Euphytica **85:** 193–202, 1995.

The manipulation and modification of tomato fruit ripening by expression of antisense RNA in transgenic plants

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Key words: carotenoids, ethylene, gene expression, *Lycopersicon esculentum* Mill., polygalacturonase, pectinesterase, phytoene synthase, ACC oxidase

Summary

The common cultivated tomato (*Lycopersicon esculentum* Mill.) provides a major focus for improvement of crop quality through genetic engineering. Identification of ripening-related cDNAs has enabled the modification of specific aspects of ripening by manipulating gene expression in transgenic plants. By utilizing 'antisense RNA' to modify expression of ripening genes, we have inhibited the production of the cell wall – metabolising enzymes polygalacturonase and pectinesterase and created transgenic plants that contain, effectively, single, targeted mutations affecting these genes. Furthermore, this approach has been used with previously unidentified cDNA clones to enable both functional identification and manipulation of genes involved in ethylene production (ACC oxidase) and carotenoid biosynthesis (phytoene synthase). The use of antisense RNA targeted to specific genes to alter ripening phenotypes and improve commercial utility of fruit by affecting shelf-life, processing characteristics and nutritional content is discussed.

We have used the extreme ripening-impaired mutant, *ripening inhibitor (rin)* to identify additional genes implicated in the ripening process. This approach has resulted in the cloning of several novel ripening-related mRNAs which are now being studied by antisense experiments. This may enable identification and manipulation of additional genes involved in processes such as softening, flavour and aroma generation and susceptibility to pathogens.

Abbreviations: ACC – 1-aminocyclopropane-1-carboxylic acid, PE – pectinesterase, PG – polygalacturonase, SAM – S-adenosyl methionine, SARs – scaffold attachment regions

Introduction

One major objective over the past few years has been to identify and clone plant genes and then modify their expression in transgenic plants. Tomato has proved particularly amenable to such molecular genetic studies for the following reasons. It has a relatively small genome, an extensive and ever-more detailed genetic map and a number of well-characterised ripening mutants that have been incorporated into isogenic backgrounds. Most importantly, tomato is readily amenable to *Agrobacterium*-mediated transformation, thus allowing the stable introduction of transgenes into explants and subsequent regeneration of transgenic plants that will yield genetically modified fruit (see review by Gray et al., 1992).

When tomato fruit ripens, it undergoes dramatic changes that lead to the production of a subtle blend of flavours, aroma and texture that make the mature fruit attractive to potential consumers, thereby aiding seed dispersal. These changes involve all cellular compartments and are brought about by altered expression of

specific genes. These 'ripening-related' genes encode a range of enzymes thought to be required to bring about the changes leading to ripe fruit. Tomato, being a climacteric fruit, shows a dramatic increase in respiration at the onset of ripening, accompanied by increased synthesis of the phytohormone, ethylene. Production of ethylene appears to be involved in the initiation, modulation and co-ordination of expression of many of the genes required for the ripening process (Picton et al., 1994). Clearly, in order to be able to manipulate genetically the ripening process, it is essential to identify and clone as many ripening mRNAs as possible. To date, over 25 such genes have been cloned. Although several of these have been assigned precise functions, the identity of many others remains to be established (Gray et al., 1992, 1994; Grierson & Schuch, 1993).

The production, growth and analysis of transgenic tomato plants expressing an 'antisense RNA', leading to a reduction in the level of expression of the endogenous gene, has allowed critical examination of the role of specific gene products in the ripening process. In addition, these experiments have led to the creation of genetically-engineered fruit with a modified ripening phenotype (reviewed by Gray et al., 1992, 1994; Grierson & Schuch, 1993). Such modifications have resulted in a fruit crop with improved commercial characteristics.

