

Reduction of Allergenic Proteins by the Effect of the *ripening inhibitor (rin)* Mutant Gene in an F₁ Hybrid of the *rin* Mutant Tomato

Mamiko KITAGAWA,¹ Tatsuya MORIYAMA,^{2,*} Hirotaka ITO,¹ Sayaka OZASA,² Atsuko ADACHI,³ Junichi YASUDA,⁴ Tetsuya OOKURA,⁴ Takahiro INAKUMA,¹ Takafumi KASUMI,^{4,**} Yukio ISHIGURO,¹ and Yasuhiro ITO^{4,†}

¹Research Institute, Kagome Co., Ltd., 17 Nishitomiya, Nasushiobara, Tochigi 329-2762, Japan

²Graduate School of Agriculture, Kyoto University, Gokasho, Uji, Kyoto 611-011, Japan

³Department of Dermatology, Hyogo Prefectural Kakogawa Hospital, 770-1 Awazu, Kakogawa-chou, Kakogawa, Hyogo 675-8555, Japan

⁴National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

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The *ripening inhibitor (rin)* mutant tomato yields non-ripening fruit, and the *rin* hybrid fruit (*RIN/rin*) shows an intermediate phenotype between the wild and mutant fruit, that is, red-ripe and extended shelf life. We found by a microarray analysis that the genes encoding possible allergenic proteins were expressed at a significantly lower level in the *rin* hybrid fruit than in the wild-type fruit. These allergenic proteins, which were β -fructofuranosidase and polygalacturonase 2A (PG-2A), were confirmed to accumulate at a lower level in the *rin* hybrid fruit than in the wild-type fruit. The immunoglobulin E (IgE) in serum from a tomato-allergic patient showed lower reactivity to the extract of the *rin* hybrid fruit than to that of the wild fruit. These results suggest that the *rin* gene has the potential to regulate allergen accumulation in tomato fruit.

Key words: *LeMADS-RIN*; *ripening inhibitor (rin)*; tomato allergen; β -fructofuranosidase; polygalacturonase

Tomatoes (*Lycopersicon esculentum*) are a widely produced and consumed vegetable worldwide. Several studies on the allergic reaction to tomato fruit have reported the clinical symptoms, cross-reactions with other allergens, and identification of possible allergens. Although the most common clinical symptom in patients is the oral allergy syndrome (OAS), in rare cases, patients exhibit a significant immediate hypersensitivity reaction such as dyspnea.^{1–3)} Petersen *et al.*⁴⁾ have reported that sensitization to tomatoes seemed to be particularly prevalent in the Mediterranean area, but that the effect was of minor importance in the more northern

parts of Europe. Cross-allergic reactions to tomatoes have been reported with latex and pollen of grass and Japanese cedar (*Cryptomeria japonica*).^{1,4–7)} Among children with an allergy to grass pollen, 39.2% reacted positively to tomatoes.⁵⁾ Japanese cedar pollinosis is one of the most common allergic disorders in Japan,⁸⁾ and cross-reactivity has been suggested between tomato fruit and Japanese cedar pollen.⁷⁾ Polygalacturonase 2A (PG-2A) derived from tomatoes has reportedly shown 40% similarity at the amino acid level to Cry j 2, a major allergen of Japanese cedar pollen.⁹⁾ To date, the following possible tomato fruit allergens have been identified through an immunoblotting analysis with the patients' sera: PG-2A, β -fructofuranosidase, superoxide dismutase, pectinesterase, suberization-associated anionic peroxidase, and profilin.^{10–12)} The allergenicity of a ripe tomato is reportedly higher than that of an unripe tomato.¹³⁾

Ripening of tomato fruit involves various biochemical and physiological changes such as increased ethylene production, fruit colour development, softening, seed maturation, flavour, texture, and aroma. A number of mutants that exhibited unusual ripening phenotypes have been identified. Of these, the *ripening inhibitor (rin)* mutant, which is the most widely studied, showed negative and pleiotropic effects on the ripening process, including fruit colour development, softening, and increased respiration and ethylene production, and its fruit failed to ripen.¹⁴⁾ This *rin* mutant carries a mutation in *LeMADS-RIN*, a gene that encodes a MADS-box transcription factor.¹⁵⁾ *LeMADS-RIN* appears to act upstream of the earliest known steps in fruit ripening and is specifically expressed during fruit ripening. The

