A Transgenic Mutant of *Lactuca sativa* (Lettuce) with a T-DNA Tightly Linked to Loss of Downy Mildew Resistance

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One hundred and ninety-two independent primary transformants of lettuce cv. Diana were obtained by co-cultivation with *Agrobacterium tumefaciens* carrying constructs containing maize *Ac* transposase and *Ds*. *R*₂ families were screened for mutations at four genes (*Dm*) for resistance to downy mildew. One family, designated dm3t524, had lost resistance to an isolate of *Bremia lactucae* expressing the avirulence gene *Avr3*. Loss of resistance segregated as a single recessive allele of *Dm3*. The mutation was not due to a large deletion as all molecular markers flanking *Dm3* were present. Loss of *Dm3* activity co-segregated with a T-DNA from which *Ds* had excised. Genomic DNA flanking the right border of this T-DNA was isolated by inverse polymerase chain reaction. This genomic sequence was present in four to five copies in wild-type cv. Diana. One copy was missing in all eight deletion mutants of *Dm3* and altered in dm3t524, indicating tight physical linkage to *Dm3*. Three open reading frames (ORFs) occurred in a 6.6-kb region flanking the insertion site; however, expression of these ORFs was not detected. No similarities were detected between these ORFs and resistance genes cloned from other species. Transgenic complementation with 11- to 27-kb genomic fragments of Diana spanning the insertion site failed to restore *Dm3* function to two ethyl methanesulfonate (EMS)-induced mutants of *Dm3* or to cv. Cobham Green, which naturally lacks *Dm3* activity. Therefore, either the T-DNA inserted extremely close to, but not within, *Dm3* and the mutation may have been caused by secondary movement of *Ds*, or *Dm3* activity is encoded by a gene extending beyond the fragments used for complementation.

Additional keywords: disease resistance.

At least 10 different resistance genes have been isolated from several species by map-based cloning or transposon tagging (reviewed in Staskawicz et al. 1995; Bent 1996). Mutants have played critical roles in the cloning of many of these genes, either as part of a transposon tagging strategy: *HMI* from corn for resistance to *Cochliobolus heterostrophus* (Johal and Briggs 1992), *N* from tobacco conferring resistance to tobacco mosaic virus (Whitham et al. 1994), *Cf9* for resistance to *Cladosporium fulvum* in tomato (Jones et al. 1994), and *L6* encoding resistance to rust in flax (Lawrence et al. 1995)—or for initial definition of the locus as well as confirmation of genes isolated by map-based cloning: *Prf* required for resistance to *Pseudomonas syringae* and sensitivity to the insecticide fenthion in tomato (Salmeron et al. 1994), and *RPS2* for resistance to *Pseudomonas syringae* in *Arabidopsis thaliana* (Bent et al. 1994). Similarities between cloned resistance genes is facilitating the isolation of additional resistance genes as candidate sequences can be recognized (Kanazin et al. 1996; Yu et al. 1996; reviewed in Michelmore 1995a). The deduced amino acid sequences of *Prf*, *RPS2*, *RPM1*, *N*, and others each have a conserved nucleotide-binding site (Grant et al. 1995). *Pto* and *Xa21* both contain motifs conserved in serine-threonine protein kinases (Martin et al. 1993; Song et al. 1995). All, except *Pto* and *HMI*, contain leucine-rich repeats that are reported to have a role in protein-protein interactions in other systems (Kobe and Deisenhofer 1994). The cloning of these genes is advancing our understanding of the molecular basis of resistance to diverse pathogens (reviewed in Bent 1996) as well as providing tools for crop improvement (reviewed in Michelmore 1995b).

We are cloning genes for resistance to downy mildew (*Dm*), with an initial focus on *Dm3*, to understand the molecular basis for resistance to *Bremia lactucae* in lettuce. *Dm* genes are located in four major clusters in the lettuce genome. *Dm3* is a member of the largest cluster that contains at least nine *Dm* genes as well as a gene for resistance to root aphid (Farrara et al. 1987). A detailed genetic map of the largest cluster has been constructed (K. Nakahara, R. Kessel, and R. Michelmore, unpublished). Deletion mutants of *Dm3* were utilized to identify new molecular markers and to construct a fine-resolution, deletion-breakpoint point map of the *Dm3* region (Okubara et al. 1994; Anderson et al. 1996). Markers unique

