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Manipulation of Plant Gene Expression by Antisense RNA

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Introduction

The development of techniques to introduce novel genes into plants has opened up the possibility of adapting crops to be more suited to the requirements of both producers and consumers. In addition to the expression of new genes conferring desirable traits such as resistance to pests and diseases, methods have also been developed for modification of the levels of expression of endogenous genes. The antisense RNA strategy is a particularly powerful tool for plant biotechnologists. It provides the opportunity to reduce the expression of specific genes and thereby produce plants with modified phenotypes. It is thus possible to inhibit characteristics that are undesirable for the contemporary applications of plant products.

Mutations, either natural or induced, have been enormously valuable sources of genetic variation for the understanding of gene activity and biochemical processes. The identification of useful mutants for an investigation usually requires recognition of a modified phenotype. Such phenotypes are not always apparent. For this reason, plant mutants have not been detected for many biochemical events. In addition, for many plant mutations, the modified gene responsible for the mutant phenotype has not been identified.

Antisense RNA can be used as an alternative strategy to create novel plant mutants. The major advantage of this technique is that it can be used to reduce expression of a specific, targeted gene. In this way, it is possible to study either the role of a gene whose function was previously unknown or the role of a protein whose gene has been identified.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; *Cab*, chlorophyll *a/b*-binding protein; CaMV, cauliflower mosaic virus; CAT, chloramphenicol acetyltransferase; CHS, chalcone synthase; DFR, dihydroflavanol reductase; DHFR, dihydrofolate reductase; GUS, β-glucuronidase; nos, nopaline synthase; PAL, phenylalanine ammonia lyase; PAT, phosphinotricin acetyl transferase; PE, pectin esterase; PG, polygalacturonase; PSII, photosystem II.

Biotechnology and Genetic Engineering Reviews — Vol. 9, December 1991
0264-8725/91/09/207-227 $20.00 + $0.00

The principle of antisense RNA

The antisense RNA strategy has been proven to be a powerful tool for the selective manipulation of gene expression in plants. The technique is based on the introduction into the cell of an RNA molecule that is complementary to the sequence of the mRNA of the target gene. This complementary RNA is known as 'antisense RNA'. Expression of the endogenous gene is inhibited in some way by the formation of a duplex between its mRNA and the antisense RNA. This blocks the availability of the mRNA for translation. The exact mechanism by which translation of mRNA is inhibited is unknown. However, duplex formation may lead to rapid RNA degradation, impaired nuclear processing, inhibition of export to the cytoplasm or obstructed translation. Experiments in plants that were designed to give an understanding of this mechanism are described in a later section.

Natural antisense RNA

Regulation of expression of specific genes by antisense RNA is a naturally occurring mechanism that was first recognized in bacteria (Simons and Kleckner, 1983; Mizuno, Chu and Inouye, 1984). The regulation of several different processes has been shown to involve short regions of antisense RNA controlling gene expression at various stages, including DNA transcription, RNA transcription and RNA translation (reviewed by Simons, 1988).

In higher eukaryotes, it has not been shown that antisense RNA is used as a natural system of gene regulation. In several cases, RNA molecules complementary to expressed genes have been identified. Small unpolyadenylated RNAs divergently transcribed from the mouse dihydrofolate reductase (DHFR) promoter with short regions of homology to the DHFR mRNA were described in 1985 (Farnham, Abrams and Schimke, 1985). Subsequently, adjacent convergent transcription units which overlap at their 3′ ends were identified in an unknown mouse gene (Williams and Fried 1986) and a *Drosophila* dopa decarboxylase gene (Spencer, Gietz and Hodgetts, 1986). In the rat gonadotrophin-releasing hormone gene, a second gene was identified which is transcribed from the other strand encoding an mRNA with significant homology (Adelman *et al.*, 1987). In addition, detailed analysis of the mouse c-*myc* gene has revealed antisense transcripts encompassing the entire gene (Nepveu and Marcu, 1986). In all these cases, the observation of the complementary RNA is reported but there is no proof of an *in vivo* regulatory role.

RNA complementary to α-amylase mRNA has been identified in barley (Rogers, 1988). These transcripts were complementary to essentially the full length of both the type A and type B α-amylase mRNAs. However, the homology was shown to be imperfect, which indicated that the antisense RNA was a transcript of a separate gene. The expression of both the α-amylase mRNA and the antisense RNA was developmentally regulated. The antisense RNA was found in developing endosperm/aleurone and mature aleurone but not in shoot or root tissue. Although the mRNA and antisense

RNA were present in equal amounts in abscisic-acid-treated aleurone tissue, there was no evidence to indicate that the antisense RNA regulated the expression of the amylase genes.

Inhibition of introduced genes

The concept of introducing synthetic antisense RNA genes into organisms to control gene expression was initially investigated in prokaryotic systems (reviewed by Green, Pines and Inouye, 1986). Subsequently, inhibition of gene expression was demonstrated in several eukaryotic cell cultures.

The first reported investigations of the antisense RNA technique in plant cells involved transient expression of both the antisense RNA and the target gene (Ecker and Davis, 1986). The expression of the bacterial chloramphenicol acetyltransferase (CAT) gene in protoplasts derived from carrot cell cultures was inhibited by co-electroporation with a CAT antisense gene. The level of inhibition was dependent on the level of expression of the antisense gene. Greater than 95% reduction of CAT activity was achieved with a 1:100 ratio of sense to antisense genes, both under the control of the nopaline synthase gene (*nos*) promoter. Approximately equal degrees of reduction were obtained with the antisense gene linked to the strong cauliflower mosaic virus 35S (CaMV 35S) gene promoter. However, fourfold less inhibition resulted from the use of the weaker carrot phenylalanine ammonia lyase (PAL) gene promoter in the antisense gene.

