Plant gene silencing regularized

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Two surprising and potentially useful phenomena of transgenic plants originated from two distinct lines of research but may converge in underlying mechanism or mechanisms (1–3). Plant transformation technology, the staple of the crop biotechnology industry, overcomes the species barrier and allows introduction into plants of genes from other plant species, genes from species of other kingdoms, and even sequences derived from the DNA synthesizer. Subsequently, the introduced sequenced does not always, are inherited as silenced Mendelian characters and exhibit an expected phenotype. It is one group of annoying exceptions to expected phenotype and inheritance that constitutes the first surprising phenomenon. Investigators, seeking to increase the expression, and thereby enhance the phenotype, of an endogenous gene, introduced additional copies of the endogenous gene as a transgene. Usually the constructions use promoters more active than the promoter of the endogenous gene. In some instances, the result was not the overexpression of the gene, as expected, but a drastically reduced or undetected expression of both the endogenous and the introduced sequences (4, 5). Much of the early research used a chalcone synthase gene, the silencing of which sometimes resulted in flowers with spectacular patterns of nonpigmented and pigmented areas. The reduced expression phenomenon is termed gene silencing. Gene silencing results in no expression or very low expression of a gene or RNA sequence that formerly was expressed, or likely would have been expressed, absent the gene-silencing phenomenon. Paradoxically, plants with multiple copies of the transgene and/or high levels of transgene transcription are more likely than plants with a single copy and low-level transcription to exhibit gene silencing (6). However, the correlation has not been consistent for some systems (7, 8). Gene silencing can be induced not only by expression of a transgene but also by transient expression from DNA geminivirus vectors (9) and RNA virus vectors (10, 11). Similarly, Agrobacterium tumefaciens-based transient expression systems can generate RNA targets for gene silencing (12, 13). Transient expression allows rapid testing of various sequences without the time-consuming process of plant transformation and regeneration. For transgenic plants, the phenotype of a silenced transgene usually is maintained through vegetative propagation or organ regeneration and can be transmitted through a graft (14, 15). However, transmission de novo and continuing expression of gene silencing to progeny through meiosis, though often complicated by the effects of multiple inserts, is unpredictable, with silencing appearing among the progeny of a silenced plant with frequencies of 2–100%. Similarly, some progeny of a silenced plant may be silenced (15–18). That is, gene silencing behaves as an epigenetic trait.

The second phenomenon appeared in the course of experiments intended to exploit pathogen-derived resistance (19, 20). As pathogen-derived resistance is applied against plant viruses, a virus genome-derived sequence is incorporated into the plant transgene. For some gene constructions, researchers noticed an unexpected inverse relationship between the degree of protection shown by a particular transformed plant and the level of expression of the introduced, virus genome-derived sequence (3, 21). Resistance exhibited by plants with constructions designed to be translation competent, and by plants with constructions designed to generate RNA that is not translatable, generated similar resistance. This finding suggests that resistance did not require the synthesis of any virus-derived protein or protein fragment (22–24). Thus the term “RNA-mediated resistance” applies. The resistance conferred by the expressed RNA usually is very robust, not being overcome by virus inoculum concentrations that are orders of magnitude greater than the concentrations that routinely infect wild-type plants. The resistance is maintained in protoplasts isolated from resistant plants (7, 22). The conferred resistance proved to be highly virus specific, failing even against viruses closely related to the virus that was the source of the transgene sequence. RNA-mediated resistance, like transgene-induced gene silencing, usually proved to be unpredictable for appearance after passing through meiosis (25). In this issue of the Proceedings, Waterhouse et al. (26) report a modified approach to gene silencing and RNA-mediated protection that generated transgenic plants and their progeny with predictable phenotypes.