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to the region are duplicated either closely around *Dm3* or distributed through the resistance gene cluster. We have also generated ethyl methanesulfonate (EMS)-induced mutants of *Dm* genes (R. Arroyo-Garcia, D. Chin, O. Ochoa, and R. Michelmore, *unpublished*). Our efforts have been primarily focused on map-based cloning of *Dm* genes because the low rate of *Ac* transposition in lettuce made a transposon tagging approach impractical (Yang et al 1993a, 1993b). However, as part of our studies on the feasibility of transposon tagging of *Dm* mutants, we generated numerous transgenic families. To identify additional mutants, we screened 192 independent R_2 transgenic families for loss of activity at each of four *Dm* genes. In this paper, we report the identification and characterization of a mutant of *Dm3* with a T-DNA inserted physically in or very close to *Dm3*.

RESULTS

Identification and genetic analyses of a mutant allelic with *Dm3*.

One hundred and ninety-two independent R_2 families, derived from self pollination of original transformed regenerants of *Lactuca sativa* cv. Diana, were screened for loss of activity at four different *Dm* loci. One transgenic line, designated dm3t524, showed loss of *Dm3* activity. Segregation of resistance was analyzed in R_2 and R_3 mutant families with *B. lactucae* isolate IM25P11. The numbers of resistant (25) and susceptible (7) R_2 seedlings did not deviate significantly from a 3:1 ratio expected for a recessive mutation of a single dominant gene ($\chi^2_{3:1} = 0.17$). A total of 55 R_3 seedlings from two different susceptible R_2 individuals were entirely susceptible to IM25P11, consistent with a homozygous recessive mutation.

Allelism tests were made by crossing homozygous dm3t524 individuals with a total of 15 other *Dm3* mutants, eight deletion mutants, two spontaneous mutants, and five EMS mutants. From 10 to 55 F_1 progeny were pooled from reciprocal crosses with each *Dm3* mutant. F_1 progeny from all crosses were susceptible to IM25P11, indicating that the mutation in dm3t524 was allelic to the other 15 *Dm3* mutations tested. Progeny of crosses with the deletion mutants could still show loss of *Dm3* activity if two or more closely linked genes were required for *Dm3* activity and both genes were missing in the deletion mutants. However, crosses between dm3t524 and the five independent EMS mutants, all of which do not lack any flanking markers and are therefore likely to carry point mutations, resulted in no additional complementation groups. Therefore, there appears to be only a single mutable gene conferring *Dm3* specificity and the mutation in dm3t524 is allelic to *Dm3*.

Loss of resistance is correlated to the presence of the pSLJ3621 T-DNA.

The segregation of the *Ac* transposase, *Ds*, and the *Ds* empty donor site in the spectinomycin resistance gene (*AadA*) was tested by polymerase chain reaction (PCR). PCR analysis confirmed the earlier observation in which spectinomycin insensitivity segregated 3:1 (20 uniformly green : 6 uniformly white) when R_2 seedlings were tested on plates containing spectinomycin; no green-white chimeras were observed (Yang et al. 1993a). Therefore, excision of *Ds* and the consequent restoration of a functional *AadA* gene had occurred early in

the transformation process, prior to the regeneration of the R_1 plant of dm3t524. *Ds* was not detectable in either *Dm3*-resistant or *Dm3*-susceptible plants by PCR with *Ds*-specific primers, indicating that *Ds* had not reintegrated after excision or been lost by somatic segregation. As expected, 16 R_2 plants that showed no sporulation with IM25P11 (either homozygous or heterozygous for *Dm3*) were segregating for both the transposase and the empty *Ds* donor site.

However, of 16 R_2 plants showing susceptibility to isolate IM25P11, all contained an empty *Ds* donor site, as indicated by the presence of a 580-bp PCR product amplified from the *Nos* promoter and a portion of the *AadA* gene. Only 13 of these individuals carried the *Ac* transposase. Therefore, loss of *Dm3* resistance co-segregated in this limited population with an empty donor site from pSLJ3621 but not with the transposase from pSLJ3232. Genetic analyses of larger R_2 and R_3 populations was not pursued because concurrent molecular studies rendered additional segregation data unnecessary.

Incomplete T-DNA insertion in dm3t524 occurred into a low copy sequence in the *Dm3* region.

