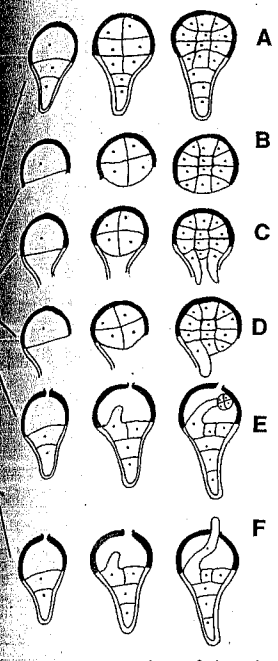

he cell wall. (A)
thallus cells. (B)
a two-cell embryo
intact. (B) The
hours, showing
s (r') at the site
. (C and D)
us cell after the
zoid wall (rw).
ıe side of the
(arrowhead) after
roduction by
l space of the
me of 48 hours
showing initial
ormer thallus wall
development (F)
contact with the
ell. Bars represent
in (E) and (F).

ged contact bet
oid wall.
nent of an
ll occasionally
ormed by the
= 6) (Figs.
ch rhizoids had
ip extension.
ard a hole-pro
wall, it contin
and remained
contrast, conta
ıtact inner wall
ed in arrested
than 24 hours
ınal axis at the
ıd F, and 4E).
ion. It is unlik
ted a barrier to
ng rhizoids
barriers by
hile maintain
onclude that

Schematic representation of the devel-
(A) intact embryos and after laser
(B) the two-cell stage of the rhizoid
wall, (C) rhizoid cell contents, (D) half
oid cell wall, and (E and F) thallus cell
(E and F) Also shown is the fate of a
produced within the thallus cell
ment after prolonged contact with the
l wall or (F) growing through a hole
cut in the cell wall. Bold lines repre-
inal thallus wall and double lines rep-
iginal rhizoid wall.

inner thallus cell wall can alter the
hizoid cells.
ments have demonstrated the
importance of position-dependent
rather than cell lineage in the
on of plant embryogenesis (*8*, *9*),
n inductive effects have not been
rated directly. Plant embryos are
to dedifferentiate and redifferenti-
3). Somatic embryos of *Daucus*
to surgical tissue removal by reor-
on of the cut region and tissue
ation (*9*, *12*). In addition to the
ed roles of diffusible morphogens
direct evidence has implicated cell
and wall-related molecules as fate
nants (*15*). We have demonstrated
ally regulated induction in a plant
and shown that fate-determining
tion is associated with the cell
ost likely the inner face of the
wall. If the original rhizoid-thallus
wall does not possess fate-deter-
roperties, the mature rhizoid or
wall should have an overriding
the fate of recently divided cells
predominantly by primary, im-
all. The factors involved in these
processes must be long-lived and
diffusible because isolated cell

wall retains its inductive properties for several days. The factors must act through the wall of the induced cell and must therefore be rendered diffusible by cell contact, possibly in response to secretions from the cell itself. The evidence presented here implicates the plant cell wall both in maintaining the differentiated state and in directing the pattern and cell fate in plant development.

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Recombination Between Viral RNA and Transgenic Plant Transcripts

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Transformed plants expressing the 3′ two-thirds of the cowpea chlorotic mottle virus (CCMV) capsid gene were inoculated with a CCMV deletion mutant lacking the 3′ one-third of the capsid gene. Although the deletion inoculum replicates in inoculated cells, systemic infections occur only if recombination restores a functional capsid gene. Four of 125 inoculated transgenic plants, representing three different transgenic lines, became systemically infected. Analysis of viral RNA confirmed that RNA recombination had united the transgenic messenger RNA and the challenging virus through aberrant homologous recombination.

The evolution of plus sense RNA viruses proceeds by natural mechanisms including errors by viral RNA polymerase, which lacks proofreading capabilities, and by homologous and heterologous RNA recombination (*1*). Recombination has generated mosaic-type defective interfering RNAs in cymbidium ringspot tombusvirus (*2*) and variants of tobacco rattle tobravirus (*3*). Recombination has been reported in the 3′ untranslated and intercistronic sequences of bromoviruses (*4*–*6*). The mechanism of plant viral RNA recombination has been addressed experimentally in both brome mosaic virus and turnip crinkle virus subviral RNAs (*7*, *8*).