These experiments demonstrated the utility of antisense RNA for gene regulation in plant cells and opened the way for subsequent experiments in which the sense and antisense genes were stably incorporated into the plant genome (*Table 1*). The ability of plants to regenerate from single cells also enabled the first multicellular organisms expressing antisense RNA to be created. Rothstein *et al.* (1987) reported that a *nos* antisense RNA gene under the control of the CaMV 35S promoter was introduced into tobacco plants that had previously been transformed with a wild-type *nos* gene. An eight- to fiftyfold reduction in nopaline synthase activity was achieved in these plants, depending on the stage of development of the tissue analysed. This was accompanied by steady-state levels of *nos* mRNA decreased by a factor of approximately eight- to tenfold. The heritability of the antisense gene and phenotype was demonstrated by backcrossing the transformants with wild-type tobacco. The reduction in *nos* activity co-segregated with the antisense gene in the progeny. This demonstration of stable inheritance of the antisense RNA effect was crucially important for the potential application of the technique to commerical crops.

In similar experiments with transgenic tobacco, up to 85% reduction in expression of a wild-type *nos* gene in leaves was achieved with antisense genes linked to the promoter from a petunia chlorophyll *a/b*-binding protein gene (Sandler *et al.*, 1988). The practicality of the antisense strategy in reducing gene expression in doubly transformed plants has been confirmed with two further model systems.

The expression of the bacterial chloramphenicol acetyltransferase (CAT)

Table 1. Antisense experiments in transformed plants

	Species	Target gene	Promoter	Antisense gene	Comments	Reference
Transient assay	Carrot	CAT	CaMV 35S *nos* PAL	Complete	Large excess of antisense vector required	Ecker and Davies (1986)
Model systems	Tobacco	*nos*	CaMV 35S	860 bp 5′	Up to 98% reduction	Rothstein *et al.* (1987)
		nos	*Cab*	Various	3′ end most effective	Sandler *et al.* (1988)
		CAT	rbs	Complete	No inhibition	Delauney, Tabaeizadeh and Verma (1988)
			CaMV 35S	Complete 172 bp 5′	5′ end most effective	
		GUS	CaMV 35S	Complete	>90% reduction	Robert *et al.* (1989)
		GUS	*Cab*	41 bp around ATG	Up to 100% reduction	Cannon *et al.* (1990)
		PAT	CaMV 35S TR2	PAT-CAT	90% reduction, transcription and translation affected	Cornelissen and Vendewiele (1989)
Endogenous genes	Tobacco	*rbc*S	CaMV 35S	322 bp 5′	Up to 63% reduction in rbcS protein	Rodermel, Abbott and Bogorad (1988)
	Petunia	CHS	CaMV 35S CHS	Various	3′ end most effective	van der Krol *et al.* (1988, 1990a,b,c)
	Potato	10 kDa PSII polypeptide	CaMV 35S	Complete cDNA	99% reduction in protein	Stockhaus *et al.* (1990)
		Starch synthase	CaMV 35S	Complete cDNA	Up to 100% reduction in enzyme activity	Visser *et al.* (1991)
	Tomato	PG	CaMV 35S	730 bp 5′	99% reduction in PG activity	Smith *et al.* (1988, 1990a,b)
		PG	CaMV 35S	Complete cDNA	90% reduction in PG activity	Sheehy, Kramer and Hiatt (1988)
		pTOM 13	CaMV 35S	1100 bp 5′	Up to 97% reduction in ethylene production	Hamilton, Lycett and Grierson (1990)
		pTOM 5	CaMV 35S	780 bp 5′		Bird *et al.* (1991)

gene in primary tobacco transformants has been reduced by up to 99% (Delauney, Tabaeizadeh and Verma, 1988) and 95% (Kanevskii and Nod, 1990). The β-glucuronidase (GUS) gene from *Escherichia coli* (Jefferson, Burgess and Hirsh, 1986) encodes an easily assayable enzyme that is widely used to study gene expression in transgenic plants. The antisense RNA technique has been used in doubly transformed plants to inhibit GUS activity by greater than 90% (Robert *et al.*, 1989) and 100% (Cannon *et al.*, 1990).

In all cases, there was considerable variation in the level of inhibition of expression between the individual transformants. The maximum level of inhibition was observed in only a few individual plants. In many transformants no reduction in marker enzyme activity was observed. This lack of correlation between transformation with the antisense RNA gene and inhibition of target gene probably represents variation in expression levels of the antisense gene. Such diversity of expression is observed commonly in transformed plants.

Co-segregation of the antisense RNA genes with the modified target gene expression provides confirmation that the inserted gene is responsible for the change of phenotype. This was demonstrated by analysis of backcross progeny in which CAT and anti-CAT genes were segregated (Cheon, Delauney and Verma, 1990). Progeny containing the CAT gene but lacking the antisense gene showed fully restored CAT activity. Efficient inactivation of the CAT gene was restored by crossing with plants expressing antisense RNA. The stability of expression of the antisense gene through two generations and the ability to introduce antisense-mediated gene inhibition through sexual crossing are important for the use of antisense RNA in commercial breeding programmes.