[†] To whom correspondence should be addressed. Fax: +81-29-838-8122; E-mail: yasuito@affrc.go.jp

* Present address: School of Agricultural Chemistry, Kinki University, 3327-204 Naka-machi, Nara-city, Nara 631-8505, Japan

** Present address: Department of Agricultural and Biological Chemistry, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-8510, Japan
Abbreviations: IgE, immunoglobulin E; OAS, oral allergy syndrome; PG-2A, polygalacturonase 2A; *rin*, *ripening inhibitor*; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

extension of the shelf life of fruit is an essential breeding objective, and *LeMADS-RIN* is a possible key factor in manipulating fruit ripening.¹⁶⁾ We have previously developed *rin* hybrid lines from crosses between wild and *rin* mutant lines in order to improve the shelf life of fruit.¹⁷⁾ We have characterized one of these hybrid lines, whose fruit exhibited extremely long shelf life and acceptable quality, as a fresh vegetable in terms of its red colouring and flavour.¹⁷⁾

In this report, we describe that the *rin* mutant gene affected the accumulation of tomato-fruit allergenic proteins and the possibility of low allergen accumulation in the *rin* hybrid fruit. We studied this fruit by a microarray assay to characterize the extended shelf life of the *rin* hybrid fruit. The analysis of the data unexpectedly revealed a low accumulation of mRNA encoding the proteins causing tomato fruit allergy. We therefore evaluated the reactivity of IgE from the serum of a tomato allergic patient with an extract of the *rin* hybrid tomato. We discuss from the results of these experiments the new possibility of utilizing the *rin* mutant gene to control the accumulation of tomato-allergenic proteins.

Materials and Methods

Plant materials. The *rin* hybrid line, Kc01-6 (*RIN/rin*), was derived from a cross between the wild-type line, PK331 (*RIN/RIN*), and the *rin* mutant line, PK353 (*rin/rin*).¹⁷⁾ The cultivar “*Momotaro*,” which is the most popular in the Japanese market, was used as a wild-type control for an immunoblotting analysis. The plants were grown in a greenhouse, and the fruits of the wild-type and the *rin* hybrid line were harvested at three different maturation stages: mature green (G), pink coloured (P; about 4 days after the fruit skin had started to show a red colour), and red ripe (R; about 3 days after the P stage). The fruits of the *rin* mutant line were harvested at the G stage, and at stages corresponding to the wild type at the P and R stages (about 5 and 9 days, respectively, after the fruit skin had started to show a yellow colour). The harvested fruits were immediately frozen in liquid nitrogen, milled with dry ice, and stored at -80°C .

Microarray analysis. Total RNA from the whole fruit was extracted with SDS/phenol, the polysaccharides were removed,¹⁸⁾ and the extract was purified by lithium chloride precipitation. cDNAs were synthesized from total RNA, hybridized at high stringency to the microarray, and detected with a 3DNATM Submicro expression array detection kit (Genisphere). The microarray used in this study was supplied by the Boyce Thompson Institute Center for Gene Expression Profiling (<http://bti.cornell.edu/CGEP/CGEP.html>). The hybridization intensity was measured with a ScanArray3000 laser scanner (GSI Luminocs) and data were analyzed by MicroArray Suite 3.5 software (Scanalytics). Each of the

microarray experiments was performed in duplicate with the dye reversed.

Real-time PCR analysis. Total RNA was extracted with an RNeasy plant mini kit (Qiagen). Reverse transcription of total RNA (200 ng) was carried out by using random 6-mer primers with the RT reagent (Takara). Real-time PCR analysis was performed with a GeneAmp5700 sequence detection system (Applied Biosystems), using the SYBR green PCR master mix (Applied Biosystems). The expressions of *β -fructofuranosidase* (GenBank accession No. Z12025) and *PG-2A* (A24194) were analyzed with the following primers: *β -fructofuranosidase*, FF-RT-F (CCGGTGACACTGATGATTATGTG) and FF-RT-R (TGACCCAGTCTAGAGGAGAGGAT); *PG-2A*, PG-RT-F (CAGGTGATGATTGTATTTC AATTGTTT) and PG-RT-R (CCATGACCTGGACCACAAGTAA). For the endogenous control, the amount of *18S rRNA* was estimated by using the following primers: 18S-RT-F (TCCTAGTAAGCGCGAGTCATCA) and 18S-RT-R (TCATTCAATCGGTAGGAGCGA).

