The Plant Cell, Vol. 8, 959–969, June 1996 © 1996 American Society of Plant Physiologists

The Tomato *Dwarf* Gene Isolated by Heterologous Transposon Tagging Encodes the First Member of a New Cytochrome P450 Family

Gerard J. Bishop, Kate Harrison, and Jonathan D. G. Jones[1]

The Sainsbury Laboratory, John Innes Centre, Norwich NR4 7UH, United Kingdom

To transposon tag the tomato *Dwarf* (*D*) gene, a tomato line that carries a T-DNA containing the maize *Activator* (*Ac*) transposable element closely linked to *D* was pollinated with a stock homozygous for the *d* mutation. Hybrid seedlings were screened for dwarf progeny, and three independent dwarf lines were obtained. Two of these lines showed inheritance of a recessive phenotype similar to that conferred by the *extreme dwarf* (d^x) allele. Variegation for the dwarf phenotype in one of these lines suggested that *D* had been tagged by *Ac*. Genomic DNA adjacent to *Ac* in these two lines was isolated by use of the inverse polymerase chain reaction, and the two insertions mapped ~2 kb apart. Partial complementation of *d* was observed when the corresponding wild-type sequence was used in transformation experiments. A cDNA clone of *D* was sequenced, and the predicted amino acid sequence has homology to cytochrome P450 enzymes.

INTRODUCTION

Dwarfism in plants is a striking trait and one of several that Mendel used to establish the laws of inheritance (Bateson, 1902). Many mutations that cause dwarfism are involved in the perception and metabolism of the plant hormone gibberellin (GA) (Reid, 1993). Other mutants generate dwarf plants through the production of abnormal cell walls (Reiter et al., 1993), mutant membranes (Lightner et al., 1994), or defects in cell expansion/elongation (Aeschbacher et al., 1995; Takahashi et al., 1995). Dwarfing genes, such as the dominant dwarf genes *Rht-1* and *Rht-2* in commercial cultivars of wheat, are of considerable economic significance (Gale and Youssefian, 1985).

The tomato *dwarf* (*d*) mutation has been known since the mid-19th century and was used to create dwarf tomato varieties such as Tiny Tim, Tom Thumb, and Dwarf Stone (Graham, 1959; C. Rick, personal communication). The stock Dwarf Champion carrying *d* was used in some of the first genetic linkage experiments in tomato (Price and Drinkard, 1908). Allelism tests between *d* and other dwarf mutants have uncovered several different *d* alleles (Rick, 1954; Stubbe, 1957, 1959), and these can be classified as strong, intermediate, or weak, with the strong alleles exhibiting the most pronounced dwarfism. Weak *d* alleles are dominant over strong alleles. Plants homozygous for the strong allele, *extreme dwarf* (d^x), have both reduced cell size and number (Nadhzimov et al., 1988). Treatment of plants homozygous for the *d* (Plummer and Tomes, 1958; Soost, 1959) or the d^x allele (Nadhzimov et al., 1988) with gibberellic acid (GA_3) does not restore a wild-type phenotype, although plant height increases. This indicates that the dwarf mutations are not GA response or biosynthesis mutants.

To gain further understanding of this interesting gene, we set out to isolate *D*, the wild-type allele of *d*, using transposon tagging. This approach, using the maize transposons *Activator* and *Dissociation* (*Ac/Ds*), has enabled the isolation of the disease resistance genes *N* from tobacco (Whitham et al., 1994), *L6* from flax (Lawrence et al., 1995), and *Cf-9* from tomato (Jones et al., 1994). Our tagging strategy took advantage of the fact that *Ac/Ds* elements transpose preferentially to genetically linked sites in tomato (Carroll et al., 1995). We used line 851Q, which has a T-DNA containing *Ac* closely linked to *D* on chromosome 2 (Thomas et al., 1994).

The D gene was tagged by a novel *Ac* element with two *Ac* 3′ ends and one 5′ end, which probably arose as the consequence of rearrangement of T-DNA sequences during T-DNA integration. Confirmation of tagging was provided by molecular analysis of two insertions in *D*, reversion of one of these alleles, and complementation of *d* with the wild-type genomic sequence. A cDNA clone and a genomic clone of *D* were sequenced, and the DWARF protein is predicted to be a cytochrome P450 (P450).

RESULTS

Crossing Strategy

The strategy adopted to transposon tag *D* is shown in Figure 1. The transgenic tomato line 851Q carries an *Ac*-containing T-DNA at a distance of 1% recombination from *D*. 851Q was produced by transformation with Agrobacterium containing the

[1] To whom correspondence should be addressed.

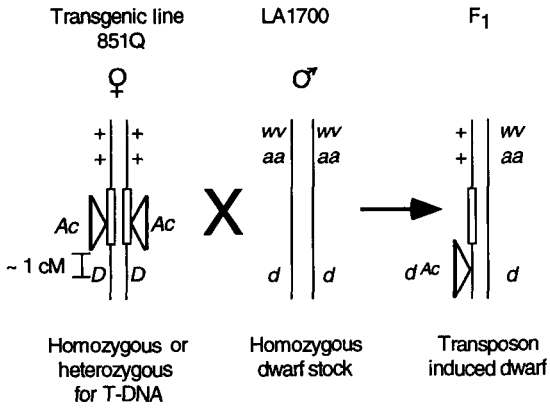

Figure 1. Tagging Strategy.

The *Ac*-containing line 851Q was pollinated with LA1700, a chromosome 2 tester stock harboring the mutations *wv*, *aa*, and *d*. Insertions of *Ac* into *D* were expected to generate dwarf progeny from this cross. cM, centimorgan.

plasmid pSLJ851, which carries a T-DNA with a neomycin phosphotransferase gene and *Ac* in a streptomycin phosphotransferase gene (Thomas et al., 1994). Individuals showing green and white variegation after germination on streptomycin-containing medium were presumed to have inherited T-DNAs with unexcised *Ac* linked to *D*. Fifteen plants harboring the 851Q T-DNA were used as female parents in crosses with the chromosome 2 tester line LA1700, which is homozygous for *d*, *white virescent* (*wv*), and *anthocyanin absent* (*aa*). These additional linked genetic markers (Figure 1) facilitated the characterization of the *Ac*-induced dwarf mutants. Of 3957 hybrid seedlings screened, 20 dwarf progeny were recovered from three different 851Q female parents (Table 1). At least three *Ac*-induced *d* alleles were generated among these 20 progeny and were named d^{Ac1}, d^{Ac2}, and d^{Ac3} (Table 1).

Genetic Analysis of the *Ac*-Induced *d* Alleles

Self-progeny from the dwarf mutants carrying d^{Ac1} or d^{Ac2} alleles segregated for a strong *d* allele, conferring a phenotype similar to that exhibited by the d^x allele. Four of the plants (1.7%) exhibiting a d^x-like phenotype among the self-progeny of the d^{Ac2} line had revertant sectors (Figures 2A to 2E). Reversion of the d^{Ac2} line provides convincing evidence that *D* was tagged; however, both the frequency of plants showing revertant sectors and the number of revertant sectors per plant are low when compared with *Ds* insertions in the tomato *Cf-9* gene (Jones et al., 1994; D. Jones, personal communication).