The commercial importance of herbicide-resistant plants has been investigated in one instance by the integration of a bialophos resistance (*bar*) gene which encodes phosphinotricin acetyl transferase (PAT) (Murakami *et al.*, 1986). After a second cycle of transformation to introduce a *bar* antisense gene, PAT activity was reduced by more than 92% (Cornelissen and Vanderwiele, 1989). It is not reported in this study whether bialophos resistance is abolished in these plants. The expression of a GUS marker gene that was linked to the *bar* gene was not inhibited. The ability to couple and uncouple the expression of linked genes by sexual crossing has several applications in breeding programmes. This may be especially valuable if the uncoupling can be achieved in specific tissues or at specific developmental stages.

Inhibition of endogenous genes

In 1988, reports of the inhibition of three endogenous plant genes by transgenic antisense RNA were published (Rodermel, Abbott and Bogorad, 1988; Sheehy, Kramer and Hiatt, 1988; Smith *et al.*, 1988; van der Krol *et al.*, 1988). The inhibition of other endogenous genes has been reported subsequently. Inevitably, these studies have involved genes involved in a wide range of biochemical and physiological processes in a variety of tissue types.

The following examples illustrate several different applications of the technique. They have demonstrated the value of the antisense RNA technique in increasing the understanding of the role of gene products in plant processes. However, it will also be clear that antisense RNA has to be used in conjunction with other techniques.

FLOWER PIGMENT GENES

Van der Krol *et al.* (1988) described the use of antisense RNA to produce modified flower pigmentation patterns in petunia and tobacco. The flavonoid pathway, which is primarily responsible for the production of floral pigments in petunia, has been widely studied. Chalcone synthase (CHS) is a key enzyme in this pathway and was chosen as the target of the first antisense RNA experiments. An antisense RNA vector was constructed in which the complete coding region and the 3′ untranslated region from a cDNA was incorporated in inverted orientation under the control of the strong, constitutive CaMV 35S promoter.

Petunia VR hybrid plants transformed with this vector showed variations of the normal pattern of uniformly pigmented corollas, tubes and anthers. Three classes of pigmentation were observed: class 1 were indistinguishable from wild-type flowers; class 2 had reduced pigmentation in the corollas, often with sectorized coloured and white patterns but normal coloured tubes; class 3 plants had colourless tubes and predominantly white corollas, often with pigmented fringes. Flavonoid pigments were shown to be absent in the white areas. However, pigmentation could be restored by feeding with naringenin-chalcone, the product of the CHS enzyme.

CHS protein and steady-state mRNA levels in the different coloured regions of the class 2 and class 3 flowers correlated with the level of pigmentation. However, there was no correlation between the level of antisense RNA in the leaves and either the number of antisense genes or the observed pigmentation pattern.

The heritability of the antisense effect was shown by backcrossing several of the transformants to the hybrid parents. Complete co-segregation of the phenotype with the antisense gene was observed.

The petunia CHS antisense vector was also effective in inhibiting flower colour in transformed tobacco, resulting in similar classes of pigmentation. The sequence divergence of CHS genes from these two species has been estimated to be 20%. This indicated that antisense RNA can be effective across species boundaries. Thus it may not be necessary to isolate genes or cDNAs from target species in order to construct antisense RNA genes. However, the lack of complete sequence specificity implies that it may not be possible to use antisense RNA to target specific genes in a multi-gene family.

Similar novel flower pigmentation patterns were observed when a CHS antisense gene under the control of the homologous CHS promoter was introduced into petunia (van der Krol *et al.*, 1990b). However, a lower proportion of the transformants (CHS promoter, 2 out of 20; CaMV

promoter, 13 out of 25) showed any effect on flower pattern. This difference may reflect the expression levels of the two promoters.

In the initial experiments, there was no clear explanation for the variety of pigmentation patterns. However, it was postulated that they must result from different expression patterns of the antisense gene (van der Krol *et al.*, 1988). This was confirmed by investigations of CHS mRNA steady-state levels in the flower, which showed a good correlation with flower pigmentation (van der Krol *et al.*, 1990a). In addition, the mRNAs for both the CHS-A and CHS-J genes (which are normally transcribed in flower tissues) were equally suppressed in white corolla tissue. The mRNA levels of two other flavonoid pathway enzymes, dihydroflavanol reductase and chalcone flavanone isomerase were not reduced.

The different flower pigmentation patterns were apparently not related to the site of insertion of the antisense genes at the chromosomal level. However, this did not preclude the possibility that the position of the antisense genes relative to other sequences was responsible for differential expression. Earlier studies had shown no correlation between leaf antisense RNA levels and flower pigmentation (van der Krol *et al.*, 1988). Only very low levels of antisense RNA could be detected in floral tissue (van der Krol *et al.*, 1990a). Again no correlation was observed with flower phenotype.

Flower patterns on individual plants were usually maintained throughout the plant. However, variable pigmentation patterns were observed on several plants. This indicated that physiological factors may be primarily responsible for the different flower phenotypes. This hypothesis was supported by the observations that spraying antisense plants with gibberellic acid increased pigmentation whereas spraying the growth retardant, B9, decreased pigmentation. Similar effects were observed with a natural petunia CHS mutant. In addition, pigmentation patterns in the antisense plants were affected by variations in light levels. CHS mRNA levels in the floral tissue were not measured after these treatments, thus it is not clear whether the pigment changes resulted from changes in efficiency of the antisense gene. It was also not clear why different transformants responded in different ways, although this may be due to the site of integration influencing the spatial expression of the antisense gene. Further experiments with antisense RNA to other flavonoid biosynthesis genes should help to elucidate further the complex genetic, physiological and environmental control of flower pigmentation in petunia.