Preparation of the tomato extract. Crude protein was extracted to analyze the protein accumulation by SDS-PAGE. Frozen, milled fruit was homogenized in ice-cold water containing a proteinase inhibitor (CompleteTM, Roche). After centrifugation at $2,400 \times g$ for 10 min, the pellet was resuspended in ice-cold 1 M NaCl containing the proteinase inhibitor, and the suspension was stirred for 3 h at 4°C . After centrifugation, the supernatant was collected as a crude extract. Protein was extracted for a subsequent immunoblotting analysis. Red-ripe tomatoes were homogenized in an extraction buffer (30 mM Tris-HCl [pH 8.0] and 1 mM EDTA). The homogenate was filtered through three layers of gauze, and then centrifuged at $15,000 \times g$ for 20 min at 4°C . The proteins in the supernatant were precipitated with 80% saturated ammonium sulfate. The precipitate was dissolved in the extraction buffer. After centrifuging again, the supernatant was desalted in a PD-10 column (Amersham Pharmacia Bioscience). The eluate was concentrated with a YM-10 Centricon (Amicon).

Human sera. Sera from patients with an allergic reaction to tomatoes were investigated in this study. Patients' sera were taken after obtaining informed consent to use for allergic research, and kept at -20°C until needed for analysis.

Electrophoresis and immunoblotting. Extracted protein from the tomatoes was subjected to SDS-PAGE. Proteins on the gel were stained with a Quick CBB kit (Wako). The gel was transferred to a Hybond-P PVDF membrane (Amersham Pharmacia Bioscience) for the immunoblotting analysis.¹⁹⁾ The transferred membrane was incubated in 10 mM sodium phosphate-buffered saline (PBS, pH 7.5) containing 0.1% Tween-20 and 5%

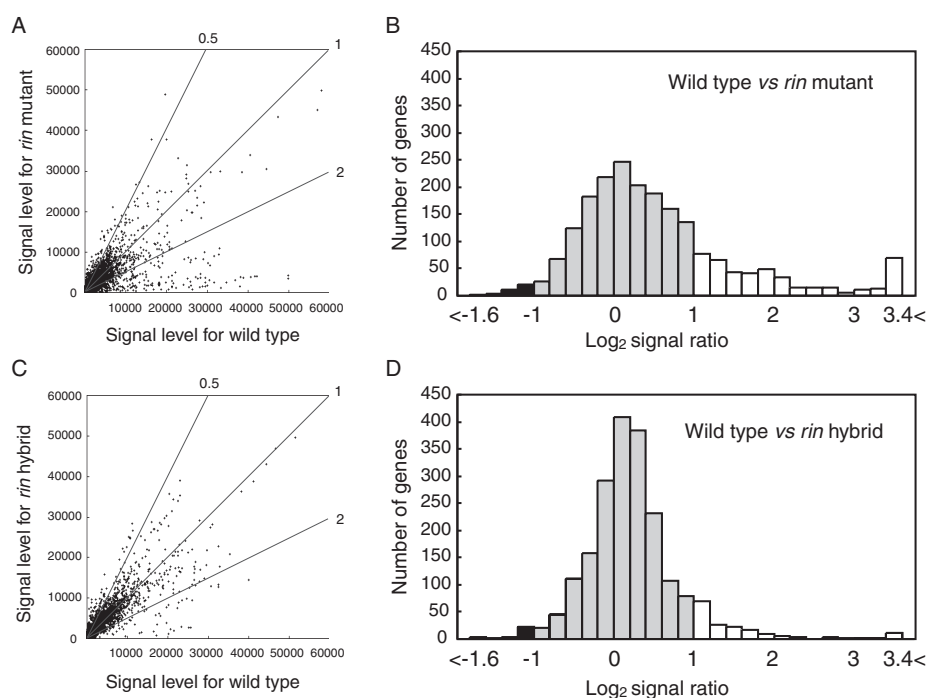


Fig. 1. Microarray Analysis Comparing the Pink-Stage Fruits of the Wild Type Versus the *rin* Mutant or the *rin* Hybrid.