The methodology of genetic engineering of plants with antisense RNA transgenes

The use of antisense RNA to manipulate gene expression

Following initial reports that the integration and expression of a specific antisense RNA transgene in tomatoes (Smith et al., 1988) and *Petunia* (van der Krol et al., 1988) led to a much-reduced accumulation of the homologous endogenous mRNA transcript, the technique has been widely used, specifically to down-regulate the expression of a number of tomato fruit-specific genes (Gray et al., 1992, 1994; Grierson & Schuch, 1993). Although the mechanism by which the expression of the antisense RNA inhibits the accumulation of the endogenous transcript is at present unclear, and will not be fully addressed here, the gene-specific action implicates nucleic acid base pairing. In brief, the technique summarised in Fig. 1, involves cloning and insertion in an inverted or 'antisense' orientation, of either a cDNA or genomic sequence or part thereof, between a promoter and terminator sequence that will be recognised and active within the plant genome. Following *Agrobacterium*-mediated transformation, the sequence between the left and right T-DNA borders (Fig. 1) is stably-integrated into the plant genome, such that when the transgene is transcribed *in planta*, an antisense RNA is produced. The transcription of the antisense RNA leads to a substantial reduction in accumulation of the endogenous sense mRNA transcript and thus a reduced level of the encoded product, effectively reducing or blocking the biochemical function of the gene. This approach has been successfully utilised to down-regulate the activity of the cell wall-metabolising enzymes polygalacturonase (Smith et al., 1988, 1990; Sheehy et al., 1988) and pectinesterase (Tieman et al., 1992; Hall et al., 1993) and has been used to bring about reduced ethylene synthesis in fruit by expression of an antisense RNA for ACC oxidase (Hamilton et al., 1990, 1991) or ACC synthase RNA (Oeller et al., 1991) and to disrupt fruit pigmentation by down-regulation of the phytoene synthase gene (Bird et al., 1991).

The use of antisense RNA as a tool for the identification of the biochemical roles played by unidentified clones

Having established the efficacy of antisense RNA expression to down-regulate genes encoding a specifically-defined enzyme of known biochemical function, it became possible to extend the use of the technique to help define the functional role of a cloned but unidentified gene. As described previously, the sequence of interest is cloned in an inverted orientation and transformed into plant tissue. Mature transgenic plants are then raised that effectively contain a single, targeted mutation in the gene of interest. Biochemical and physiological analysis of such plants can aid the functional identification of the gene being studied. This approach was used successfully to identify fruit ripening clones isolated from the ripe fruit TOM cDNA library (Slater et al., 1985), as encoding the enzymes phytoene synthase (Bird et al., 1991) and ACC oxidase (Hamilton et al., 1990). Attempts to define the function of an additional ethylene-regulated, fruit-ripening gene, E8 (Lincoln et al., 1987) by expression of an antisense transgene, led to the production of fruit with altered ethylene over-production but failed to establish unequivocally the biochemical function of the encoded product (Peñarrubia et al., 1992).