What are other similarities of RNA-mediated resistance and gene silencing (27)—of highly virus-resistant plants and, for example, plants with flowers showing unusual pigmentation? Two sorts of gene silencing have been documented for plants. In transcriptional silencing, messenger RNA synthesis is greatly reduced or absent (28). In posttranscriptional gene silencing (PTGS), messenger RNA, or messenger RNA precursors, are synthesized but apparently are degraded rapidly or improperly processed or both (7, 18, 25, 29, 30). PTGS was demonstrated for chalcone synthase by analyses of RNA synthesized by isolated nuclei, i.e., in “run-on” experiments (6, 8). The accumulation of transcripts in the nucleus but not in the cytoplasm (31) also suggests that transcription is not altered significantly and that PTGS may occur in the cytoplasm. Plant RNA viruses, which have been the usual targets of RNA-mediated resistance, replicate in the cytoplasm. The elegant experiments of English et al. (32) directly connected RNA-mediated resistance and PTGS. The RNA genome of potato virus X (PVX) is tolerant of the insertion of an additional gene, although the burden of the inserted nucleotide sequences slows the replication process (33). PVX with inserted Escherichia coli β-glucuronidase gene was infectious to wild-type tobacco plants but not to plants from transgenic tobacco lines that are posttranscriptionally silenced for β-glucuronidase. Results from experiments with PVX inserts corresponding to various fragments of the β-glucuronidase-encoding sequences allowed the target of the silencing to be mapped to the 3′ region of β-glucuronidase ORF. PTGS, unlike transcriptional gene silencing, is correlated with RNA-mediated resistance against RNA plant viruses (7). In both phenomena, although the targets are different, silencing affects both the target and the expressed sequence that triggered the silencing, justifying the term “cosuppression” (7, 34).
The variety of similarities between PTGS and RNA-mediated resistance, e.g., a high degree of sequence specificity for affected targets, correlation of the silencing with high copy number of the transgene, and the unpredictability of retaining the phenomenon after passing through meiosis (25), justify using the term PTGS collectively for both phenomena. PTGS can be a tool for both research and crop improvement, giving the investigator the power to prevent expression of a specific gene or to create plant resistance against specific viruses. However, just as the occurrence of gene silencing may be the unwanted outcome when the goal is overexpression, when the objective is useful PTGS, unfortunately only a few silenced plant lines may appear, and such lines generally will not hold to character when propagated by seed.

An alternative PTGS is "antisense suppression," a technology applied before "sense"-mediated gene silencing was known. Antisense technology, like PTGS, is intended to reduce the expression of a target gene, but by expression in the transgenic plant of sequences complementary to, rather than the same polarity as, the target sequence. Researchers presumed that the expressed antisense sequence would form a double-helical complex with the target RNA, preventing the target RNA from acting as a messenger RNA, and perhaps marking the target RNA for degradation (18). Are antisense suppression and sense RNA-mediated PTGS related phenomena, in at least some instances? The stringent sequence specificity exhibited by both phenomena suggests that this is the case. PTGS can be effective regardless of which polarity of target RNA is inserted in a virus RNA vector, and one example of PTGS has been shown to be specific for the complementary strand RNA, rather than the RNA strand expressed from the transgene (7, 12). Analyses for both strands of RNA in extracts of PTGS plants sometimes have revealed antisense as well as sense RNA (8, 16). The detailed phenotypes of sense and antisense plant constructions were found to be mostly similar but with some differences (35, 36). These results suggest a common intermediate for antisense RNA-mediated and sense RNA-mediated gene silencing. Logically, this intermediate would be double-stranded RNA (28, 37, 38). For PTGS, such an RNA might be generated from the transgene transcript by the action of a plant RNA-dependent RNA polymerase (21, 39).

