The dominant inhibitory chalcone synthase allele \textit{C2-Idf (Inhibitor diffuse)} from \textit{Zea mays} (L.) acts via an endogenous RNA silencing mechanism

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AY728478 \textit{c2} gene chalcone synthase (wild type) \textit{C2-W22};

AY728476 \textit{Zea mays} L. \textit{C2-Idf} allele; gene copies \textit{C2-Idf-I} and \textit{C2-Idf-II};

AY728477 \textit{Zea mays} L. \textit{C2-Idf} allele; gene copy \textit{C2-Idf-III}
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ABSTRACT

The flavonoid pigment pathway in plants has been used as a model system for studying gene regulatory mechanisms. C2-Idf is a stable dominant mutation of the chalcone synthase gene, c2, which encodes the first dedicated enzyme in this biosynthetic pathway of maize. Homozygous C2-Idf plants show no pigmentation. This allele also inhibits expression of functional C2 alleles in heterozygotes, producing a less pigmented condition instead of the normal deeply pigmented phenotype. To explore the nature of this effect, the C2-Idf allele was cloned. The gene structure of the C2-Idf haplotype differs substantially from that of the normal c2 gene in that three copies are present. Two of these are located in close proximity to each other in a head-to-head orientation and the third is closely linked. Previous experiments showed that the lower level of pigmentation in heterozygotes is correlated with reduced enzyme activity and low steady-state mRNA levels. We found that c2 transcription occurs in nuclei of C2-Idf/C2 heterozygotes, but mRNA does not accumulate, suggesting that the inhibition is mediated by RNA silencing. Infection of C2-Idf/C2 heterozygotes with viruses that carry suppressors of RNA silencing relieved the phenotypic inhibition, restoring pigment production and mRNA levels. Finally, we detected small interfering RNAs (siRNAs) in plants carrying C2-Idf, but not in plants homozygous for the wild-type C2 allele. Together, our results indicate that the inhibitory effect of C2-Idf occurs through RNA silencing.
INTRODUCTION

The maize flavonoid pigment pathway offers an excellent model system for studying the regulation of gene expression. The pathway is genetically well-characterized and most of the structural and regulatory genes have been cloned (DOONER et al. 1991). Genetic and molecular studies of a wide spectrum of mutants have revealed that the distribution and level of pigment accumulation accurately reflect activity of these genes (COE et al. 1988). In maize, several genes involved in anthocyanin biosynthesis have been identified. Among these, regulatory genes encoding a suite of transcription factors, as well as structural genes encoding biosynthetic enzymes have been extensively characterized at the molecular level (DOONER et al. 1991). From these data, detailed knowledge about the regulation, mode of interaction and function of maize anthocyanin genes has been assembled.

One of the structural genes, colorless2 (c2), encodes chalcone synthase, the enzyme responsible for the first dedicated step in the pathway. In combination with appropriate regulatory alleles, a normal C2 allele leads to pigment production in many parts of the plant, including the pericarp, the aleurone layer of the endosperm, tassels, and vegetative organs such as ear husks and leaf sheaths. A colorless mutant, initially called Inhibitor diffuse (Idf), was isolated from Peruvian lines as a dominant inhibitor of pericarp pigmentation (BRINK and GREENBLATT 1954). Later, when the mutation was mapped to the c2 locus, the allele became known as C2-Idf (BRINK and GREENBLATT 1954). When heterozygous with a normal C2 allele, C2-Idf reduces pigmentation not only in the pericarp, but also in the aleurone. At the enzyme level, the effect on aleurone pigmentation is due to a lack of chalcone synthase enzyme activity (DOONER 1983). At the RNA level, c2-homologous RNA is not detectable in either tassels or aleurone of C2-Idf homozygotes (FRANKEN et al. 1991).
Unlike cases of paramutation (for reviews see Chandler and Stam 2004; Della Vedova and Cone 2004), C2-Idf inhibition is not meiotically heritable. These observations suggest that the absence of enzyme activity in C2-Idf/C2 heterozygotes results from a reduction of C2 mRNA accumulation. In this work, we investigated the mechanism of inhibition of the C2 allele by C2-Idf.

Similar semi-dominant types of mutations in chalcone synthase genes have been described and analyzed in Antirrhinum majus L. (niv-535 allele) and soybean (chalcone synthase allele I) (Coen and Carpenter 1988; Todd and Vodkin 1996; Tuteja et al. 2004). In these inhibitory mutants, the dominant negative effect likely results from either gene duplication events of the chalcone synthase gene (as shown in the case of soybean, Tuteja et al. 2004) or to the production of antisense transcripts (as proposed for the niv-525 allele of Antirrhinum majus L., Coen and Carpenter 1988). The inhibitory effects of these mutants was mimicked phenotypically in transgenic petunia lines; plants carrying multiple insertion copies of the chalcone synthase gene showed reduction of normal gene activity, caused by a cosuppression effect that involves RNA silencing (Napoli et al. 1990; Van der Krol et al. 1990; Jorgensen et al. 1996; Metzlafl et al. 1997).

RNA silencing refers to a homology dependent type of gene silencing that employs RNA to mediate the targeted degradation of homologous transcripts. The process is triggered by the production of aberrant RNA, which is usually at least partially double-stranded. Double-stranded RNA is recognized by an RNase-III like enzyme, referred to as Dicer (Bernstein et al. 2001), and cleaved into small double-stranded RNAs of 21-26 nt known as small interfering RNAs (siRNAs) (Hamilton and Baulcombe 1999). These siRNAs are incorporated into a ribonucleo-protein complex known as the RNA Induced Silencing Complex (RISC), which targets homologous transcripts, catalyzing their degradation (Hammond et al. 2001). Thus, any transcript homologous to the aberrant RNA
is destroyed, essentially silencing the expression of the cognate gene. The RNA silencing machinery can also participate in chromatin modifications, whereby siRNAs may recognize homologous DNA loci and induce remodeling of the surrounding chromatin into a more restrictive state, effectively silencing transcription from that locus (CAO et al. 2003; CHAN et al. 2004; PAL-BHADRA et al. 2002; VOLPE et al. 2002; VERDEL et al. 2004).

RNA silencing has likely evolved as a defense mechanism against invasive nucleic acids (HERBERT 2004). For example, in many plant viruses with a single-stranded RNA genome, replication involves a double-stranded RNA intermediate produced by a virally-encoded RNA-dependent RNA polymerase (BAULCOMBE 1996). This double-stranded transcript is recognized as aberrant by the host plant and triggers RNA silencing. The virus infection is thus controlled by degradation of its RNA, preventing further cycles of replication (AL-KAFF et al. 1998). However, as a counterdefense, many plant viruses carry genes that encode proteins capable of suppressing the plant’s RNA silencing machinery. A number of such suppressors of RNA silencing have been identified, but the mechanism by which suppression is achieved remains unclear for most (AHLQUIST 2002). The two best-described viral suppression proteins are P1/HC-Pro from potyviruses and p19 from tombusviruses. P1/HC-Pro appears to prevent Dicer cleavage of the aberrant precursor RNA while p19 binds siRNAs and thus prevents them from acting as guides for degradation of homologous transcripts (DUNOYER et al. 2004; LAKATOS et al. 2004).

