differential mechanisms of tolerance are governed by nuclear and (or) cytoplasmic interactions. During the course of normal breeding and maintenance of germplasm, we could observe the non-Mendellian inheritance of DM tolerance in poppy indicating the involvement of plastids in disease tolerance. Plastid-encoded genes are transcribed by 2 types of RNA polymerases, a nuclear-encoded single subunit enzyme (NEP) homologous to bacteriophage T7 RNA polymerase and a plastid-encoded multisubunit enzyme (PEP) homologous to eubacterial RNA polymerase (Oikawa et al. 2000). Chloroplast genome from several plant species have been sequenced, revealing *rpoA*, *rpoB*, *rpoC1*, and *rpoC2* genes, which encode proteins homologous to α, β, β', and β'' subunits of bacterial RNA polymerase (Stern et al. 1997). Transcription rates of chloroplast genome increase dramatically during chloroplast development, and RNA polymerase increases in parallel with transcription activity (Stern et al. 1997). Transcription and RNA level of *rpoB-rpoC1-rpoC2, rps-16*, rRNA, and some tRNAs are differentially elevated at this stage relative to genes encoding proteins of the photosynthetic apparatus (Rapp et al 1992; Mullet 1993). But PEP specificity is provided by σ-like factors encoded in the nucleus (Tanaka et al. 1996) that recognize promoters similar to those of *E. coli* RNA polymerase with −35 and −10 consensus sequences (Stern et al. 1997).

In the present study, we report for the first time, the identification and cloning of full-length plastid-encoded *rpoC1* gene encoding RNA polymerase β' subunit in opium poppy. We also observed base substitutions in this gene in DM-resistant genotypes of opium poppy that had a common maternal ancestor. The study discusses the possible role of these differences in the plastid-encoded *rpoC1* gene in the plant defense response to DM.

Materials and Methods

Plant Material and Disease Evaluation

The National Gene Bank of Medicinal and Aromatic Plants at Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, possesses a diverse germplasm collection of opium poppy accessions that are collections, mutants, and selections, mostly constituting a homogenous population and narrow genetic base with the exception of few identifiable morphological characters. A preliminary random amplified polymorphic DNA analysis with 20 medicinal and aromatic plant (MAP) primers revealed a maximum polymorphism of 5% (data not shown). These germplasm accessions were evaluated against DM under field and glasshouse conditions during 3 crop seasons (2003–2004, 2004–2005, and 2005–2006). Disease reactions were scored, by observing the natural infection in the field as well as in the glasshouse by artificial inoculations on a 0–9 disease intensity index scale as described earlier (Dhawan et al. 1998) (score 0–1.0 = 0%–10% infection, 1.1–2.0 = 11%–20%, 2.1–3.0 = 21%–30%, through 8.1–9.0: 80%–90% infection on individual plant basis). Disease severity index (DSI) was calculated for each germplasm accession by the following formula (Kim et al. 1999).

$$\mathrm{DSI} = \frac{\sum(\text{rating of each plant}) \times 100}{9 \times \text{number of plants rated}}.$$

Two accessions, Pps-1 (DSI = 11.1, highly resistant to DM) and H-9 (DSI = 66.7, highly susceptible to DM), were selected for comparative study of disease resistance to DM. The genotype Pps-1 spontaneously originated from the DM-resistant inbred accession I-14 of opium poppy having deep-fringed leaf margins, and it has been inbred for 6 selfing cycles to make it completely distinct, uniform, and stable (Dhawan et al. 2007). The parental genotype I-14 had also been inbred for 9 selfing cycles and was developed from the recurrent backcross progeny where the resistance and deep-fringed leaf character from a wild collection SPS-20 was introduced into a genotype SPS-49 using SPS-20 as the donor and SPS-49 as recurrent parent. On the other hand, H-9 is an inbred line developed by a selection of a spontaneous leaf color mutant in the progeny of SPS-49 having slightly fringed leaf margins (Figure 1). Chloroplasts isolated from bulked populations (more than 500 seedlings) of these 2 accessions constituted the material for DNA isolation.

Genetic Study of Chloroplast Variant and DM Resistance

To study the inheritance of DM resistance and leaf color characters, reciprocal crosses involving the mutant (H-9) and resistant (Pps-1) genotypes were made during 2003–2004, and F_1 generations of the crosses were raised during 2004–2005. Seeds for F_2 generation were obtained by selfing the F_1s. All generations of the crosses (reciprocal F_1s and F_2s) along with their parents were grown in the field in randomized block design with 3 replications during 2005–2006 crop season. A row-to-row distance of 50 cm and plant-to-plant distance of 10 cm was maintained. Standard agronomic practices were followed, and observations were made on single plant basis. Chi-square analysis was applied to test the goodness of fit for frequency distributions in the F_2 generations for leaf color traits. *t*-test (paired and unpaired) was applied on calculated DSI values of reciprocal F_1 and F_2 generations for DM resistance.