There are indications that plant RNAs have recombined with replicating viruses. Several potato leafroll virus isolates contain sequences homologous to an exon of tobacco chloroplast RNA (*9*). Additionally, a deletion mutant of red clover necrotic mosaic virus was restored by recombination with transgenically expressed viral RNA (*10*). The rarity of reported recombination events between viral RNA and host mRNA may reflect their infrequency or the failure of products to be viable.

Virus resistance can be conferred on transgenic plants by expression of segments of viral genome, such as capsid genes (*11*). Transgenic plants expressing a viral capsid protein exhibit resistance to that virus and closely related strains (*12*) but remain susceptible to other viruses.

Plants frequently resist viral attack by restricting virus movement rather than inhibiting replication (*13*). Therefore, plants challenged by viruses that are not patho-

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gens of that particular species may support viral replication. Thus, in virus-resistant transgenic plants, replicating pathogenic and nonpathogenic viruses may come in contact with a pool of viral RNA transcribed by the plant that is available for RNA recombination. Such events could generate a virus with properties that differ from either progenitor virus (*14*).

The following experiments sought to determine if mRNA expressed in a transgenic host is available for recombination with a replicating virus. Cowpea chlorotic mottle bromovirus (CCMV) consists of two monocistronic RNAs 1 and 2 that encode replication proteins and a dicistronic RNA 3 that encodes the putative movement protein, 3a, and capsid protein. Infectious transcripts are produced from complementary DNA (cDNA) clones of these RNAs (*15*). Transformed plants expressing the 3′ two-thirds of the CCMV capsid gene were inoculated with a CCMV deletion mutant lacking the 3′ one-third of the capsid gene. This deletion prohibits systemic infections. If recombination occurs within the central third of the capsid gene, a segment shared by both the transgenic and inoculation RNAs, a functional capsid gene could be restored that supports systemic infection. With this system, we demonstrate RNA recombination between mRNA derived from the host chromosome and replicating viral RNA.

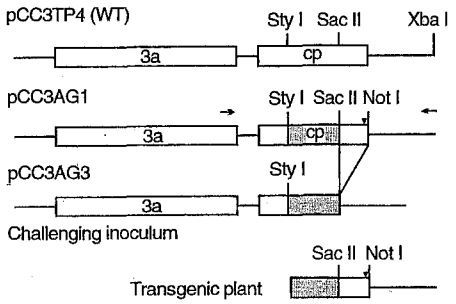

Fig. 1. Plasmid construction. The oligonucleotide TCTTCAGCGGCCGCTAATAGACCGGAGT was used to direct mutagenesis of pCC3TP4. The resulting plasmid, pCC3AG1, contained a silent mutation (G to C) at position 1926 (triangle) and changes at positions 1933 and 1935 (T to C, C to G), which introduced a Not I site at the 3′ end of the capsid gene. The 119-nucleotide Sac II–Not I fragment was removed from pCC3AG1, the sticky ends were filled with Klenow, and the plasmid was ligated to form pCC3AG3. Plasmid pCC3AG1 was digested with Sty I and Xba I, and the 697-nucleotide fragment was ligated into the Xba I site of transformation vector pGA643. This ligation was facilitated by first treating the vector ends with alkaline phosphatase, completing the Xba I ligation, then blunting the incompatible ends with Klenow and completing the ligation to form pGACCMV. Nucleotide numbering is given in (*23*). Arrows indicate positions of oligonucleotides used during PCR amplification of virion RNA.

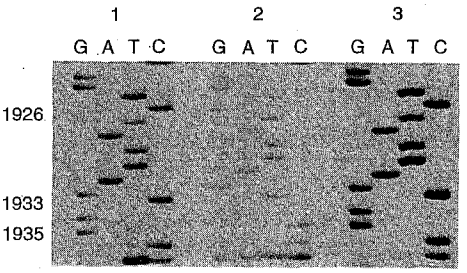

Fig. 2. Sequence analysis of PCR products generated from virion RNA of a transgenic plant infected with WT CCMV and plant 5-58. Full-length first-strand cDNA was made from virion RNA with oligonucleotide 3′-CCMV, CAGTCTAGATGGTCTCCTTAGAGAT. A 1096-bp fragment containing the intercistronic region, capsid gene, and 3′ untranslated region of RNA3 was amplified by PCR with oligonucleotides TAAAATCGCCGTAACCGC and 3′-CCMV. The amplified product was cloned into the Sma I–Xba I sites of pUC18 and sequenced with USB Sequenase. The sequences of plasmid pCC3AG1, and cloned PCR products from the WT infected plant and 5-58 are shown in panels 1 to 3, respectively. Nucleotide numbers denote positions of marker mutations present only in pCC3AG1 and viral RNA recovered from 5-58.