A conserved feature of RNA silencing is that it is triggered by double-stranded RNA molecules which, in some cases, originate from transcription of repeated DNA segments (MUSKENS et al. 2000; WASSENEGGER 2000). Two recently published descriptions of RNA silencing in rice and soybean involve endogenous alleles that are composed of multiple genes arranged in inverted repeat orientations (KUSABA et al. 2003; TUTEJA et al. 2004).
this study, we addressed the basis of silencing by \textit{C2-Idf} in maize and conclude that it involves an RNA based silencing mechanism.

**MATERIALS AND METHODS**

**Genetic stocks:** Genetic and molecular analyses of the \textit{C2-Idf} mutant line were carried out in W22 or Mo17 inbred backgrounds carrying \textit{R-scm2} and \textit{C1} or in the original genetic \textit{C2-Idf} background (\textit{A1, C1, R1, P-wr/W22}). For comparison, a color converted W22 carrying \textit{A1, A2, C1, C2} and \textit{R1} (Line C, \textit{Wienand et al.} 1986) was used; in the present study, this normal \textit{c2} allele is designated \textit{C2-W22}. For analysis of husk RNA levels, \textit{C2} and \textit{C2-Idf} in a W22 inbred background were backcrossed three times to a stock, which is homozygous for \textit{R-g, C1, B-I, Pl-Rhoades} and \textit{P-ww}. With \textit{C2} homozygous, these plants are deep purple. The negative controls were plants homozygous for \textit{R-r; c1; B-I; pl-0; P-ww}. The \textit{pl-0} mutation prevents any accumulation of anthocyanin in vegetative tissue. With \textit{C2} homozygous, these plants are green (P. Cooper and K. Cone, unpublished results). All stocks used in this study were homozygous dominant for \textit{white pollen1 (whp)}, a duplicate of the \textit{c2} gene which influences pollen viability. \textit{C2-Idf} plants have normal pollen.

**Probes:** \textit{c2}-specific probes used in DNA and/or RNA analysis were generated from restriction fragments or PCR-amplified products. All PCR products were cloned into pCR-TOPO (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two probes specific for the \textit{c2} promoter were produced. A 388 bp product (-452 to -65; \textit{C2-P1}) was amplified by PCR with the primers \textit{C2PromF} (5'-AATTTACCACGGACGACGAGACGACG-3') and \textit{C2PromR} (5'-TCAGTTGGACGGGCGATGG-3') and a 753 bp \textit{Hinfl} fragment (-1427 to -675) was subcloned from a genomic \textit{c2} clone (\textit{C2-P2}; \textit{Wienand et al.} 1986). A probe specific for the 5' UTR was made by PCR amplification of a 303-bp fragment with forward primer \textit{C2 gs 5'}
F (5’-GGCTTCTCGTCTCTCCCACCACAG-3’) and reverse primer C2 gs 5’ R (5’-CGCGCTGGGCTTCCACCTCCTCC-3’). Two probes specific for the c2 intron were made: a 1294-bp AvaI fragment (Wienand et al. 1986) and an 884-bp PCR product amplified with forward primer C2st2F (5’-CTCGCGCCATGCACAAAGAC-3’) and reverse primer C2st3R (5’-GCGCGCTAGAAGAAGAAGAGGT-3’). A 164-bp probe specific for the 3’ UTR of C2 was amplified by PCR with the primers C23’F (5’-CTCCACAGCGTCCCATA-3’) and C23’R (5’-ACACACGACAATTATAGCAGAGA-3’).

**Genomic DNA isolation and Southern hybridization:** Genomic DNA was isolated from maize leaves as described (Cone et al. 1986). Ten micrograms of genomic DNA were incubated with 10 U of each of the appropriate restriction endonuclease(s) for 4 h at the suggested temperature and were subjected to agarose gel electrophoresis and Southern blotting. Hybridizations were performed with randomly labeled radioactive probes under stringent conditions according to Sambrook and Russell (2001).

**Lambda cloning:** To obtain full-length C2-Idf clones, two independent lambda libraries were generated. To clone a longer sequence of the C2-W22 allele, one lambda library was generated. To produce these libraries, DNA was isolated from young seedlings from either C2 or C2-Idf mutant backgrounds as previously described (Cocciolone and Cone 1993; Cone et al. 1986). Genomic DNA was fragmented by partial digestion with the restriction endonuclease MboI as described previously (Sambrook and Russell 2001). In order to select for fragments in the range of 9-23 kb, the DNA was fractionated on a 10-40% sucrose gradient or on a 5-20% sodium acetate gradient and subjected to centrifugation at 23,500 rpm for 20 h at 20°C. Fractions in the correct size range were selected as previously described (Sambrook and Russell 2001). DNAs were ligated either by using the Lambda Fix® II/XhoI partial fill-in Vector Kit (Stratagene, La Jolla, CA) or into the EMBL4 λ-
vector (Frischauf et al. 1983). For packaging, either the Gigapack® III Gold Packaging Extract (Stratagene, La Jolla, CA) system or self-made packaging extracts were used. Phage were plated in top agar either with XL1-Blue MRA (P2) or with K803 E. coli cells. Plaque lifts and hybridization to detect positive clones were performed as previously described (Cocciolone and Cone 1993; Sambrook and Russell 2001) using either the C2-P1 or the C2-P2 probe. DNA was isolated from phage of interest using a Qiagen Lambda Maxi Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and was subcloned using standard procedures. In order to sequence large clones, the Locus Pocus™ subcloning System (Novagen, Schwalbach, Germany) was used with clones of the EMBL4 λ-library according to the manufacturer’s instructions.

**Fosmid cloning:** The CopyControl™ Fosmid Library Production Kit (Epicentre, Madison, WI) was employed to obtain larger size clones than possible with lambda cloning. DNA was isolated by cesium chloride centrifugation. Library production was performed using the manufacturer's instructions. Colonies were screened using the protocol for screening bacterial colonies as described by Sambrook and Russell (2001). Filters were hybridized as for plaque lifts, except that the 3' UTR of c2 was used as a probe. For positive colonies, the plasmids were induced to high copy number using the manufacturer's protocol. DNA was isolated for restriction enzyme mapping and sequencing using a Qiagen Plasmid Maxi Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. In order to expedite sequencing of the large inserts, an *in vitro* transposition system was employed. The GeneJumper™ Primer Insertion Kit for Sequencing (Invitrogen, Carlsbad, CA) was used to introduce bacteriophage *Mu* into random positions in the cloned *C2-Idf* containing DNA.