GENES INVOLVED IN PHOTOSYNTHESIS

The assembly of the photosynthetic apparatus of green plants involves a complex interaction of many proteins that are encoded on both nuclear and chloroplast genomes. Many natural mutants of photosynthesis genes have been identified. Modified chlorophyll pigmentation patterns have often indicated photosynthetic mutations, although in many cases the mutated gene has not been identified. The use of antisense RNA to inhibit the production

of proteins involved in photosynthesis was one of the first applications of the technique in plants.

Rodermel, Abbott and Bogorad (1988) used antisense RNA to study the regulation of the assembly of the large and small subunits of ribulose bisphosphate carboxylase (*rbc*). Tobacco plants (*Nicotiana tabacum*) were transformed with an antisense RNA vector that consisted of an almost full length *rbc* small-subunit (*rbc*S) cDNA from *Nicotiana sylvestris* cloned in antisense orientation under the control of the CaMV 35S promoter. The *rbc*S genes from the two tobacco species are more than 99·7% homologous.

Five independent transformants were selected for further study. Four of these were identified as having single gene inserts. In these plants *rbc*S mRNA and protein levels were reduced by a maximum of 75% and 51% of that in controls, respectively. The fifth transformant contained at least four inserts, mRNA and protein levels were reduced by 88% and 63%, respectively. Only extremely low levels of antisense RNA could be detected.

The reductions in *rbc*S mRNA levels had little effect on the levels of the chloroplast-encoded *rbc* large-subunit mRNA. However, the levels of both *rbc*S and *rbc*L polypeptides were equally depressed by up to 60%. Thus the normal stoichiometry of pool size was maintained in the antisense plants. The accumulation of holoenzyme appears to be related to the *rbc*S mRNA levels rather than the *rbc*L mRNA levels. In addition, the accumulation of *rbc*L protein appears to be regulated by translational and post-translational factors that are influenced by the expression of the *rbc*S genes.

The reduction in *rbc* protein by antisense RNA has an effect on the growth rate of the plants. Self-fertilized progeny with the largest number of antisense genes were severely stunted. There was an apparent correlation between growth rates and *rbc*S mRNA levels.

In a further study of a photosynthesis gene using antisense RNA, Stockhaus *et al.* (1990) have inhibited the formation of the 10 kDa polypeptide associated with the water-splitting apparatus of photosystem II (PSII) in transgenic potato plants. A full-length cDNA was used to construct an antisense gene under the control of the CaMV 35S promoter. In 3 of the 36 transformants tested, the amount of the 10 kDa protein was reduced to 1–3% of that in wild-type controls. The level of 10 kDa protein was closely related to the steady-state levels of mRNA. However, there was no obvious correlation between the amount of antisense RNA and the suppression of the 10 kDa transcript levels. The inhibition of expression of the 10 kDa protein gene did not affect transcript levels of other nuclear genes encoding chloroplast-located proteins. In addition, the steady-state levels of other polypeptides that are components of the photosystem II oxygen-evolving complex were not reduced.

The reduction of 10 kDa protein to less than 1% of normal did not result in any morphological differences in the plants. In addition, there was no effect on the ultrastructure of the chloroplasts. However, electrochemical analysis revealed changes in oxidation rates in PSII and reduced photosynthetic efficiency. This study has confirmed that the 10 kDa protein has a role in PSII. Further work will, however, be required to clarify its function in

relation to other PSII polypeptides. The use of antisense RNA targeted against other PSII proteins has considerable potential for functional analysis of the complex. However, on its own, the technique will not give a complete understanding of function.

FRUIT-RIPENING GENES

The biochemical and physical changes that occur during fruit ripening require co-ordinated modifications in the expression of many genes. The antisense RNA technique has been used to create novel gene-specific mutants for the study of their role in tomato fruit-ripening. In addition to aiding the understanding of the ripening process, these modified plants have been shown to have properties of considerable commerical importance (Schuch *et al.*, 1991).

The major tomato cell wall enzyme, polygalacturonase (PG), has been considered to have a major role in fruit-softening (Hobson, 1964, 1965; Brady *et al.*, 1982). It is primarily responsible for changes to the pectin fraction of the cell wall during ripening (Crookes and Grierson, 1983). Plants with reduced levels of PG activity due to the expression of antisense RNA (Sheehy, Kramer and Hiatt, 1988; Smith *et al.*, 1988) have helped to clarify the role of the enzyme (Smith *et al.*, 1990a).

PG antisense genes were constructed using a 730 bp fragment from the 5′ end of an almost full-length cDNA (Smith *et al.*, 1988) and a 1·6 kb cDNA fragment containing the entire PG open reading frame (Sheehy, Kramer and Hiatt, 1988). These fragments were fused in antisense orientation under the control of the CAMV 35S promoter with either the 3′ end of the *nos* gene (Smith *et al.*, 1988) or the transcript 7 3′ termination region (Sheehy, Kramer and Hiatt, 1988). The constructs were integrated into the tomato genome by *Agrobacterium*-mediated transformation. In both cases, transformed plants were shown to have reduced levels of PG mRNA in ripening fruit. Smith *et al.* (1988) identified a plant with only 6% of the PG mRNA that would normally be present in orange fruit from an untransformed plant. Fruit from this plant were shown to have only 10% of the normal PG activity, which was correlated with a reduction in PG protein. Sheehy, Kramer and Hiatt (1988) identified a plant with approximately 10% of the PG mRNA and 20% of the PG activity in control fruit.

Both groups showed that there was considerable variation in the level of reduction of PG activity in individual transformants. In neither case could a relationship be established between the level of PG antisense RNA in green tissue and the degree of inhibition of PG activity. Detectable antisense RNA in transformed fruit decreased as ripening progressed and was always less than the level of PG mRNA (Sheehy, Kramer and Hiatt, 1988; Smith *et al.*, 1988). However, analysis of transcription rates in isolated nuclei indicated that the antisense RNA gene was transcribed at a higher rate than the tomato PG gene (Sheehy, Kramer and Hiatt, 1988). Transcription of the endogenous PG gene did not appear to be inhibited by PG antisense RNA.