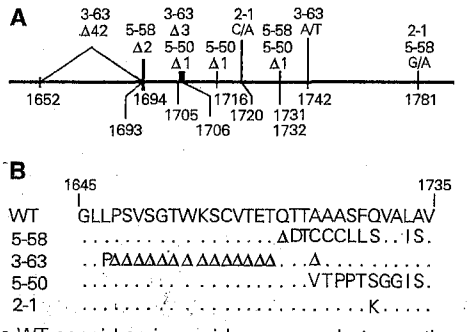

Fig. 3. Mutations in capsid genes of recombinant viruses 5-58, 3-63, 5-50, and 2-1. (**A**) The nature and extent of each nucleotide mutation is denoted above the horizontal line, which represents a segment of the WT capsid gene between nucleotides 1652 and 1781. Delta (Δ) indicates deleted nucleotides. Wild-type nucleotides and substitution mutations are separated by slanted lines. Exact positions of mutations are noted below the horizontal lines. Where adjacent or overlapping changes occur, the top recombinant corresponds to the top nucleotide number. (**B**) Amino acid deletions (Δ) and changes in the recombinants are compared with the WT capsid amino acid sequence between the Gly and Val codons beginning and ending at the indicated nucleotides. Dots indicate identity. See (*24*) for amino acid abbreviations.

Three marker mutations were introduced near the junction of the capsid gene and the 3′ untranslated region of the full-length cDNA clone of CCMV RNA3, pCC3TP4, to form pCC3AG1 [transcript AG1 (Fig. 1) (*16*)]. These mutations did not affect the viability of CCMV (*17*), and pCC3AG1 was used for construction of both the transgenic *Nicotiana benthamiana* and the challenging CCMV inoculum.

Deletion inoculation plasmid pCC3AG3 [transcript AG3 (Fig. 1)] was prepared by deletion of 119 nucleotides from the 3′ terminus of the capsid gene of pCC3AG1. When full-length transcripts of CCMV RNAs 1 and 2, C1 and C2, were coinoculated with AG3, neither cowpea nor *N. benthamiana* became systemically infected, but AG3 replication was observed in protoplasts.

A truncated capsid protein gene lacking 118 nucleotides from the 5′ end of the coding region, but containing the full-length 3′ untranslated region of RNA3 was cloned into transformation vector pGA643 (*18*) to generate pGACCMV (Fig. 1). This construct, which contains the neomycin phosphotransferase (NPT) II marker gene, was used to transform *N. benthamiana* (*19*).

From 57 kanamycin-resistant regenerated plants, 6 were selected on the basis of high levels of NPT II expression as judged by enzyme-linked immunosorbent assay. A CCMV-specific probe (6) hybridized to a single band on Northern blots of total RNA extracted from the six plants that were clonally propagated for recombination experiments. Transgenic plants challenged with either CCMV virions or wild-type (WT) RNA transcripts, C1, C2, and C3, became systemically infected. Thus, the transgenic transcript was insufficient to provide *N. benthamiana* with CCMV resistance.

The two youngest expanded leaves of 60 transformants at the six-leaf stage of development were inoculated with C1, C2, and AG3. Fourteen days after inoculation, plants were screened for systemic infections. Total RNA extracted from the fifth leaf above the inoculated leaf was probed for CCMV RNA. A positive hybridization to extracts from plant 5-58 suggested recombination.

Virion RNA was extracted from plant 5-58, and cDNA extending from the 3′ end of the 3a gene to the 3′ terminus of putative recombinant RNA 3 was synthesized and amplified by polymerase chain reaction (PCR) (Fig. 1). The recovered fragment was cloned and sequenced. All three mutations that were originally present only in the transgenic RNA were identified in the nucleotide sequence of the PCR gene (Fig. 2). Only wild-type sequences were recovered from control transgenic plants inoculated with WT CCMV. The

e that the systemic infection of 5-58
d from recombination between
expressed by the plant and the
ging deletion inoculum.
eral deletions within 5-58 shifted the
open reading frame (ORF) 13 codons
). Despite these amino acid substi-
, sap extracts from 5-58 initiated
CCMV systemic infections in both
as and *N. benthamiana*, and normal
of virion RNA were recovered from
pecies. Therefore, RNA recombina-
n 5-58 produced a mutant form of
/ by aberrant homologous recombi-
within the overlapping region
transgenic mRNA and the viral
um.

