with recolonization by piscivores could thus create the variability in predation pressure needed to promote the evolution of an inducible defense in crucian carp.

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10. In May 1991, the ponds were divided into two sections of equal size with a plastic curtain attached to vertical stakes at 2- to 3-m intervals; the curtain extended 30 cm above the water. The lower edge of the curtain was folded and wedged to form a tube that was filled with gravel. The tube sank down into the soft sediment to create a tight bottom seal, which was confirmed by a scuba diver. Crucian carp were sampled with two trap nets placed overnight in each pond half in May and September, and body lengths (total length, measured to the nearest millimeter) and body depths (just anterior to the dorsal fin, measured to the nearest millimeter) were measured. On 14 to 19 June, pike were introduced to one sector of each pond. Pike were caught by electrofishing in nearby ponds and measured and weighed before release. We added 15 pike (total biomass: 5.85 kg, 117 kg/ha; lengths: 168 to 570 mm) to Mats' Pond and 20 pike (total biomass: 5.17 kg, 103.3 kg/ha; lengths: 136 to 595 mm) to Severin's Pond. The resulting densities are typical of those in nearby ponds (*11*). Gut content analysis in July revealed that the pike were feeding on crucian carp (*11*). Young-of-the-year crucian carp were not measured in 1991, but in May 1992, carp less than 40 mm in length (young-of-the-year 1991) showed no divergence in body depth (*11*).
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12. Each of 15 165-liter aquaria was divided into two compartments of equal size by a plastic screen partition (mesh size: 20 mm). Three sides of the aquaria were covered externally with black plastic. Aquaria were filled with dechlorinated water that was continually filtered (one filter per aquarium), and their bottoms were covered with a thin layer (2 cm) of sand. One-third of the water was exchanged every week. Aquaria were aerated and kept at 20° ± 1°C with a regime of light to darkness of 10:14 hours. Crucian carp were collected on 10 to 11 October 1991 in Severin's Pond (section without pike) with trap nets. Length and body depth were measured. The experiment was started on 11 October when nine crucian carp were introduced into one of the compartments of each aquarium. Aquaria were randomly assigned to treatments: (i) low food, (ii) high food, or (iii) low food + pike; each treatment had five replicates. Initial body depth-length ratios were similar between treatments (0.295 ± 0.002, 0.295 ± 0.004, and 0.296 ± 0.002, respectively; mean ± SD). Pike were introduced to the second compartment of aquaria used for the treatment with pike. Crucian carp were fed daily with a mixture of trout pellets and frozen chironomids at two different rations, low or high. The low-food ration was initially set at 3% of crucian carp dry weight per day, whereas the high ration was 6% per day. Rations were increased to 6 and 12%, respectively, after 1 month, because low-food carp decreased in weight over this period. Pike were fed three times weekly with one crucian carp. The experiment ended after 58 days, when all fish were measured.
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$$V_d = 1 - \sum_{w=0}^{d} W$$

where W is the frequency of pike mouth widths (w) in the population. Because we knew the exact size distribution of pike introduced into ponds, we could calculate mouth widths from $w = 0.087\,(TL) - 1.38$, where TL = total length. Equations are from K. D. Hambright, R. W. Drenner, S. R. McComas, N. G. Hairston, Jr., *Arch. Hydrobiol.* **121**, 389 (1991).
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Activation of a Plant Gene by T-DNA Tagging: Auxin-Independent Growth in Vitro

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A transferred DNA (T-DNA) tagging vector with the potential to produce dominant mutations was used with cocultured *Agrobacterium tumefaciens* and protoplasts to tag genes involved in the action of the plant growth substance auxin. Transgenic calli were selected for their ability to grow in the absence of auxin in the culture media. From one experiment, 12 calli that displayed this phenotype were recovered, of which 11 were able to regenerate into plants. In one plant studied in detail, protoplast division in the absence of auxin genetically cosegregated with a single T-DNA insert. A messenger RNA encoded by a 6.4-kilobase sequence of plant genomic DNA rescued from the mutant is overexpressed relative to untransformed plants. The genomic DNA, as well as a cognate complementary DNA, once transfected into protoplasts promote growth and cell division in vitro in the absence of exogenously added auxin.

