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Update on Biotechnology

Modification of Fruit Ripening by Suppressing Gene Expression

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Ethylene is one of the simplest organic molecules with biological activity. Its effects on plant tissue are spectacular and commercially important (1, 2). This hydrocarbon gas is generally considered to be the fruit-ripening hormone (2, 3). Because of its effects on plant senescence, large losses of fruits and vegetables are incurred annually in the United States. The losses are much greater in developing countries because of the lack of sufficient refrigeration and transportation. Consequently, it has always been a goal of plant biologists and of postharvest physiologists, in particular, to be able to prevent or delay fruit ripening in a reversible manner by controlling ethylene action or production. Thus, an understanding of ethylene action and biosynthesis is of fundamental as well as applied significance. This update summarizes the recent advances in manipulating key genes in the ethylene biosynthetic pathway to prevent ethylene production and fruit ripening.

Methionine is the biological precursor of ethylene in all higher plants (22) and is converted to ethylene according to the following sequence:

$$\text{Methionine} \xrightarrow{(1)} \text{AdoMet}^{1} \xrightarrow{(2)} \text{ACC} \xrightarrow{(3)} \text{Ethylene}.$$

The rate-limiting step in the pathway is the formation of the amino acid ACC from AdoMet, catalyzed by ACC synthase (reaction 2). The final step is the conversion of ACC to ethylene catalyzed by ACC oxidase (reaction 3). Molecular cloning approaches and expression in heterologous systems allowed the isolation of the genes encoding AdoMet synthase (step 1 [14]), ACC synthase (step 2 [11, 16, 21]), and ACC oxidase (step 3 [6, 7, 18, 19]).

INHIBITION OF FRUIT RIPENING USING REVERSE GENETICS

Ethylene is thought to regulate fruit ripening by coordinating the expression of genes that are responsible for a variety of processes, including enhancement of a rise in the rate of respiration, autocatalytic ethylene production, Chl degradation, carotenoid synthesis, conversion of starch to sugars, and increased activity of cell-wall degrading enzymes (5). Throughout the years, various methods for prolonging fruit senescence have been employed, such as ventilation with air under hypobaric pressures (4). This procedure accelerates the escape of ethylene and, by reducing the oxygen tension, also lowers the fruits' sensitivity to hormone. Inhibitors of ethylene action also have been used, such as silver ions and carbon dioxide (22). These approaches are expensive and fail to prevent fruit senescence satisfactorily. In a few cases, however, such as apple, controlled atmosphere storage has been a commercial success. A more desirable solution to the problem will be the construction of a mutant plant whose fruits do not ripen unless they are treated with ethylene. Tomato (*Lycopersicon esculentum*) ripening mutants exist, but their phenotype is not reversible by ethylene (10).

The cloning of genes induced during fruit ripening and of genes involved in ethylene biosynthesis opened the road to the construction of ripening mutants in tomato using reverse genetics. In the absence of gene-replacement technology in plants, antisense RNA technology and overexpression of an ACC-metabolizing enzyme became the tools of choice (5, 9).

Antisense RNA

Initially, attempts to inhibit tomato fruit softening by antisense RNA to the gene for PG, an enzyme thought to be responsible for cell-wall hydrolysis during ripening, failed to give a strong effect (17, 18). Expression of PG antisense RNA dramatically inhibited PG mRNA accumulation and enzyme activity, suggesting that PG is not the sole determinant of cell-wall hydrolysis (18).

Another approach to prevent fruit ripening is to inhibit ethylene production. Hamilton et al. (7) inhibited ACC oxidase activity with antisense RNA. In plants that were homozygous for the antisense gene, ethylene production was inhibited by 97% in ripening fruit. In these antisense fruits, the color change was initiated at about the normal time, however, the extent of reddening was reduced. Antisense fruits stored for several weeks at room temperature were more resistant to overripening and shriveling than control fruits (7). More recently, Oeller et al. (12) used antisense RNA to ACC synthase to inhibit tomato fruit ripening. This approach led to severe inhibition of ethylene production (below 0.1 nL/g·h; 99% inhibition), resulting in a tomato fruit mutant with a striking phenotype (Fig. 1). This dramatic inhibition of ethylene production can be attributed to the short half-life of ACC synthase (8). Antisense experiments are intrinsically "leaky," allowing some mRNA to be translated. Consequently, the stability of the encoded polypeptide is an im-

[1] Abbreviations: AdoMet, *S*-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; PG, polygalacturonase.

portant factor for successful gene inactivation by antisense RNA (12).

During tomato fruit ripening, two ACC synthase genes are expressed, *LE-ACC2* and *LE-ACC4* (13, 15). Expression of antisense RNA derived from the cDNA of the *LE-ACC2* gene resulted in an almost complete inhibition of mRNA accumulation of both ripening-induced ACC synthase genes (12). Control fruits kept in air begin to produce ethylene 50 d after pollination and fully ripen after another 10 d (Fig. 1). The red coloration resulting from Chl degradation and lycopene biosynthesis is inhibited in antisense fruits (Fig. 1). Antisense fruits kept in air or on the plants for 90 to 150 d eventually develop an orange color but never turn red and soft or develop an aroma.