Arbitrarily-primed polymerase chain reaction of cpDNA and Cloning of Unique Amplicon

Chloroplast DNA (cpDNA) was isolated from 7- to 10-days-old seedlings using the protocol described by Triboush et al. (1998) with a modified sieve size (43 cm) for chloroplast separation from cell debris. cpDNA of the 2 selected accessions (Pps-1 and H-9) was amplified using 80 decamer oligonucleotide primers (20 MAP primers [Shasany et al. 2005], and 60 operon primers [OPA, OPB, and OPO series; Operon Technologies, Alameda, CA]). Amplification was carried out as per the protocol of Shasany et al. 2005. The specific amplicon obtained with MAP-9 primer was excised from a low-melting agarose gel using standard procedures

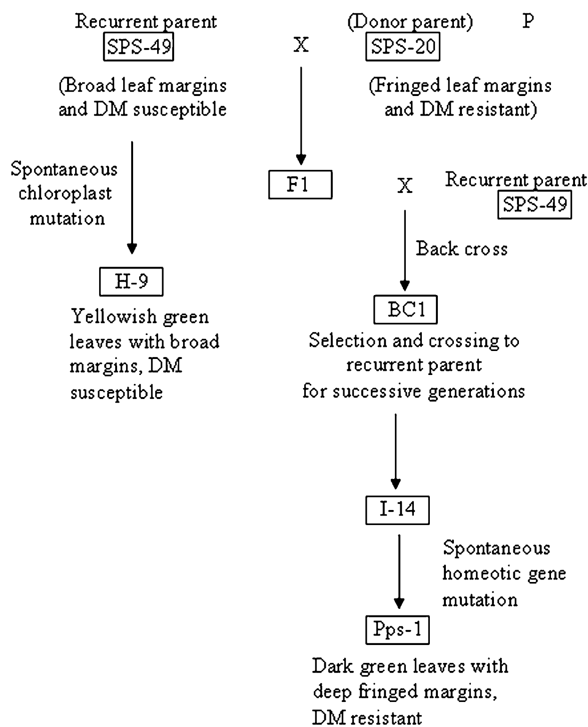

Figure 1. Graphic representation of development of DM-resistant genotype Pps-1 and -susceptible genotype H-9, showing that both genotypes developed from the same progenitor SPS-49. H-9 is an inbred line developed from a selection of a chlorophyll mutant of "SPS-49" having broad leaf character, whereas the genotype Pps-1 spontaneously originated from the DM-resistant inbred accession I-14 of opium poppy having deep-fringed leaf margins. The parental genotype I-14 was developed from the recurrent backcross progeny where the resistance and deep-fringed leaf character from a wild collection SPS-20 was introduced into a genotype SPS-49 using SPS-20 as the donor and SPS-49 as recurrent parent.

(Sambrook et al. 1989), purified using the QIA Quick Gel Extraction Kit (Qiagen Inc., Valencia, CA) using manufacturer instructions, restricted with *Bam*HI (this site was present in the MAP-9 primer), ligated to *Bam*HI-digested pUC19 vector, and transformed into DH5α-competent cells. Recombinants were selected using blue/white screening, and a particular white clone was used for plasmid DNA isolation. The recombinant plasmid was checked for the presence of insert of appropriate size and was further used for automated DNA sequencing of the insert using Big Dye Terminator v 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The sequence was analyzed using the National Center for Biotechnology Information (NCBI) BLAST (Altschul et al. 1990).

Sequencing of Full-Length *rpoC1* DNA

The full *rpoC1* gene from both the genotypes (Pps-1 and H-9) was cloned in 3 fragments. Primer pairs were designed from the *rpoC1* sequence of *Arabidopsis thaliana* (accession number NP051050). To amplify the 5′ region, middle region, and 3′ region, primer pairs, 5′-ATGATTGATCAGTATAAACA-3′ and 5′-AAACCTTATTATGACCATCC-3′, 5′-ATTAATGAACTCTATAGAAGAGTT-3′ and 5′-AATAAGCATATCTTGCGTTGGTAC-3′, and 5′-GGAAGGACGCACTATTTGTTTACATC-3′ and 5′-TTAGGTATCATATGAACAGGCTTGAGAAA-3′, were used, respectively. Amplifications were carried out using cpDNA as template with Platinum *Taq* Polymerase (Invitrogen, Carlsbad, CA). The PCR was programmed as earlier (Shasany et al. 2005) except that it was carried out for 35 cycles with different annealing temperatures for the 5′ region (55 °C), middle region (55 °C), and 3′ region (50 °C). The amplified products were cloned on sequencing vectors (TOPO/pUC19/pGEMT) for downstream sequencing of inserts. The full-length *rpoC1* gene sequences were constructed by aligning the overlapping insert sequences. The full-length *rpoC1* gene sequences from both the genotypes (Pps-1 and H-9) were verified using the BLAST and compared using CLUSTALW (Thompson et al. 1994).

Inheritance Study of DM-Linked Nucleotide Substitutions in *rpoC1*

In order to study inheritance of the base differences identified in the DM-resistant (Pps-1) and -susceptible (H-9) genotypes of *P. somniferum*, differentiating primers were designed based on the *rpoC1* gene sequences obtained from both the genotypes. Two sets of forward primers and one set of reverse primers were designed, whereby the forward primers in a set differed by a single nucleotide at the 3′-terminal and the reverse primers differed by 2 nucleotides at the 3′ terminal (Figure 2A).