In plants, auxins and cytokinins are required to induce cell division (*1*) and affect plant growth and development (*2*), although little is known of the molecular basis by which normal plant cells synthesize, perceive, or respond to plant growth substances (*3*). Several plant pathogens are able to induce growth and division of infected plant cells as a result of the synthesis of growth substances (*4*), and in the case of the tumor-inducing soil bacteria *Agrobacteria tumefaciens*, neoplastic growth results from the integration of a defined sequence of bacterial DNA, the T-DNA, into the genome of the infected plant cell (*5*). T-DNA encodes proteins that interfere with the normal biosynthetic pathways of plant growth substances (*6*). T-DNA is used as a transformation vector (*7*) and gene tag (*8*). We describe here a tagging vector derived from T-DNA that produces dominant mutations and thus allows selection for specific mutations from the population of primary transformants.

The T-DNA tagging vector pPCVICEn4HPT (Fig. 1A) contains multiple transcriptional enhancers derived from the cauliflower mosaic virus (CaMV) 35S RNA promoter located near the right border sequence. After insertion into the plant genome by *Agrobacterium*-mediated transformation, genes present in the plant DNA

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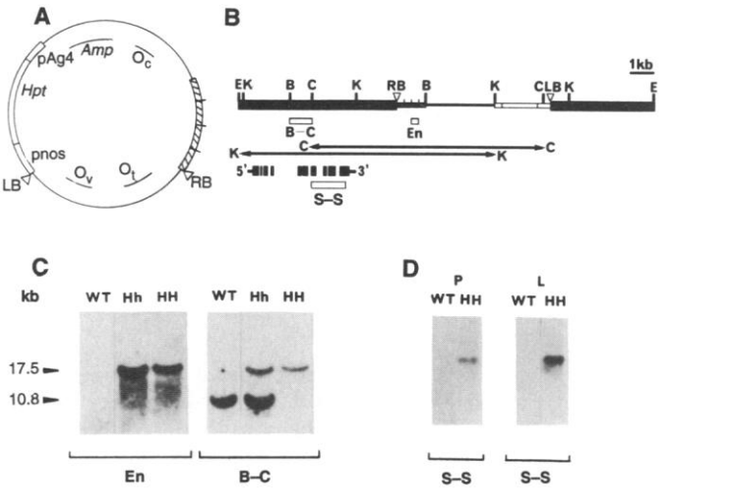

Fig. 1. Organization of T-DNA and tagged plant sequences. (**A**) pPCVICEn4HPT (*13*) contains a hygromycin resistance selectable marker (open box) that consists of the structural gene (*Hpt*), the nopaline synthase promoter (pnos), and the polyadenylation signal from gene 4 of T-DNA (pAg4). Four transcriptional enhancers (hatched boxes) derived from the CaMV 35*S* RNA promoter are in tandem approximately 20 bp from the right border sequence. LB and RB are the left and right border sequences, respectively, of the T-DNA; O_v and O_t, origins of replication and plasmid conjugational transfer of the wide host range plasmid RK2 that are functional in *Agrobacterium*; O_c, *E. coli* ColE1 plasmid origin of replication; *Amp*, ampicillin resistance gene. (**B**) Structure of pHH159 (*14*). Plant-derived sequences are depicted as thick lines, the T-DNA sequences are depicted as in (A), and selected restriction sites are indicated: B, Bam HI; C, Cla I; E, Eco RI; and K, Kpn I. Deletion analysis showed that the region (K-K) is able to direct hormone-independent growth, whereas the region (C-C) is unable to do so. Sequences found in common between genomic DNA and the cognate cDNA are indicated as black boxes with the 5′ and 3′ ends of the mRNA indicated. The 850-bp Bam HI–Cla I fragment of plant DNA used in Southern blot analysis and cDNA library screening is indicated by the open box B-C, the 350-bp Eco RV fragment containing the CaMV 35*S* RNA enhancer sequence used in Southern blot analysis and genomic DNA library screening is shown by the open box En, and the 800-bp Sph I fragment of the cDNA used in Northern analysis is indicated by the open box S-S. S, restriction site used to generate S-S, the hybridization probe (Sph I). (**C**) Southern blot analysis of SR1 and *axi* 159 genomic DNA (*15*). Eco RI–digested plant DNA from wild-type *N. tabacum* SR1 (WT), a heterozygotic *axi* 159 individual (Hh), and a homozygotic *axi* 159 individual (HH) were hybridized with the 350-bp 35*S* RNA enhancer sequence (En) or the B-C fragment of pHH159. (**D**) Northern blot analysis of SR1 and *axi* 159 RNA (*12*). RNA isolated from leaf tissue (L) and 2-day-old protoplasts (in the absence of auxin) (P) of SR1 (WT) and a homozygotic *axi* 159 individual (HH) was hybridized with the S-S fragment of the cDNA.