We isolated sequences flanking the T-DNA from pSLJ3621 by inverse PCR (IPCR). IPCR with primers specific to the right T-DNA border (Table 1) resulted in the longest product of approximately 1,200 bp, designated IPCR₁₂₀₀, that included 800 bp of lettuce DNA (designated IPCR₈₀₀; hatched box in Figure 1). PCR products unique to dm3t524 were not obtained with primers for the left T-DNA border, suggesting that normal insertion of the left border of the T-DNA had not occurred.

The IPCR₁₂₀₀ fragment was used as a probe in Southern blots of wild-type cv. Diana and dm3t524 DNA (Fig. 2A). In Diana, single 4.5-kb *EcoRI* or 6.0-kb *HindIII* + *NotI* bands were observed; in dm3t524, additional bands of 8.0 kb (with *EcoRI*) and 9.5 kb (with *HindIII* + *NotI*) were detected. The presence in dm3t524 of more than one genomic fragment hybridizing to IPCR₁₂₀₀ suggested that the T-DNA had inserted into one copy of a multi-copy sequence. The additional band in each digest was only 3.5 kb larger, rather than the 7.2 kb expected for insertion of a full-length copy of the T-DNA of pSLJ3621 from which *Ds* had excised. Neither *EcoRI* nor *HindIII* + *NotI* cleaves within this T-DNA. The size disparity suggested that the insertion of the T-DNA was either incomplete, or was complete but accompanied by a deletion of lettuce genomic DNA. Hybridization with T-DNA sequences demonstrated that the T-DNA was only present in the larger *EcoRI* and *HindIII* + *NotI* bands of dm3t524 (Fig. 2B).

A variety of endonucleases were tested for their ability to resolve multiple members of the IPCR₈₀₀ sequence. Digestion of DNA from Diana with one enzyme, *AccI*, detected multiple copies of the IPCR₈₀₀ sequence, confirming the presence of a small family of sequences (Fig. 3). At least four major *AccI* bands were seen in cv. Diana. A 4.8-kb fragment (Fig. 3, band 3) was absent in all of the *Dm3* deletion mutants tested. dm3t524 also lacked this band and instead showed two novel bands of 2.6 and 2.3 kb, explained by the presence of an *AccI* site close to the right border of the T-DNA. Deletion breakpoint analysis showed that the IPCR₈₀₀ family members were distributed between molecular markers known to be tightly linked to *Dm3* (Anderson et al. 1996). The two *Dm3* mutants with the largest deletions lacked all detectable copies of

IPCR₈₀₀. Therefore, this sequence family is tightly clustered around *Dm3*. We have screened approximately 65,000 amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) markers against the deletion panel; only one marker was missing in all deletion mutants of *Dm3* (D. Lavelle, *unpublished*). Assuming random distribution of markers through the lettuce genome, the average spacing between the markers screened is 42 kb. Therefore, the copy of IPCR₈₀₀ on the 4.8-kb *AccI* fragment is likely to be physically close to *Dm3*.

The number of copies of the IPCR₈₀₀ sequence in the lettuce genome was also estimated by measuring the relative intensities of hybridization with a phosphorimager. DNA of dm3t524 was digested with either *EcoRI* or *HindIII* and hybridized to 660 bp of lettuce DNA from the IPCR₁₂₀₀ fragment. Amounts of radiolabeled probe hybridizing to both the T-DNA tagged copy or the faster-migrating untagged copy(s) were quantified with a phosphorimager linked to MACBAS 2.0. Twofold more radiolabel was measured in the smaller *EcoRI* band than in the larger, T-DNA-containing band, and two- to threefold more radiolabel was measured in the smaller *HindIII* band. Assuming that the T-DNA-tagged band was single copy, we estimated three to four copies of the IPCR₈₀₀ sequence in dm3t524. This is consistent with Southern blots of *AccI*-digested DNA (Fig. 3) that indicated four to five different fragments hybridizing to the IPCR₈₀₀ sequence.

Characterization of BAC clones carrying the IPCR sequence.