Forward primer set 1
5′-CCAGTCCTTCCCCCCGAC-3′: Pps-F (based on Pps-1 *rpoC1* gene sequence)
5′-CCAGTCCTTCCCCCCGAA-3′: H9-F (based on H-9 *rpoC1* gene sequence)
Forward primer set 2
5′-GTGGATTACCCCGCGAAATG-3′: Pps-F(1) (based on Pps-1 *rpoC1* gene sequence)
5′-GTGGATTACCCCGCGAAATA-3′: H9-F(1) (based on H-9 *rpoC1* gene sequence)
Reverse primer set 1
5′-CAAACTAATGGATGTAAACAAATAGTG-3′: Pps-R (based on Pps-1 *rpoC1* gene sequence)
5′-CAAACTAATGGATGTAAACAAATAGCA-3′ H9-R (based on H-9 *rpoC1* gene sequence)

Primer combinations, Pps-F/Pps-R, Pps-F(1)/Pps-R, H9-F/H9-R, and H9-F(1)/H9-R, were used with DNA templates from parental genotypes (Pps-1 and H-9), their progenitors (I-14, SPS-20, and SPS-49), and their reciprocal F_1 and F_2 progenies to analyze the inheritance pattern of the base differences. The primer combinations Pps-F/Pps-R and H9-F/H9-R targeted the amplification of a 640-bp (808–1448 bp) fragment, and Pps-F(1)/Pps-R and H9-F(1)/H9-R targeted the amplification of a 264-bp (1184–1448 bp) fragment of the *rpoC1* gene. The PCR reactions were carried out as done earlier (Shasany et al. 2005) except that number of cycles was 35 and primer-annealing temperature was 65 °C.

A

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                                          Pps-F
Pps-1   GCAGAATGGATGGTTTTGTGCCTATTACCAGTCCTTCCCCCGACTTGAGACCGATCATT 840
                                          H9-F
H-9     GCAGAATGGATGGTTTTGTGCCTATTACCAGTCCTTCCCCCGAATTGAGACCGATCATT 840
        ******************************************* ****************

Pps-1   CAAATAGATGGGGGTAAACTGATGAGTTCGGATATTAATGAACTCTATAGAAGAGTTATC 900
H-9     CAAATAGATGGGGGTAAACTGATGAGTTCGGATATTAATGAACTCTATAGAAGAGTTATC 900
        ************************************************************

Pps-1   TATCGGAACAATACTCTTACCGACCTATTAACAACAAGTAGATCTACGCCAGGGGAGTTA 960
H-9     TATCGGAACAATACTCTTACCGACCTATTAACAACAAGTAGATCTACGCCAGGGGAGTTA 960
        ************************************************************

Pps-1   GTTATGTGTCAAGAAAAATTAGTGCAAGAGGCCGTGGATACACTTCTTGATAATGGGATC 1020
H-9     GTTATGTGTCAAGAAAAATTAGTGCAAGAGGCCGTGGATACACTTCTTGATAATGGGATC 1020
        ************************************************************

Pps-1   CGCGGACAACCAATGAGGGATGGTCATAATAAAGTTTATAAGTCTTTTTCAGATGTAATT 1080
H-9     CGCGGACAACCAATGAGGGATGGTCATAATAAAGTTTATAAGTCTTTTTCAGATGTAATT 1080
        ************************************************************

Pps-1   GAAGGCAAAGAAGGAAGATTTCGTGAGACTTTGCTTGGTAAACGTGTCGATTATTCGGGG 1140
H-9     GAAGGCAAAGAAGGAAGATTTCGTGAGACTTTGCTTGGTAAACGTGTCGATTATTCGGGG 1140
        ************************************************************
                                                    Pps-F(1)
Pps-1   CGTTCCGTAATTGTCGTGGGCCCCTCACTTTCATTACATCGATGTGGATTACCCCGCGAA 1200
                                                    H9-F(1)
H-9     CGTTCCGTAATTGTCGTGGGCCCCTCACTTTCATTACATCGATGTGGATTACCCCGCGAA 1200
        ************************************************************

Pps-1   ATGGCAATAGAGCTTTTCCAGACATTTGTAATTCGTGGTCTAATCAGACAGCAGGTTGCT 1260

H-9     ATAGCAATAGAGCTTTTCCAGACATTTGTAATTCGTGGTCTAATCAGACAGCAGGTTGCT 1260
        ** *********************************************************

Pps-1   TCAAACATAGGAGTTGCTAAAAGTAAAATAAGGGAAAAAGAACCTATTGTATGGGAAATA 1320
H-9     TCAAACATAGGAGTTGCTAAAAGTAAAATAAGGGAAAAAGAACCTATTGTATGGGAAATA 1320
        ************************************************************

Pps-1   CTTCAAGAAGTTATGCAAGGGCATCCTGTATTGCTGAATAGAGCACCCACTCTGCACAGA 1380
H-9     CTTCAAGAAGTTATGCAAGGGCATCCTGTATTGCTGAATAGAGCACCCACTCTGCACAGA 1380
        ************************************************************
                                                    Pps-R
Pps-1   TTAGGCATACAGGCATTCCAACCCATTTTAGTGGAAGGACGCACTATTTGTTTACATCCA 1440
                                                    H9-R
H-9     TTAGGCATACAGGCATTCCAACCCATTTTAGTGGAAGGACGTGCTATTTGTTTACATCCA 1440
        *****************************************  *****************