Fig. 2. Growth of *axi* 159 protoplasts in the absence of auxin. Protoplasts were isolated (*16*) from 6-week-old plants of SR1 tobacco (left) and *axi* 159 (right), cultured in the presence (top) or absence (bottom) of auxin, and embedded in agarose 1 week after isolation. The photograph was taken 4 weeks later.

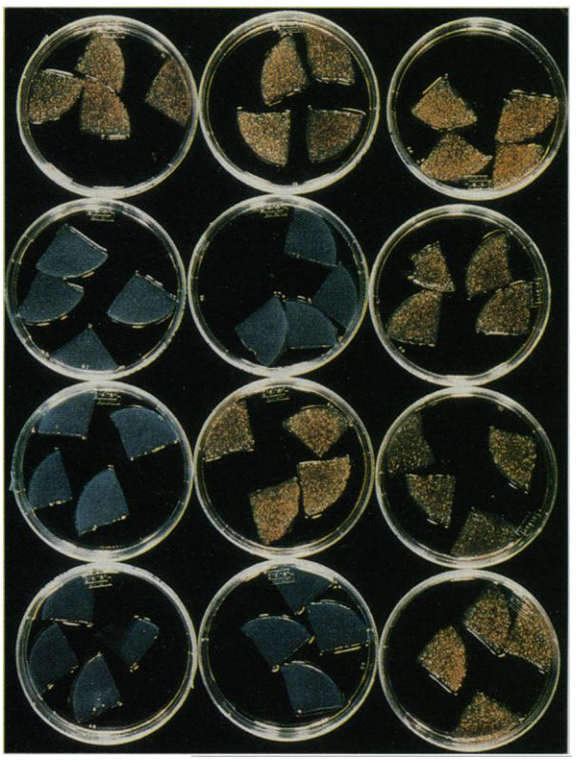

Fig. 3. Transfection of DNA rescued from *axi* 159 confers auxin-independent growth. Equimolar amounts of DNA of either pPCVICEn4HPT (9.6 μg) or pHH159 (17.5 μg) were used to transfect (*17*) 3×10^5 protoplasts isolated from SR1 tobacco. Left column, untransformed SR1 control protoplasts; middle column, protoplasts transfected with pPCVICEn4HPT; right column, protoplasts transfected with pHH159. Top row, protoplasts cultured in the presence of cytokinin and auxin (NAA, 1 mg/ml); second row, lacking auxin; third row, with auxin and hygromycin (15 μg/ml); bottom row, lacking auxin but containing hygromycin. Protoplasts were embedded in agarose a week after isolation and photographed 4 weeks later.

flanking the T-DNA insert become overexpressed.

Plant protoplasts have an absolute requirement for exogenously applied auxins in order to divide and grow (*1*). We selected for growth of transgenic calli in the absence of auxin. *Agrobacterium*-containing pPCVICEn4HPT were cocultivated with *Nicotiana tabacum* (SR1) protoplasts to generate calli that were selected for their ability to grow in the presence of hygromycin and in the absence of exogenously applied auxin. In one experiment involving the cocultivation of 30×10^6 protoplasts, where the transformation efficiency was approximately 20%, 12 calli were recovered. Control experiments that involved the culture of the protoplasts in the absence of bacteria or with *Agrobacterium* harboring pPCVHPT, a plasmid related to pPCVICEn4HPT that contains the hygromycin resistance cassette but that lacks the multiple enhancer sequences, failed to yield any calli that could grow in the absence of auxin. Of the 12 transgenic calli, 11 could be regenerated into plants. From four that contained single T-DNA inserts, one plant line, *axi* (auxin-independent) 159 is described here.