A and C show scatter plots of the signal intensity for pink fruit cDNAs of the wild type versus the *rin* mutant and the wild type versus the *rin* hybrid, respectively. The spots showing a signal intensity of the wild type of more than 2000 were further analyzed to calculate the ratio of the signal intensity value, and the number of spots for each class of the ratio is shown in B for the wild type versus the *rin* mutant and in D for the wild type versus the *rin* hybrid. White bars show the number of spots having a more than 2-fold-stronger signal in the wild fruit and black bars show the number in the mutant or the *rin* hybrid fruit. Gray bars show the number of spots having a log₂ signal ratio between -1 and 1.

skim milk for blocking. The membrane was incubated overnight at 4 °C with the patient's serum diluted 1:20 in the same buffer. Bound IgE antibodies were detected by using HRP-conjugated goat anti-human IgE (Kirkegaard and Perry Laboratories) and an ECL western blotting kit (Amersham Pharmacia Bioscience).

Analysis of the N-terminal amino acid sequence. After electrophoresis, the SDS-PAGE gel was blotted to a Immobilon-PSQ PVDF membrane (Millipore), before the membrane was stained with CBB R-250. The objective protein bands were excised and subjected to N-terminal amino acid sequencing with an HP G1000A protein sequencer (Hewlett Packard).

Results

Screening of the suppressed genes in the *rin* hybrid fruit by the microarray analysis

To analyze the *rin*-genotype effect on the ripening fruit, the gene expression pattern of the wild-type fruit harvested at the P stage was compared with the *rin* hybrid and the *rin* mutant fruits by a cDNA microarray of tomato.²⁰⁾ Genes expressed at a higher level in the wild-type fruit than in the mutant had the potential to contribute to fruit ripening. Of these genes, those expressed at a low level in the *rin* hybrid fruit may be responsible for the extended shelf life of the *rin* hybrid

fruit. Figures 1A and C respectively show the results of the microarray assay comparing pink fruit cDNAs of the wild type versus the *rin* mutant and of the wild type versus the *rin* hybrid. Guide lines are provided in the plots showing when the signal ratios are 1, 2, and 0.5. The data show that many genes were expressed at a higher level in the wild-type fruit than in either the *rin* hybrid or mutant. In addition, the scatter was less for the *rin* hybrid than for the *rin* mutant. The spots whose signals were detected at a low level showed low reproducibility, so those spots showing signals of the wild type at more than 2000 in Fig. 1A were analyzed further. The signal ratios of these 2047 spots were analyzed and are shown in Figs. 1B and D. In the wild-type versus the mutant fruits, the difference in expression pattern was obvious, with 458 spots showing more than a 2-fold stronger signal in the wild-type fruit (Fig. 1B). In the wild-type versus the *rin* hybrid fruits, 174 spots showed more than a 2-fold stronger signal in the wild-type fruit, although the *rin* hybrid fruit showed a more similar gene expression pattern to the wild-type fruit than the *rin* mutant fruit did (Fig. 1D). Of these 174 spots, down-regulation was confirmed in several genes by the northern blotting analysis or real-time PCR analysis. These genes were as follows: *PG-2A* (DDBJ accession No., A24194), *LeACS2* (X59145), *LeACS4* (M88487), *LeACO1* (A35021), *LeEXP1* (U82123), β -fructofuranosidase (Z12025), and the short-chain alco-

hol dehydrogenase-like gene (clone ID, cLED6A8). Of these, β -fructofuranosidase and PG-2A had previously been identified as tomato allergenic proteins.¹⁰⁾ On the microarray spots of β -fructofuranosidase (clone ID, cLEC6K14) and PG-2A (cLEG12J1), the signal intensities of the *rin* hybrid fruit were 8.3% and 33% of the wild-type fruit.