Pps-1   TTAGTTTGTAAGGGGTTCAATGCAGACTTTGATGGGGATCAAATGGCTGTTCATGTACCT 1500

H-9     TTAGTTTGTAAGGGGTTCAATGCAGACTTTGATGGGGATCAAATGGCTGTTCATGTACCT 1500
        ************************************************************
```

B

Figure 2. (**A**) Alignment of partial sequences of *rpoC1* gene of Pps-1 and H-9 genotypes using CLUSTALW indicating the positions of the nucleotide variations and the primer sequences used to detect them. Direction of arrow indicates the orientation (5′-3′) of primer sequences. (**B**) Detection of identified base differences at 4 positions in *rpoC1* gene of progenitors (I-14, SPS-20, SPS-49) of DM-resistant (Pps-1) and -susceptible (H-9) genotypes and in their reciprocal F_1 and F_2 progenies. I = primer combination Pps-F/Pps-R, II = Pps-F(1)/Pps-R, III = H9-F/H9-R, IV = H9-F(1)/H9-R, M = molecular weight marker (100-bp ladder), 1 = H-9, 2 = SPS-49, 3 = Pps-1, 4 = I-14, 5 = SPS-20, 6 = Pps-1 × H-9 (F_1), 7 = H-9 × Pps-1(F_1), 8–13 = Pps-1 × H-9 (F_2), 14–19 = H-9 × Pps-1(F_2).

Results and Discussion

Two morphologically distinguishable accessions of *P. somniferum* L., Pps-1 (dark green and deep-fringed leaves, highly resistant to DM) (Figure 3A) and H-9 (yellowish green and slightly fringed leaves, highly susceptible to DM) (Figure 3B), were selected from a pool of about 150 accessions of opium poppy having varied degree of resistance to DM. Both these accessions originated from a common progenitor SPS-49. However, H-9 had a distinct yellowish green leaf color (total chlorophyll content = 7.86 mg/g fresh weight) and was selected as a spontaneous variant, whereas Pps-1 had dark green leaf color (total chlorophyll content = 16.10 mg/g fresh weight) and was developed as a spontaneous variant from the open-pollinated progenies of I-14, which were selected in the recurrent backcross generations of the crosses of SPS-49 (recurrent) and SPS-20 (donor) (Figure 1). Local DM symptoms on H-9 included irregularly shaped chlorotic pinkish brown lesions on leaves and stems, which expanded in size to generate large necrotic patches (Figure 3C). Systemic infection of the disease causes hypertrophy of the leaves, and a dense felt of sporangiophores with sporangia were produced on the abaxial leaf surface (Figure 3D). However, these symptoms were extremely restricted on the leaves of Pps-1 (Figure 3A) thereby exhibiting a very high level of resistance to DM.

Genetic Analysis of DM-Resistant and -Susceptible Genotypes

Inheritance patterns of the DM-resistant and -susceptible genotypes were studied by genetic analysis of F_1 and F_2 generations of their reciprocal crosses. The phenotypic characteristics of all the F_1 plants in the reciprocal crosses

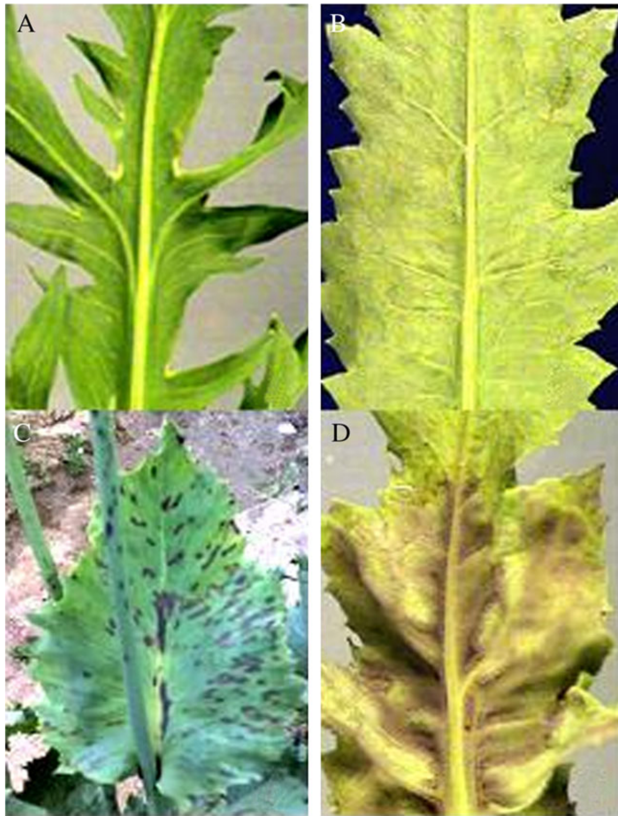

Figure 3. (**A**) Pps-1 leaf. (**B**) H-9 (chlorophyll variant) leaf. (**C**) Topical DM symptoms showing irregularly shaped chlorotic lesions on leaf and stem of susceptible genotypes H-9. (**D**) Systemic symptoms of DM showing curls on thickened and deformed leaf of H-9 covered with conidia of *Peronospora arborescens*.

involving H-9 and Pps-1 during 2 years (2004–2005 and 2005–2006 crop seasons) showed dark green leaf color thereby indicating that the typical yellowish green leaf color was a recessive character. Segregation pattern of the F_2 populations of both the reciprocal crosses also provided good fit of the monogenic (3:1) Mendelian ratio ($P \geq 0.50$–0.30) for normal leaf color (dark green) and the variant trait (yellowish green) indicating that variant character is controlled by a single recessive nuclear gene (Table 1). Similar results for mutant leaf color have been reported by Singh et al. (2002). Besides, earlier reports in *A. thaliana* suggested that chloroplast development requires nucleus-encoded transcription factor Sigma B (Shirano et al. 2000) that is in line with our observations of nuclear control of leaf color variation.