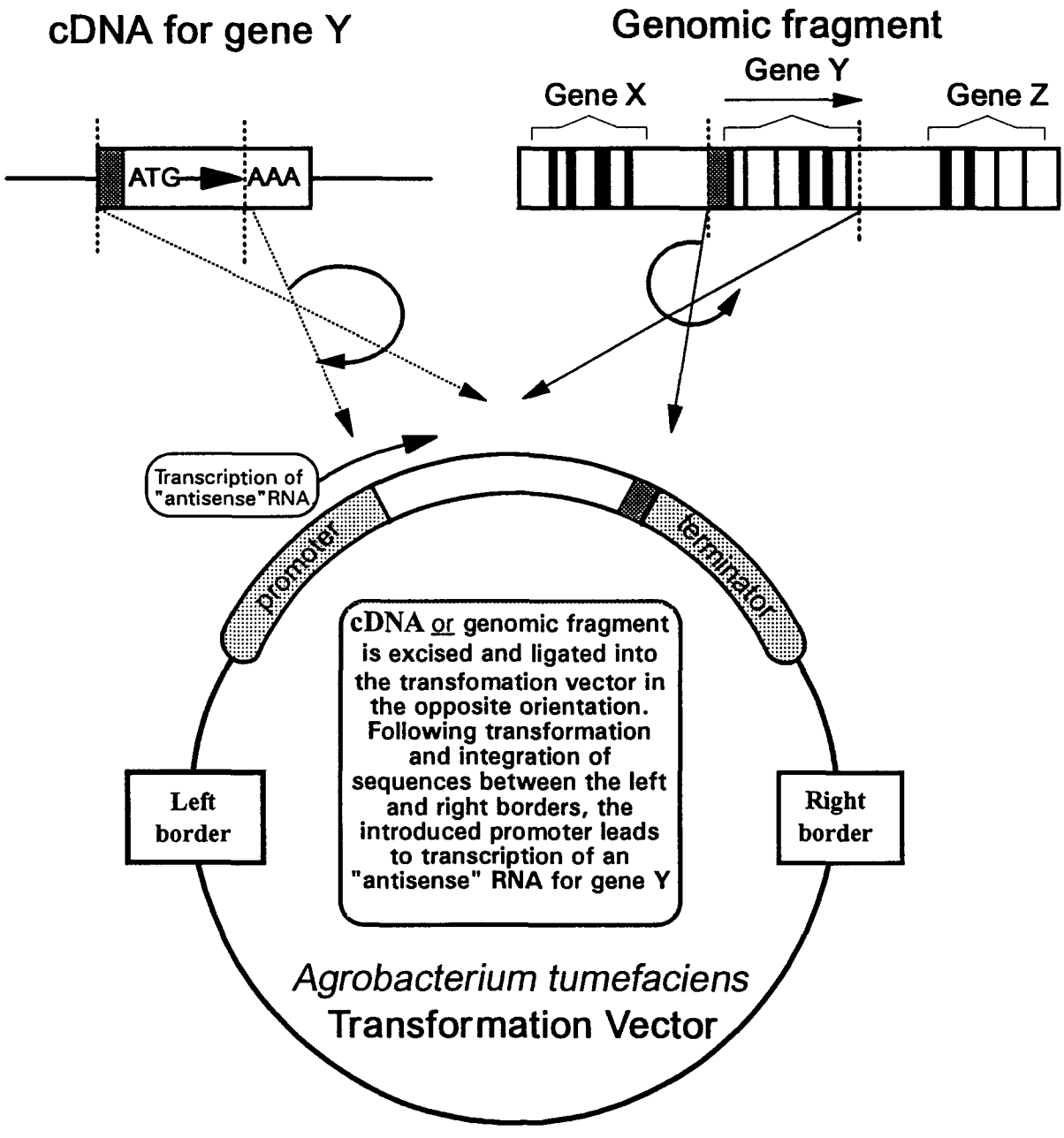

Fig. 1. Schematic representation of the creation of a transformation vector for integration and expression of antisense RNA in transgenic plants. The left and right border sequences indicated are derived from the *Agrobacterium tumefaciens* Ti plasmid. (After Picton et al., 1994.)

Manipulation of the expression of genes encoding the cell wall metabolising enzymes polygalacturonase and pectinesterase

Polygalacturonase

The first experiments to isolate, characterise (Grierson et al., 1986a, 1986b; DellaPenna et al., 1986; Sheehy et al., 1987) and genetically manipulate (Smith et al., 1988, 1990; Sheehy et al., 1988; Giovannoni et al., 1989) a tomato fruit-ripening gene, involved the cell wall-hydrolysing enzyme, polygalacturonase (PG). Much circumstantial evidence suggested that PG was the major determinant of the softening that occurs in ripe tomato fruits (Hobson, 1965; Crookes & Grierson, 1983) and as such, had attracted much interest