If double-stranded RNA is formed in plants expressing antisense or sense polarity sequences, is that RNA critical to PTGS or is it merely a dead-end product? As reported by Waterhouse and coworkers (26) in this issue of the Proceedings, the authors set out to test directly the possible involvement of double-stranded RNA in gene silencing by preparing constructions designed to generate double-stranded RNA in transformed plants. They introduced, into separate tobacco lines, gene constructions designed to express the sense polarity or the antisense polarity of the coding sequences from a potato virus Y (PVY) protease gene. For these control constructions, tobacco lines immune or resistant to PVY were obtained at a frequency of less than 10%. Sampled immune or resistant lines contained 3–9 copies of the transgenes, whereas susceptible lines had 1–3 copies, which is consistent with the observations of others concerning a correlation of gene silencing with multiple inserts of the transgene. In contrast, reliable resistance was obtained when protease-encoding sequences of both polarities were transferred to the same line. That is, five hemizygous, PVY-susceptible tobacco lines with sense-orientation, single-copy protease insert were selected. When these lines were selfed, and when two of the lines were crossed, all progeny were susceptible to PVY. Two hemizygous, PVY-susceptible lines with antisense orientation, single-copy protease insert similarly were selfed and crossed without the appearance of PVY-resistant progeny. However, when progeny from each of 10 crosses, of sense orientation line to antisense orientation line, were challenged with PVY, among the 20 progeny analyzed from every cross were at least three that were immune or resistant to PVY. A total of 45 of the 200 progeny showed resistance or immunity. In the plants tested for transcript, immunity against PVY was correlated with the accumulation of both the sense and the antisense transcripts, possibly protected from degradation because of the formation of perfect duplexes. The authors note that the parents for these crosses have distinct sense and antisense loci, making it unlikely that the consistent appearance of the resistance phenotype is caused by interactions at the chromosome level. Rather, the effect probably is the result of interaction of transcripts, i.e., formation of double-stranded RNA. Indeed, the authors detected transcripts of both polarities in the PVY-resistant, progeny tobacco plants.

The reliability of protection, in the hybrid transgenic plants generating RNA of both polarities of the target sequence, is supported by experiments in which plants were transformed with tandem gene constructions, one gene for expression of sense orientation protease sequence and the other for antisense. About half of the transformants derived from these tandem constructions were resistant or immune to PVY. There was no obvious bias in favor of multiple insertions of the tandem constructs for the resistant lines compared with the sensitive lines, and progeny of the resistant lines inherited resistance in a Mendelian fashion. These results stand in contrast to the results from transformation with the corresponding single gene sense or antisense constructions, with less than 10% of such lines showing resistance against PVY, the above-mentioned bias toward multiple insertions, and inheritance of the resistance phenotype to only about 10% of the progeny. The results suggest that double-stranded RNA indeed is a critical intermediate in the development of PTGS and that at least some examples of antisense suppression are in fact examples of PTGS.

Double-stranded RNA-mediated down-regulation of specific genes has been demonstrated even more directly, by using a transient assay in the nematode Caenorhabditis elegans. Double-stranded RNA corresponding in sequence to the target gene was injected into the nematode and the RNA either or both of the component RNA strands alone into the nematode did not give a strong effect. Double-stranded RNA injection has become standard for the investigation of the effects of specific gene expression in this model organism.

The authors of the work that stimulated this commentary (26) propose a self-perpetuating mechanism for RNA degrada- tion, a mechanism that is consistent with a central role for double-stranded RNA in PTGS and for the observed efficiency of silencing. Neither the model nor their results shed light on one phenomenon that seems to be closely correlated with PTGS: DNA methylation. Transgene nucleotide sequences corresponding to the sequences targeted in PTGS become methylated during the induction of PTGS (7, 32, 37). Is this methylation a central feature or simply a byproduct of PTGS? Demonstration of methylation in the absence of transgene-mediated PTGS supports but does not prove the byproduct hypothesis. DNA copies of the RNA pathogen, potato spindle tuber viroid, were altered to make transcripts inactive for replication and were introduced into the tobacco genome. Infection of the plants with the viroid, which replicates in the nucleus, resulted in the methylation of the viroid sequences but not surrounding DNA sequences (27).

Waterhouse and coworkers (26) seem to have uncovered a reliable path to the construction of transgenic plants in which the replication of a specific virus is restricted or expression of a specific gene is suppressed. Correlation and possibly functional connections to gene silencing-associated phenomena, such as methylation of coding sequences and developmental state at which the silenced condition is established, must be demonstrated before we will know whether heretofore
has been generally recognized as PTGS has indeed been converted into a Mendelian trait.

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