However, inheritance pattern of the DM resistance did not follow the typical Mendelian ratios in all the segregating generations of the reciprocal crosses (Table 1), which is in conformity with earlier reports in which a strong evidence of cytoplasmic control and paternal/biparental transmission of DM resistance has been found (Dhawan et al. 1998). These observations led us to understand that the degree of DM resistance in opium poppy is under the control of interaction between cytoplasmic and nuclear genes. As leaf color was governed by nuclear genes and DM resistance was found to be maternally inherited, no correlation was established between these 2 traits.

arbitrarily-primed polymerase chain reaction–Based Polymorphism Analysis of cpDNA

Interestingly, Arbitrarily-primed polymerase chain reaction analyses of cpDNA of these 2 accessions (using 80 random primers) could generate a unique polymorphic fragment (with primer MAP-9) in DM-resistant genotype Pps-1 (Figure 4). Sequence analysis of the cloned Pps-1–specific DNA fragment revealed that it was a part of the *rpoC1* gene encoding the β′ subunit of plastid-encoded RNA polymerase.

Plastid-Encoded RNA Polymerase β′ Subunit Analysis

The full-length DNA sequence of opium poppy *rpoC1* gene was found to be 2707 bp long with a 658-bp intron (436–1093) and an open reading frame of 2049 bp encoding a polypeptide of 682 amino acid residues. The exon-intron boundary of the DNA sequence was determined by aligning the sequence with known *rpoC1* sequences from GenBank database (NCBI). Using the online tool "compute pI/MW," (www.expasy.org) the isoelectric point (pI) and molecular weight of the deduced protein were predicted to be 8.38 and 78.44 kDa, respectively. BLAST analysis showed that the deduced protein sequence had high (96%) homology with plastid-encoded RNA polymerase β′ subunit (RpoC1) of a number of plants thereby indicating that *rpoC1* is highly conserved in different plant species. The conserved domain analysis of the protein sequence using the NCBI "Conserved Domain Architecture Retrieval Tool" and protein BLAST showed that the RpoC1 subunit consisted of 3 RNA polymerase (Rpb) domains, namely RNA_pol_Rpb1_1 (aa4–aa371), RNA_pol_Rpb1_2 (aa373–aa515), and RNA_pol_Rpb1_3 (aa518–aa682), respectively. Rpb 1 represents the clamp domain, which is mobile and involved in positioning the DNA and nascent RNA stand and maintenance of transcription bubble. Rpb 2 contains the active site and the conserved motif "NADFDGD" that binds the active site magnesium ions. Rpb 3 represents the pore domain, which acts as a channel through which nucleotide enters the active site.

Comparative Analysis of *rpoC1* Gene From Pps-1 and H-9

Comparative sequence analysis of the *rpoC1* gene from both the genotypes, susceptible (H-9) and resistant (Pps-1), revealed single-nucleotide substitutions at 4 positions that

Table 1. Segregation pattern of leaf color variance and DM-resistance in different generations of the reciprocal crosses involving Pps-1 and H-9[a]

		Number of plants observed for leaf color					Number of plants observed for DM-resistance							
							Resistant		Susceptible					
Genotype/cross	Generation	Dark green leaves	Yellowish green leaves	Segregation ratio	χ^2	*P*	R	T	S	HS	Total	DSI (mean)	*t*-test (unpaired)	*t*-test (paired)