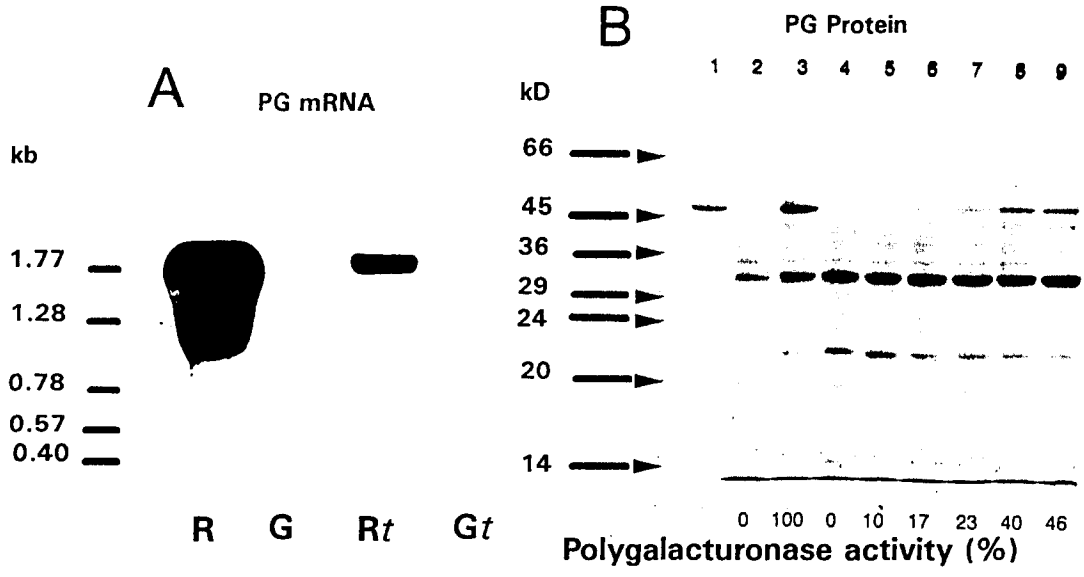

Fig. 2. Reduction of the level of PG mRNA, protein and enzyme activity in a number of independently-transformed primary (hemizygous) PG antisense plants. A. Northern blot showing PG mRNA levels in green (G) and red (R) wild-type fruit and in transformed green (Gt) and red (Rt) fruit. Size of the RNA markers run in an adjacent lane are indicated on the left. B. Levels of PG protein detected in controls and a number of primary transformed lines. Lane 1, purified PG protein, Lane 2, untransformed green fruit, Lane 3, untransformed red fruit, Lane 4, transformed green fruit, Lanes 5–9, red fruit obtained form independently-transformed primary lines. Molecular weight markers run in an adjacent lane are shown on the left. PG enzyme activity, measured in fruit extracts from each of the displayed lines, is shown below. (After Smith et al., 1988 (A) and Grierson & Schuch, 1993 (B).)

at both the biochemical and physiological level over a number of years.

A ripe tomato fruit cDNA library was prepared (the TOM clones), differentially screened against green fruit, and 19 non-homologous classes of mRNAs identified that showed increased accumulation during ripening (Slater et al., 1985). Comparison of a partial amino acid sequence obtained from purified PG enzyme with nucleic acid sequence obtained from the TOM clones, identified TOM 6 as encoding the PG enzyme (Grierson et al., 1986a, 1986b). To address specifically the role of this enzyme in the fruit-ripening process, experiments were undertaken to modify the normal pattern of expression of the PG gene in transgenic plants.

An antisense construct (Fig. 1) was prepared containing a 730 bp 5′ fragment of the TOM 6 cDNA and this was used to transform plants. Analysis of a number of primary transformed lines showed that, in some, the expression of the endogenous PG gene was reduced (Smith et al., 1988). Separate experiments were performed independently with full length PG cDNA and similar results obtained (Sheehy et al., 1988). Examination of primary transformed plants (hemizygotes) showed a reduction in the level of accumulation of the PG mRNA transcript (Fig. 2A) and a range of reduction in PG protein and PG activity (Fig. 2B). Self-pollination of the primary transformed lines gave rise to a segregating population of plants that inherited zero, one or two copies of the transgene, the introduced sequence being stably integrated into the plant genome and inherited in a normal Mendelian fashion (Smith et al., 1988, 1990). Homozygous lines derived from primary transformants with low PG activity, inherited two copies of the PG antisense transgene and showed a further reduction in level of both PG mRNA and PG enzyme activity (Fig. 3A and 3B) (Smith et al., 1990). In such plants, other aspects of ripening such as ethylene production, fruit pigmentation and onset and speed of ripening appeared unaffected, demonstrating the gene-specific action of the antisense construct (Smith et al., 1990).

Despite the dramatic reduction in PG mRNA accumulation and PG enzyme activity, little difference was