Axi 159 plants are morphologically similar to untransformed SR1 tobacco plants; they flower, self-fertilize, and set seed nor-

Fig. 4. Deduced amino acid sequence of cDNA able to promote hormone-independent growth. Once subcloned into a plant expression vector (*11*), the cognate cDNA corresponding to pHH159 is able to promote hormone-independent growth in transfected protoplasts. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The GenBank accession number for the sequence is L03208.

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   1  MKRRKQRSHE KLFLVIQIIL HTLIFKRRRR IHHLLPLLAA LTGGILFFFV
  51  LFSFPITSQH HHLINPIWFN NGTELQMNLQ KEHVFRVPMG GGSLSGDIWT
 101  SKQSILYHGC SNSSYKFPSA DVNTHPNRYL MIATSGGLNQ QRTGIVDAVV
 151  AAHIINAVLV VPKLDQKSYW KDSSNFSEIF DVDRFISHLS KDVKIIRDIP
 201  RIGDKVITPY TTRVPRKCNA KCYQTRILPI LKKKHAVQLT KFDYRLSNRL
 251  DIDMQKLRCR VNFHALKFTD PIIEMGRKLV ERIRMKSKHF VALHLRFEPD
 301  MLAFSGCYYG GGDKETKELG KIRKRWKTLH ATNPDKERRH GKCPLTPEEI
 351  GLMLRALGFG NDVHIYVASG EIYGGKETLA PLKALFPNFY SKETIASKEE
 401  LAPFSSFSSR MAALDFMVCD ESDVFVSNNN GNMARMLAGR RRYFGHKPTI
 451  RPNAKKLYKL FLSRNNMTWE EFASQVRTSQ IGFMGEPMEV KPGRGEFHEN
 501  PSACICADSD ANDREDASLF GTHSIISEID TGSSTNSARR DMVEVTDGQA
 551  SEEEQEWSDT EYMETELEI
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mally. However, protoplasts isolated from *axi* 159 individuals are able to divide and form calli in vitro in the absence of auxin (Fig. 2). T2 individuals, produced by self-fertilization of the original transformant (T1), segregate the hygromycin resistance marker contained on the T-DNA in a 3:1 ratio (216 resistant; 73 sensitive). T2 and T3 individuals, produced by the crossing of T2 individuals, were tested for cosegregation of the T-DNA and the ability of the protoplasts to form calli in the absence of auxin. To do this, we germinated seeds from plants that were judged heterozygous or homozygous by segregation of the hygromycin resistance marker and Southern (DNA) blot analysis and used leaf tissue as a source of protoplasts. Plants were also tested for their ability to root on media that contained hygromycin. In all cases, the hygromycin resistance marker and the ability of protoplasts to grow in the absence of auxin cosegregated.

In *axi* 159 individual plants, the T-DNA of pPCVICEn4HPT was contained on a single 17.5-kb Eco RI fragment (Fig. 1C). For the rescue of plant sequences that flanked the T-DNA insertion, *axi* 159 genomic DNA was digested with Eco RI, fractionated by size, and used to construct a λDASH library. Positive clones were selected by hybridization to a T-DNA–specific probe. The ampicillin resistance gene and the *Escherichia coli* origin of replication present within the T-DNA facilitated the rescue of flanking plant DNA sequences that were subcloned from the positive λ clones that produced pHH159 (Fig. 1B). This pHH159 contains the T-DNA of pPCVICEn4HPT and approximately 10.8 kb of plant genomic sequences that were linked to the T-DNA in *axi* 159.