Pps-1 × H-9	F_1 ($P_1 \times P_2$)	165	0	—	—	—	—	—	60	—	60	61.47	6.90[b]	6.97[b]
H-9 × Pps-1	rF_1 ($P_2 \times P_1$)	172	0	—	—	—	—	—	64	—	64	66.57		
Pps-1 × H-9	F_2 ($P_1 \times P_2$)	958	299	3:1	0.492	0.50–0.30	35	150	135	25	345	52.04	1.40[c]	11.70[b]
							10.15%	**43.47%**	**39.14%**	**7.24%**				
							53.62%		**46.38%**					
H-9 × Pps-1	rF_2 ($P_2 \times P_1$)	774	280	3:1	1.376	0.50–0.30	25	130	137	38	330	55.58		
							7.58%	**39.39%**	**41.51%**	**11.52%**				
							46.97%		**53.03%**					

Bold numbers denote percentage frequency. R = resistant, T = tolerant, S = susceptible, HS = highly susceptible, rF_1, rF_2 = F_1 and F_2 generations of reciprocal cross combinations.

[a] Paired and unpaired *t*-tests were applied on the calculated DSI values to observe the significant differences between normal and reciprocal cross combinations.

[b] Significant value at 1% level of significance.

[c] Significant value at 5% level of significance.

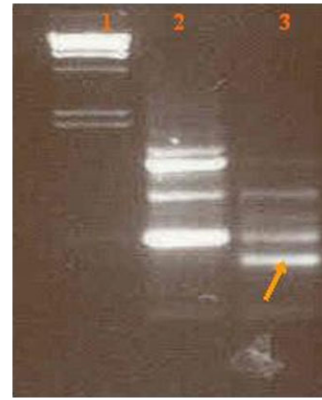

Figure 4. cpDNA amplification with primer MAP-9. Lane 1 = molecular weight marker (λ DNA digested with HindIII). Lane 2 = amplification product of cpDNA of DM-susceptible genotype H-9. Lane 3 = amplification product of cpDNA of DM-resistant genotype Pps-1 showing the unique cpDNA fragment.

caused 3 amino acid changes in the protein sequence—1) A to C transversion at position 825 (Glu275Asp), 2) A to G transition at position 1203 (Ile401Met), and 3) T to C transition at position 1422 and G to A transition at position 1423 both in same codon (Ala475Thr) (Figure 5). The RpoC1 protein sequence obtained from both the genotypes in the present study was compared with the RpoC1 protein sequence from other plants (*Nandina domestica, Eucalyptus globulus* subsp. *globulus, A. thaliana, Vitis vinifera, Solanum lycopersicum, Pinus koraiensis*) and bacteria (*Escherichia coli* K12 and *Escherichia coli*) available in GenBank (NCBI) (Figure 6). It was interesting to note that the RpoC1 protein sequence at amino acid position 275 and 401 from the DM-susceptible genotype (H-9) was similar to the conserved RpoC1 protein sequences from other plant sources but not bacterial sources, whereas at position 475 it was similar to both plant and bacterial sources except RpoC1 protein sequence of *A. thaliana* (NP_051050.1). The RpoC1 protein sequence from the DM-resistant genotype (Pps-1), which had 3 amino acid substitutions, was different from the RpoC1 protein sequences of plant sources but similar to bacterial RpoC1 protein sequences at positions 275 and 401. However, at position 475, the RpoC1 protein sequence of Pps-1 was similar only to the RpoC1 protein sequence of *A. thaliana* (NP_051050.1) (Figure 6). This investigation is the first report indicating base differences in the plastid-encoded *rpoC1* gene in DM-resistant and -susceptible genotypes of *P. somniferum.*

Analysis of Inheritance Pattern of Base Substitutions in *rpoC1* Linked to DM Resistance

In this study, we have used gene-specific primers that form A-C (H9-F–Pps-F), A-G [H9-F(1)–Pps-F(1)], and CA-TG

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Pps-1 MIDQYKHQHLRIGSVSPEQISAWAKKILPNGEVVGEVTKPYTFHYKTNKPEKDGLFCERI 60
H-9   MIDQYKHQHLRIGSVSPEQISAWAKKILPNGEVVGEVTKPYTFHYKTNKPEKDGLFCERI 60
      ************************************************************

Pps-1 FGPIKSGICACGNYRVIGDEKEDPKFCEQCGVEFVDSRIRRYQMGYIKLACPVTHVWYLK 120
H-9   FGPIKSGICACGNYRVIGDEKEDPKFCEQCGVEFVDSRIRRYQMGYIKLACPVTHVWYLK 120
      ************************************************************