Expression analysis of β -fructofuranosidase and PG-2A in the *rin* hybrid

The gene expression of β -fructofuranosidase and PG-2A during fruit ripening was quantified by real-time PCR. In the wild-type fruit, the expression of β -fructofuranosidase mRNA was observed during ripening, the highest accumulation being detected at the R stage (Fig. 2A). In contrast, little transcription was detected in the mutant fruit throughout ripening (Fig. 2A). In the hybrid fruit as well as in the wild fruit, the mRNA accumulation increased as the fruit ripened, although, at the R stage, the amount was less than half that in the wild fruit (Fig. 2A). PG-2A mRNA rapidly increased at the P stage and decreased at the R stage in the wild fruit, whereas little transcription was detected in the mutant fruit throughout ripening (Fig. 2B). In the hybrid fruit, as well as in the wild

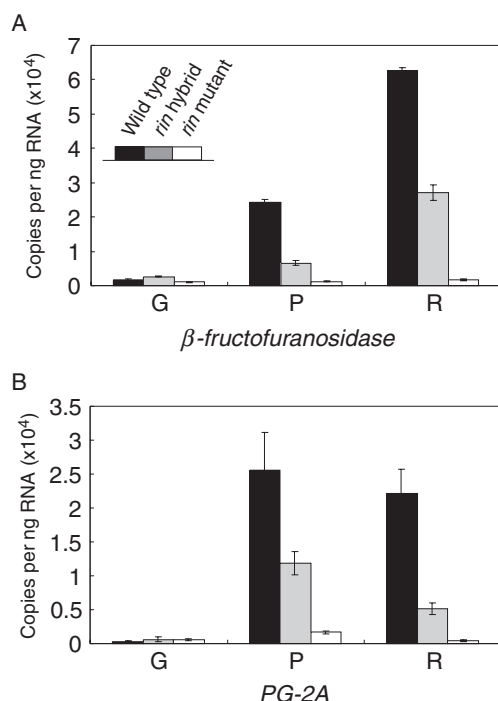


Fig. 2. mRNA Quantification of β -Fructofuranosidase and PG-2A in Tomato Fruit of the Wild-Type Parent, *rin* Hybrid, and *rin* Mutant Parent by a Real-Time PCR Method.

mRNAs of β -fructofuranosidase and PG-2A were analyzed in fruits at the G, P and R stages. The copy number of mRNA molecules per ng of extracted RNA was obtained from the normalization by the amount of 18S rRNA (X51576) in each sample. Error bars denote the standard deviation of the measurement of three independent reactions.

fruit, the level of PG-2A mRNA increased at the P stage, although the amount in the hybrid fruit was less than half that in the wild fruit at the P stage (Fig. 2B).

The protein accumulation in the *rin* hybrid fruit was compared with that in the wild fruit and the mutant fruit. Aliquots of crude protein extracts from equal amounts of fruit tissue were subjected to SDS-PAGE. As shown in Fig. 3A, the protein bands of 50-kDa and 22-kDa shown in the wild fruit at the P and R stages were faint in the hybrid fruit (shown as "a" and "c" in Fig. 3A). The 46-kDa band in the wild fruit at the R stage was also faint in the hybrid fruit (shown as "b" in Fig. 3A). β -Fructofuranosidase had previously been identified as IgE-reacted protein that was detected at 22, 25, 50 and 52-kDa in the SDS-PAGE assay, and PG-2A was also identified at 36- and 46-kDa.^{4,10,21)} We therefore tried to identify the three protein bands indicated as "a," "b," and "c" in Fig. 3A. As shown in Fig. 3B, the N-terminal amino acid sequences of the 50-kDa and 22-kDa proteins showed almost complete similarity to tomato β -fructofuranosidase, and that of the 46-kDa protein showed similarity to PG-2A.