To prepare DNA for sequencing, the R.E.A.L.® Prep 96 Kit (Qiagen, Valencia, CA) was used with a number of modifications of the manufacturer's instructions. Precultures were grown in 1 mL 2XYT plus 10 µg/mL kanamycin for 16 h at 37° with shaking at 175
rpm. A 200 µL aliquot of the precultures was added to 2.5 mL of fresh 2XYT containing 25 µg/mL chloramphenicol and 2X CopyControl™ Induction Solution (Epicentre, Madison, WI); these cultures were grown for 16 h at 37° with shaking at 175 rpm. The cells were harvested by centrifugation for 10 min at 1000 x g. Resuspension, lysis, precipitation of cellular debris and lysate clearing were performed as per manufacturer's instructions. To precipitate the DNA, 0.7 volumes of room temperature isopropanol and 2 µg glycogen were added to each well. Samples were centrifuged at 2254 x g for 60 min to pellet the DNA. Ethanol wash and redissolving DNA was performed as per manufacturer's instructions. To complete the sequencing of C2-Idf-I/II a 6.1 kb PstI fragment, containing sequence between the inverted promoters, was selected by gel purification and subcloned.

**DNA sequencing:** DNA sequencing reactions were performed either by the University of Missouri DNA Core facility or by the Wienand laboratory with Applied Biosystems 377 automated DNA sequencers using Applied Biosystems Prism BigDye Terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA). Computer assisted sequence assembly was performed using EditSeq (DNAStar, Madison, USA) and the programs provided by the ExPASy proteomics server ([http://www.expasy.ch/](http://www.expasy.ch/)). Alignments were calculated with the CLUSTAL X 1.83 program (THOMPSON *et al.* 1997) and manually edited using GeneDoc 2.6 (NICHOLAS and NICHOLAS 1997).

**RNA blot analysis:** Total RNA was isolated from husks or leaf sheaths just after silk emergence using Trizol Reagent (Invitrogen) according to the manufacturer’s instructions. RNA was stored at -20° in 100% formamide at a concentration of 500 ng/µL. Total RNA was fractionated on formaldehyde gels as described (SAMBROOK and RUSSELL 2001), except that blots were stained with 0.04% methylene blue; 0.5 M sodium acetate for 5 min. The blots were rinsed with deionized water and then destained in 0.2 x SSC; 1% SDS. RNA was blotted to Magnagraph Nylon Transfer Membrane (Osmonics, Inc, Minnetonka, MN) and
hybridized as previously described (CONE et al. 1986). Blots were hybridized with a probe specific for the 3’ untranslated region of c2. RNA blots were also hybridized with a maize actin probe (SHAH et al. 1983) as a loading control. Signal was detected by exposure to a Fuji Bas-III S imaging plate followed by detection by a Fuji Bas-1000 phosphorimager. c2 RNA levels were normalized to actin by subtracting the background signal from both c2 and actin and then dividing the c2 signal by the actin signal.

**Slot blots:** For use in nuclear run-on transcription the following probes were linearized: a 1.5-kb c2 cDNA, 2.5-kb maize actin cDNA, a 200-bp fragment from the c2 promoter and empty pCR-TOPO as negative control. For the slot blots, 20 µg of each linearized plasmid was denatured in 0.1 N NaOH. The total volume was increased to 1 mL with 6 x SSC and 250 µL of each sample was added to a slot of a Bio-Dot SF Microfiltration Apparatus (Bio-Rad, Hercules, CA) containing nitrocellulose hydrated with TE and equilibrated with 2 x SSC. The membrane was dried and baked under vacuum at 80° for 90 min.

**Nuclear run-on transcription:** Nuclei were isolated from husks just after silk emergence as described previously (CONE et al. 1993a; HOEKenga et al. 2000). Nuclear run-on transcription was performed using approximately 5 x 10⁶ nuclei per reaction. Counts per minute (cpm) were determined as described previously (CONE et al. 1986) and labeled RNA (3-5 x 10⁶ cpm) was added to slot blots in hybridization solution. Hybridization was carried out for 2-4 days at 42°. Following hybridization, membranes were washed with 2 x SSC for 15 min at room temperature followed by four washes 15 min each in 0.1 x SSC; 0.1% SDS at 50°. Signal was detected by exposure to a Fuji Bas-III S imaging plate followed by detection by a Fuji Bas-1000 phosphorimager. Transcription levels were normalized to actin by subtracting empty vector signal from both c2 and actin and then dividing the c2 signal by the actin signal.
**Viral inoculation methods:** Inoculum for *Maize necrotic streak virus* (MNeSV) (LOUIE *et al.* 2000) was prepared by grinding a small amount of virus-infected leaf material in three volumes of 0.1 M potassium phosphate, pH 7, and collecting the supernatant from a 15,000 g centrifugation. Maize seeds were inoculated with MNeSV by vascular puncture inoculation of embryos (LOUIE 1995). Briefly, seeds were soaked (2.5 h) in water at 28°, then arranged on 4 layers of wet paper towels in a petri dish. Three to five microliters of virus extract was placed on the embryo and 5 minuten pins were pushed through the inoculum into the vascular tissue on each side of the embryo. After 2 days at 28°, the seeds were planted and transferred to a greenhouse without supplemental light. When the plants were mature, virally-infected streaks were excised for RNA extraction. Similar tissues were processed as control from uninfected plants.

Inoculum for *Maize dwarf mosaic virus*-A (MDMV-A) infection was prepared by grinding a small amount of virus-infected leaf tissue with roughly four volumes of 10 mM phosphate buffer (pH 7.5) and a small amount of carborundum. The slurry was manually applied to maize plants at the three leaf stage by rubbing the slurry onto the top two leaves. The plants were then rinsed with water. Inoculation was judged to be successful if visible symptoms of infection--chlorotic mosaic and streaks on leaves and leaf sheaths--were apparent after 7-10 days. Plants not showing symptoms of infection by 14 days after inoculation were removed from the experiment. Uninfected control plants were kept separate from infected plants and any noninoculated plants showing symptoms of infection at any time were removed from the experiment. In the field, the uninfected and infected plants were in separate locations and the same criteria were used to select plants to be analyzed. MDMV-A was propagated by sequentially infecting young plants from previously infected plants or by inoculation with infected tissue that had been stored at -80°. At maturity, husks were harvested for RNA isolation.
**siRNA detection:** RNA was isolated with Trizol (Invitrogen) and the final pellet was resuspended in 2.5 mL water in a 15 mL Corex tube. High molecular weight RNA was precipitated by adding 0.5 mL 5M NaCl and 2 mL 20% PEG-8000; 30 mM MgCl₂ and incubating overnight at 0° (on ice in a 4° refrigerator). Following precipitation, the RNA was subjected to centrifugation at 4° in a Beckman JS13.1 rotor at 10,000 x g for 30 min. The supernatant, containing RNA roughly 200 nt and smaller, was transferred to a 30 mL Corex tube and extracted with 100:100:1 phenol:chloroform:isoamyl alcohol followed by centrifugation at 4° in a JS13.1 rotor for 10 min at 10,000 x g. The aqueous phase was transferred to a new 30 mL Corex tube and the RNA was precipitated by adding 0.5 mL 3M sodium acetate and 15 mL absolute ethanol and storing overnight at -20°. RNA was pelleted at 4° in a JS13.1 rotor at 10,000 x g 4° for 1 h. The pellet was suspended in 250 µL of water and the total amount of RNA was determined spectrophotometrically. The samples were transferred to a 1.5 mL microcentrifuge tube and the RNA was precipitated by adding 10 µL 3M sodium acetate and 330 µL ethanol and storing at -20° overnight. The RNA was pelleted by centrifugation in a microcentrifuge at maximum speed for 1 h and the pellet was resuspended in water to a concentration of 2.5 – 5 µg/µL. The samples were mixed with an equal volume of deionized formamide and denatured by heating at 95° for 5 min. An aliquot of 25-50 µg of RNA was fractionated on 20% polyacrylamide; 8M urea denaturing gels. The acrylamide gels were blotted to Hybond N+ nylon membranes (Amersham Biosciences, Piscataway, NJ) using a Trans-blot Cell (BioRad) at 22V overnight. Pre-hybridization and hybridization were performed as described previously (CONE et al. 1986) except that approximately 6 x 10⁶ cpm of probe was added to each blot. Blots were washed twice in 1 x SSC; 0.1% SDS for 20 min at 50° followed by two washes in 0.5 x SSC; 0.1% SDS for 1 h at 50°. Signal was detected by autoradiography.
RESULTS