In a screen of over 50,000 bacterial artificial chromosome (BAC) clones, representing two genome equivalents of lettuce, four BAC clones, designated BACH15, BACH18, BACH19, and BACE42, hybridized to the 660-bp fragment from IPCR₁₂₀₀. All of the clones carried a 4.0-kb *AccI* fragment; none carried

the 4.8-kb fragment that was missing or altered in the *Dm3* mutants. It was surprising that all four of the BAC clones only contained the smallest *AccI* fragment seen in genomic Southern blots of wild-type Diana and *Dm3* mutants. The pattern of IPCR₈₀₀-containing fragments in genomic Southern blots might be attributed to differential digestion of genomic DNA by *AccI*, which recognizes the degenerate palindromic sequence GTA/CG/TAC and is subject to site-dependent deoxyguanosine methylation (e.g., Webb and Chaplin 1990). Such methylation would not occur on plasmid DNA replicated in *Escherichia coli*. Therefore, the *AccI* digestion pattern of the BAC clones did not

Fig. 1. Diagram of the genomic regions flanking the T-DNA integration site. A 6.6-kb *HindIII* fragment was subcloned from BACH15. Approximately 6.2 kb was sequenced (GenBank accession numbers AF018069 and AF018070); a 0.4-kb region that has so far proved recalcitrant to sequencing is shown as a dotted line. The T-DNA insertion site in dm3t524, 800 bp of lettuce DNA contained in the original inverse polymerase chain reaction product (hatched box above the line), and a 50-bp region that is absent in dm3t524 (black rectangle below the line) are shown. Opening reading frames (ORF1, ORF2, ORF3) are indicated as arrows. The position of microsatellite marker MSAT15-34 is marked by *. The 3.5-kb T-DNA insertion and the segment to the right of the 0.5-kb break are not drawn to scale.

Table 1. Oligonucleotide primers used for polymerase chain reaction (PCR)

Purpose and origin	Designation	Sequence (5' to 3')	Reference
Detection of <i>Ac</i> transposase			
35S 5'	C13	ATCTCCACTGACGTAAGGGATGACG	Yang et al. 1993a
<i>Ac</i> 5'	J	CCTCTCGGCCTCAGCTGTTGATGATGA	
Detection of <i>Ds</i>			
<i>Ac</i> 5'	D1	AGTCGGAGAGAGCAGGCGAGAGCGA	Yang et al. 1993a
<i>Ac</i> 5'	D4	ACTGCAATATGGCCAGCTCCAACCTC	
Detection of <i>Ds</i> donor site			
<i>AadA</i>	C14	AGTTGAGTCGATACTTCGGCGATCAC	Yang et al. 1993a
<i>Nos</i> 5'	C15	GCGGGTTTCTGGAGTTAATGAGCTAAG	
Inverse PCR (IPCR) of T-DNA right border	C31	GGGGCATCGCACCGGTGAGTAA	Thomas et al. 1994
	C32	TTGTGGGCCTGTGGTCTCAAGATG	
	D6	GCGGGTTTCTGGAGTTAATGAGCTAAG	
	D57	ATACGTCAGAAACCATTATTGCGCG	
IPCR of T-DNA left border	D14	AGGTAATGGGCTACACTGAATTGG	Thomas et al. 1994
	D43	CGCATAATCTCAGACCAATCTGAAG	
	D58	GCTTGGGTCCACATTTTCGCACGTAG	
	D59	GGCAAACAGGAATGCTCAAGCCAGG	
Amplification of lettuce genomic DNA			
390-bp genomic fragment	D23A	GTCACCTGTGCACATGT	
	D36B	CCAAGTCAACAAAAGAG	
660-bp genomic fragment	D23A	GTCACCTGTGCACATGT	
	D36D	GAGTCACCAAATTCAAAG	
T-DNA integration site	KSF1	GGAAAGTAGCGGAACGGGGA	
	KSR2	GAGGGTGATGTTGTGCGGGA	
Amplification of microsatellite marker			
BACH15	MSAT15-34F	GTATCACATCCCAAACCTCTC	
BACH15	MSAT15-34R	GCAAACAACAGTTGAACCTGCC	

allow us to identify the copy of the IPCR sequence that was tagged in dm3t524.