To confirm that the rescued plant DNA was able to confer auxin-independent cell division and growth, we used pHH159 to transform tobacco protoplasts by polyethylene glycol–mediated DNA uptake. Protoplasts transfected with DNA of pHH159 were able to grow in the absence of exogenously applied auxins and in the presence of hygromycin, whereas untransfected protoplasts or protoplasts transfected with pPCVICEn4HPT were not (Fig. 3). Subsequent transformations with the use of deletion derivatives of pHH159 generated by restriction digestion (Fig. 1B) followed by religation mapped this property to a 6.4-kb sequence of plant DNA located between the right border sequence of the T-DNA and the Kpn I site farthest to the left end of the sequence.

A 2.1-kb cDNA synthesized from polyadenylate [poly(A)] RNA isolated from leaf tissue of *axi* 159 (9) and selected by hybridization to the 850-bp fragment of pHH159 (B-C) was found by sequence analysis (*10*) to contain a single open reading frame bearing sequence homology to a gene located on pHH159 (Fig. 1B). The deduced transcription start site located approximately 6.2 kb from the T-DNA that is inserted downstream of the gene (Fig. 1B). An 800-bp fragment of this cDNA (S-S) was used to probe poly(A) RNA isolated from leaf tissue or protoplasts of control SR1 or *axi* 159 plants. An mRNA of approximately 2.5 kb was identified that was present in larger amounts in *axi* 159 leaf tissue and protoplasts than in SR1 (Fig. 1D). The deduced amino acid sequence from the cDNA reveals a highly basic protein (Fig. 4). When subcloned between the CaMV 35*S* RNA promoter and the polyadenylation site of pRT101 (*11*), the cDNA confers the ability of protoplasts to divide in vitro in the absence of auxin.

The use of transcriptional enhancers in an *Agrobacterium*-based T-DNA tag allows the production of dominant, overexpression mutants. This strategy, coupled with the ability to produce large numbers of transformants by protoplast cocultivation, could be used to tag any gene whose enhanced activity can be selected in vitro. Here, we describe the isolation of a plant gene that upon overexpression is able to promote the growth and division of protoplasts in vitro in the absence of exogenously applied auxins. Although the importance of auxin in plant growth and development has long been recognized (*1*, *2*), information concerning its mode of action at the molecular level is scant (*3*). Further analysis of *axi* 159 may shed light on the molecular mechanisms that underlie the action of the plant growth factor auxin and its role in the differentiation and development of plants.

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15. DNA from leaf tissue of SR1 and *axi* 159 plants

was digested with Eco RI, fractionated on 0.7% agarose, blotted onto Hybond-N (Amersham), and hybridized as described [J. Sambrook, A. Frisch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989)].
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Map-Based Cloning of a Gene Controlling Omega-3 Fatty Acid Desaturation in *Arabidopsis*

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A gene from the flowering plant *Arabidopsis thaliana* that encodes an omega-3 desaturase was cloned on the basis of the genetic map position of a mutation affecting membrane and storage lipid fatty acid composition. Yeast artificial chromosomes covering the genetic locus were identified and used to probe a seed complementary DNA library. A complementary DNA clone for the desaturase was identified and introduced into roots of both wild-type and mutant plants by Ti plasmid–mediated transformation. Transgenic tissues of both mutant and wild-type plants had significantly increased amounts of the fatty acid produced by this desaturase.

The small crucifer *Arabidopsis thaliana* (L.) is suitable for the application of map-based cloning methods because it has a small nuclear genome that is almost devoid of interspersed, highly repetitive DNA (*1*). The five chromosomes have a total DNA content of about 70,000 kb (*1*). The average distance from any gene to the nearest restriction fragment length polymorphism (RFLP) marker (*2*, *3*) is about 225 kb, and several yeast artificial chromosome (YAC) genomic libraries are available for *Arabidopsis* (*4*, *5*). Here, we used these resources to isolate a gene from *Brassica napus* that complements a mutant of *Arabidopsis* deficient in omega-3 unsaturated fatty acids.