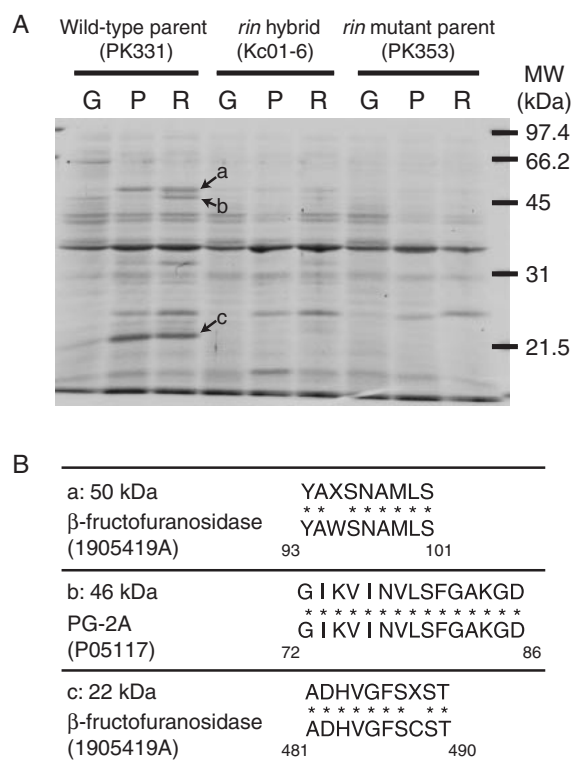


Fig. 3. Effect of the *rin* Mutant Gene on the Protein Accumulation in Tomato Fruit.

A, SDS-PAGE analysis of a fruit extract of the wild-type parent, *rin* hybrid, and *rin* mutant parent harvested at the G, P and R stages. Protein bands displayed as "a," "b" and "c" were subjected to N-terminal amino acid sequencing. B, Comparison of the N-terminal amino acid sequence with the known protein sequence. The N-terminal amino acid sequences of the proteins indicated as "a," "b" and "c" in Fig. 3A were determined. The accession number of each protein is indicated under the protein name. Stars indicate identical amino acids.

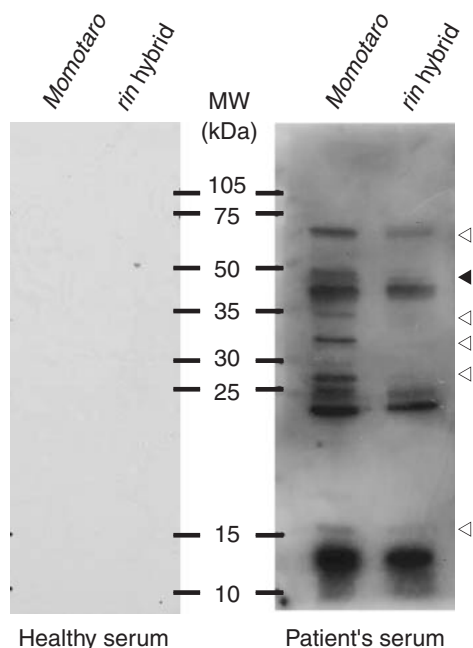


Fig. 4. Immunoblotting Analysis of the Red-Ripe Tomato Fruit Extract Using the Serum from a Tomato-Allergic Patient.

Tomato fruit extracts (100 µg) of the *rin* hybrid and wild-type cultivar, “*Momotaro*,” were electrophoresed and analyzed with the sera from a healthy control and from the patient. The filled arrowhead shows the 50-kDa band which is putative β -fructofuranosidase. Unfilled arrowheads show undetermined IgE reactive proteins whose accumulation was reduced in the *rin* hybrid.

IgE reactivity of the rin hybrid fruit in an OAS patient

To evaluate the IgE reactivity of the *rin* hybrid fruit, a protein blot of the red-ripe fruit extract was incubated with the serum of a patient with OAS to tomatoes. The patient was a 44-year-old female who demonstrated OAS after ingesting vegetables and fruits, particularly tomato, peach, melon, water-melon and cucumber, and who also demonstrated pollinosis symptoms from Japanese cedar, *Dactylis glomerata*, and *Ambrosia artemisiaefolia*. As shown in Fig. 4, the IgE reactivity to the hybrid-fruit extract was significantly lower than that to “*Momotaro*,” the most common cultivar in the Japanese market. In this patient, the approximately 50-kDa band that was detected in the wild-fruit extract was clearly absent in the hybrid-fruit extract (Fig. 4, filled arrowhead). Additionally, several bands detected in the blot of the wild-fruit extract were either absent or faint in that of the hybrid-fruit extract (Fig. 4, unfilled arrowhead).