The BAC clones could be distinguished on the basis of restriction fragment profiles and the size of an adjacent microsatellite marker. BAC15H differed from the others in restriction patterns with *EcoRV*, *AseI*, and *ScaI* (data not shown). The other three BAC clones had identical restriction enzyme restriction patterns with these enzymes. However, these BACs have currently been placed in two different contigs on the basis of AFLP fingerprints and their hybridization patterns to a molecular marker, AC15 (B. Meyers, *unpublished*). Sequencing of the genomic region flanking the T-DNA identified a compound microsatellite sequence, (CA)₉T(AG)₁₅(AAG)₃(AG)₅, 864 bp from the T-DNA integration site. This provided a highly informative marker that distinguished three classes of BAC clones (Fig. 4). BACH15 and BACH18 each had unique microsatellite array sizes; BACH19 and BACE42, which seem to be overlapping clones in the same genomic contig (B. Meyers, *unpublished*) contain the same size of microsatellite array. The microsatellite array size in BACH15 was absent in all of the *Dm3* deletion mutants (Fig. 4). Therefore, BACH15 was likely to contain the copy of the IPCR₈₀₀ sequence most closely linked to *Dm3*.

Characterization of the T-DNA insertion site.

A 6.6-kb *HindIII* fragment containing the IPCR₈₀₀ sequence was subcloned from BACH15 and approximately 6.2 kb was

sequenced (Fig. 1). PCR with primers KSF1 and KSR1, designed from sequences of the 6.6-kb and IPCR₈₀₀ fragments (Table 1), resulted in amplification of a 400-bp fragment containing the wild-type insertion site from Diana and an approximately 4-kb fragment containing the insertion site plus a truncated T-DNA from dm3t524. Presence of the T-DNA in the amplified product was confirmed by hybridization with the *AadA* gene. Comparison of the genomic sequences flanking the T-DNA insertion site in dm3t524 with those of wild-type cv. Diana showed that about 50 bp of lettuce DNA was missing in dm3t524. Lettuce sequences were adjacent to the *NptII* gene; the left border of the T-DNA was missing. This data is consistent with the disparity in sizes of the restriction fragments detected on Southern blots (Fig. 2) and with the lack of IPCR products derived from primers specific for the left border. The selection of transgenic lettuce lines containing pSLJ3621 was made with spectinomycin rather than kanamycin (Yang et al. 1993a); therefore, a T-DNA lacking a functional *NptII* gene was possible in dm3t524.

T-DNA insertion has interrupted a deduced ORF.

The nucleotide sequence of the IPCR product and of the wild-type sequence from the *HindIII* BAC subclone indicated that the T-DNA insertion occurred within a putative 92 amino-acid open reading frame (ORF), designated ORF2 (Fig. 1). ORF2 showed similarity [$P_{(N)} = 5.6 \times 10^{-7}$] to zinc finger transcription factors from various eukaryotes, including *Droso-*

Fig. 2. Integration of a partial T-DNA results in an increased fragment size in a multigene family. Southern blots of genomic DNA from wild-type Diana (D) and dm3t524 were probed with a 390-bp genomic fragment adjacent to the integration site (A) or with a T-DNA-specific probe, *Nos:AadA* (B). Approximately 2 to 5 μ g of Diana DNA and 5 μ g of dm3t524 DNA were digested with either *EcoRI* (E) or *HindIII* + *NotI* (H + N). After hybridization with IPCR₈₀₀, the membrane was stripped and re-probed with *Nos:AadA*.

phila and yeast in a BLASTX search (Altschul et al. 1990). A second ORF of 138 deduced amino acids, designated ORF1, started at the beginning of the subcloned 6.6-kb fragment in the reverse direction to ORF2 and terminated 1.58 kb from the T-DNA integration site (Fig. 1). A third ORF (ORF3) of 250 amino acids started 3.1 kb away from the T-DNA integration site and was oriented in the same direction as ORF2. No significant similarity was detected, using BLASTX (Altschul et al. 1990), between ORFs 1 or 3 and any sequences in GenBank. There were four other shorter ORFs within the 6.2-kb nucleotide sequence. No motifs, such as nucleotide-binding domains, obvious leucine-rich repeats, or domains conserved in protein kinases, that are present in disease resistance genes from other plants (Staskawicz et al. 1995; Bent 1996), were evident in the 6.2-kb sequence. Much of the sequence was AT rich.