Variegation for wild-type sectors in an extreme dwarf background indicates that the physiological effect of *D* is not freely diffusible around the plant, which is consistent with reciprocal grafting experiments in which extreme dwarf material was not restored to wild-type phenotype by use of wild-type grafts (Stubbe, 1957). However, the sector boundaries were diffuse and found mainly at the location of a physical boundary, such as the veins (Figures 2C to 2E). This observation indicates that *D* does not act completely cell autonomously, and this implies that a small number of genotypically revertant cells may generate relatively large phenotypically revertant sectors. Transmission of revertant material through the gametes was poor, with self-seed from variegating lines producing mainly mutant progeny (99%) with rare revertants (1%). This pattern can also be explained by the nonautonomy of the effect of *D* between cell layers. Reversion in the epidermal (L1) or vascular cell layer (L3) could restore a wild-type phenotype, without a reversion having taken place in the layer that gives rise to the gametes (L2). Evidence for such layer-specfic sectors can be seen in Figure 2. For example, the main stem in plant V363 (Figure 2B) is revertant, but the majority of leaves are mutant, suggesting that the L3 is revertant but the L2 and L1 are mutant. No revertants were recovered from inflorescences arising from tissue with this phenotype.

Both d^{Ac3} individuals had reduced fertility, and no extreme dwarf progeny were recovered. The majority of the F_2 progeny from the two d^{Ac3} mutants were white virescent, due to the *wv* mutation linked to the reference *d* allele. This suggested that gametes carrying the d^{Ac3} event are poorly transmitted and that in these individuals, *D* sequences were lost through a deletion or other rearrangements.

Table 1. Dwarf Progeny Obtained from the Cross 851Q × LA1700[a]

851Q Female Parent Plant Number	Progeny Screened	Dwarf Plants Recovered[b]
K77	133	0
K78	313	17 d^{Ac1}
K80	6	0
K82	471	0
K83	244	0
K84	196	0
K85	451	0
K86	219	1 d^{Ac2}
K87	101	0
K88	172	0
K89	234	0
K90	202	0
K91	689	2 d^{Ac3}
K92	162	0
K93	344	0

[a] Each entry indicates seedlings screened from independent crosses.

[b] $d^{Ac1/2/3}$ refer to designation of the new *d* alleles carried by the transposon-induced dwarf lines.

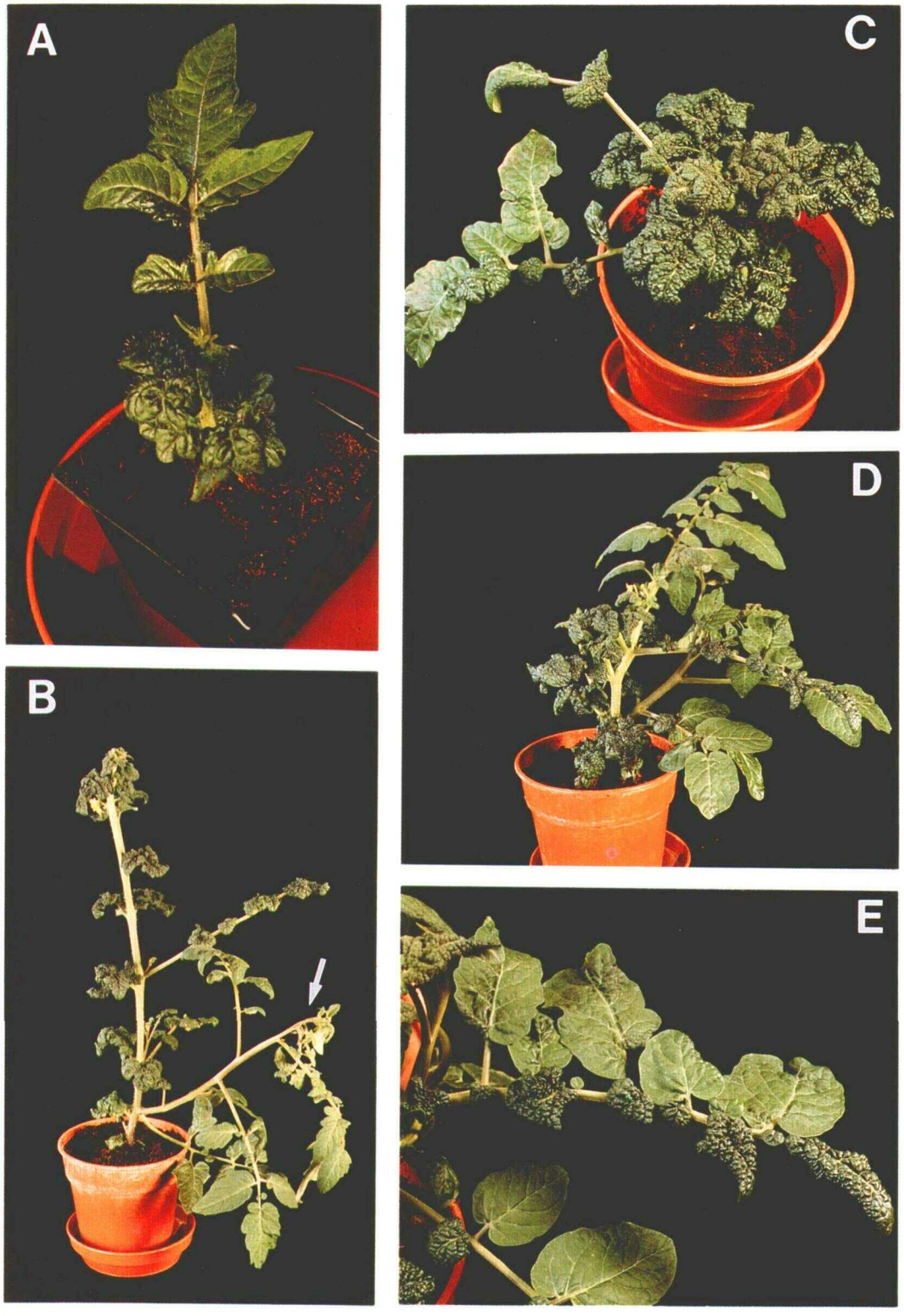

Figure 2. Transposon-Induced Dwarf Lines Showing Revertant Sectors.

Variegating F_2 individuals from the d^{Ac2} line are shown.

(A) A 3- to 4-week-old plant showing a revertant wild-type leaf.

(B) Plant V363 with a main shoot showing reversion in the stem but not in the leaves and a revertant side shoot (indicated with an arrow).

(C) Plant V364 with a revertant sector limited to three leaflets and petiole of one leaf and the petiole of another.

(D) Plant V362 showing revertant sectors.

(E) A close-up of a leaf from plant V362, with half of the leaf having a revertant phenotype and the other half being mutant.

In **(B)** to **(E)**, plants are 3 to 4 months old.