Discussion

β -Fructofuranosidase and PG-2A have been identified as allergens in tomato fruit,^{4,10,21)} and Kondo *et al.*¹⁰⁾ have reported that the sera of most patients with OAS to tomato reacted to β -fructofuranosidase (9 of 11 patients) and to PG-2A (8 of 11). These results suggest that tomato with a low-level accumulation of β -fructofur-

anosidase and PG-2A reduced the allergic potential towards many patients. In the microarray assay shown here, part of the genes related to ripening showed lower expression in the *rin* hybrid fruit (Fig. 1D). We found from an analysis of the data that β -fructofuranosidase and PG-2A were included in these genes. *LeMADS-RIN* regulates the expression of various ripening-related genes including PG-2A.^{22–24)} Since *LeMADS-RIN* showed incomplete dominance, the expression of most of the genes regulated by *LeMADS-RIN* in the hybrid fruit was at a lower level than that in the wild type.¹⁷⁾ The results of the assays of the *rin* hybrid fruit suggest that the low-level protein accumulation of β -fructofuranosidase and PG-2A was caused, at least partially, by transcriptional control, and that these changes were a heterozygous effect of the *rin* gene. We confirmed that the hybrid fruit exhibited lower reactivity to IgE in the serum from the OAS patient (Fig. 4). Moreover, in our preliminary experiments, 10 of 11 patients demonstrating OAS to tomato as proven by a prick test, immunoassay, radioallergosorbent test (RAST), or subjective symptoms also reduced the IgE reactivity with the *rin* hybrid tomato in the immunoblotting analysis (data not shown). We therefore regarded this hybrid as a possible low-allergenicity tomato.

In the immunoblotting assay presented here, several bands were either absent or significantly faint in the hybrid fruit (Fig. 4). Although one band seemed to correspond to one of our targets (the 50-kDa protein), others corresponded to neither of our targets. These bands might have been other processed forms of β -fructofuranosidase or PG-2A. Another possibility is that these bands were another kind of protein whose expression was controlled by *LeMADS-RIN*. The results of the microarray analysis indicate that 174 spots exhibited significantly lower signal intensity in the hybrid fruit than that in the wild fruit (Fig. 1D). It has been reported that many N-glycosidic proteins derived from plants reacted with IgE of patients' sera^{25–27)} and that the N-glycosidic structure was important to be recognized by IgE.^{4,28)} Indeed, β -fructofuranosidase and PG-2A that were focused on in this study are known as N-glycosidic proteins.^{29,30)} The total amount of N-glycosidic proteins obviously increases in tomato fruit during ripening,¹³⁾ suggesting that the accumulation of N-glycosidic proteins in fruit would also be affected by *LeMADS-RIN*. Thus, the N-glycosidic proteins are candidates for the undetermined IgE-reactive bands in Fig. 4. Further analysis is necessary to identify these proteins.

Various plants with a low amount of allergy-causing protein have been developed by using genetic engineering techniques or by the selection of mutants.^{31–35)} Although these approaches are highly effective in eliminating a target protein, they are limited to only one allergen. The result of this study, in contrast, suggests that multiple allergenic proteins accumulated at a significantly low level in the *rin* hybrid fruit (Fig. 4),

although the expression of each gene was not completely suppressed (Fig. 2). Since proteins reactive to IgE depend on the individual patient, the fruit of the *rin* hybrid might be acceptable to a wide range of patients. *LeMADS-RIN* and its *rin* mutant gene have attracted considerable attention as the key factor for controlling fruit ripening.¹⁶⁾ In addition, the results of this study open the new possibility that the *rin* mutant gene can be used to control tomato allergenicity. As shown here, developing the *rin* hybrid lines is one of the practical methods to breed low-allergenicity tomatoes. An investigation of the clinical reaction of tomato-allergic patients to this hybrid fruit is now being planned.

Acknowledgments

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