The possibility that *Dm3* encoded a zinc finger protein as new class of resistance genes led to attempts to identify a cDNA or transcript. However, Northern (RNA) blots, cDNA library screens, and rapid amplification of cDNA ends (RACE) failed to identify transcripts corresponding to ORF2. PolyA RNA from cv. Diana and related cultivars did not hybridize to either IPCR₁₂₀₀ or BAC clones carrying wild-type copies of the 6.6-kb *Hind*III fragment. cDNA clones (1.4×10^6) from a cv. Diana leaf mRNA library were screened under various stringencies with a combination of BAC clones as probes. These clones carried copies of the 6.6-kb *Hind*III fragment (Fig. 1). None of the probe combinations showed

significant and reproducible hybridization. The cDNA library and leaf RNA were also used for 5' RACE and 3' RACE with ORF2-specific primers. No identifiable amplification products were obtained. Therefore, ORF 1, ORF2, and ORF3 encode low-abundance transcripts or are not transcribed. These experiments also indicated that no other abundant transcripts are encoded by BACH15.

Transgenic complementation experiments with the region flanking the T-DNA insertion site.

BACH15 contained the microsatellite array that was missing in all the deletion mutants; therefore, it was judged to be the most likely of the IPCR₈₀₀-containing BAC clones to contain *Dm3* (see above, Fig. 4). Overlapping 11-, 16- and 27-kb fragments of BACH15, each containing the complete 6.6-kb *Hind*III fragment with the wild-type T-DNA insertion site, were introduced by *Agrobacterium*-mediated transformation into cv. Cobham Green (no known *Dm* genes) and two EMS-induced mutants of *Dm3* in cv. Diana (R. Arroyo-Garcia, *unpublished*). T₂ families from 27 independent transgenic plants were tested for resistance to *Bremia lactucae*. None of the families showed resistance to the isolate IM25P11 (*Avr3*). The EMS-induced mutants were resistant and Cobham Green was susceptible to the three other isolates (CG1, R60, C83M47) as expected. Therefore, either T-DNA insertion had occurred close by but not within *Dm3*, or *Dm3* extends beyond the segments of genomic DNA tested.

Fig. 3. The T-DNA integrated into a fragment that is missing in all *Dm3* deletion mutants. Southern blot of genomic DNA from wild-type Diana (D) and various *Dm* mutants was probed with IPCR₁₂₀₀. Each lane contains about 2.5 to 5 µg of *Acc*I-digested DNA. A 4.8-kb band (arrow) is present in both wild-type Diana and a *Dm7* mutant (dm7r240), but is missing in all the *Dm3* deletion mutants (dm3r129 to 2022). In dm3t524, two novel bands (*) are observed due to an *Acc*I site in the T-DNA.

DISCUSSION

The dm3t524 mutant, a transgenic line of lettuce that has lost *Dm3* activity, has provided a more detailed understanding of the organization and complexity of the *Dm3* region. It led to the identification of molecular markers, including a region-specific microsatellite marker, very closely linked to *Dm3*. These markers have been useful for the isolation and characterization of BAC clones in the *Dm3* region (B. Meyers, Z. Zhang, K. Shen, and R. Michelmore, *unpublished*).

The T-DNA inserted either in or very close to *Dm3*. The T-

DNA originally contained a *Ds* element that had excised and been lost from the genome; it is impossible to determine if *Ds* excised, re-integrated close by, and then re-excised prior to being lost. Therefore, the mutation might have resulted from either T-DNA insertion into the *Dm3* gene or from insertion of *Ds* into *Dm3* and subsequent excision, leaving a "footprint" of approximately 8 bases that is difficult to locate. Such a footprint would result in a frameshift mutation and loss of *Dm3* activity.

Transcript analyses provided no indications of gene activity at the site of T-DNA insertion. Absence of detectable transcripts of ORF1, ORF2, or ORF3 in multiple experiments suggests that they are either rare or are not transcribed in the leaf. Alternatively, the AT-rich nature of the IPCR product and flanking sequences indicates that much of the 6.6-kb fragment sequenced could be part of an intron. Several consensus sequences for GT-AG splice junctions are present throughout the *Hind*III fragment. Insertion of the T-DNA would prevent correct processing and cause a loss of *Dm3* activity. In this case, ORF2 is coincidental or is a remnant of genome evolution. It is unlikely that the whole gene is represented by the 6.6-kb region sequenced. ORF1 was located at one end of the region sequenced and lacked an ATG at the beginning; therefore, it could be part of a larger ORF. However, there were no sequence motifs that indicated a function for ORF1 or ORF3.