**Phenotypic characterization of the C2-Idf mutation:** The inhibitory effect of the C2-Idf allele is clearly visible as a reduction of aleurone pigmentation in kernels of reciprocal crosses between C2-Idf and a normal line (Figure 1A). Kernels of a color converted W22 line (LC) are dark red whereas homozygous C2-Idf mutant kernels are colorless. Due to the triploid nature of the aleurone, either one or two C2-Idf alleles are present in kernels of the reciprocal crosses. An increase in dosage of C2-Idf alleles resulted in a dosage-dependent decrease in aleurone pigmentation. However, even a single dose of C2-Idf produces a less pigmented aleurone phenotype than does a single dose of a recessive loss-of-function allele (DOONER 1983). The phenotype of the C2-Idf mutant allele in vegetative tissues is shown in Figure 1B. Plants homozygous for a functional C2 allele produce anthocyanin pigments in leaf sheaths and husks. A single copy of the dominant C2-Idf allele nearly eliminates this pigmentation. C2-Idf/C2-Idf homozygotes are completely lacking anthocyanin.

**The c2 locus is substantially rearranged in the C2-Idf mutation:** As an explanation of the dominant inhibitory effect of the C2-Idf mutation, an epigenetic silencing mechanism was postulated as a working hypothesis. Such effects might result from gene duplication events or rearrangements of the c2 locus. To address this hypothesis, the C2-Idf locus was investigated by detailed Southern and sequence analysis.

In order to examine the c2 locus arrangement in C2-Idf, Southern analyses with DNA probes derived from the c2-intron (Figure 2) and the c2-promoter (data not shown) were performed. The c2 gene of the line LC consists of two exons and an intron of 1523 bp (Figure 2; WIENAND *et al.* 1986). Hybridizations with the c2-intron probe revealed a higher number of c2 hybridizing fragments in the C2-Idf line as compared to LC. Restriction analyses with different endonucleases in most cases revealed three fragments in C2-Idf DNA.
and only one fragment in LC DNA (Figure 2). In some cases, only two c2 homologous fragments were detectable (e.g. EcoRI+Aval, Figure 2); however, one of these fragments showed a stronger hybridization signal suggesting comigration of two of the three putative c2-homologous fragments. These data suggested that three different c2 homologous regions (or c2 gene copies) are present in the C2-Idf allele.

Cloning and genomic structure of the C2-Idf allele: Approximately 46 kb of the genomic C2-Idf locus were cloned and sequenced. Four independent c2-hybridizing clones were identified during screening of two lambda phage libraries. Moreover, three longer genomic DNA clones were identified during screening of a fosmid C2-Idf library. A segment of 2.1 kb not covered by the cloned DNA fragments was amplified by PCR. For comparison, 7.2 kb of the C2-W22 allele, a functional c2 allele, were cloned from a lambda genomic DNA library and sequenced (Figure 3A). As expected, the corresponding parts of this sequence were almost identical to the previously published 3.8 kb of C2 from Line C (FRANKEN et al., 1991).

Assembly of overlapping C2-Idf sequences generated two contigs of roughly 23 kb each. Three different copies of the c2 gene could be identified on these clones (Figure 3A). The three copies were designated as C2-Idf-I, C2-Idf-II and C2-Idf-III, respectively. Two copies (C2-Idf-I and C2-Idf-II) were found to be oriented head-to-head with a distance of approximately 3.5 kb between the postulated promoter regions (Figure 3A). These regions were defined based on the similarity of their sequences to the corresponding sequenced region upstream of the C2-W22 protein coding sequence. For the third copy, C2-Idf-III, a position relative to C2-Idf-I and C2-Idf-II could not be determined because no sequence overlaps were present in the two contigs. Based on the lengths of non-coding sequences that border the three C2-Idf gene copies on both contigs, the minimal distance between the C2-Idf-III gene copy and the C2-Idf-I and -II gene cluster was calculated to be at least 6.7 kb.
However, the fact that $C2-Idf$ has segregated genetically as a stably transmitted single locus for over seven generations of backcrossing to inbreds Mo17 and W22 (our unpublished data) argues that all three $C2-Idf$ gene copies are likely to be located in close proximity to each other. The sizes of the predicted restriction fragments of the sequenced $C2-Idf$ allele were identical to the sizes of fragments detected by Southern analysis (Figure 2). These data demonstrate that the $C2-Idf$ locus indeed consists of three different $c2$ gene copies.

The genomic structure of the individual $C2-Idf$ gene copies is very similar to that of $C2-W22$ and contains two exons. The protein coding regions of $C2-Idf-I$ and $C2-Idf-II$ show only two single nucleotide exchanges in comparison to $C2-W22$; neither change would alter the amino acid sequence of a putative $C2-Idf$ chalcone synthase protein. $C2-Idf-III$ is truncated and is missing 77 bp at the 3′ end of the second protein encoding segment. This truncation is due to an insertion of a PREM-2/Ji-like (SanMiguel et al. 1996; Turcich et al. 1996) retrotransposon of approximately 9.2 kb. The 3′-long terminal repeat (LTR) of this element, adjacent to the remaining part of the $C2-Idf-III$ gene copy, introduces a new stop codon, such that translation of this gene copy would result in a C2 protein shortened by 21 amino acids and containing four changed amino acids at its C-terminal end (Figure 3A).

Because the C-terminus is highly conserved in chalcone synthases (Niesbach-Klösgen et al. 1987), it is very likely that this 3′ truncation would have a negative effect on a putative $C2-Idf-III$ protein. However, theoretically, translation from the $C2-Idf-I$ and $C2-Idf-II$ copies could produce active, functional C2 proteins.

At its 5′-LTR, the PREM-2/Ji-like element is flanked by an Opie-like retrotransposable element (Meyers et al. 2001). A 5.5 kb portion of this Opie-2-like element was sequenced without reaching its putative 5′-LTR. The exact border between both elements could not be determined by sequence similarity analysis. Both elements are members of retroelement-families that are highly abundant in maize (Meyers et al. 2001).
The intron lengths of the three C2-Idf gene copies were 1730 bp for C2-Idf-I and C2-Idf-II and 1740 bp for C2-Idf-III, respectively. All three introns were highly similar to each other (98-99% identity). The C2-Idf introns differ from the 1520-bp C2-W22 intron by the presence of several small insertions/deletions (indels) and insertion of a 239 bp sequence stretch that is specific for C2-Idf (Figure 3A, C2-Idf intron ‘insertion’). This insertion has no significant homology to known sequences.