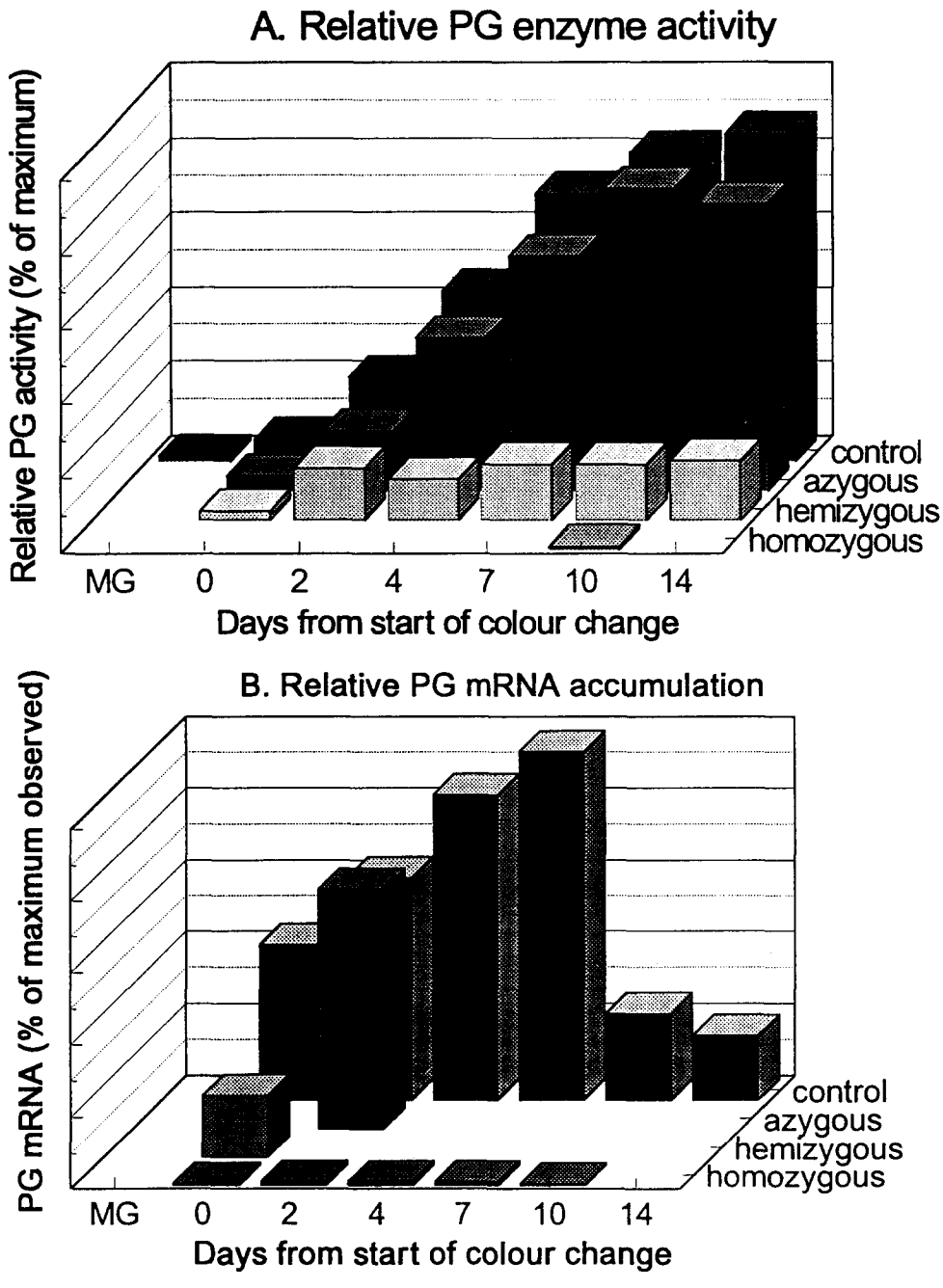

Fig. 3. Reduction in PG enzyme activity (A) and PG mRNA accumulation (B). Fruit was obtained from wild-type 'Ailsa Craig' controls and transformed homozygous, hemizygous and azygous plants prior to the onset of ripening (MG), at the onset of ripening (colour change, day 0) and at days from the start of colour change as indicated. All data is presented relative to the maximum level observed during the normal ripening of control fruit. (Data from Smith et al., 1990.)

observed between the 'softening' of homozygous PG antisense and wild-type fruit as assessed by probe penetration studies on ripe fruit pericarp (Smith at al., 1990; Schuch et al., 1991). This suggested that PG was not the sole, or even a major determinant, of the process of fruit softening. This observation was strengthened by reports that expression of the PG enzyme in a fruit mutant that does not normally express the PG

gene, yields transgenic fruit that continues to remain hard during the ripening process (Giovannoni et al., 1989). More recently, trials of PG antisense fruit have demonstrated that the engineered fruit does, however, show increased resistance to the shrivelling and splitting associated with over-ripening (Schuch et al., 1991; Gray et al., 1992) and does have small but statistically significant changes in firmness at the later stages of ripening (Grierson & Schuch, 1993). Additionally, the fruit has new characteristics that improve its value for the processing market. Bostwick viscosity, an index measure of paste yield potential, is increased by over 80% in transgenic fruit where PG activity is reduced by 99% (Grierson & Schuch, 1993). Independent experiments on low PG fruit of a different variety, containing a full length PG antisense transgene, confirm these findings (Kramer et al., 1990, 1992) and also showed increased resistance of the low PG fruit to several post-harvest pathogens.

The economic potential of this PG antisense fruit has already been realised in the United States where Calgene, following extensive product testing and analysis (Redenbaugh et al., 1992), is marketing the fresh fruit under the banner 'FlavrSavr^{TM}'. Processed tomato products based on low PG fruit arising from collaboration between Nottingham University and Zeneca Seeds, are planned to be introduced into Europe and the United States marketplace in 1995 or 1996 (Grierson & Schuch, 1993).