Fatty acid desaturases catalyze the O_2 and electron donor–dependent insertion of double bonds into fatty acids. Because the amount of membrane lipid unsaturation affects the physical properties of membranes, the desaturases are thought to affect the ability of plants to survive extreme temperatures (*6*). Also, the nutritional quality of edible plant oils is largely determined by the composition of storage triacylglycerols. Except for the stearoyl-ACP desaturase, plant desaturases are thought to be membrane proteins and have been difficult to characterize by conventional biochemical methods (*7*). Information about the number and properties of the various desaturases in *Arabidopsis* has been obtained by the isolation of an extensive collection of mutants with altered membrane and storage lipid unsaturation (*8*). One of the mutations, designated *fad3*, resulted in reduced accumulation of linolenic acid ($18{:}3^{\omega 3,6,9}$), and a corresponding increase in the amount of linoleic acid ($18{:}2^{\omega 6,9}$), in extrachloroplast membrane and storage lipids (*9*). These metabolic effects suggested that the *fad3* locus encoded an ω3 linoleate desaturase.

The *fad3* locus was genetically mapped by scoring the fatty acid composition of 137 progeny (F2) of a cross between plants of the *fad3* mutant line BL1 (Landsberg race) (*9*) and wild type (Niederzenz race). Because the *fad3* phenotype is only weakly evident in chlorophyllous tissues, but is strongly expressed in root or seed tissue, the fatty acid phenotype of each F2 plant was scored by gas chromatographic analysis of the fatty acid composition of each of ten seeds obtained by self-fertilization of F2 plants. The RFLP genotype of F2 progeny was determined by analysis of DNA preparations from F2 plants and F3 families (*2*, *3*). The *fad3* mutation mapped on chromosome 2 adjacent to the RFLP markers M220 (*2*) and ASA2 (*10*) (Fig. 1). When used to screen YAC libraries, M220 hybridized with YAC EG4E8 (*4*) and ASA2 hybridized with YACs EW7D11 and EW15G1 (*5*). The YACs were ordered by analysis of hybridization of end-specific probes on Southern (DNA) blots of the YAC clones. The resultant, approximately 340 kb, contig of four YACs extended a minimum of 170 kb to the right of ASA2 (Fig. 1). As the maximum distance between M220 and ASA2 was 180 kb (the sum of the inserts in EG4E8 and EW15G1), we estimated that 1 centimorgan (cM) was equivalent to less than an average value of 105 kb in this region. As *fad3* was 0.4 cM to the right of ASA2, we estimated that the YAC contig extended far enough to the right to include *fad3*.

Stearoyl-ACP desaturase, an enzyme that catalyzes a chemically equivalent reaction, is encoded by a moderately abundant mRNA in developing seeds of oil-accumulating plants such as *Ricinus communis* (*11*). Therefore, we used the YACs to isolate moderately abundant cDNA clones. DNA from one YAC, EW7D11, was isolated from a low-melting agarose-clamped homogeneous electric field (CHEF) gel (*12*) and used to probe a λgt11 cDNA library made from developing seeds of the closely related crucifer *Brassica napus*. The *B. napus* library was used because we did not have a suitable library from developing seeds of *Arabidopsis*, and genes from *B. napus* are highly homologous to the corresponding *Arabidopsis* genes (*13*). Of 31 positive plaques among 2×10^5 screened, 17 cross-hybridized at high stringency and, therefore, appeared to be derivatives of the same gene. None of the other clones were highly represented among the 31 positive clones. The largest insert (1.4 kb) representing the abundant transcript was

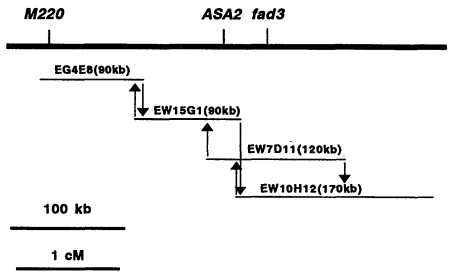

Fig. 1. Genetic map of the region of chromosome 2 (thick line) that contains the *fad3* locus. The YACs that correspond to this region of the genome are shown below. The sizes of the inserts (in parentheses), were determined by pulsed-field gel electrophoresis (*12*). Arrows indicate regions of overlap demonstrated by hybridization of end-specific probes. Scales of genetic distance (centimorgans, cM) and kilobases (kb) are indicated.

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