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SILENCING THE RIPENING-ASSOCIATED ENZYME 1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE (ACO) IN APPLE USING AN ENDOGENOUS ANTISENSE RNA CONSTRUCT

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INTRODUCTION

The plant hormone ethylene exerts a diverse and complex effect on plants, including developmental and stress-related responses (STEPNOVA; ALONSO, 2009). Physiological, biochemical and molecular evidences indicate that ethylene is the principal hormone controlling ripening, a complex developmental process that involves the formation of taste, aroma, establishment of skin color and fruit softening by cell wall hydrolysis. Upon ethylene perception, the receptor complexes inactivate a kinase signaling partner, which, in turn, regulates the activity of several transcriptional regulators, leading to extensive changes in gene expression (revision in YOO et al., 2009). Genetic engineering is an interesting alternative to modify ethylene production/action in plants to generate fruits tolerant to longer storage periods and develop gain- and loss-of-function systems to study basic aspects of ripening processes by high-throughput approaches. Thus, we have employed the antisense sequence of the endogenous *Malus x domestica* gene coding for 1-aminocyclopropane-1-carboxylate (ACC) oxidase (ACO), the final enzyme converting ACC, an S-adenosyl-L-methionine derivative, to ethylene, to produce genetically modified apple plants. A modified transformation protocol allowed us to recover 12 independent transgenic lines of ‘Royal Gala’. The engineered plants exhibit reduced ACO protein levels and represent an important tool for future studies on ripening.

MATERIAL AND METHODS

Lateral meristems of field-grown ‘Royal Gala’ plants were surface disinfected with ethanol 70% (v/v) followed by immersion in 1% (v/v) sodium hypochlorite. The explants were inoculated

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in flasks containing MS (MURASHIGE; SKOOG, 1962) medium, supplemented with 3% (w/v) sucrose, 0.6% (w/v) agar and 1 $mg.L^{-1}$ 6-benzylaminopurine (BAP) and cultured under 16h-photoperiod at 75 $\mu mol.m^{-2}.s^{-1}$ at 23°C ± 2°C, for shoot propagation.

Two weeks prior to transformation, plants were transferred to the dark. Leaf explants (~1 cm diameter) were excised from etiolated plants, pre-conditioned as described (PINO et al., 2010) and transformed by co-cultivation with low density cultures of *Agrobacterium tumefaciens* EHA105 (Figure 1). Transformed shoots were selected on selective medium (50 $mg.mL^{-1}$ kanamycin), as described (LIU et al., 2001). Individualized shoots exhibiting kanamycin resistance were checked for the transgene presence by PCR, employing vector specific primers (Figure 2). PCR positive plants, able to produce vigorous roots in kanamycin-containing medium were acclimated *ex-vitro* by transfer to cover-protected cups containing an autoclaved mixture of vermiculite: horticulture substrate (1:1). Gradually, relative humidity inside the covers was reduced by opening holes, until the complete removal of the covers four weeks after *ex-vitro* transplantation. Fully acclimated plants were transferred to biosafety greenhouse with humidity (70%) and temperature (25°C) control.

The leaves from genetically modified plants were pre-induced by physical injury of the mesophyll with a scalpel blade prior to protein extraction and ACO immunoblotting. Total proteins were phenol-extracted and precipitated by methanolic ammonium acetate (HURKMAN; TANAKA, 1986). Extracts were quantified for protein concentration and equal amounts were loaded on SDS–8% PAGE (AUSUBEL et al., 1994). Proteins were electro-blotted to nitrocellulose membranes and probed with polyclonal antibody against apple ACO, as previously described by ROMBALDI et al. (1994).

RESULTS AND DISCUSSION

The modifications introduced in the standard leaf-disc transformation protocol for apple leaf-discs, namely the use of etiolated explants, pre-conditioning with auxin and use of low-density *A. tumefaciens* cultures, allowed us to obtain 12 independent transgenic lines, as shown in Table 1 and Figure 1.

Figure 1 - Transformation of *M. x domestica* cv. Royal Gala with antisense ACO (αACO).**A**: Leaf discs on selective regeneration medium two weeks after co-cultivation, **B**: regenerating plants on selective medium; **C**: putative transformants on selective medium.

The plants were further grown and acclimated *ex vitro* and the transformed status was confirmed by PCR employing vector-specific primers (Figure 2).

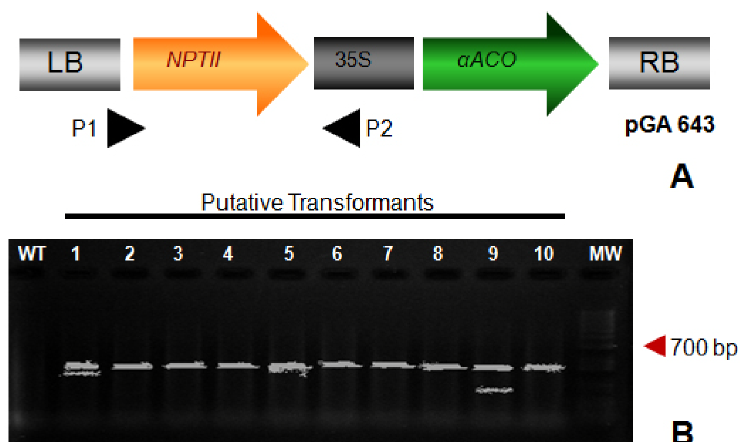

Figure 2 - A: Schematic representation of transformation vector pGA643, carrying the anti-sense sequence of *M. x domestica ACO* gene. Primers used to screen the regenerants are represented by P1 (NOS terminator) and P2 (35S). **B**: PCR analysis of ten putative transformants.

Table 1 - Genetic transformation of 'Royal Gala' apple plants with the anti-sense sequence of an endogenous *ACO* gene. Transformants correspond to plants positive for rooting in kanamycin-containing medium and PCR amplification of binary vector-specific sequences.

Cultivar Royal Gala	Transformation		Efficiency (%)
	co-cultured explants	transformants	
Exp.1	438	6	1.37
Exp. 2	301	5	1.67
Exp. 3	54	1	1.89
Total	793	12	1.64

The expression of the antisense mRNA of apple *ACO*, driven by the constitutive promoter CaMV 35S, reduced the levels of ACO protein in wounded apple leaves (Figure 3).

Figure 3 - Silencing of ACO protein in 'Royal Gala' plants transformed with the antisense sequence of an endogenous apple *ACO* gene by immunoblotting. Lane numbering from 1 to 6 correspond to independent transgenic lines (Royal Gala αACO), 7 and 8 correspond to wild-type

'Royal Gala' control. Letters A and B represent Non-induced and Wounded (induced) leaves, respectively.

CONCLUSIONS

Apple plants with reduced levels of the ripening-associated enzyme ACO were generated by genetic engineering using the antisense sequence of an endogenous gene. The use of a plant sequence may help to reduce the public concerns on the safety of genetically modified fruits. The transgenic plants represent an interesting tool to investigate several aspects of flesh fruit ripening and ethylene-related processes.

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