Pectinesterase

A similar experimental approach by two independent research groups has been used to reduce the accumulation of another cell wall-metabolizing enzyme, pectinesterase (PE) (Tiemann et al., 1993; Hall et al., 1993). Detailed analysis of PE antisense fruit suggests that PE, like PG, is not the major factor associated with fruit softness. However, the engineered fruit has altered characteristics, such as increased serum viscosity, that improves the 'gloss' of fruit extracts, and thus increases the suitability of this fruit for processing into products such as pastes, sauces and soups (Grierson & Schuch, 1993). PE antisense fruit has also been reported to show a substantial increase in the level of soluble solids in the fruit (Tieman et al., 1993).

Unlike the PG expressed in fruit, which appears to be encoded by a single gene (Bird et al., 1988), PE is encoded by a small multigene family whose members show differential patterns of expression and enough divergence in nucleic acid sequence, that the introduction of a single PE antisense transgene does not lead to reduction in all fruit PE mRNAs.

The possibility of combinatorial improvements in fruit quality by down-regulation of more than one gene in a single transgenic line have been explored by two mechanisms. Firstly, low PG/PE lines have been created by crossing low PG and low PE parental lines (Grierson & Schuch, 1993) and, secondly, a chimeric antisense construct containing both a PE and PG antisense transgene has been introduced into plants (Grierson & Schuch, 1993 but see also Seymour et al., 1993). These lines exhibit phenotypic changes associated with each of the independent transgenes and additionally show an increase in soluble solids (termed Brix), thus producing fruit in which the most important determinant of processing quality has been increased (Grierson & Schuch, 1993).

Identification and manipulation of genes encoding ACC oxidase and phytoene synthase

ACC oxidase

The pathway of ethylene biosynthesis in higher plants involves the conversion of S-adenosyl methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) and the subsequent conversion of ACC to ethylene (Yang & Hoffman, 1984; Yang, 1985). These steps are catalysed by the enzymes ACC synthase and ACC oxidase, respectively. The use of antisense RNA expression has allowed the identification of a clone encoding ACC oxidase formerly known as the ethylene-forming enzyme (Hamilton et al., 1990, 1991), a previously elusive enzyme (John, 1992). The mRNA homologous to the tomato fruit clone, TOM 13, was identified as increasing rapidly at the onset of ripening and in response to wounding of plant tissues (Slater et al., 1985; Smith et al., 1986; Holdsworth et al., 1987, 1988), and during foliar senescence (Davies & Grierson, 1989), situations that involve a rapid increase in the synthesis of ethylene.

A TOM 13 antisense transgene was introduced into tomato plants and these were self-pollinated to yield a segregating population of plants containing zero, one or two copies of the antisense gene. Fruit and leaf tissue of the hemi- and homozygous transgenic plants was analysed. The increased ethylene synthesis associated with wounding of leaf tissue (Fig. 4A) and ripening of fruit (Fig. 4B) was significantly reduced in a transgene dose-dependent manner and was shown to be associ-