Molecular Analysis of the *Ac*-Induced *d* Alleles

The presence of *Ac* in the new dwarf lines was tested by DNA gel blot analysis. Hybridization of HpaI-digested DNA with *Ac* sequences indicated that four of the d^{Ac1} lines had the same 4.9-kb *Ac*-hybridizing band, suggesting that they carry the same transposition event (Figure 3, lanes 2 to 5). All 17 d^{Ac1} lines were found to carry a common *Ac*-hybridizing band on a gel blot of HindIII-digested DNA (data not shown). The d^{Ac2} mutant had a slightly larger 4.95-kb *Ac*-hybridizing band (Figure 3, lane 6). One of the two d^{Ac3} mutants lacked *Ac*-hybridizing sequences, and the other contained *Ac* (Figure 3, lanes 7 and 8). The d^{Ac3} lines did not transmit a transposon-induced phenotype and were not investigated further.

If *D* were tagged, then the d^{Ac1} and d^{Ac2} alleles should be linked in *cis* to the T-DNA. DNA gel blot analysis of 11 individuals exhibiting the extreme dwarf phenotype from a d^{Ac1} F_2 population and five from a d^{Ac2} F_2 population was performed with the tomato genomic sequences adjacent to the 851Q T-DNA as a probe. The results showed that they are also homozygous for the T-DNA (data not shown), indicating close linkage in *cis* between the T-DNA and the d^{Ac1} and d^{Ac2} alleles as predicted.

Isolation of the *D* Gene

Inverse polymerase chain reaction (IPCR; Ochmann et al., 1988; Triglia et al., 1988) was used to isolate the sequences adjacent to *Ac* in the lines d^{Ac1} and d^{Ac2}. Primary amplification with primers B34 and B39 (Figure 4A) on HpaI-digested and self-ligated DNA from d^{Ac1} and d^{Ac2} individuals generated PCR products of 401 bp for d^{Ac1} and 467 bp for d^{Ac2}, which is consistent with the length predicted by DNA gel blot analysis (Figure 3). Reamplification of these PCR products with nested primers D71 and D73 (Figure 4A) produced the predicted 45-bp reduction in product length, and the sequence of the cloned IPCR products indicated that the 5′ end of *Ac* is adjacent to 99 and 165 bp of tomato genomic DNA for the d^{Ac1} and d^{Ac2} insertions, respectively. However, for both insertions, the 3′ end of *Ac* is adjacent to the cauliflower mosaic virus 35S and T-DNA right-border sequences, suggesting a complex transposition product that may have been derived from a duplication of the T-DNA right end. A larger 1.4-kb IPCR product was obtained adjacent to the 5′ end of the d^{Ac2} insertion by using BglII-digested and self-ligated DNA as the template DNA for primers D60 and D73.

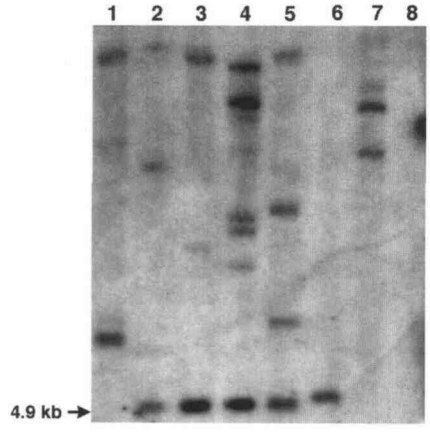

Figure 3. DNA Gel Blot Analysis of the Primary Dwarf Mutants.

DNA was digested with HpaI. The blot was hybridized with *Ac* sequences. Lanes 2 to 5 contain DNA from four of the 17 d^{Ac1} primary mutants, lane 6 contains DNA from the d^{Ac2} primary mutant, and lanes 7 and 8 contain DNA from the two d^{Ac3} primary mutants. DNA of a wild-type sibling plant of the d^{Ac1} primary mutant is present in lane 1. The arrow at 4.9 kb indicates the *Ac*-hybridizing band common to the d^{Ac1} mutants.

To obtain the tomato genomic sequences corresponding to the d^{Ac1} and d^{Ac2} insertion sequences, a binary vector cosmid genomic library of tomato (Dixon et al., 1996) was screened. The vector used allows the cloning of ~20 kb of DNA between the T-DNA left- and right-border repeats; this sequence can then be directly transferred into plant cells by using Agrobacterium-mediated transformation. Primers DW8 and DW7, which are homologous with the tomato genomic DNA sequence of the longer 1.4-kb d^{Ac2} IPCR product, were used in PCR screening of DNA extracted from 144 pools of the library (~1500 clones per pool). A single pool exhibited a PCR product of the predicted length. Cells from this pool were plated, and colony hybridizations using the d^{Ac2} IPCR product as the probe enabled the isolation of a single positive clone, which was called GB17-12. The tomato genomic sequences of both the d^{Ac1} and d^{Ac2} IPCR products hybridized with DNA of this cosmid (data not shown) and mapped ~2 kb apart (Figure 4B).

Primer DW8 was also used to amplify ~1 kb of cDNA by 3′ rapid amplification of cDNA ends (RACE; Frohman et al., 1988), and this PCR product was cloned and shown to hybridize with GB17-12 cosmid DNA (data not shown). The 3′ RACE product was also used to probe a tomato leaf cDNA library, and a single 1.6-kb cDNA was obtained after 500,000 plaques were screened. This low frequency of positive plaques was expected because RNA gel blot analysis of 10 μg of poly(A)-enriched RNA showed very weak hybridization with a band of ~1.7 kb when this probe was used (data not shown).

A 6-kb XhoI fragment from the cosmid GB17-12, containing the sequences corresponding to the points of *Ac* insertion in d^{Ac1} and d^{Ac2}, was subcloned and used to generate the genomic sequence of *D*. The cDNA was sequenced and used to obtain the predicted amino acid sequence of *D*. A schematic diagram of the structure of *D*, which is based on this sequence analysis, is shown in Figure 4A. *D* has nine exons, and the *Ac* insertions occurred in exon 2 for the d^{Ac2} mutation and exon 8 for the d^{Ac1} mutation.