125 transgenic plants tested, four
minant viruses have been verified
hree different transgenic plant lines.
e attempts to favor homologous re-
nation by providing 338 overlapping
tides between the transgenic viral
and genomic RNA of the challeng-
us, sequences derived from recombi-
revealed that each resulted from a
tly different aberrant homologous re-
nation event (Fig. 3). Therefore, pre-
combination was not required to
virus viability.

vious bromovirus studies have dem-
ed RNA recombination only within
ding regions (*4–6*). This report dem-
es intragenic recombination in 3%
transgenic plants inoculated. Regen-
n of a functional ORF must provide
nt selection pressure on recombina-
oducts.

e factor that may contribute to re-
nation is the presence of the com-
3′ untranslated sequence from
/ RNA3 in the mRNA transcript.
the viral replicase complex initi-
inus strand RNA synthesis on this
al sequence (*20*), its presence may
replication to begin on the mRNA
ript and then switch to the RNA
um to complete synthesis. Thus,
resence of 3′ untranslated se-
e may target the transcript to the
tion complex and enhance the
ility of recombination. This would
nsistent with the template-switch-
odel for RNA replication (*21*). Be-
the 3′ untranslated region of the
nay lend stability to the viral RNA,
frequently included in transgenic
uctions.

combination during RNA virus repli-
contributes to the rapid evolution of
viruses and could affect host range or
specificity, traits that have been
ted to capsid proteins of several plant
s (*22*). As transgenically expressed
nRNA is available to recombine with
ting RNA viruses, RNA recombina-
tion should be considered when analyzing the risks posed by virus-resistant transgenic plants.

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16. To avoid potential spurious mutations, we substituted a 359–base pair (bp) Sac II–Xba I fragment containing the introduced mutations for the similar fragment in the original plasmid, pCC3TP4, to form pCC3AG1. The fidelity of all constructs was confirmed by sequence analysis.
17. Effects of mutations on virus infectivity were ascertained by inoculation of both cowpea, *Vigna sinensis* (Torner) Savi, and *Nicotiana benthamiana* (Domin) with full-length plasmid-derived transcripts of WT CCMV RNAs 1 and 2 and either WT RNA 3 or pCC3AG1, referred to hereafter as C1, C2, C3, and AG1. Plants became systemically infected within 14 days, and no differences were observed in either the quantity or stability of recovered virions or viral RNA.
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19. This placed the CCMV sequence under the control of the constitutive 35*S* promoter and within the T-DNA region of pGA643. The pGAC-CMV plasmid was introduced by tri-parental mating into *Agrobacterium tumefaciens* strain LBA4404 for use in leaf disk transformation of *N. benthamiana*. Transformed explants were selected for kanamycin resistance in tissue culture.
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24. Single-letter abbreviations for amino acid residues are as follows: 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.

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High-Resolution Molecular Discrimination by RNA

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Species of RNA that bind with high affinity and specificity to the bronchodilator theophylline were identified by selection from an oligonucleotide library. One RNA molecule binds to theophylline with a dissociation constant K_d of 0.1 μM. This binding affinity is 10,000-fold greater than the RNA molecule's affinity for caffeine, which differs from theophylline only by a methyl group at nitrogen atom N-7. Analysis by nuclear magnetic resonance indicates that this RNA molecule undergoes a significant change in its conformation or dynamics upon theophylline binding. Binding studies of compounds chemically related to theophylline have revealed structural features required for the observed binding specificity. These results demonstrate the ability of RNA molecules to exhibit an extremely high degree of ligand recognition and discrimination.

The conformational complexity of libraries of random-sequence single-stranded oligonucleotides offers the opportunity to search for molecules that show high-affinity binding to biomedically important targets (*1*). A procedure called SELEX (systematic evolution of ligands by exponential enrichment) permits the iterative isolation and amplification of RNA or DNA oligonucleotides with selective affinity for defined targets, which represents a route to drug discovery (*1*). With this technique, RNA oligomers have been isolated that have high affinity and specificity for a variety of both protein and small molecule targets, including bacteriophage T4 DNA polymerase (*1*), R17 coat protein (*2*), human immunodeficiency virus (HIV) reverse transcriptase (*3*), HIV Rev protein (*4*), basic fibroblast growth factor (*5*), adenosine triphosphate (*6*), and several amino acids (*7*). Oligomers of DNA that recognize thrombin (*8*) and oligomers of RNA and DNA that bind to organic dyes (*9*) have also been identified.

Many of these SELEX-generated oligo-

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