Transgenic complementation experiments failed to restore *Dm3* activity. Therefore, either the T-DNA did not integrate into *Dm3* or the regions tested are not sufficient to confer *Dm3* activity. To determine if the IPCR₈₀₀ sequence or the 6.6-kb *Hind*III fragment is actually a portion of *Dm3*, or is necessary for *Dm3* activity, requires more extensive analysis of ORFs in the region and complementation with additional segments of genomic DNA.

MATERIALS AND METHODS

Plant material and genetic stocks.

Transformation procedures were as described previously (Michelmore et al. 1987). Cotyledons or leaf pieces of cv. Diana were sequentially co-cultivated first with *Agrobacterium tumefaciens* carrying Ti plasmid pSLJ3232, encoding CaMV 35S promoter:*Ac* transposase, followed by a second co-cultivation with *A. tumefaciens* carrying Ti plasmid pSLJ3621, encoding *Ds* (Yang et al. 1993a). Fungal isolates C83M47 (avirulent on *Dm1*), IM25P11 (avirulent on *Dm3*), R60 (avirulent on *Dm7*), and CG1 (avirulent on *Dm8*), and screens for loss of *Dm* activity in R₂ populations were as described previously (Okubara et al. 1994). Eight *Dm3* deletion mutants, two spontaneous mutants, and five EMS mutants were used for allelism tests (Table 2).

Analysis for *Ac*, *Ds*, and molecular markers in the *Dm3* region.

DNA from R₂ and R₃ seedlings was analyzed by PCR for the presence of maize *Ac* transposase sequences, *Ds*, the *Ds* empty donor site (Yang et al. 1993a), and molecular markers in the *Dm3* region as described (Paran and Michelmore 1993). Oligonucleotide primers used for PCR are listed in Table 1.

Isolation of dm3t524 genomic DNA flanking the T-DNA and of the corresponding sequence from wild-type Diana.

Lettuce genomic sequences that flanked the T-DNA inser-

Fig. 4. A microsatellite marker 864 bp from the T-DNA integration site detects several loci that are tightly clustered around *Dm3* and distinguishes bacterial artificial chromosome (BAC) clones from the region. Microsatellite marker MSAT15-34 was amplified from genomic DNA of Diana (D), seven deletion mutants of *Dm3*, dm3t524, and four BAC clones from the *Dm3* region. Each BAC clone contained a single array size. Different deletion mutants lacked different array sizes. The largest array that was missing in all deletion mutants was present in BACH15.

tion site in dm3t524 were isolated initially by IPCR. Nuclear DNA was digested with *Bam*HI and either *Bc*II, *Bg*III, or *Bst*YI prior to ligation. Nested primers specific to the right and left borders are shown in Table 1. PCR products obtained from dm3t524 but not from wild-type Diana were inserted into the *Eco*RV site of pBSKII- (Stratagene, La Jolla, CA). To obtain additional sequences flanking the T-DNA insertion site, genomic DNA from wild-type Diana and from dm3t524 was amplified with primers designed from the 1,200-bp IPCR product flanking the T-DNA, obtained above. A 1-kb PCR product from dm3t524, containing a portion of pSLJ3621 plus adjacent lettuce DNA, was cloned in pAMP1 (Gibco BRL, Gaithersburg, MD).

Analysis of *Dm3* mutants by hybridization to IPCR probes in Southern blots.

From 2.5 to 5 µg of DNA from cv. Diana and the *Dm3* mutants was digested with restriction enzymes, then transferred to Zeta Probe nylon membranes (BioRad, Hercules, CA) and hybridized to the 1,200-bp IPCR product (including a portion of the T-DNA right border and 800 bp of flanking lettuce DNA) or 390- or 660-bp fragments (containing only lettuce sequences) amplified by PCR from the cloned IPCR₁₂₀₀ product. Probes were radiolabeled with the Multiprime DNA Labeling System (Amersham, Arlington Heights, IL) to specific activities of 2 to 4 × 10⁶ cpm of ³²P per ng of DNA. Hybridization was carried out in 50% formamide.