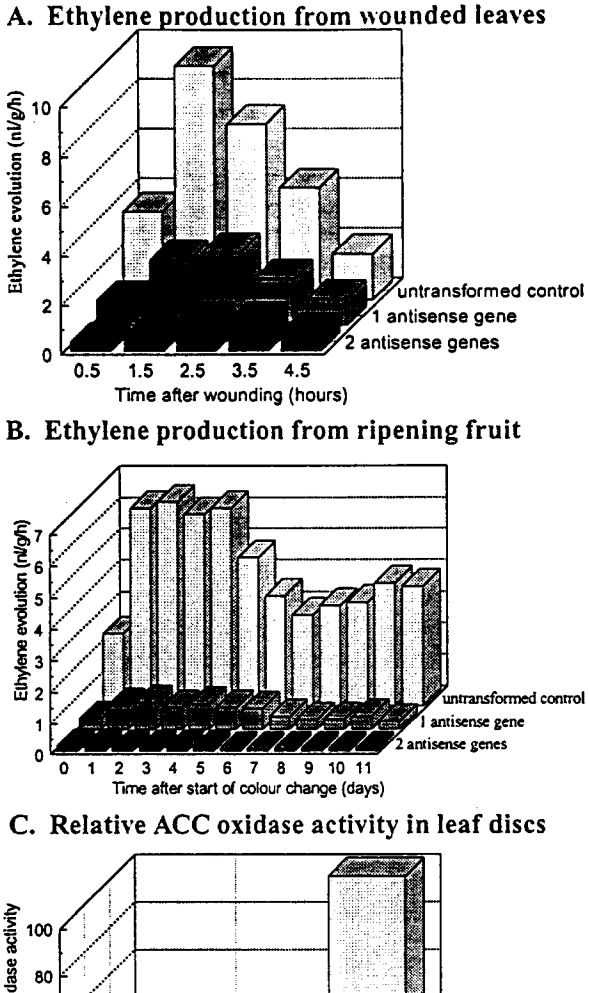

Fig. 4. Ethylene evolution and ACC oxidase activity in hemizygous and homozygous ACC oxidase antisense plants. A. Ethylene evolution from mechanically-wounded, mature leaves of wild-type and transgenic plants at the times indicated. B. Ethylene evolution from ripening fruit obtained from plants indicated, at various stages after the onset of ripening (colour change). C. ACC oxidase enzyme activity assayed in extracts from tomato leaf discs obtained from plants indicated. (Data from Hamilton et al., 1990, 1991.)

ated with a decrease in the activity of ACC oxidase (Fig. 4C) (Hamilton et al., 1990). This implicated the TOM 13-encoded product as playing a role in synthesis of ethylene. The introduction and expression of a full length TOM 13 cDNA in yeast conferred the ability on the transformed cells to synthesise ethylene

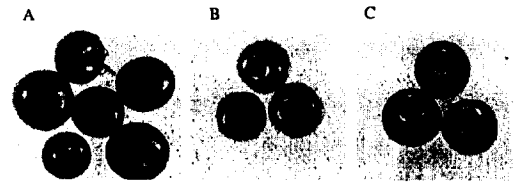

Fig. 5. Phenotypic changes in homozygous ACC oxidase antisense fruit. Fruit was picked from wild-type (A) or homozygous ACC oxidase antisense plants (B, C) prior to the onset of ripening. Prior to photography, fruit was allowed to ripen for 14 days in air (A, B) or with the addition of 20 μl/l ethylene (C). ACC oxidase antisense fruit incubated in air failed to achieve normal pigmentation, remaining yellow (B). (After Gray et al., 1991.)

from its immediate precursor, ACC, thus giving final proof that the TOM 13 product was indeed the elusive ACC oxidase (Hamilton et al., 1991). This was also demonstrated by injection of a TOM 13-related mRNA into *Xenopus* oocytes and the subsequent production of ethylene from ACC (Spanu et al., 1991).

The ACC oxidase antisense fruit displayed an altered ripening phenotype (Hamilton et al., 1990; Bouzayen et al., 1992; Gray et al., 1992; Picton et al., 1993a). Fruit ripening on the plant appeared superficially normal but accumulation of lycopene, responsible for the red coloration of ripe fruit, was reduced as was the level of shrivelling and splitting associated with over-ripening (Hamilton et al., 1990; Gray et al., 1992; Picton et al., 1993a). When fruit was detached at the onset of ripening and allowed to ripen in air, a more extreme phenotype was observed, with substantial reduction in lycopene accumulation resulting in yellow-orange fruit (Fig. 5) that displayed increased storage longevity. The accumulation of a number of ripening-related mRNAs, including that encoding the carotenoid biosynthetic enzyme, phytoene synthase, was shown to be reduced. The phenotypic and molecular alterations observed were partially restored by application of ethylene to the detached fruit (Fig. 5); however, full reversal was not achieved and the ethylene-treated fruit still displayed resistance to shrivelling and splitting (Picton et al., 1993a). Large scale glasshouse evaluation of the ACC oxidase antisense fruit has indicated that some of the phenotypic changes observed may be of commercial value, as low ethylene fruit has much-improved handling characteristics (Murray et al., 1993; Grierson & Schuch, 1993).

Phytoene synthase

The same experimental approach, that is, the expression of an antisense construct derived from a previously-cloned, but functionally-unidentified gene, also led to the biochemical identification of another important ripening-associated gene. The fruit clone TOM 5 (Slater et al., 1985) was sequenced (Ray et al., 1987) and found to be homologous with bacterial genes involved in the production of phytoene (Armstrong et al., 1990). This suggested that the TOM 5 product may encode a protein involved in the synthesis of coloured carotenoids during the ripening of tomatoes. The expression of a TOM 5 antisense transgene in tomato plants confirmed that the TOM 5 product is involved in carotenoid biosynthesis. Analysis of the antisense fruit and its phytoene precursors demonstrated that the TOM 5 product is the enzyme phytoene synthase (Bird et al., 1991). Following its identification, the cDNA has been further exploited to complement a naturally-occurring fruit mutant, *yellow flesh*, which fails to accumulate β-carotene and lycopene as a result of a mutation in the phytoene synthase gene, thus identifying the gene responsible for the mutation (Fray & Grierson, 1993).