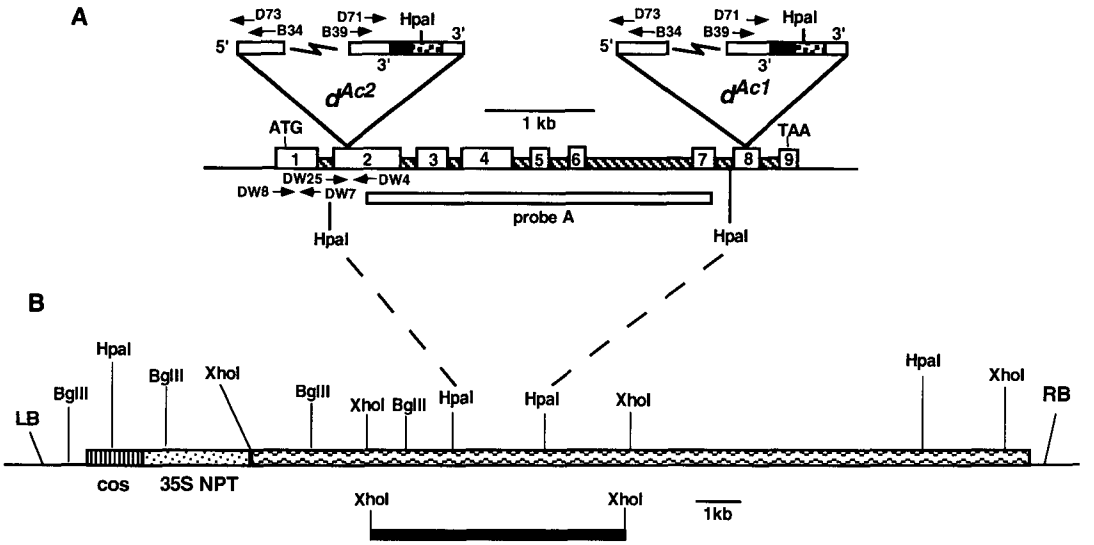

Figure 4. Diagram of the Structure of *D*, the Location of Transposon Insertions, and a Restriction Map of the T-DNA Region of Plasmid GB17-12.

(A) A comparison of the genomic and cDNA sequences of *D* indicates that *D* has nine exons (numbered boxes) and eight introns (diagonally striped bars). The locations of transposon insertions for the d^{Ac1} and d^{Ac2} lines are indicated. Both insertions consist of a full-length *Ac* transposon (unfilled boxes; not drawn to scale), with the 3′ end adjacent to the 35S (black boxes) and T-DNA right-border sequences (speckled boxes) and an additional 196 bp of 3′ *Ac* sequence. The approximate locations of primer sequences are shown by arrowheads. The open box indicates the extent of probe A used in DNA gel blot analysis (Figure 6A).

(B) Shown is a restriction map of the T-DNA region of the binary vector cosmid GB17-12, which contains ~20 kb of tomato genomic DNA (wavy lined box) plus a 35S:neomycin phosphotransferase (35S:NPT) gene fusion (speckled box) and the λ phage *cos* sequences (cos) (striped box) in between the T-DNA left- and right-border sequences (LB and RB). The 6-kb XhoI fragment shown as a black box was subcloned and used to obtain the genomic sequence of *D*.

Complementation of the *d* Mutation

To confirm that *D* had been isolated, complementation of the *d* mutation was performed with the use of the plasmid GB17-12 to transform LA1525 (a line homozygous for *d*). Partial complementation of *d* was observed in four of the seven transformants generated, and linkage of this phenotype to the T-DNA was observed in testcross progeny from transformants 17-12A and 17-12B (Figures 5A and 5B) and in the F_2 progeny of all four complementing transformants (data not shown). Plants containing the T-DNA are all taller, the height to the first inflorescence is greater, and the sixth true leaf length is longer than in plants lacking the T-DNA (Figure 5C). These differences were analyzed by Student's *t* tests, and all were found to be significant at $P = 0.001$. The complemented plants, however, were not fully restored to the wild-type phenotype. They are shorter than the wild type (Figure 5A) and still have a slightly rugose leaf phenotype (Figure 5B).

Isolation and Analysis of a Revertant

A putative germinal revertant was obtained at a frequency of 1.5% from self-seed of a variegating d^{Ac2} line. This revertant was analyzed using DNA gel blot and PCR methods (Figure 6). Tomato genomic sequences adjacent to the 3′ end of the d^{Ac2} insertion (probe A in Figure 4A) were hybridized with DNA digested with NcoI and EcoRV (Figure 6A). Wild-type DNA yielded a single 4.5-kb hybridizing band (lane 1). An extreme dwarf sibling of the germinal revertant had 4.5-, 5.2-, and 9.8-kb hybridizing bands (lane 2), which suggests 0.7- and 5.3-kb insertions into the 4.5-kb wild-type sequence. The germinal revertant lacked the 9.8-kb band but had both the 5.2-kb band and the wild-type 4.5-kb band (lane 3). This observation suggests that two different types of excisions can occur from the d^{Ac2} insertion: either *Ac* excises, leaving behind 0.7 kb comprising the 35S promoter, T-DNA right border, and additional *Ac* 3′ end, or the whole 5.3-kb unit excises to generate the 4.5-kb wild-type band.

This observation was verified by PCR amplification of the insertion sequences by using primers DW4 and DW25 (Figure 4A). The revertant gives rise to the 849- and 158-bp PCR products (Figure 6B, lane 3). Sequence analysis of the 849-bp showed the presence of the 35S promoter, the T-DNA right border, and *Ac* 3′ sequences; the sequence of the 158-bp product was identical to that of the wild type (data not shown). The phenotypic extreme dwarf individual of the d^{Ac2} line gives rise to a 849-bp product and a product slightly larger than 158 bp

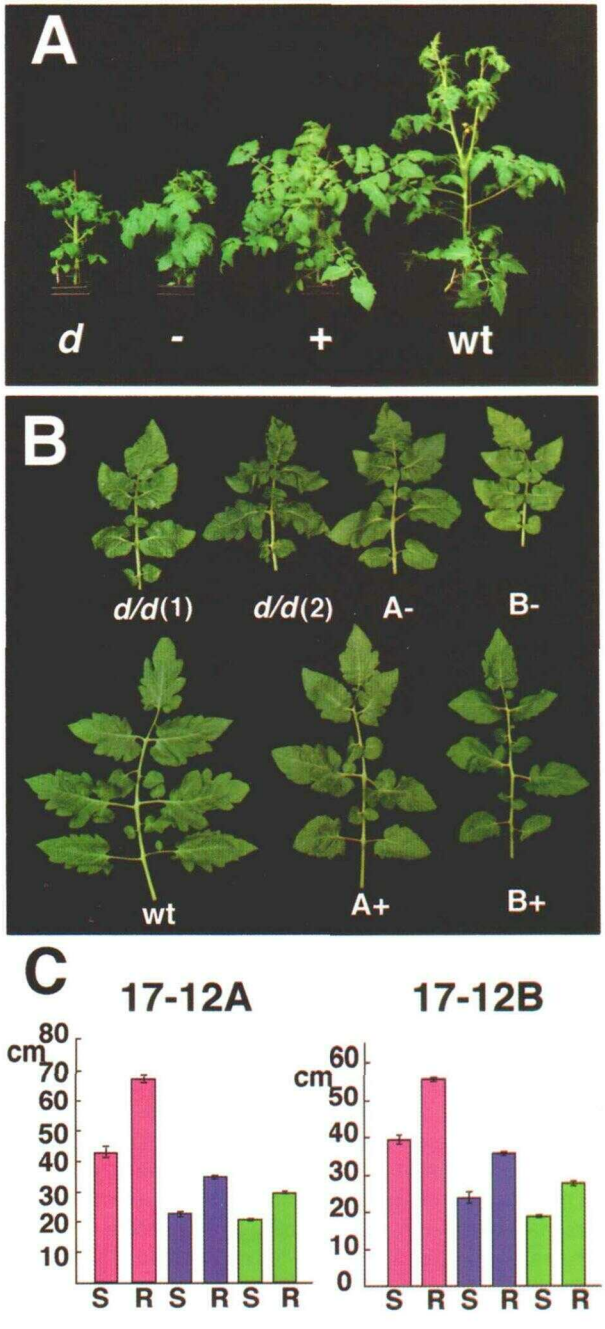

Figure 5. Complementation of *d*.