The reduction in PG activity segregated with the PG antisense gene in

self-fertilized progeny of a transformed plant with 10% of normal PG activity (Smith *et al.*, 1991a). This plant had previously been shown to have an antisense gene inserted at a single locus. Plants homozygous for the antisense gene had less than 1% of normal PG activity, whereas heterozygous plants had approximately 20% and azygous plants had normal PG activity. Self-fertilized progeny of a homozygous plant also had 1% PG activity (Smith *et al.*, 1990a), whereas progeny from a backcross to the wild-type parent had approximately 20% PG activity (Bird *et al.*, unpublished). Thus the phenotype of the PG antisense gene was stably inherited through at least two generations.

The reduction in accumulation of PG activity during ripening did not affect other ripening-related processes, such as ethylene production, lycopene accumulation, invertase activity and pectin esterase activity (Smith *et al.*, 1990a). However, the changes in pectin that normally accompany ripening were modified. The levels of soluble pectin were unaltered, but the weight-average M_r of soluble pectin was significantly higher in ripe fruit from the antisense plants. This clearly indicated that PG was involved in the depolymerization of soluble pectin, but did not apparently have a role in the solubilization of pectin that normally occurs during ripening.

Reductions of PG activity to less than 1% of that in normal fruit did not result in any visual differences in the phenotype of the ripening fruit. In addition, analysis of large numbers of fruit indicated that there was no significant difference in the firmness of fresh or stored fruit (Schuch *et al.*, 1991). However, the PG antisense fruit were significantly less susceptible to both cracking and infection in storage and damage during transport. The modified fruit have considerable potential for reduced post-harvest losses. The reduction in PG activity also gives the fruit enhanced processing characteristics.

Antisense RNA has also been used to inhibit the expression of the gene for pectin esterase (PE), another tomato cell wall enzyme (Hall *et al.*, paper in preparation). PE catalyses the de-methylation of pectin; however, the physiological role of this activity is not understood. Several transgenic plants with reduced PE activity have been identified. These plants are being analysed for the biochemical and physiological changes that result from low PE activity. These studies should shed light on the role of the isoenzymes of PE in cell wall metabolism.

In a further study using antisense RNA to understand fruit ripening, Hamilton, Lycett and Grierson (1990) have inhibited the expression of genes related to the ripening-enhanced cDNA known as pTOM13 (Slater *et al.*, 1985). pTOM13 is the product of a small family of genes (Holdsworth, Schuch and Grierson, 1988) that show differential expression during fruit ripening as well as after wounding of fruit and leaves (Smith, Slater and Grierson, 1986; Holdsworth, Schuch and Grierson, 1988). Increases in steady-state levels of pTOM13 RNA were closely associated with enhanced ethylene biosynthesis. Transgenic tomato plants expressing pTOM13 antisense RNA under the control of the CaMV 35S promoter had significantly reduced levels of ethylene biosynthesis in ripening fruit and wounded tissues (Hamilton, Lycett

and Grierson, 1990). In plants homozygous for the pTOM13 antisense gene, ethylene biosynthesis was reduced by up to 97% in ripening fruit and to 90% in wounded leaves. This was paralleled by reductions in pTOM13 homologous mRNA. However, the effect on expression of individual members of the multigene family has not yet been reported.

Biochemical analysis of the ethylene biosynthesis pathway indicated that the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase was drastically reduced. Limited homology exists between the protein encoded by pTOM13 and several oxidases that catalyse potentially similar reactions. However, further analysis is required for confirmation that reduced ACC oxidase activity is the primary effect of pTOM13 antisense RNA.

The pTOM13 antisense fruit do not develop red colour as rapidly as normal ripening fruit. In addition, preliminary experiments indicate that the fruit show delayed deterioration during storage. Although several natural tomato mutants have reduced ethylene biosynthesis and do not ripen normally, it is not clear which genes are mutated. The availability of novel mutants in which ethylene biosynthesis is specifically inhibited should enhance the understanding of the role of ethylene in controlling gene expression during ripening.

Further novel mutants in which fruit colouration has been inhibited have been generated by the expression of antisense RNA to another ripening enhanced cDNA, pTOM5 (Bird *et al.*, 1991). Fruit from these plants turn yellow during ripening and have considerable reductions in carotenoid levels. In fruit from some plants, lycopene, which is primarily responsible for the red pigmentation in normal fruit, is undetectable. The identification of the role of the pTOM5 gene in carotenoid biosynthesis had not been predicted prior to the antisense RNA experiments. This demonstrates the potential of the technique for the identification of the role in plant development of genes of unknown function.

INHIBITION OF OTHER PLANT GENES

Antisense RNA has been used to inhibit the expression of a granule-bound starch synthase gene in potato (Visser *et al.*, 1991). Significant reductions in enzyme activity (70–100%) were reported which resulted in a reduction in the amylose content of starch in the tubers.

Reports of the use of antisense RNA to probe the function of genes and their products in plants is likely to increase in the near future. Recent conference proceedings have reported several experiments with antisense RNA targeted against a wide range of genes: calmodulin (Wang *et al.*, 1990), vacuolar ATPase (Gogarten *et al.*, 1990) and peroxidase (Rothstein, Rice and Lagrimini, 1990).