The antisense phenotype can be reversed by treatment with ethylene or propylene, an ethylene analog. The treated fruits are indistinguishable from naturally ripened fruits with respect to texture, color, aroma, and compressibility. The duration of ethylene treatment required to reverse the antisense phenotype is 6 d. Antisense fruits treated for 1 or 2 d with ethylene do not develop a fully ripe phenotype compared with control fruits treated similarly.

ACC Deaminase

Klee et al. (9) used a different approach to inhibit ethylene production. They overexpressed the gene for ACC deaminase, which metabolizes ACC to α-ketobutyrate from *Pseudomonas* sp. in transgenic tomato plants. This approach led to 90 to 97% inhibition in ethylene production during ripening. Reduction in ethylene synthesis in transgenic plants did not cause any apparent vegetative phenotypic abnormalities. However, fruits from these plants showed significant delays in ripening, and they remained firm for at least 6 weeks longer than the nontransgenic control fruits (9).

Three important conclusions can be inferred from the tomato fruit mutants. (a) The ethylene-mediated ripening process requires continuous transcription of the necessary genes, which may reflect a short half-life of the induced mRNAs or polypeptides. (b) Ethylene is indeed autocatalytically regulated. (c) The hormone acts as a rheostat rather than as a switch for controlling the ripening process. The accumulated evidence from the antisense and the deaminase experiments indisputably demonstrated that the Yang cycle is solely responsible for ethylene synthesis during ripening and that ethylene is the key regulatory molecule for fruit ripening and senescence, not the by-product of ripening (2).

The mutant fruits producing low levels of ethylene have proven to be excellent experimental material for assessing which of the ripening-induced genes so far cloned are indeed ethylene inducible. The expression of PG and pTOM13 (ACC oxidase) genes, which were previously thought to be ethylene regulated, have been found to be ethylene independent (12). It is surprising that, although antisense fruits express large amounts of PG mRNA, they fail to accumulate the PG polypeptide, suggesting that ethylene may control the translatability of PG mRNA or the stability of the PG polypeptide (20).

The mutants also show that at least two signal transduction pathways are operating during tomato fruit ripening. The ethylene-independent (developmental) pathway is responsible for the transcriptional activation of genes for enzymes such as PG, ACC oxidase, and chlorophyllase. The ethylene-dependent pathway, on the other hand, is responsible for the transcriptional and posttranscriptional regulation of genes involved in lycopene and aroma biosynthesis, respiratory

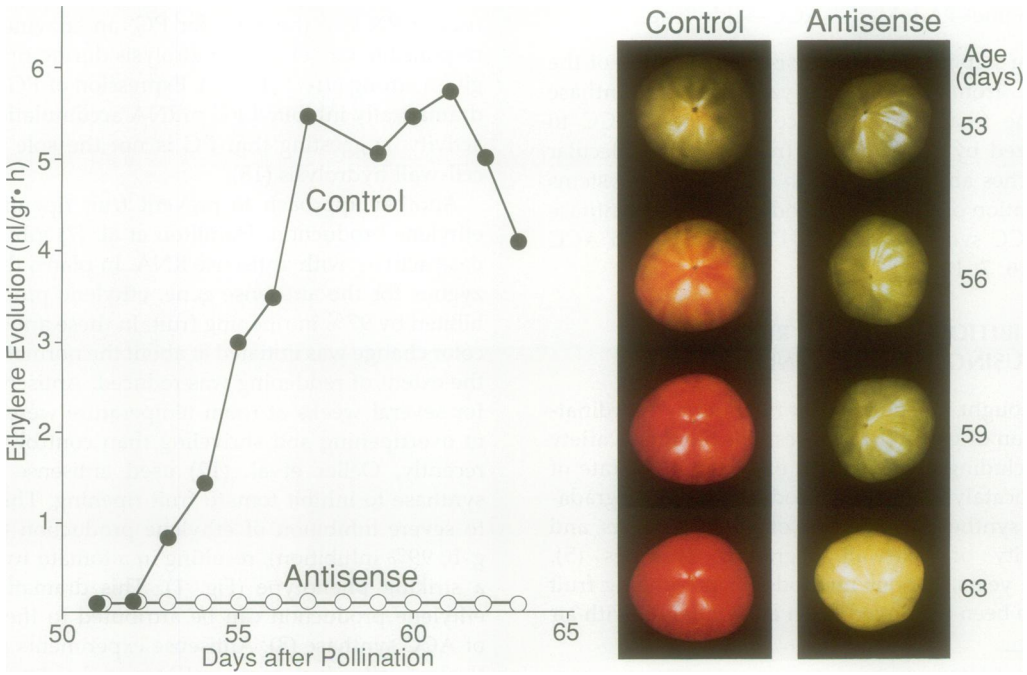

Figure 1. Inhibition of tomato fruit ripening and ethylene evolution in detached tomato fruits by antisense ACC synthase RNA (12).

metabolism, ACC synthase gene expression, and translatability of developmentally regulated genes such as PG (20).

THE FUTURE

The use of antisense technology and overexpression of metabolizing enzymes such as ACC deaminase in controlling fruit ripening is only the first step toward controlling fruit senescence. Expression of antisense RNA using regulated promoters may eliminate the use of exogenous ethylene for reverting the mutant phenotype. However, the development of gene transplacement technology by homologous recombination should allow the creation of nonleaky ripening mutants with long-term storage potential. The prospect arises that inhibition of ethylene production using reverse genetics may be a general method for preventing senescence in a variety of fruits and vegetables.

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