Conclusions and future directions

The ability to isolate, clone, sequence and subsequently modify the expression of individual genes involved in ripening of tomatoes by expression of antisense transgenes is of major scientific and commercial interest. So far the technique has enabled studies on the developmental and hormonal regulation of gene expression, allowed the physiological and biochemical function of individual genes to be identified, and has created valuable genetically-engineered fruit with altered ripening characteristics.

The process of antisense expression effectively allows the creation of a single, targeted mutation in the gene of interest. When applied to fruit ripening, the process has been used to alter the expression of the cell wall-metabolising enzymes PG and PE and has enabled both the identification and manipulation of the enzymes ACC oxidase and phytoene synthase. The technique has also been applied to studies on ACC synthase (Oeller et al., 1992) and the functionally-unidentified clone E8 (Peñarrubia et al., 1992). Gentically modified plants have produced fruit with increased utility for the fresh and processed fruit markets and indicate that further improvements to the crop are possible. Clearly, the continued success of this approach to improve fruit quality and modify nutritional and storage qualities, will rely on continued biochemical and molecular analysis of key changes and identification of enzymes and genes responsible for ripening. It is also clear that further ripening-related genes remain to be identified. To this end, we have recently isolated and cloned several novel mRNAs whose expression appears to be severely repressed in the naturally-occurring, extreme ripening-impaired mutant, *ripening inhibitor (rin)* (Picton et al., 1993b, 1993c; Gray et al., 1994). This mutant *rin* fruit shows no respiratory rise, little ethylene production at the onset of ripening, abnormal pigment accumulation and 'ripens' extremely slowly over the course of several months, remaining resistant to many of the common post-harvest pathogens (Grierson et al., 1987; Picton et al., 1993b; Gray et al., 1994). By expression of antisense transgenes derived from these new clones, we aim to identify and modify their function in tomato. Such transgenic fruit may display novel ripening phenotypes that may also prove to be of scientific and commercial interest.

At present, one limitation of the approach outlined above is the extreme variability observed in the 'strength' of the antisense effect. Primary transformants, derived from experiments introducing an identical transgene, show a large range of levels of transgene expression. This so-called 'position effect' may result from the actual site of integration of the transgene into the plant genome. It has even been suggested that the majority of transformation events actually lead to very low expression of the transgene and in many cases the successful transfer of DNA may even go undetected (Peach & Velten, 1991). In the case of metabolically-essential genes, a range of 'leaky' antisense mutations may allow the production and analysis of an otherwise lethal line. In some cases, however, it may prove more beneficial to achieve maximum down-regulation of the targeted gene in all transformants. Reports on reduction of position effects in transgenic plants, by making the introduced transgene appear more like a natural gene, by inclusion of nuclear scaffold attachment regions (SARs) (Breyne et al., 1992; Allen et al., 1993) mean that the variability of antisense inhibition observed at present may be overcome (Allen et al., 1993) achieving a 20-fold increase in the level of transgene expression by flanking the introduced DNA with SARs. Position effects may also be overcome by using natural plant gene promoters with high transcrip-

tional activity in the target tissue. Nicholas et al. (1994) have delineated a 5′ promoter region of the PG gene that is responsible for extremely high levels of fruit-specific reporter gene expression in transgenic plants. Introduction of antisense constructs containing several copies of the transgene may increase the effect, as can transformation techniques that favour multiple insertion of the single, introduced transgene, as illustrated by Oeller et al. (1991) who inserted an estimated 10 copies of an antisense ACC synthase gene into tomato in order to achieve maximum gene inactivation.

With further studies it may be possible to increase the efficacy of transgene expression by either one, or a combination of the methods outlined above. Once the expression of the introduced transgene can be increased and controlled, the use of expression of antisense RNA transgenes to inhibit specific genes *in planta* may prove to be an even more powerful tool for genetic improvement of crop plants than is realised at present.

Acknowledgements

This work was supported by grants from the BBSRC, SERC Biotechnology Directorate and the Gatsby Charitable Foundation. All work with transgenic plants was performed under MAFF licences. The authors wish to acknowledge the published work of Chris Smith, Andrew Hamilton, Rupert Fray and others in the Nottingham laboratory, cited in this article.

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