The promoter sequences of the C2-Idf copies are identical over a sequence range between -1500 and -1 relative to the predicted transcription start with the exception of two small indels in C2-Idf-I and an insertion of a 1163 bp transposable element in C2-Idf-II. This element belongs to the CACTA-type family and is inserted at position -211 bp relative to the putative start of transcription. A 3-bp target site duplication (TAG), which is typical for the CACTA family elements, is present at the site of insertion of the element (Figure 3B). A footprint of this transposable element (TAGCTAG) is found at identical positions in C2-Idf-I and C2-Idf-III (Figure 3B). Based on this finding, it seems likely that the CACTA-element was already present before the first duplication event of the c2 gene in the C2-Idf allele. This conclusion is further supported by sequence analysis of the regions 3’ of the C2-Idf-I and II genes. The 3’-region following the C2-Idf-II protein coding sequence is almost identical to the corresponding region of C2-W22 (~1.3 kb; Figure 3A). In contrast, the sequence identity between the 3’-regions of C2-Idf-I and C2-W22 spans only 556 bp. Further downstream in C2-Idf-I are clusters of repetitive sequences that probably originated from the insertion/activity of additional mobile elements (Figure 3A). The C2-Idf-II copy seems to be the progenitor in this cluster and the C2-Idf-I and C2-Idf-III copies most likely duplicated from this copy.

A comparison between the promoter regions of C2-Idf-I and C2-W22 revealed overall identity of 98% with two deletions of a CGCGC motif at -105 and -147 respectively,
one insertion of GCTA at -209 and four single nucleotide exchanges. Each promoter of the C2-Idf copies and of the C2-W22 version contained a small defective CACTA-like Isb transposable element at identical positions but with some minor sequence variations in the element (Figure 3A). Isb was previously identified to be of ancient origin and is present in a number of different anthocyanin genes (TECHEN et al. 1999). The high degree of similarity between the C2-W22 promoter and the promoters of C2-Idf-copies extends close to the respective Isb elements (Figure 3A, promoter region).

Several transposable element-like sequences were identified in the intergenic region between C2-Idf-I and C2-Idf-II. Two imperfect direct repeats of approximately 100 bp each were identified at -2092 and -2603 relative to the putative transcription start of C2-Idf-I (+1 C2-Idf-I; Figure 3A). Both repeats have significant similarity to a part of the 300 bp ZmSINE1 element (YAO et al. 2002; GenBank AC: AF434193). Additionally, a 308 bp heartbreaker element (MITE-like) (Hbr, ZHANG et al. 2000) was identified at position -3304 (relative to +1 of C2-Idf-I, Figure 3A). The 3-bp target site duplication of this element (TTT) is identical to the previously described target site duplication of Hbr22 (ZHANG et al. 2000). Another putative TE was identified by the presence of two nearly identical 56-bp inverted repeats (putative TIRs) at positions -4946 and -5046, relative to +1 of C2-Idf-I and separated by 46 bp (putative novel TE; Figure 3A). Similar TIR-like sequences, enclosing sequences of variable lengths, were also found in other genomic sequences of Zea mays subsp. mays and subsp. parviglumis (data not shown). Hence, these sequences might be parts of a novel genetic element.

**C2-Idf promoter analysis:** The presence of multiple c2 gene copies in the C2-Idf allele prompted us to ask which might be capable of driving expression. In plants, transcriptionally active genes have loosely packed chromatin, which is associated with low levels of cytosine methylation on DNA. Conversely, transcriptionally silent genes have
more tightly packed chromatin organization and higher levels of cytosine methylation (Jenuwein and Allis 2001; Jackson et al. 2002; Hoppe et al. 2002). To assess the expression potential of the C2-Idf genes, we used methylation-sensitive restriction enzyme digests to compare the methylation status of the C2-Idf gene promoters to that for the wild-type C2-W22 allele. To provide a reference, DNA was first digested with the non-methylation sensitive enzyme, NdeI. Probing with the c2 promoter probe yielded a single hybridizing band of 2.1 kb in C2-W22, which is the predicted fragment based on sequence data (Figure 4). In C2-Idf, there were two hybridizing bands; based on sequence data, these represent all three copies, with C2-Idf-I and C2-Idf-III producing 2.1-kb fragments and C2-Idf-II producing a larger 3.2-kb fragment due to the CACTA element insertion. When digested subsequently with a methylation-sensitive enzyme, these fragments should be cleaved into smaller products if the restriction sites are unmethylated. If the sites are methylated, the larger fragments will remain uncut.

In the normal allele, all of the sites tested are unmethylated, which is the expectation in a transcriptionally active allele (Figure 4). The C2-Idf allele has a more complex pattern of methylation (Figure 4). In C2-Idf-I and C2-Idf-III, the sites in the promoter nearest to the putative transcription start site (Clal, PstI, HinfI, Sac1 and AatII) are methylated, which is typical of a transcriptionally silent allele. In C2-Idf-II, which has a CACTA element inserted in the promoter region, sites upstream of the insertion are methylated, as in C2-Idf-I and C2-Idf-II; however, the two sites closest to the start of transcription (SacI and AatII) are unmethylated, as would be expected if this gene were transcriptionally active. This unmethylated region appears to be localized, because Southern analysis with a c2 intron probe (Figure 2) showed that the intron regions of all three C2-Idf gene copies are subject to methylation; in an EcoRI/AvaI digest, none of the three expected C2-Idf fragments (C2-Idf-I
and II: 1494 bp; C2-Idf-III 1505 bp) could be detected, whereas the expected fragment from the wild-type allele was detected in LC DNA (1295 bp).

In some cases of silencing, the methylation pattern of one allele can be transferred to a second, homologous allele (Luff et al. 1999; Walker and Panavas 2001). To ask whether the promoter methylation pattern in C2-Idf is transferred to the normal allele, methylation-sensitive restriction enzyme analysis was carried out in heterozygotes (Figure 4). In these plants, restriction fragments characteristic of both alleles are seen, indicating that trans-methylation of the wild-type C2 allele did not occur. This result fits with the genetic behavior of C2-Idf, in that C2-Idf does not heritably alter the expression of the normal allele.

**RNA blot analysis:** To investigate the nature of the C2 silencing by C2-Idf, steady state RNA levels were determined. RNA was isolated from husks of C2-Idf/C2 and C2/C2, blotted and hybridized with a probe derived from the 3’ untranslated region of c2 and an actin cDNA (Shah et al. 1983) as a loading control (Figure 5A). In husks from C2-Idf/C2 plants, c2-homologous transcript accumulated at roughly 20% of the C2/C2 level (Figure 5B). In homozygous C2-Idf/C2-Idf plants, RNA levels were nearly as low as in the negative control plants (Pl-0/Pl-0). These data confirm that C2-Idf inhibition occurs at the RNA level.