(A) Examples of plant phenotypes observed 3 months after sowing. Independent transformants of dwarf stock LA1525 were generated using Agrobacterium containing plasmid GB17-12 and sequentially named 17-12A, 17-12B, and so forth. *d*, dwarf stock (LA1525); −, kanamycin-sensitive segregant from primary transformant 17-12A crossed to LA1700; +, kanamycin-resistant segregant from 17-12A crossed to LA1700; wt, wild-type cultivar Moneymaker.

(Figure 6B, lane 2), which possibly represents excision of the complete transposon, but with the retention of all or some of the 8-bp duplication.

To confirm that the revertant was not the product of contaminating pollen, the zygosity of the linked T-DNA was analyzed by DNA gel blot analysis. The revertant was found to be homozygous for the linked T-DNA (data not shown) and therefore could not have been derived from contaminating pollen lacking the 851Q T-DNA.

Homology of DWARF to Cytochrome P450 Enzymes

The predicted amino acid sequence of *D* was determined (Figure 7). The DWARF protein would be 464 amino acids long with a molecular mass of ~54,000 D. BLAST data base searches (Altschul et al., 1990) indicated that DWARF is homologous with cytochrome P450 enzymes that are members of a superfamily of proteins acting as the terminal oxidases in electron transfer chains. P450s are heme thiolate proteins, and the sequence surrounding the heme binding cysteine is conserved in most P450 enzymes at a location ~50 amino acids from the C terminus (Nelson et al., 1996). DWARF has this sequence in the appropriate location, and on this basis it has been classified as a P450. However, the sequence homology of DWARF to other P450 enzymes is not limited to the heme binding domain. Another similarity includes a conserved threonine in helix I preceded by an acidic residue (glutamic acid) (Figure 8, Domain A). Other similarities are summarized in Figure 8, which provides a comparison of the DWARF sequence to known plant P450s. In addition, DWARF has 23 hydrophobic residues at the N terminus that may function as the membrane anchor region. Currently, the most homologous sequence detected in the data bases is an Arabidopsis P450 sequence whose function is unknown (CYP90 GenBank accession number X87367) (Figure 9). This sequence has 38% identity with DWARF but, according to P450 classification, represents a different family of P450 (Nelson et al., 1996). In fact, DWARF represents the first member of CYP85, a new family of P450 enzymes (D. Nelson, personal communication).

(B) Phenotypes of the sixth true leaf obtained from 3-month-old plants. *d/d*(1), F_1 heterozygote for the two dwarf stocks LA1700 and LA1525; *d/d*(2), dwarf stock LA1525; A− and B−, kanamycin-sensitive segregants from the cross of lines 17-12A and 17-12B to LA1700, respectively; wt, wild-type cultivar Moneymaker; A+ and B+, kanamycin-resistant segregants from the cross of lines 17-12A and 17-12B to LA1700, respectively.

(C) Shown are mean measurements of plant height (pink), height to first inflorescence (blue), and length of the sixth true leaf (green) of 3-month-old progeny derived from the cross of transformants 17-12A and 17-12B to LA1700. Twenty-four individuals were analyzed for line 17-12A and 17 for 17-12B. S, kanamycin-sensitive individuals; R, kanamycin-resistant individuals. Standard error bars are shown.

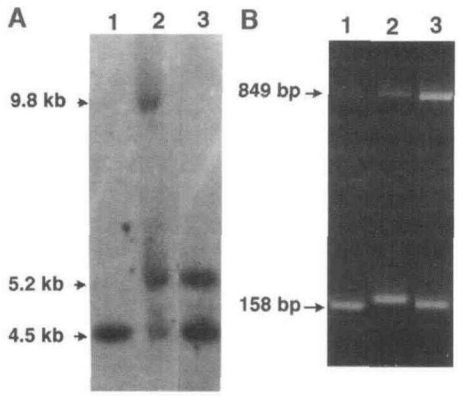

Figure 6. Reversion.

(A) DNA gel blot analysis. DNA was digested with NcoI and EcoRV. Blots were hybridized with probe A (see Figure 4A). Lane 1 contains DNA from a wild-type plant; lane 2, DNA of an extreme dwarf sibling of the d^{Ac2} revertant; lane 3, DNA from a putative d^{Ac2} germinal revertant. See text for details of the 4.5-, 5.2-, and 9.8-kb hybridizing bands. **(B)** PCR analysis. An ethidium bromide–stained agarose gel shows PCR products generated, using primers DW4 and DW25, which amplify the d^{Ac2} insertion site. Lanes 1 to 3 contain PCR products derived from the same DNA used in the corresponding lanes of the DNA gel blot analysis. See text for details of the 158- and 849-bp PCR products.

DISCUSSION

Transposon Tagging and Gene Isolation

We isolated a specific tomato gene by transposon tagging. This strategy has been successful for isolating the disease resistance genes *Cf-9*, *L6*, and *N* (Jones et al., 1994; Whitham et al., 1994; Lawrence et al., 1995), and the addition of *D* to this list confirms its efficacy. *D* was tagged at a high frequency (5.1×10^{-4}), presumably because of the proximity of the *Ac* donor locus (1% recombination) to the *D* gene. This frequency is similar to that at which *Cf-9* was tagged (3.4×10^{-4}) with a linked *Ds* element (3% recombination) (Jones et al., 1994).

Initial evidence suggesting that *D* was tagged was obtained from the inheritance of two *Ac*-induced alleles of *D*, d^{Ac1} and d^{Ac2}, conferring an extreme dwarf phenotype in the homozygous state. These insertions are likely to have generated complete loss-of-function alleles of *D* similar to the d^{x} allele reported previously. Somatic reversion of the d^{Ac2} allele generated variegation for wild-type somatic sectors. The third *Ac*-induced mutation, d^{Ac3}, probably represents deletion of *D* or a chromosome breakage event. This is not unusual because mutations derived by deletion of target genes have been observed previously (Peterson and Yoder, 1993) and are thought to be the consequence of *Ac/Ds*–induced chromosome breakage.