Inhibition of viruses

The development of plants that are resistant to pests and diseases is one of the major commercial applications of the introduction of foreign genes into plants. Virus resistance is particularly important since agrochemicals are

often not very effective against viruses. Chemical protection against viruses often targets the vector that spreads the virus rather than the virus. Several strategies have been used to introduce virus resistance into plants. The first successful mechanism was the expression of the viral coat protein gene (Powell-Abel *et al.*, 1986) which has achieved very high levels of protection.

Two papers from Monsanto in 1988 reported the use of viral coat-protein antisense RNA to give resistance against two RNA viruses (Cuozzo *et al.*, 1988 and Hemenway *et al.*, 1988). Cuozzo *et al.* (1988) produced transgenic tobacco plants that expressed antisense RNA to cucumber mosaic virus (CMV) coat protein. Hemenway *et al.* (1988) expressed potato virus X coat protein antisense RNA in transgenic tobacco. Both papers reported resistance to low inoculum concentrations of the specific virus. However, in each case the antisense RNA strategy was found to be considerably less effective than expression of the coat protein. A similar result was obtained for tobacco plants expressing tobacco mosaic virus antisense RNA (Powell *et al.*, 1989). In this case, plants expressing antisense RNA to the coat protein coding region and the 3′ untranslated region were protected from infection by TMV at low levels of inoculum. However, plants expressing antisense RNA to the coat protein coding sequence alone were not protected from infection. The 3′ untranslated region is thought to be a replicase binding site. Interference with this site by the antisense RNA may be responsible for the protection.

Antisense RNA to cucumber mosaic virus RNA was also expressed in transgenic tobacco by Rezaian, Skene and Ellis, (1988). This group used three antisense genes constructed from different parts of the CMV genome. None of these included coat protein sequence. Only plants from one line transformed with one construct showed resistance to relatively low inoculums of CMV. Other plants transformed with the same construct showed no resistance. The authors concluded that resistance would not be correlated with the expression of antisense RNA.

It is not clear why antisense RNA, which has been so successful for inhibiting plant genes, has not given greater levels of protection against viruses. All of the viruses tested with this system have been RNA viruses and in most cases the coat protein gene has been included as a target for the antisense RNA. Evidence on the mechanism of antisense RNA inhibition of nuclear genes indicates that RNA interactions in the nucleus are important. Since RNA viruses are translated in the cytoplasm, this may not be the optimal site for inhibition by antisense RNA.

Mode of action of antisense RNA

The underlying hypothesis of antisense experiments is the formation of duplexes between sense and antisense strands which interferes with gene expression. The formation of RNA duplexes could interfere with several events in the expression pathway: transcription and processing within the nucleus; transcript transport to the cytoplasm; translation. The experiments with antisense RNA in plants have not yet resolved the mode or site of action.

It is perhaps likely that antisense RNA will interfere with all stages to a greater or lesser extent.

The first demonstration of the regulation of gene expression by antisense RNA in plant cells used co-electroporation of the target and the antisense genes (Ecker and Davis, 1986). These experiments required 50–100-fold excess of the antisense gene. Similar excess of antisense genes has not been found to be essential when both genes are incorporated into the host plant genome. This implies that the mode of action of antisense RNA in the co-electroporation experiments was not identical to that of subsequent experiments.

A decrease in the steady-state level of target gene mRNA has been reported in all experiments where antisense RNA has successfully down-regulated gene expression. This has been accompanied in many cases by a reduction in the steady-state levels of antisense RNA. For example, Smith *et al.* (1988) showed that the level of PG mRNA was reduced to 6% of normal in ripe fruit of transformed plants with 10% of normal enzyme activity. The level of detectable antisense RNA in the ripe fruit was reduced to approximately 30% of that found in green fruit. These results may indicate that production or export of RNA from the nucleus have greater roles than inhibition of translation. However, the low levels of sense and antisense RNA could indicate that double-stranded RNA is more susceptible to degradation.

The steady-state levels of antisense RNA expressed with constitutive promoters in tissues in which the target gene is not normally expressed do not, in general, correlate with the extent of modification of the phenotype. Van der Krol *et al.* (1988) showed that independent transgenic plants with the same levels of antisense CHS RNA in leaf tissue had different flower pigmentation patterns. In addition, plants with very low levels of antisense CHS RNA had pronounced changes in floral pigmentation. Stockhaus *et al.* (1990) could not demonstrate any correlation between the levels of antisense RNA to the 10 kDa PSII protein in roots and the reduction of protein in the leaf.

Although these studies of steady-state RNA levels demonstrate that there is no requirement for excess antisense RNA over sense RNA, they cannot identify the primary mode of action. Sheehy, Kramer and Hiatt (1988) used nuclear run-off transcription experiments to study the rates of transcription of sense and antisense PG RNA. Although they demonstrated that the level of transcription of PG mRNA was much lower than that of the antisense RNA, the results indicated that the rate of transcription of the PG gene was similar in both transgenic and control fruit. These results suggest a mechanism exerted at the post-transcriptional level.

The hypothesis that antisense RNA can inhibit more than one point of the expression pathway is supported by experiments on the antisense control of a bialophos-resistance gene (Cornelissen, 1989; Cornelissen and Vanderwiele, 1989). A thirteenfold reduction in phosphinotricin acetyl-transferase synthesis was not matched by the fourfold reduction in *bar* mRNA. The reduced synthesis of PAT per *bar* mRNA may be due to either nuclear or cytoplasmic events. Studies of the levels of *bar* mRNA in nuclear and cytoplasmic RNA

preparations indicated that although the total level of *bar* mRNA was reduced, the residual was almost entirely located in the cytoplasm. This implied that cytoplasmic translation of mRNA was inhibited by antisense RNA. In further experiments, the half-life of cytoplasmic *bar* mRNA in the presence of transcriptional inhibitors was found to be independent of the presence of antisense RNA. The reduction of extractable *bar* mRNA by antisense RNA was thus likely to be the result of events not occurring in the cytoplasm. This suggested that the inhibition of translation of the *bar* mRNA and the reduction of *bar* mRNA steady-state levels take place in different cellular compartments and are thus independent events.