**Nuclear run-on transcription:** To determine whether C2-Idf is transcribed, we performed nuclear run-on transcription on nuclei isolated from husks. In run-on transcription, nascent nuclear transcripts are radioactively labeled and used as hybridization probes on DNA slot blots. This experiment allows measurement of the amount of transcription from genes of interest (C2 and C2-Idf) relative to negative (empty vector) and positive (actin) controls. If C2-Idf is transcribed and has no influence on C2 transcription, then the signal from C2-Idf/C2 heterozygotes should be greater than 50% that of C2/C2. Alternatively, if C2-Idf represses transcription of the C2 allele, then signal from C2-Idf/C2 heterozygotes should reflect the levels of steady-state transcript accumulation.
Run-on transcription revealed that the C2-Idf locus is transcribed (Figure 5C and D). In C2-Idf/C2-Idf homozygotes, the level of transcription was 44% of the C2/C2 level and significantly higher than the level of the negative control (pl-0/pl-0), indicating that the C2-Idf allele produces c2-homologous transcripts, albeit at a lower level than C2. In C2-Idf/C2 heterozygotes, c2 transcription was 73% that of C2/C2, indicating that C2-Idf does not transcriptionally silence the normal allele.

We should note that for this assay, the DNA used on the slot blots was a C2 cDNA clone. Transcripts hybridizing to the cDNA could come from either the c2 gene or from a duplicate gene called white pollen1 (whp). The whp gene is 94% identical to c2 within the coding region (FRANKEN et al. 1991) and is expressed at low levels in husks. If whp transcription is unaffected by the C2-Idf genotype, then in each sample, the same fraction of the signal is expected to represent whp transcription. In fact, the low level of transcription observed in the pl-0/pl-0 negative control is probably due to whp. There are two lines of evidence that suggest that C2-Idf does not affect whp expression. First, plants that lack chalcone synthase activity in pollen are self-sterile due to defective pollen. If C2-Idf silences whp1, then C2-Idf homozygotes would be self-sterile; this is not the case. Secondly, in a homozygous intensifier1 (in1) mutant background, whp1 is expressed in the kernel aleurone resulting in colored phenotype in a recessive loss-of-function c2 mutant. Plants homozygous for the in1 mutant and C2-Idf produce kernels with a colored aleurone, indicating that C2-Idf does not affect whp expression (our unpublished data). Thus, the presence of whp signal does not alter the conclusions from this experiment.

In some cases of silencing, ectopic transcription of promoter sequences leads to production of an aberrant RNA that triggers transcriptional silencing of the gene through chromatin modifications to the promoter (METTE et al. 1999). To test whether the C2-Idf promoter is transcribed, we hybridized labeled nuclear transcripts to a c2 promoter DNA
fragment. There was no signal in any genotype (data not shown), indicating that the promoters are not transcribed at a detectable level.

**Viral suppression of silencing by Maize necrotic streak virus (MNeSV):** A number of viruses encoding proteins that are known to effectively inhibit RNA silencing are common maize pathogens. MNeSV has been tentatively identified as the first monocot-infecting tombusvirus. It encodes a protein highly homologous to the p19 protein from dicot-infecting tombusviruses, such as Tomato bushy stunt virus (TBSV) and Carnation Italian ringspot virus (CIRV) (LOUIE et al. 2000). The p19 protein acts as a suppressor of RNA silencing, and for TBSV and CIRV, has been shown to bind siRNAs (VARGASON et al. 2003; YE et al. 2003), presumably inhibiting their incorporation into the RISC and curtailing downstream roles in silencing (LAKATOS et al. 2004).

To assess whether infection with this virus relieved C2-Idf inhibition of the C2 allele, C2-Idf mutant and C2 control plants were infected with MNeSV. If C2-Idf inhibits the functional C2 allele by RNA silencing, then infection of C2-Idf/C2 heterozygotes with this virus should result in a higher level of steady-state transcript and a more pigmented phenotype. Infected C2-Idf/C2 heterozygotes exhibited anthocyanin accumulation that corresponded to the MNeSV-infected lesions on the leaf sheath (Figure 6A). There was no anthocyanin accumulation in infected pl-0/pl-0 plants, nor did we observe anthocyanin accumulation in C2-Idf/C2 plants infected with the unrelated Maize chlorotic mottle virus (MCMV), a machlomovirus (data not shown). The latter result indicates that viral infection per se does not activate the anthocyanin pathway and that MCMV does not suppress C2-Idf silencing.

Total RNA was extracted from MNeSV-infected streaks and from uninfected control tissue and subsequently analyzed by RNA blot hybridization with a probe derived from c2. MNeSV-infected C2-Idf/C2 heterozygotes accumulated twice as much c2 mRNA as...
uninfected plants (Figure 6B). In contrast, infected C2/C2 and C2-Idf/C2-Idf plants showed a slight decrease in c2 mRNA, relative to controls. Infection of pl-0/pl-0 plants did not alter c2 mRNA levels. Together, the pigment phenotypes and the RNA levels indicate that infection with MNeSV relieves C2-Idf inhibition of C2.

**Viral suppression of silencing by Maize dwarf mosaic virus-A** (MDMV-A): As a second test for viral suppression of RNA silencing, we infected homozygous C2-Idf mutant and C2 control plants with MDMV-A, a potyvirus, which produces the P1/HC-Pro polyprotein. P1/HC-Pro has been shown to act as an effective inhibitor of RNA silencing in other systems (ANANDALAKSHMI et al. 1998; KASSCHAU and CARRINGTON 1998). In infected C2-Idf/C2 heterozygotes, suppression of the typical colorless or faintly colored phenotype first became evident approximately four weeks after inoculation as dark red or purple streaks along the sheaths in a pattern characteristic of the viral infection pattern (Figure 7A). By anthesis, strong purple pigmentation was visible on leaf sheaths, husks, some adult leaves and tassel glumes.

Infected C2-Idf/C2 heterozygotes accumulated more than three times the amount of steady-state c2 mRNA in husks than did uninfected heterozygotes (Figure 7B). The mRNA level in the infected C2-Idf/C2 plants was about 65% of the mRNA level in C2/C2 husks. A slight but lesser increase of c2 mRNA level was also observed between infected and uninfected C2-Idf/C2-Idf homozygotes. This coincides with the slight phenotypic suppression of silencing in the infected C2-Idf/C2-Idf homozygote plants. Neither C2/C2 nor pl-0/pl-0 homozygotes exhibited a significant difference in transcript accumulation upon MDMV-A infection.

The increase in steady-state transcript levels in MDMV-A infected plants carrying C2-Idf could be explained either by an increase in transcription or by a decrease in mRNA degradation. To distinguish between these possibilities, we used run-on transcription assays
to measure the amount of transcription in husks of infected and uninfected plants. As shown in Figure 7C, MDMV-A infection did not significantly alter transcription. These results indicate that the increase in steady-state mRNA levels in MDMV-A infected plants containing C2-Idf alleles is likely the result of reduced transcript degradation.