Additional evidence for the tagging of *D* was suggested by the proximity (~2 kb) of the two independent transposon insertions generating a d^{x}-like phenotype. However, both insertions were unusual because they were composed of an intact *Ac* adjacent to the 35S promoter and T-DNA right-border

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   1 ATGGCCTTCTTCTTAATTTTTCTTTCATCCTTTTTTGGCCTATGTATCTTTTGTACTGCT
     M  A  F  F  L  I  F  L  S  S  F  F  G  L  C  I  F  C  T  A

  61 TTATTAAGATGGAATCAAGTCAAGTATAACCAAAAAAACTTGCCCCCTGGTACTATGGGT
     L  L  R  W  N  Q  V  K  Y  N  Q  K  N  L  P  P  G  T  M  G

 121 TGGCCACTTTTTGGTGAAACTACTGAGTTTCTTAAACTTGGTCCAAGTTTCATGAAAAAC
     W  P  L  F  G  E  T  T  E  F  L  K  L  G  P  S  F  M  K  N

 181 CAAAGAGCCAGATATGGGAGTTTTTTTAAATCACACATACTTGGTTGTCCAACAATTGTT
     Q  R  A  R  Y  G  S  F  F  K  S  H  I  L  G  C  P  T  I  V

 241 TCAATGGATTCAGAACTGAACAGATATATACTAGTGAATGAAGCGAAAGGACTGGTCCCA
     S  M  D  S  E  L  N  R  Y  I  L  V  N  E  A  K  G  L  V  P
                 dAc2
 301 GGATACCCACAGTCTATGATAGATATTTTAGGAAAATGTAATATTGCAGCTGTCAATGGT
     G  Y  P  Q  S  M  I  D  I  L  G  K  C  N  I  A  A  V  N  G

 361 TCAGCTCACAAGTACATGAGGGGTGCATTGTTATCCCTAATTAGCCCTACAATGATCAGA
     S  A  H  K  Y  M  R  G  A  L  L  S  L  I  S  P  T  M  I  R

 421 GACCAACTTTTGCCTAAAATTGATGAGTTTATGAGATCCCACTTAACCAATTGGGATAAT
     D  Q  L  L  P  K  I  D  E  F  M  R  S  H  L  T  N  W  D  N

 481 AAAGTTATTGACATTCAAGAGAAAACCAATAAGATGGCATTTCTATCATCGTTGAAGCAA
     K  V  I  D  I  Q  E  K  T  N  K  M  A  F  L  S  S  L  K  Q

 541 ATTGCTGGTATTGAATCTACCTCTTTAGCTCAAGAATTCATGTCTGAATTTTTCAATCTA
     I  A  G  I  E  S  T  S  L  A  Q  E  F  M  S  E  F  F  N  L

 601 GTGCTAGGCACTCTTTCACTACCTATCAATCTTCCAAACACCAACTATCATCGCGGATTT
     V  L  G  T  L  S  L  P  I  N  L  P  N  T  N  Y  H  R  G  F

 661 CAGGCAAGGAAAATTATTGTGAACTTATTACGAACACTCATAGAAGAGAGAAGAGCTTCA
     Q  A  R  K  I  I  V  N  L  L  R  T  L  I  E  E  R  R  A  S

 721 AAGGAAATTCAACATGATATGCTTGGTTACCTGATGAATGAGGAAGCAACACGATTCAAA
     K  E  I  Q  H  D  M  L  G  Y  L  M  N  E  E  A  T  R  F  K

 781 TTAACAGATGATGAGATGATTGATTTAATTATAACTATTTTGTACTCTGGATATGAAACT
     L  T  D  D  E  M  I  D  L  I  I  T  I  L  Y  S  G  Y  E  T

 841 GTTTCCACCACTTCTATGATGGCTGTGAAATATCTTCATGATCATCCAAAAGTTCTTGAA
     V  S  T  T  S  M  M  A  V  K  Y  L  H  D  H  P  K  V  L  E

 901 GAACTTAGAAAAGAACACATGGCTATTAGAGAAAAGAAAAAACCTGAGGATCCTATCGAT
     E  L  R  K  E  H  M  A  I  R  E  K  K  K  P  E  D  P  I  D

 961 TACAACGATTACAGGTCAATGCGGTTCACACGAGCTGTGATTTTAGAGACCTCCAGGTTA
     Y  N  D  Y  R  S  M  R  F  T  R  A  V  I  L  E  T  S  R  L

1021 GCAACAATAGTAAATGGGGTTTTGAGAAAAACAACTCAAGATATGGAAATAAATGGGTAT
     A  T  I  V  N  G  V  L  R  K  T  T  Q  D  M  E  I  N  G  Y

1081 ATCATTCCTAAAGGATGGAGAATATACGTATATACAAGGGAGTTGAATTACGATCCAAGA
     I  I  P  K  G  W  R  I  Y  V  Y  T  R  E  L  N  Y  D  P  R

1141 CTTTATCCTGATCCATATTCGTTCAATCCATGGAGATGGATGGATAAGAGCCTGGAACAC
     L  Y  P  D  P  Y  S  F  N  P  W  R  W  M  D  K  S  L  E  H
                                                 dAc1
1201 CAAAACTCATTTTTGGTATTTGGAGGTGGTACTAGACAATGTCCTGGAAAGGAACTTGGT
     Q  N  S  F  L  V  F  G  G  G  T  R  Q  C  P  G  K  E  L  G

1261 GTAGCAGAAATTTCCACATTTCTTCATTACTTCGTAACAAAATACAGATGGGAAGAAATA
     V  A  E  I  S  T  F  L  H  Y  F  V  T  K  Y  R  W  E  E  I

1321 GGTGGAGATAAACTGATGAAATTCCCAAGAGTTGAAGCACCAAATGGTCTACGGATTAGA
     G  G  D  K  L  M  K  F  P  R  V  E  A  P  N  G  L  R  I  R

1381 GTTTCAGCTCAC 1392
     V  S  A  H   464
```

Figure 7. cDNA and Predicted Amino Acid Sequence of *D*.

The amino acid sequence predicted from the cDNA sequence of *D* is shown in boldface letters, and amino acids homologous with the heme binding consensus sequence of cytochrome P450 enzymes are underlined with dots. The 8-bp sequences, duplicated as a consequence of transposon insertion in d^{Ac1} and d^{Ac2}, are double underlined. This sequence has GenBank accession number U54770.

Domain A

```
Dwf   LIITILYSGYETVS
Pet   LLLNLFTAGTDTSS
Avo   VILDMFSGGTDTTA
Art   LYIVENVAAIETTL
```

Domain B

```
Dwf   MRFTRAVILETSRL
Pet   LPYLRAICKETFRK
Avo   LHYLKLIIKETLRL
Art   LPYLQAVVKETLRL
```

Domain C

```
Dwf LNYDPRLYPDPYSFNPWRW
Pet IGRDPEVWENPLEFYPERF
Avo IGRDPKSWENAEEFLPERF
Art LANNPDQWKKPEEFRPERF
```

Domain D (heme binding)