To estimate the number of copies of the IPCR sequence in the lettuce genome, a Southern blot of dm3t524, digested with either *Eco*RI or *Hind*III, was hybridized with the 1,200- or 660-bp IPCR fragments. A 580-bp probe consisting of the nopaline synthase (*Nos*) promoter and a portion of *AadA* gene for resistance to spectinomycin was used for detection of T-DNA-associated sequences. Radiolabel that hybridized to both T-DNA-tagged and untagged copies under high-stringency conditions was quantified with a Fujix BAS 1000 phosphorimager. Quantitation was carried out by scanning lanes and measuring areas under peaks (Profile, MACBAS 2.0, Fujix, Stamford, CT), or by the direct counting of pixels in defined areas (Quant, Fujix).

Table 2. Mutants of *Dm3* used for allelism tests with mutant dm3t524

Designation	Inducing agent	Flanking makers	Previous designation ^a
dm3r129	Fast neutron	Some missing	dm3a
dm3r621	Fast neutron	Some missing	dm3b
dm3r794	Fast neutron	Some missing	dm3c
dm3r1208	Fast neutron	Some missing	dm3d
dm3r1403	Fast neutron	Some missing	dm3e
dm3r1608	Fast neutron	Some missing	dm3f
dm3r1885	Fast neutron	Some missing	dm3h
dm3r2022	Fast neutron	Some missing	dm3i
dm3e310	EMS ^b	All present	
dm3e407	EMS	All present	
dm3e1368	EMS	All present	
dm3e1534	EMS	All present	
dm3e1591	EMS	All present	
dm3s218	Spontaneous	Some missing	
dm3s1977	Spontaneous	All present	

^a The names of mutants reported previously (Okubara et al. 1994; Anderson et al. 1996) have been revised.

^b Ethyl methanesulfonate.

BAC clones containing IPCR sequences.

Over 50,000 BAC clones from two different libraries of cv. Diana, representing approximately two genome-equivalents (Frijters et al. 1997), were screened with the IPCR 660-bp probe. Four positive BAC clones, designated BACH15, BACH18, BACH19, and BACE42, were treated with *Acc*I, *Ase*I, *Eco*RV, or *Sca*I, diagnostic for different copies of the IPCR sequence. *Hind*III fragments of about 6.6 kb from BACH15, BACH18, and BACH19 that hybridized to the IPCR 660-bp probe in Southern blots were inserted into a low-copy plasmid vector, pBeloBAC11 (Shizuya et al. 1992).

BAC clones were distinguished with a microsatellite sequence identified adjacent to the T-DNA integration site. Primers were end labeled with γ³²P ATP. Microsatellites were amplified with *Taq* DNA polymerase, 10 pmol of each primer (Table 1), 50 ng of genomic DNA, and 5 mM dNTPs in a reaction volume of 20 µl for 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C. Reaction products were separated on a 8% denaturing polyacrylamide gel.

Nucleotide sequencing and database searches.

Portions of the *Hind*III subclones derived from BAC clones carrying the IPCR sequence and clones of amplified genomic DNA from Diana and dm3t524 were sequenced with an ABI373 Automated DNA Sequencer (Applied Biosystems, Foster City, CA). Primers used for sequencing are listed in Table 1. Nucleotide and deduced amino acid sequences were analyzed for similarities to known sequences in GenBank with BLASTX (Altschul et al. 1990).

Constructs for transgenic complementation.

DNA from BACH15 was partially digested with *Hind*III, size fractionated by gel electrophoresis, and cloned into the binary vector pCGN1557 (McBride and Summerfelt 1990). Clones that contained fragments with the T-DNA insertion site were identified by restriction analysis and hybridization. These were introduced into lettuce by *Agrobacterium*-mediated transformation, using previously published protocols (Micheltore et al. 1987) with *Agrobacterium* strain LBA4404. The presence of the insert in lettuce was confirmed by PCR with primers for *NptII* and growth of seedlings on kanamycin (200 mg/liter). All plants tested were transgenic. The presence of the 6.6-kb fragment was demonstrated in Cobham Green with the microsatellite marker MSAT15-34. It was not possible to test for the insert in the EMS-induced mutants of cv. Diana as they already had the wild-type sequence except presumably for a point mutation in the *Dm3* gene. At least 50 T₂ seedlings from each transgenic plant were tested for resistance to each of the following isolates: IM25P11 (avirulent on *Dm3*); C83M47 (avirulent on *Dm1*); R60 (avirulent on *Dm7*); and CG1 (avirulent on *Dm8*).

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