**Detection of small interfering RNAs:** Accumulation of siRNAs, processed from double-stranded RNAs by Dicer, is a hallmark of RNA silencing. To determine whether c2-homologous siRNAs are present in plants carrying a C2-Idf allele, we analyzed RNA from husks of C2/C2, C2-Idf/C2 and C2-Idf/C2-Idf plants on RNA blots using probes from different parts of the c2 gene (Figure 8A). The siRNAs were not detected in normal C2/C2 with any of the probes or in any genotype with the promoter probe. However, all three probes derived from the transcribed regions of the c2 gene detected siRNAs in husks of plants carrying C2-Idf (Figure 8C). There were two classes of siRNAs present. Multiple size classes of siRNAs have been described previously and may play different roles in silencing (HAMILTON et al. 2002).

We also assayed for the presence of siRNAs in MDMV-A infected plants. The three probes from the transcribed region of c2 detected siRNAs in husks from both infected and uninfected C2-Idf plants (heterozygotes and homozygotes); however, while this assay is not strictly quantitative, in most cases the siRNA levels appeared to be lower in the infected plants. This reduction is consistent with current thinking about how P1/HC-Pro suppresses RNA silencing. The viral protein is thought to interfere with either production of the aberrant double-stranded precursor or processing of siRNAs (DUNOYER et al. 2004); either possibility would be consistent with reduced levels of siRNAs in infected plants.

**DISCUSSION**
C2-Idf inhibits expression of normal C2 alleles to produce a colorless phenotype that is distinct from the deeply pigmented phenotype of the normal plants. The results of our experiments indicate that this phenotypic inhibition occurs by RNA silencing. The sequence analysis of the C2-Idf allele revealed that the structural difference between the mutant and a normal functional allele is the presence of three nearly full-length copies of the c2 gene in C2-Idf. The C2-Idf allele is transcribed, as assessed by nuclear run-on transcription assays, but there is little accumulation of full-length transcripts. Instead, plants carrying C2-Idf produce siRNAs with homology to the transcribed portions of the c2 gene. Furthermore, infection of C2-Idf/C2 heterozygotes with either MNeSV or MDMV-A partially relieves silencing, leading to production of more pigment and higher levels of steady-state c2 mRNA than in uninfected plants. In the case of MDMV-A infection, the increase in c2 mRNA accumulation is not due to higher levels of transcription, but instead is correlated with lower levels of siRNA accumulation.

Two previously described cases in which a dominant mutant induces RNA silencing of an endogenous gene bear similarities to C2-Idf silencing. In the first case, a rice mutant, Low glutelin content1 (Lgc1), contains a deletion of sequences between two tail-to-tail inverted repeat gene segments. The deletion leads to read-through transcription from one gene into the inverted repeat, producing a double-stranded RNA capable of inducing RNA silencing. When Lgc1 is crossed to wild-type, gene expression from the wild-type allele is reduced and the reduction is correlated with production of siRNAs (Kusaba et al. 2003). In soybean, the Inhibitor mutation, which is a dominant negative variant of the chalcone synthase (CHS) locus, contains multiple CHS gene copies, some arranged as inverted repeats (Tuteja et al. 2004). When Inhibitor is crossed to wild-type, expression of the wild-type gene is silenced. Silencing is accompanied by reduction of CHS mRNA and production of
CHS-homologous siRNAs. Furthermore, infection with viruses carrying suppressors of silencing partially restore CHS expression in heterozygotes (SEDA et al. 2004).

The C2-Idf allele is also composed of multiple c2 gene copies. C2-Idf-I and C2-Idf-II are arranged as a head-to-head inverted repeat and a novel CACTA transposable element is found in the promoter of C2-Idf-II. C2-Idf-III is truncated in the second exon by a Jil/Prem-2 retrotransposon. The presence of these transposable elements suggests a possible sequence of events that led to the present structure of the C2-Idf allele. First, a CACTA element inserted into the ancestral c2 gene and then this gene was duplicated. This event was followed by excision of the CACTA from one c2 copy followed by a second duplication of this gene, leading to production of C2-Idf-I and C2-Idf-III with identical transposon footprints. The CACTA element remains in the C2-Idf-II gene copy. It is not clear which duplication event coincided with production of the head-to-head arrangement of C2-Idf-I and C2-Idf-II. Later, C2-Idf-III was modified by retroelement insertion or by a deletion event to abut the 3’ end of the gene to a retroelement. It is also possible that transposition events of the CACTA element might have occurred independently after duplication.

Although there are three c2 gene copies in the C2-Idf allele, it is not clear which gene or genes are transcribed. The results of run-on transcription assays, which showed that transcription in C2-Idf homozygotes is about 40% of that in C2 homozygotes, indicate that it is unlikely that all three genes are each transcribed at normal levels to produce RNA homologous to the c2 coding region. Methylation analysis showed that the only gene with an unmethylated promoter is C2-Idf-II, the copy carrying a CACTA element. However, the influence of the CACTA-insertion at position -211 is difficult to predict, as insertions of CACTA elements in the promoter regions of c2 and other genes can have either positive or negative effects. For example, in the c2-m1-130 allele, insertion of an En/Spm CACTA-like element at position -94 results in a complete loss of C2 expression (WIENAND et al. 1986).
In contrast, CACTA-like Doppia elements located upstream of coding sequences in some alleles of the anthocyanin regulatory genes r1 (WALKER et al. 1995; MAY and DELLAPORTA 1998; BERCURY et al. 2001) and plI (CONE et al. 1993b) appear to be essential for proper promoter activity.

The nature of the RNA that triggers RNA silencing in C2-Idf is not known. Unlike the silencing associated with the soybean CHS genes or the rice, Lgc1 gene, the C2-Idf genes are not arranged in the type of tail-to-tail inverted repeat that would lead to read-through production of an antisense transcript. However, in C2-Idf, one possible source of triggering RNA could be transcription from the Prem-2/Ji retroelement inserted at the end of the second exon in C2-Idf-III. The orientation of this element is such that transcription from its 3' LTR could produce an antisense c2 transcript that could associate with RNA produced from the transcribed C2-Idf-II gene to induce silencing.

In plants carrying the C2-Idf mutation, two species of siRNAs are present—one of 21-22 nt and a second of 24-25 nt. These two size classes of siRNAs play different roles in silencing (HAMILTON et al. 2002). The short siRNAs, which are typically produced from silenced transgenes, are required for mRNA degradation. In contrast, the long siRNAs, produced from silenced transgenes and from retroelements, result in modification of the chromatin in sequences with homology to degraded transcripts; this results in silencing of homologous loci (AUFSATZ et al. 2002; CAO et al. 2003; VOLPE et al. 2002; VOLPE et al. 2003). The presence of these RNAs raises the possibility that one or more gene copies in C2-Idf are transcriptionally silenced to some degree. However, the available evidence indicates that the effect of C2-Idf on normal C2 is post-transcriptional and that this is the process suppressed by viral infection. The respective roles of the two classes of siRNAs in the observed silencing for C2-Idf are currently unknown.
The finding that $C2-Idf$ inhibition of $C2$ involves an RNA silencing mechanism and that this silencing is suppressed by some maize viruses establishes a phenotypic system in which silencing mechanisms can be easily studied. This reporter system should allow the detection of mutations defective in silencing functions and will facilitate the study of silencing suppression by maize viruses.