```
Dwf NSFLVFGGGTRQCPGKELGV
Pet FELIPFGAGRRICAGTRMGI
Avo FQLIPFGAGRRGCPGIAFGI
Art FRYLPFGVGRRSCPGIILAL
```

Figure 8. Homology of DWARF to Conserved Regions of Known P450 Enzymes.

The predicted amino acid sequence of *D* (Dwf) is shown aligned to the predicted amino acid sequences of other known plant P450 enzymes, within four conserved domains described by Kalb and Loper (1988). Residues conserved between most cloned P450s are shown in boldface letters; identical residues are underlined. Sequences were aligned using the Genetics Computer Group PILEUP program. Pet, petunia (Holton et al., 1993); Avo, avocado (Bozak et al., 1990); Art, artichoke (Teutsch et al., 1993).

sequences and an additional ~200 bp of *Ac* 3′ end sequences. This structure was detected in F_2 progeny of the primary transformant as well as in wild-type siblings of the tagged dwarf lines (data not shown). It seems likely therefore that such a structure was not generated through an aberrant transposition event but occurred earlier, possibly during T-DNA integration. However, we have not characterized the structure of the 851Q T-DNA insertion to determine whether the T-DNA carries a duplicated right end.

The transposon insertions into *D* generated typical 8-bp duplications, and at least one revertant arose by perfect excision of the transposon. Perfect excision has been reported for tagged alleles of *N* (Dinesh-Kumar et al., 1995) and of the fatty acid elongation gene (James et al., 1995). This suggests that although perfect excision of *Ac/Ds* in heterologous hosts may be uncommon, it may not be as rare as it is in maize (Baran et al., 1992). The low frequency of perfect excision may explain the relatively low number of plants exhibiting reversion.

Additional proof that *D* had been isolated was obtained by partial complementation of line LA1525, which was homozygous for the *d* mutation. The lack of complete complementation is not fully understood but could be due to position effects on

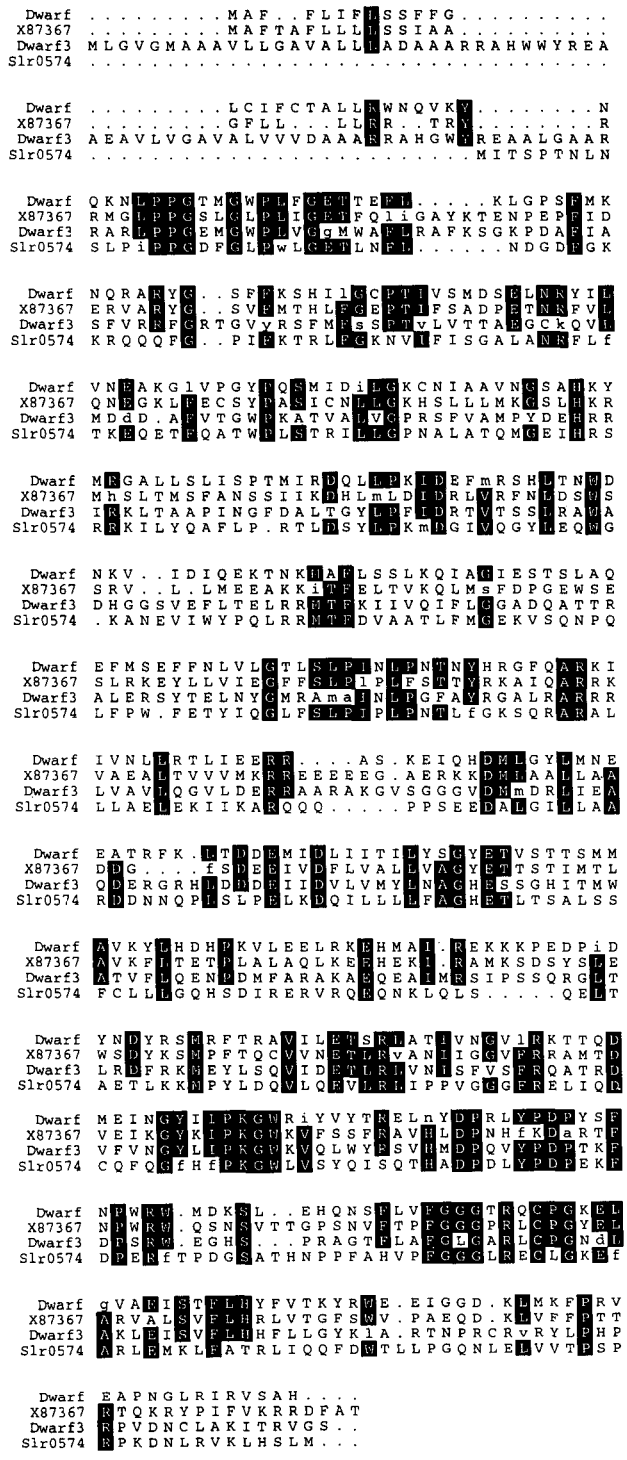

Figure 9. Homology of DWARF to Non-Class A Plant P450s and a Cyanobacterial Sequence.

Shown is a multiple alignment of the predicted amino acid sequence of DWARF to the predicted amino acid sequences of the most homologous sequences at the National Center for Biotechnology Information

the introduced *D* gene and inappropriate or insufficient expression. A possible means for testing this hypothesis would be to express *D* under the control of a promoter less susceptible to position effects.

DWARF, a Cytochrome P450

The homology between the predicted amino acid sequence of *D* and the conserved sequences of P450s, especially the heme binding sequence, provides convincing evidence that *D* encodes a cytochrome P450. P450s are so named because the photoreversible binding of molecular carbon monoxide to the heme group present in P450 enzymes produces a characteristic Soret absorption maximum of 450 nm. The inhibition of P450 activity by carbon monoxide has been used to identify plant P450s in many biochemical pathways (Bolwell et al., 1994). Few plant P450 genes have been cloned by protein purification methods because P450 enzymes are predominantly membrane bound, expressed at low levels, and are relatively unstable. The isolation of *D* represents one of the few plant P450 genes that is associated with known mutations. Other mutations associated with P450 function include dwarfism in maize (Winkler and Helentjaris, 1995) and flower color in petunia (Holton et al., 1993).

The maize *Dwarf3* (*D3*) gene encodes a P450 that is believed to catalyze the 13-hydroxylation of GA_{12} to GA_{53} (Winkler and Helentjaris, 1995). D3 has 30% amino acid identity to DWARF (Figure 9) and is a member of a different family of P450, CYP88. Further differences between D3 and DWARF are apparent. First, the phenotype of the maize *d3* mutation can be restored by using GAs, whereas the phenotypes of the *d* and d^x mutations of tomato are not restored to that of the wild-type by using GA_3 (Plummer and Tomes, 1958; Soost, 1959; Nadhzimov et al., 1988). Second, lines homozygous for the d^x allele of tomato have increased levels of GA_{20} (Nadhzimov et al., 1988), which is a GA produced downstream from the action of *D3*. The increased levels of GA_{20} might suggest that *D* encodes the 3β-hydroxylase that converts GA_{20} to the active GA_1. This possibility is unlikely, however, because in Arabidopsis this reaction is catalyzed by a dioxygenase and not by a P450 (Chiang et al., 1995). These observations suggest that *D* is probably not involved in the biosynthesis of GA.

What reaction does DWARF catalyze? Plant P450 enzymes catalyze many different reactions in secondary metabolism involving lipids, phenylpropanoids, flavonoids, terpenoids, alkaloids, and cyanogenic glucosides, and they can also metabolize xenobiotics (e.g., herbicides; reviewed in Bolwell et al., 1994). Assigning a biochemical function to DWARF by homology to known P450 enzyme sequences is difficult, but there are some clues to the possible function of DWARF.

First, the recovery of variegated plants suggests that DWARF catalyzes a reaction that either utilizes a substrate or generates a product that is not freely diffusible. DWARF may therefore be involved in the biosynthesis of a fatty acid or a steroid.

Second, two major classes of plant P450 enzymes exist. Class A P450s perform plant-specific reactions and have the consensus sequence PFGG(ASV)GRRC(PAV)G around the heme binding cysteine (Durst and Nelson, 1995). Non-class A P450s, which are more similar to animal, lower eukaryote, and bacterial sequences, lack this motif. Non-class A P450s in plants are thought to perform reactions similar to those occurring in other kingdoms. DWARF appears to be a non-class A P450, as shown in the sequence comparison of DWARF to the most homologous sequences in the data base (Figure 9). The relatively high homology of DWARF to a cyanobacterial sequence (Figure 9) adds weight to the idea that DWARF is catalyzing a reaction similar to those in other systems, which includes reactions such as steroid and fatty acid production. One possibility may be that DWARF could be involved in the metabolism of brassinosteroids, which are known to affect plant growth (Sakurai and Fujioka, 1993).

