Note

Ethylene Biosynthesis Regulation in Tomato Fruit from the F1 Hybrid of the ripening inhibitor (rin) Mutant

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Received November 14, 2005; Accepted December 7, 2005; Online Publication, July 23, 2006
[doi:10.1271/bbb.50611]

We have previously shown a significant decrease in the ethylene production in tomato fruit from the Rin/rin genotype. In this present study, we evaluated the amount of 1-aminocyclopropane-1-carboxylic acid (ACC) and the gene expression and enzymatic activities of ACC synthase (ACS) and ACC oxidase (ACO) to find which type of regulation influenced this low ethylene production. The results suggest that the decreased ethylene production was due to transcriptional regulation of the ACS and ACO genes by the heterozygous effect of the rin gene.

Key words: LeMADS-RIN; ripening inhibitor (rin); ethylene; ACC synthase (ACS); ACC oxidase (ACO)

Extension of the shelf life of fruit is an essential objective in tomato breeding, and the LeMADS-RIN gene, a fruit-ripeness-regulating gene, is a possible key factor in controlling fruit ripening.1) LeMADS-RIN encodes a transcription factor belonging to the MADS-box family, and is expressed specifically during fruit ripening.2) The ripening inhibitor (rin) mutation in this locus shows negative and pleiotropic effects on the ripening of tomato fruit.3) Hence, we developed a northern blotting assay.4) In this present study, to clarify which type of regulation on the ethylene biosynthetic pathway caused the low ethylene production in the F1 hybrid fruit, mRNAs of the ACS and ACO genes were more precisely quantified by a real-time PCR assay, using multiple samples for reproducible results. The amount of ACC and the enzymatic activities of ACS and ACO were also evaluated.

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC synthase; ACO, ACC oxidase; rin, ripening inhibitor
The F₁ hybrid line, Kc01-6 (RIN/rin), was derived from a cross between wild-type line PK331 (RIN/RIN) and rin mutant line PK353 (rin/rin).⁴¹ The fruits of the wild type and the F₁ hybrid were harvested when mature green, pink colored (about 4 d after the breaker stage) and red ripe (about 7 d after the breaker stage), and the fruits of the rin mutant parent (PK353) were harvested at the mature green stage and at the stages corresponding to the wild type at the pink colored and red ripe stages (about 5 d and 9 d respectively after the fruit skin had started to show a yellow color). ACC was extracted from frozen whole fruits by the method described by Kato et al.¹² and the amounts were measured by the method described by Lizada and Yang.¹³ The extraction and enzyme assays for ACS and ACO were performed according to Nakatsuka et al.¹⁴ and Mathooko et al.¹⁵ respectively. To evaluate the expression of the genes encoding the ACS and ACO enzymes, the mRNA accumulation of *LeACS2*, *LeACS4*, and *LeACO1*, which have been reported to be mainly expressed during fruit ripening,⁵ were analyzed by a real-time PCR assay with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and GeneAmp 5700 (Applied Biosystems). As shown in Fig. 1, fruits at the mature green stage of all three lines accumulated ACC at a very low level ([< 0.2 nmol ACC/g of fruit] (Fig. 1A). The mRNA accumulation and activities of ACS (Fig. 1B) and ACO (Fig. 1C) at the mature green stage were also low in all three cultivars. The fruit at the pink colored stage of the wild type produced ethylene abundantly,⁴ and rapid elevation in the mRNA accumulation of *LeACS2*, *LeACS4* (Fig. 2B) and *LeACO1* (Fig. 2C) was followed by elevation in the ACS (Fig. 2B) and ACO (Fig. 2C) activities and a rise in ACC (Fig. 2A). In the F₁ hybrid fruit, ethylene production was significantly lower than that in the wild type.³ The F₁ hybrid fruit accumulated about 38% of the ACC accumulated by the wild type (Fig. 2A). The accumulation of *LeACS2* and *LeACS4* mRNA was respectively about 26% and 28% that of the wild type, and the ACS activity was about 25% that of the wild type (Fig. 2B). The accumulation of *LeACO1* mRNA was about 31% and the ACO activity was about 40% that of the wild type (Fig. 2C). At the red ripe stage, ethylene production by the wild type declined slightly and that of the F₁ hybrid fruit also declined to the basal level.⁴ The ACC accumulation of the F₁ hybrid fruit decreased to about 23% that of the wild type (Fig. 3A). The accumulation of *LeACS2* and *LeACS4* mRNA was about 38% and 17% that of the wild type, respectively, and the ACS activity was about 52% that of the wild type (Fig. 3B). The accumulation of *LeACO1* mRNA was about 46% and the ACO activity was about 40% that of the wild type (Fig. 3C). In the rin mutant fruit, the amount of ACC, the mRNA accumulation, and the activities of ACS and ACO remained very low during ripening (Fig. 2, 3). A similar trend was observed with all sample lots in every assay (data not shown).

These results show clear differences in the activation of the ethylene-biosynthetic pathway between the ripening fruits of the wild type and F₁ hybrid. Although the wild type fruit producing ethylene abundantly accumulated the mRNAs of ACS and ACO at high levels and possessed obvious ACS and ACO activities, the F₁ hybrid fruit, which showed low ethylene productivity, accumulated the mRNAs of these genes at significantly lower levels than those of the wild type, and possessed lower ACS and ACO activities (Fig. 2, 3).
These results suggest that the low ethylene production in the F1 hybrid fruit was due to low transcription of the genes encoding ACS and ACO, and that this low transcription was caused by the heterozygous effect of the rin gene. Among the multigene family of the ACS gene, LeACS2 and LeACS4 have been reported to be expressed mainly in ripening tomato fruit. The real-time PCR assay estimated the mRNA accumulation of LeACS2 during ripening and that LeACS2 would therefore contribute primarily to the ACS activity during fruit ripening. MADS-box transcription factors are known to bind to one of two classes of binding sites based on the central consensus motifs of 5'CC(A/T)3GG-3' and 5'CTA(A/T)3TAG-3'. The LeACO1 promoter region of 1,952 bp (DDBJ accession no. X58273) contains three sites of the latter motif at −1,898 to −1,889, −1,715 to −1,706, and −1,007 to −998, but no site of the former motif. We could not find these two motifs in either the LeACS2 promoter region of 3,055 bp (X59139) or LeACS4 of 2,283 bp (M88487).

In our preliminary experiment, the LeMADS-RIN protein exhibited binding activity to the latter motif in vitro. Therefore, LeACO1 might be a direct target of LeMADS-RIN. Our preliminary data showed that an exogenous ethylene treatment increased the red coloring of F1 hybrid fruits, suggesting that the low level of ethylene production in the F1 hybrid fruit affected fruit ripening. Therefore, transcriptional regulation of the ACS and ACO genes by a heterozygous effect of the rin gene must be one of the key factors in controlling the ripening process of the F1 hybrid fruit. Determination of the direct targets of LeMADS-RIN and the biochemical function of the rin mutant protein are the important subjects for future study.
Acknowledgments

The authors are grateful to Professor H. Mori of Nagoya University for his valuable comments. This research was supported in part by grant-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a grant from the Ministry of Agriculture, Forestry, and Fisheries of Japan (Development of Novel Processing/Distribution Technology for the Enhancement of Domestic Agricultural Products).

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