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FIGURE 1. - C2-Idf phenotypes. (A) Phenotypes of maize kernels from the mutant line C2-Idf, a normal line (LC) and reciprocal crosses of both lines. The corresponding number of C2-Idf alleles in the triploid aleurone is given. (B) Vegetative phenotype of C2/C2 and C2-Idf/C2 plants.

FIGURE 2. - Analysis of C2-Idf allelic structure. Southern blot of genomic DNA from homozygous C2-Idf and C2 (LC) leaves, respectively. DNA samples were digested with the restriction enzyme EcoRI and with a second enzyme as indicated. DNA was fractionated on an agarose gel, blotted to a nylon membrane and was hybridized with an intron-specific probe that was generated by AvaI restriction of a genomic C2 clone from LC (Wienand et al. 1986). The schematic structure of this normal C2-W22 gene is given (exons = black, intron = gray; transcription start = +1). Sizes of fragments in kilobases are indicated at left.

FIGURE 3. - Schematic comparison between the C2 and the C2-Idf allelic structures. (A) Genomic regions of the C2-Idf allele were cloned from corresponding λ- and fosmid-libraries, respectively. Sequences were assembled and analyzed with the help of bioinformatic software. For comparison, 7.2 kb of the C2 gene from the normal line W22 were cloned and sequenced. The C2-Idf allele consists of three C2 gene copies, C2-Idf-I, C2-Idf-II and C2-Idf-III. Two of the gene copies, C2-Idf-I and C2-Idf-II are located in close proximity to each other in a head-to-head orientation on the same contig (23,255 bp). C2-Idf-III was identified on a second contig of 22,981 bp. Both contigs contained no overlapping flanking sequences so that the relative position of C2-Idf-III within the C2-Idf allele is unknown. For comparison, corresponding regions of C2-Idf-II and the normal C2
gene are aligned (dotted lines). Putative positions of the transcription start sites of the C2-Idf
gene copies are marked according to the experimentally determined start site of the normal
C2 gene (FRANKEN et al. 1991). Major and minor characteristic sequences of the C2-Idf
allele are given as boxes or triangles. (B) Insertion of the CACTA-type element in the
promoter of C2-Idf-II and footprints of the insertion/excision event in C2-Idf-I and C2-Idf-
III. The insertion of the element generated a 3 bp target site duplication (TAG) in C2-Idf-II.
The identical footprint of this transposon was found in C2-Idf-I and C2-Idf-III. One
additional nucleotide [C] was present in both sequences. No such footprint was found in
normal c2 promoters of LC and W22.

FIGURE 4. - Promoter methylation. DNA from C2-W22, C2-Idf and C2-Idf/C2-W22
heterozygotes was digested with the methylation-insensitive enzyme NdeI. Samples were
then digested with methylation-sensitive enzymes. (A) A diagram of restriction sites
assayed. Open circles represent unmethylated sites and closed circles represent methylated
sites. Because the 3' most Hinfl site is located very close to the 3' NdeI site, its methylation
status could not be determined by Southern analysis. (B) Representative Southern blot
probed with the c2 promoter.

FIGURE 5. - RNA levels in C2-Idf mutants (A) Representative RNA blot probed with the
C2 3'UTR. The blot was stripped and re-hybridized with an actin probe as a loading control.
(B) Relative steady-state c2 transcript levels normalized against actin (n=6-8; error bars are
the standard errors of the means). (C) Representative slot blots probed with radioactively
labeled nuclear RNA. (D) Relative transcription rate normalized against actin (n=6; error
bars represent the standard errors of the means).
FIGURE 6. - The effect of infection with MNeSV on C2-Idf silencing. (A) Phenotypes of MNeSV infected (+) and uninfected plants (-). (B) Relative steady-state c2 transcript levels normalized against actin (n=3; error bars are the standard errors of the means).

FIGURE 7. - The effect of infection with MDMV-A on C2-Idf silencing. (A) Phenotypes of uninfected control plants (top) and MDMV-A infected plants (bottom). (B) Steady-state c2 transcript levels normalized against actin (n=6-8; error bars are the standard errors of the means). (C) Relative c2 transcription rate was measured by run-on transcription assays of infected plants and uninfected controls (n=3, error bars are the standard errors of the means).

FIGURE 8. - siRNA analysis from plants infected with MDMV-A. (A) Diagram of the c2 gene showing location of hybridization probes (hatched boxes). Gray boxes represent untranslated regions; black boxes represent protein encoding regions. (B) Ethidium bromide stained image of RNA gel to show comparable loading in all lanes. (C) RNA blots hybridized with probes from A. RNA was isolated from MDMV-A infected plants (+) and uninfected controls (–). Position of 21-nt size standard in gel is indicated to the left of each blot.
Figure 1

A

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B

C2/C2  C2-Idf/C2
Figure 2

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Southern probe:
1.3 kb Avai fragment of C2-W22

C2-W22

+1

Inton

[1523 bp]

1 kb

Coding region

Inton

kb

21 -

12 -

10 -

8 -

6 -

5 -

4 -

3 -

2 -

1.6 -
Figure 3

A

putative transcription start

| coding region | CACTA-like [length bp] | putative novel TE |
| Intron [length bp] | heartbreaker [hbr] | L1b element |
| C2-Idf-I ‘insertion’ | retroelement | repetitive region |
| TOA | retroelement LTR | |
| translation stop | | |
| promoter region | | |

3’ region identical to C2-W22

B

C2-Idf-II

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<th>TAG</th>
<th>TAGTAC</th>
<th>3’</th>
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</table>

C2-LC

| 5’ | TAG | TAC | 3’ |

C2-W22

| 5’ | TAG | 3’ |
Figure 4

A

C2-W22

C2-Idf-I

C2-Idf-III

C2-Idf-II

A = AatII, C = Clal, H = HinfI, N = NdeI, P = PstI, S = SacII

probe

CACTA element

5' UTR

coding region

intron

methylated site

unmethylated site

B

NdeI

AatII

ClaI

HinfI

PstI

SacII

kb

C2

C2-Idf/C2

C2-Idf/C2

C2-Idf/C2

C2-Idf/C2

C2-Idf/C2

C2-Idf/C2
Figure 5

A

B

c2 3’UTR

actin

C

D

C2/C2  C2-Idf/C2  C2-Idf/C2-Idf  pl-0/pl-0

C2/C2  C2-Idf/C2  C2-Idf/C2-Idf  pl-0/pl-0

C2/C2  C2-Idf/C2  C2-Idf/C2-Idf  pl-0/pl-0

plasmid

actin

plasmid

actin
Figure 6
Figure 7

A

Uninfected

Infected

B

C

\(c_2/\text{actin}\)

\(C2/C2\) \(C2-\text{Idf}/C2\) \(C2-\text{Idf}/C2-\text{Idf}\)

\(C2/C2\) \(C2-\text{Idf}/C2\) \(C2-\text{Idf}/C2-\text{Idf}\)  \(pl-0/pl-0\)

\(C2/C2\) \(C2-\text{Idf}/C2\) \(C2-\text{Idf}/C2-\text{Idf}\)

\(\text{Uninfected}\) \(\text{Infected}\)
Figure 8

A

promoter

5'UTR

intron

3'UTR

B

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