Conceivably, generating transformed lines that increase or decrease the level of expression of *D* in wild-type tomatoes may allow the production of larger and smaller plants. Controlling plant morphology in this way may prove to be beneficial in crop production. Furthermore, once the function of DWARF is known, it may prove useful in the biosynthesis or degradation of economically important chemicals.

METHODS

Tomato Lines

The transgenic tomato line 851Q has been described previously (Thomas et al., 1994). GB17-12 transformants of stock LA1525 were obtained by *Agrobacterium tumefaciens*–mediated tomato transformation, as described previously (Horsch et al., 1985; Fillatti et al., 1987). Dwarf stocks LA1700 (*wv-aa-d*) and LA1525 (*aa-d*) were obtained from C. Rick (University of California at Davis).

Nucleic Acid Extraction and Analysis

DNA extraction and DNA gel blot analysis were performed as described previously (Thomas et al., 1994). Blots were washed with an aqueous

Figure 9. (continued).

data base. The alignment was created by using the PILEUP program (Genetics Computer Group), and sequence homology is shown by using the PRETTYBOX program. Black shading indicates identical residues, and lowercase letters indicate residues that are conservative substitutions. Gaps introduced to improve the alignment are represented by dots. Dwarf, tomato DWARF (CYP85); X87367, GenBank accession number X87367 for CYP90 from Arabidopsis; Dwarf3, D3 (CYP88) from maize (Winkler and Helentjaris, 1995); and Slr0574, GenBank accession number D64003, for a sequence from the cyanobacterium *Synechocystis*. All plant P450 sequences are non-class A P450 sequences.

solution of 30 mM sodium chloride, 3 mM sodium citrate, and 1% (w/v) SDS at 65°C. DNA adjacent to *Activator* (*Ac*) in tomato line 851Q was obtained by inverse polymerase chain reaction (IPCR; Ochmann et al., 1988; Triglia et al., 1988), as described previously (Thomas et al., 1994). PCR was performed as recommended by Perkin-Elmer (Norwalk, CT). DNA sequencing was performed both on double-stranded plasmid DNA purified using tip 20 columns (Qiagen, Chatsworth, CA) and on gel-purified PCR products. Sequencing reactions were performed with the use of cycle-sequencing dye terminator kits (Applied Biosystems, La Jolla, CA), according to the manufacturer's instructions. Sequencing reactions were analyzed with an ABI 373A sequencing system. Plasmid templates for sequencing included nested deletions in either orientation of a 6-kb XhoI fragment from GB17-12 and plasmid GB030-5, which harbors the ~1.6-kb *Dwarf* (*D*) cDNA (Figures 4A and 4B). Contiguous sequence was generated with Macintosh-based ABI software Autoassembler. Compiled sequences were analyzed further with the Genetics Computer Group (Madison, WI) sequencing programs, and version 8.0. BLAST programs (Altschul et al., 1990) were used to detect homology of DWARF to P450 enzymes in the nonredundant nucleotide and peptide data bases at the National Center for Biotechnology Information (Bethesda, MD).

Primer Sequences

The 5′ to 3′ sequences of primers used are as follows: DW4, GTGAGCTGAACCATTGACAGCTG; DW7, GTTGGACAACCAAGTATGTGTGAT; DW8, TGAGGTGCATCAATGGCCTTC; DW25, TCACACATACTTGGTTGTCCAAC; B34, ACGGTCGGTACGGGATTTTCCCAT; B39, TTTCGTTTCCGTCCCGCAAGTTAAAT; D60, GTGATCCAGATGTGAGCAAG; D71, CCGTTACCGACCGTTTTCACTCCTA; and D73, TTTCCCATCCTACTTTCATCCCTG.

Binary Vector Cosmid and cDNA Isolation

The binary vector cosmid library, distributed in 144 pools as described by Dixon et al. (1996), was screened by use of primers DW8 and DW7 (Figure 4A). Positive pool 17 was screened by colony blot hybridization by using the tomato genomic sequences adjacent to d^{Ac2} as the probe. A single positive clone was isolated and designated GB17-12. A cDNA library (Jones et al., 1994) was screened by using the 3′ rapid amplification of 3′ ends (RACE) product as the probe on plaque filter lifts. A single positive plaque was isolated, and the cDNA insert was subcloned into pdarkBluescript and designated GB030-5.

Plant Growth and Analysis

Plants were grown under standard glasshouse conditions. The presence of T-DNA was tested by assaying for neomycin phophotransferase activity as described by McDonnell et al. (1987). Student's *t* test was performed on plant height and leaf length measurements, as described by Bailey (1984).

ACKNOWLEDGMENTS

We thank Charles Rick for providing the dwarf stocks. We are grateful to Colwyn Thomas for providing the cDNA library and Mark Dixon and James Keddie for providing the binary vector cosmid library. David Nelson of the P450 nomenclature committee kindly classified the putative DWARF protein as CYP85. We thank Sara Perkins, Jake Darby, and Margaret Schailer for plant care and Peter Scott and Andrew Davies for photography. We are also very grateful for David Jones's frank and constructive criticism of the manuscript. This project was supported by the Gatsby Foundation and Biotechnology and Biological Sciences Research Council grant No. 83/P01834.

Received February 20, 1996; accepted April 25, 1996.

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NOTE ADDED IN PROOF

Recent publications by Sekeres et al. (1996) and Li et al. (1996) describe the isolation of the *CPD* and *DET-2* genes of Arabidopsis that are involved in brassinosteroid biosynthesis. *CPD* encodes CYP90, which was classified in the main text as function unknown. The high sequence homology between CYP85 and CYP90 (see Figure 9) plus the similar mutant phenotype strengthen the suggestion that *Dwarf* could be involved in brassinosteroid production. We are investigating the Arabidopsis CYP85 homolog, and current analysis shows it to have ~68% amino acid similarity to the tomato sequence (data not shown), which confirms CYP85 as a first member of a new family of P450s.

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