The investigations that have currently been reported do not yet give a full understanding of the mechanism of action of antisense RNA. However, it seems likely that inhibition of post-transcriptional nuclear events and/or transport to the cytoplasm are responsible for the reduced steady-state levels of sense and antisense RNA. It is not clear whether this is a result of either increased degradation of double-stranded RNA or other factors. Interactions between residual sense and antisense RNA in the cytoplasm may have a compounding effect on the reduction of mRNA. The relative contributions of these events have not been determined and may vary for individual target gene–antisense interactions.

Optimization of gene regulation by antisense RNA

For both experimental and commercial applications, the optimization of antisense inhibition of gene expression may be required. Antisense RNA is akin to a leaky mutation and may not be able to abolish completely the expression of the target gene. However, in several cases the level of expression has been reduced to less than the minimum sensitivity of the assay (e.g. Cannon *et al.*, 1990). In some situations, maximum inhibition may not be required and it may even be lethal for some genes. Several factors that affect the efficiency of gene inhibition have been investigated.

The origin of the DNA for antisense RNA production has usually been a cDNA for the endogenous target gene. However, no clear indications have emerged on the optimal region of the cDNA to be used. In many cases, the incorporation of an almost full-length cDNA in antisense orientation has given efficient inhibition (e.g. Sheehy, Kramer and Hiatt, 1988; van der Krol *et al.*, 1988). Other groups have successfully used fragments from the 5′ end of the cDNA (e.g. Rodermel, Abbott and Bogorad, 1988; Smith *et al.*, 1988). Cannon *et al.* (1990) reported 100% inhibition of GUS activity using antisense RNA with only a 41-base homology spanning the translation start codon. Antisense RNA to the translation start codon is not required for the inhibition of all genes. Flower pigmentation was modified by antisense CHS genes encoding half- or quarter-length RNA complementary to the 3′ end of the CHS mRNA (van der Krol *et al.*, 1990b). In this case a construct containing the 5′ half of the cDNA proved ineffective.

Most reports of the expression of antisense RNA in transformed plants have shown a range of effectiveness in individual transformants. For example,

a small population of transgenic plants containing a GUS antisense gene showed a range of GUS inhibition from 0 to 100% (Cannon *et al.*, 1990). The specific levels of inhibition were stably inherited over two generations. It is likely that the site of insertion of the antisense gene into the host plant genome has a significant effect on the expression of the gene. The range of expression levels that can be achieved due to this position effect will provide the opportunity to select plants with the desired phenotype.

Antisense RNA gene dosage has been shown to affect the level of inhibition of the target gene. Smith *et al.* (1990a) demonstrated a range of PG activities in self-fertilized progeny of an individual transformant with 10% of normal activity. Progeny heterozygous for the antisense gene had approximately 20% activity whereas homozygous progeny had about 1% activity. Similar enhancements of the antisense effect have been observed in homozygous progeny of tomato plants containing antisense pTOM13 genes (Hamilton, Lycett and Grierson, 1990) and tobacco plants with antisense GUS genes (Cannon *et al.*, 1990). Gene dosage effects have not been clearly identified for primary transformants containing more than one antisense gene, presumably due to differential position effects at individual sites of insertion.

The 'design' of transformed plants for a specific function may require the inhibition of gene expression to be confined to defined organs or cell types. For this purpose the antisense technique has an advantage over specific gene mutations since the antisense RNA can be expressed in the required cell type. Cannon *et al.* (1990) demonstrated leaf-specific inhibition of a constitutively expressed GUS gene. The light-regulated *Cab* promoter from *Arabidopsis thaliana* was used to control expression of the GUS antisense gene in *Nicotiana*. In some transformants, GUS activity was inhibited in the leaves but not significantly in roots.

The use of inducible promoters in antisense experiments also increases the flexibility of the technique. Robert *et al.* (1989) showed that it was possible to inducibly restore GUS activity in plants in which antisense RNA had previously been used to inhibit activity. As well as constitutively expressed sense and antisense GUS genes, these plants had additional copies of the GUS gene under the control of the *Drosophila melanogaster hsp70* heat inducible promoter.

Further flexibility of the antisense technique was demonstrated by the inhibition of CAT gene expression by a bifunctional transcript that contained the coding region of a hygromycin-resistance gene as well as CAT antisense sequences (Delauney, Tabaeizadeh and Verma, 1988). The ability to fuse antisense sequences to other genes could have significant advantages in reducing the complexity of insertions in transgenic plants.

Inhibition of gene expression by sense genes

Recently, reports of the down-regulation of endogenous plant gene expression by sense-RNA transcripts have appeared in the literature. In most cases, these surprising results came from experiments designed to increase gene expression by increasing the gene copy number.

Full-length dihydroflavanol reductase (DFR) and CHS sense-gene constructs were introduced into petunia to evaluate the effect of increased gene copy number on flower colour (van der Krol *et al.*, 1990c). CHS sense genes were constructed using either the complete gene and various lengths of 5′ and 3′ gene-flanking region or a full-length cDNA driven by the CaMV 35S promoter. The DFR construct used a full-length cDNA linked to the CaMV 35S promoter. In the majority of plants transformed with DFR or CHS sense genes, an increase in the level of the respective mRNA was observed. However, no effect on flower colour was seen in these transformants. Unexpectedly, in 25% of the transformants flower colour was affected in a manner not unlike the effects seen in plants transformed with antisense CHS constructs (van der Krol *et al.*, 1988).