Pps-1 RLPSYIANLLDKPLKELEGLVYCDVSFARTVAKKPTFLRLRGSFEYEIQSWKYSIPLFFT 180
H-9   RLPSYIANLLDKPLKELEGLVYCDVSFARTVAKKPTFLRLRGSFEYEIQSWKYSIPLFFT 180
      ************************************************************

Pps-1 TQGFDTFRNREISTGASAIREQLADLDLRLIIDCSLVEWKELGEEGPTGNEWEDRKIGRR 240
H-9   TQGFDTFRNREISTGASAIREQLADLDLRLIIDCSLVEWKELGEEGPTGNEWEDRKIGRR 240
      ************************************************************

Pps-1 KDFLVRRMELAKHFIRTNVEAEWMVLCLLPVLPEDLRPIIQIDGGKLMSSDINELYRRVI 300
H-9   KDFLVRRMELAKHFIRTNVEAEWMVLCLLPVLPEELRPIIQIDGGKLMSSDINELYRRVI 300
      **********************************:*************************

Pps-1 YRNNTLTDLLTTSRSTPGELVMCQEKLVQEAVDTLLDNGIRGQPMRDGHNKVYKSFSDVI 360
H-9   YRNNTLTDLLTTSRSTPGELVMCQEKLVQEAVDTLLDNGIRGQPMRDGHNKVYKSFSDVI 360
      ************************************************************

Pps-1 EGKEGRFRETLLGKRVDYSGRSVIVVGPSLSLHRCGLPREMAIELFQTFVIRGLIRQQVA 420
H-9   EGKEGRFRETLLGKRVDYSGRSVIVVGPSLSLHRCGLPREIAIELFQTFVIRGLIRQQVA 420
      ****************************************:*******************

Pps-1 SNIGVAKSKIREKEPIVWEILQEVMQGHPVLLNRAPTLHRLGIQAFQPILVEGRTICLHP 480
H-9   SNIGVAKSKIREKEPIVWEILQEVMQGHPVLLNRAPTLHRLGIQAFQPILVEGRAICLHP 480
      ******************************************************:*****

Pps-1 LVCKGFNADFDGDQMAVHVPLSLEAQAEARLLMFSHMNLLSPAIGDPISIPTQDMLMGLY 540
H-9   LVCKGFNADFDGDQMAVHVPLSLEAQAEARLLMFSHMNLLSPAIGDPISIPTQDMLMGLY 540
      ************************************************************

Pps-1 VLTIGNRRGICANRYNPCNHLNYQNEKIDDNNYKYTKEKEPYFCSSYDALGAYRQKRINL 600
H-9   VLTIGNRRGICANRYNPCNHLNYQNEKIDDNNYKYTKEKEPYFCSSYDALGAYRQKRINL 600
      ************************************************************

Pps-1 DSPLWLRWRLDQRVIGSREVPIEVQYDSFGTYHEIYGHYLIVRSVKKETLCIYIRTTVGH 660
H-9   DSPLWLRWRLDQRVIGSREVPIEVQYDSFGTYHEIYGHYLIVRSVKKETLCIYIRTTVGH 660
      ************************************************************

Pps-1 ISFYREIEEAIQGFSQACSYDT 682
H-9   ISFYREIEEAIQGFSQACSYDT 682
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Figure 5. Alignment of plastid-encoded RpoC1 amino acid sequences of Pps-1 and H-9 using CLUSTALW showing differences in amino acids at positions 275, 401, and 475—(Glu275Asp), (Ile401Met), (Ala475Thr).

(H9-R–Pps-R) mismatches at the 3′ end corresponding to base differences in the *rpoC1* gene. As previously reported, PCR can be adapted for the rapid detection of known single-base differences in DNA using specially designed oligonucleotides in a method known as amplification-refractory mutation system (Bottema et al. 1993). The principle of this method is based on the fact that a perfectly matched primer set amplifies the desired gene, whereas the mismatched gene does not amplify due to its mismatch at (or near) the 3′ end of the specific primer (Sarkar et al. 1990; Cantafora et al. 1998).

Primer combinations Pps-F/Pps-R and Pps-F(1)/Pps-R) produced amplicons of 640 and 264 bp, respectively, with DM-resistant genotypes Pps-1, I-14, SPS-20, and F_1/F_2 progenies of Pps-1 × H-9 crosses, whereas no amplification product was obtained with DM-susceptible genotypes H-9, SPS-49, and F_1/F_2 progenies of the reciprocal cross H-9 × Pps-1. On the other hand, primer combinations H9-F/H9-R and H9-F(1)/H9-R produced amplicons of 640 and 264 bp, respectively, with DM-susceptible genotypes H-9, SPS-49, and F_1/F_2 progenies of H-9 × Pps-1 cross, whereas no amplification product was obtained with DM-resistant genotypes Pps-1, I-14, SPS-20, and F_1/F_2 progenies of Pps-1 × H-9 cross (Figure 2B). These results indicate that the base differences detected in the DM-resistant genotype Pps-1 were also present in the DM-resistant progenitors I-14 and SPS-20 but were absent in the DM-susceptible progenitor SPS-49. The results also suggested that these base differences were inherited maternally in F_1 and F_2 progenies of normal and reciprocal crosses of Pps-1 and H-9 genotypes.