Corollas of plants transformed with either DFR or CHS genes had white sectors where anthocyanins and flavonols could not be detected indicating blockage of the flavonoid pathway. The reduced flower pigmentation was shown to be accompanied by a specific reduction in the levels of mRNA derived from both the endogenous and the introduced gene in colourless tissue. In the case of CHS, the effect was independent of the promoter used to drive expression of the sense transgene. Increased light levels used during the growth of plants with altered flower colouration further changed the pattern of pigmentation.

A CHS chimeric gene consisting of the CaMV 35S promoter and a full-length coding region was used in a similar over-expression study (Napoli, Lemieux and Jorgensen, 1990) using three varieties of petunia. All varieties, when transformed with the sense gene construct, gave individuals with white or sectored flowers. The reduced flower colour phenotype was shown to be heritable and associated with the transforming DNA. In one transformant with white flowers, the level of steady-state mRNA for the endogenous CHS gene was shown to be fiftyfold lower than that in conrol plants at each stage of flower development. The level of mRNA derived from the introduced gene remained relatively constant.

Some transformants gave rise to branches which regained the natural violet colour. Analysis of the mRNA from these revertant flowers showed that the levels of mRNA for both endogenous and introduced CHS genes were thirty- to fiftyfold higher than the level in white flowers from the original transformant.

Flower pigmentation in tobacco has also been affected by the introduction of a chimeric sense-gene construct (Elkind *et al.*, 1990). A bean phenylalanine ammonia lyase (PAL) gene under the control of the wild-type promoter plus CaMV 35S enhancer elements, was introduced into tobacco plants. Three classes of phenotypes were observed: class I had no visible phenotypic lesions, class II had altered flowers and class III had altered flower colouration plus further vegetative abnormalities. PAL activity in leaves and flowers of class III transformants was much lower than that observed in wild-type control tissue. The additive effect of two copies of the heterologous sense transgene on PAL activity was demonstrated in homozygous progeny from one self-fertilized transformant. The level of tobacco PAL mRNAs in leaf

tissue of class III transformants was much lower than in control tissue. Accumulation of phenylpropanoids in leaf tissue was also associated with low PAL activity in class III transformants. As in the CHS system described above (van der Krol *et al.*, 1990c), expression of the phenotype was affected by environmental factors, increased temperatures and light intensities stimulated more pronounced versions of the phenotypic lesions.

The incorporation of a full-length coding region into the sense gene is not essential for efficient down-regulation of the endogenous gene. Expression of a partial copy of the PG gene has been shown to inhibit the expression of PG in ripening tomato fruit (Smith *et al.*, 1990b). A chimeric partial sense gene was constructed using the CaMV 35S promoter and the first 730 bp of the cDNA coding for PG. In fruit from transformants shown to express the sense transgene at the unripe stage, the level of PG mRNA was greatly reduced in ripe fruit when compared to the level observed in untransformed ripe fruit. The level of mRNA derived from the transgene was also reduced in ripe fruit. There were similarities in the size of low molecular weight mRNAs related to PG mRNA in ripe fruit from plants transformed with either a sense transgene or an antisense transgene. This may imply that site-specific cleavage of aberrant mRNAs is part of a mechanism common to down-regulation of endogenous plant genes by either antisense RNA or sense RNA.

A mechanism for the inhibition of gene expression by sense RNA has been postulated by Cameron and Jennings (1991). They used short sense and antisense RNAs homologous to the 5′ end of CAT in a monkey kidney cell transient expression system to down-regulate a previously introduced CAT gene. It is possible that introduced sense RNA forms an RNA:RNA hybrid with the endogenous message by intermolecular base-pairing in an 'antisense manner'. Stem loop regions where secondary structure may be expected to be important for stability of the mRNA are possible targets for short regions of sense RNA to base pair to the endogenous message. Although such binding may not be particularly strong, a destabilized mRNA of this type may become more susceptible to breakdown.

The insertion of additional genes homologous to endogenous genes does not always inhibit expression. Lagrimini, Bradford and Rothstein (1990) successfully expressed a peroxidase cDNA in transgenic tobacco. This resulted in enhanced levels of peroxidase activity in the plants.

Thus in some transgenic plants where additional copies or partial copies of endogenous genes have been introduced, the level of expression of the endogenous gene has been unexpectedly down-regulated. The mechanism for inhibition of expression may be related to the modification of gene expression by antisense RNA. A mechanism for the destabilizing effect of the presence of sense RNA on the level of endogenous target mRNA may be indicated in Cameron and Jennings (1991). However, the elucidation of the process whereby destabilized sense-RNA:target-mRNA hybrids are formed and subsequently broken down poses technical problems.

Future developments

In the past few years, the reports appearing in the literature have clearly demonstrated the ability of antisense RNA to reduce expression of specific target genes in transformed plants. The inhibition of endogenous plant genes in several of these studies has proved to be a useful tool for the investigation of the role of the gene and its product in plant metabolism. The ability to generate novel mutants in which a target gene is specifically inhibited will become increasingly important in biochemical and physiological studies.

The most exciting developments to stem from the use of antisense RNA will be modifications to the phenotype of plants to give improved agricultural and industrial products. Several commercial organizations are investigating these opportunities. Products may include tomatoes with improved processing characteristics, flowers with modified colour patterns or caffeine-free coffee beans.

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