It has been described earlier in literature that chloroplast-governed factors are involved in disease resistance and HR-like cell death in plants through the generation of reactive oxygen species (ROS) (Kariola et al.

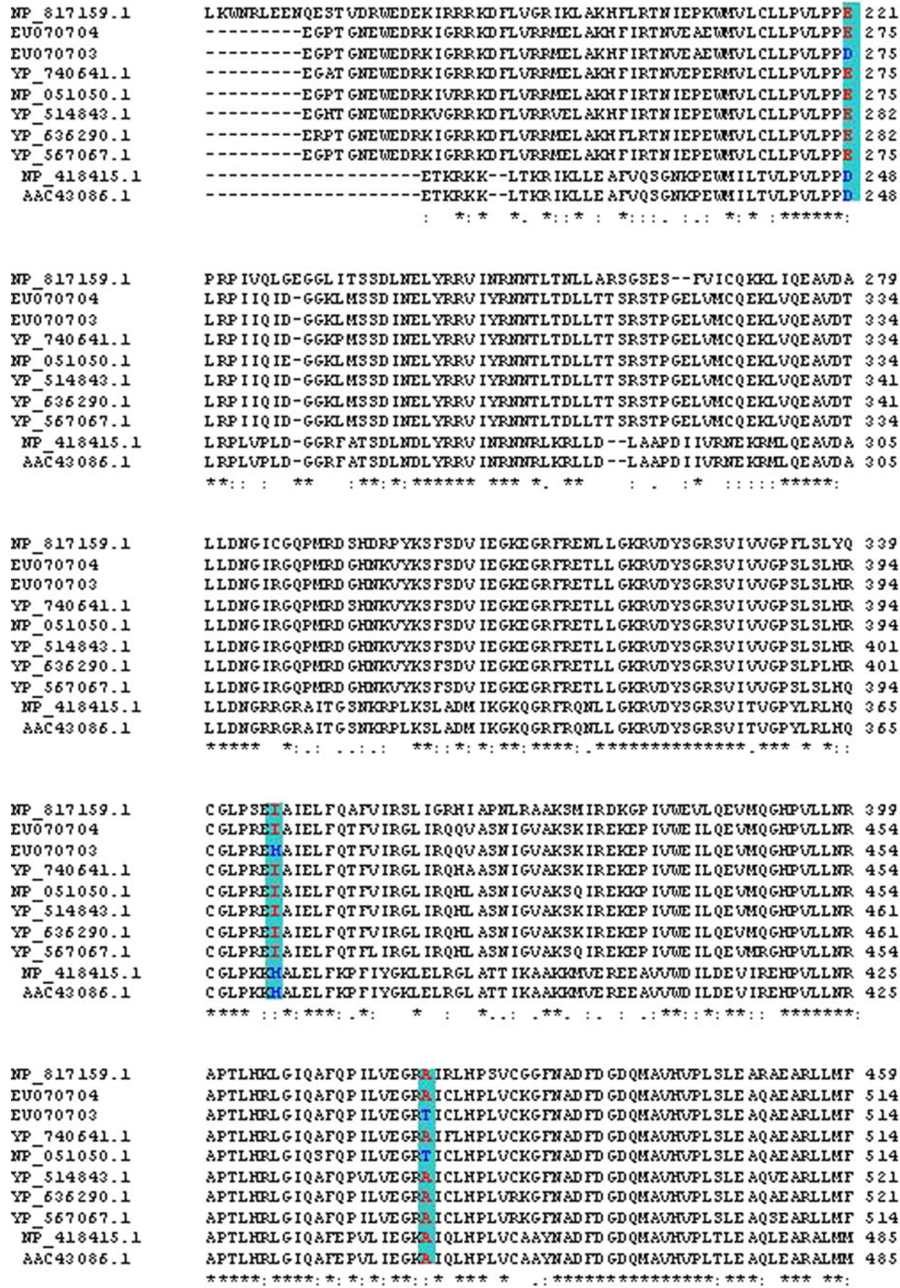

Figure 6. Alignment of plastid-encoded RpoC1 amino acid sequences of DM-resistant Pps-1 (EU071703) and DM-susceptible H-9 (EU071704) genotypes of opium poppy with diverse plant species of different families—*Nandina domestica* (YP_740641.1), *Eucalyptus globulus* subsp. *globulus* (YP_636290.1), *Arabidopsis thaliana* (NP_051050.1), *Vitis vinifera* (YP_567067.1), and *Solanum lycopersicum* (YP_514843.1); gymnosperm—*Pinus koraiensis* (NP_817159.1); and bacteria like *Escherichia coli* K12 (NP_418415.1) and *Escherichia coli* (AAC43086.1) using CLUSTALW program. Amino acid residues identical at a given position are denoted with an asterisk, gaps are denoted by dash, and similar residues are by a colon or dots. The 3 positions in the RpoC1 protein sequence indicating the differences are highlighted (275, 401, and 475).

2005; Liu et al. 2007) and other mechanisms (Kachroo et al. 2001; Yaeno et al. 2004). The photosynthetic electron transport system in the thylakoid membrane of chloroplast is a major source of ROS in plants (Foyer et al. 1994). Although we could not find any direct evidence of the involvement of ROS for DM disease resistance in genotype Pps-1, the variations in the amino acid sequence of *rpoC1* gene may have a role to play in development of this

resistance as this gene is responsible for the transcription of most of the plastid genes, including those for proteins involved in photosynthesis and electron transport chain (Palmer 1991). Of the 3 amino acid variations in the RpoC1 protein sequence of DM-resistant accession Pps-1, 1 was in the Rpb1 domain and the other 2 were in the Rpb2 domain. It may be hypothesized that variations in these RpoC1 domains may have a role in the development of DM disease resistance as chloroplast RNA transcription, processing, stability, and translation are closely coupled and a defect in any of these processes could affect the others. Another observation in line with these results is that *rpoC1* gene of chloroplast shows sufficient homology with eubacterial RNA polymerase genes (Hu and Bogorad 1990; Mullet 1993) (more so in the case of DM-resistant genotype Pps-1 as discussed earlier), and in the case of *E. coli* it has already been shown that a mutation in the *rpoC* gene makes it resistant to microcin J25 (MccJ25), a bactericidal 21-amino acid peptide active against Gram negative bacteria (Delgado et al. 2001). Because the chloroplast genome in both the resistant and susceptible genotypes (that have been inherited from the same maternal parent) was expected to be same, the differences obtained in the *rpoC1* gene may have implications in the differential behavior of the 2 genotypes to DM infection. However, the involvement of other genes in the development of DM resistance in *P. somniferum* could not be ruled out. Further studies will provide evidence for the direct functional involvement of the nucleotide variations in the *rpoC1* gene in DM disease resistance in opium poppy.

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