Enhancement of fruit shelf life by suppressing *N*-glycan processing enzymes

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In a globalized economy, the control of fruit ripening is of strategic importance because excessive softening limits shelf life. Efforts have been made to reduce fruit softening in transgenic tomato through the suppression of genes encoding cell wall–degrading proteins. However, these have met with very limited success. *N*-glycans are reported to play an important role during fruit ripening, although the role of any particular enzyme is yet unknown. We have identified and targeted two ripening-specific *N*-glycoprotein modifying enzymes, α-mannosidase (α-Man) and β-D-*N*-acetylhexosaminidase (β-Hex). We show that their suppression enhances fruit shelf life, owing to the reduced rate of softening. Analysis of transgenic tomatoes revealed ≈2.5- and ≈2-fold firmer fruits in the α-Man and β-Hex RNAi lines, respectively, and ≈30 days of enhanced shelf life. Overexpression of α-Man or β-Hex resulted in excessive fruit softening. Expression of α-Man and β-Hex is induced by the ripening hormone ethylene and is modulated by a regulator of ripening, *rin* (ripening inhibitor). Furthermore, transcriptomic comparative studies demonstrate the down-regulation of cell wall degradation- and ripening-related genes in RNAi fruits. It is evident from these results that *N*-glycan processing is involved in ripening-associated fruit softening. Genetic manipulation of *N*-glycan processing can be of strategic importance to enhance fruit shelf life, without any negative effect on phenotype, including yield.

α-mannosidase | β-D-N-acetylhexosaminidase | fruit softening | RNAi

The postharvest losses of fruits and vegetables in the developing countries account for almost 50% of the produce. India, the world's second largest producer of fruits and vegetables, loses 35–40% of produce because of excessive softening. The softening that accompanies ripening of fruits exacerbates damage during shipping and handling processes. It plays a major role in determining the cost factor, because it has a direct impact on palatability, consumer acceptability, shelf life, and postharvest disease/pathogen resistance (1–3). Generally, reduction in fruit firmness due to softening is accompanied by increased expression of cell wall–degrading enzymes acting upon proteins and carbohydrates (4). However, many efforts to suppress expression of cell wall–degrading enzymes have not provided the insight needed to genetically engineer fruits whose softening can be adequately controlled (5–11). Previous studies have shown that polygalacturonase, pectin methylesterase, β-glucanase, and β-galactosidase are not sufficient to significantly impact texture (5–10, 12). This may be due to the presence of functionally redundant components of a complicated metabolic process (6, 7, 10, 13). It also suggests that the suppression of enzymes acting on cellulose, hemicellulose, and pectin is not sufficient to prevent softening. The improvement in fruit shelf life achieved to date is not adequate, and therefore the identification of new targets is required.

N-glycoproteins are commonly found in plant cell walls, and free *N*-glycans occur as the precursors of glycosylation or glycoprotein proteolysis. The biologic activity of free *N*-glycans has been noted: injection of $Man_3(Xyl)GlcNAc(Fuc)GlcNAc$ and $Man_3GlcNAc$ into mature green tomatoes stimulated ripening, as measured by the red coloration and ethylene production (14). Free *N*-glycans constitute a significant fraction of the soluble oligosaccharide pool in the tomato pericarp. They are present in the pericarp tissue at all stages of tomato development, and the amount increases particularly during ripening. Moreover, the blocking of *N*-glycosylation delayed fruit ripening, which suggests that *N*-glycan processing may be important in the ripening process (15). Therefore, among the suite of enzymes involved in carbohydrate metabolism, we targeted the *N*-glycan processing enzymes α-mannosidase (α-Man; EC 3.2.1.24) and β-D-*N*-acetylhexosaminidase (β-Hex; EC 3.2.1.52). α-Man and β-Hex, members of glycosyl hydrolase families 38 and 20, respectively, are known to break the glycosidic bonds between carbohydrates, as well as between carbohydrate and noncarbohydrate (16, 17). α-Man cleaves the terminal α-mannosidic linkages from both the high mannose type and plant complex type *N*-glycans present in glycoproteins (18), whereas, β-Hex cleaves the terminal *N*-acetyl-D-hexosamine residues and generates the paucimannosidic *N*-glycans present in most plant glycoproteins (19, 20). Moreover, α-Man and β-Hex are present at high levels during the ripening of many fruits, including the climacteric fruit tomato (16, 17). However, their molecular function remains to be elucidated. Here we report the isolation and functional characterization of α-Man and β-Hex and their use to produce transgenic fruits with enhanced shelf life.

Results and Discussion

Identification and Cloning of Tomato Ripening–Specific α-Man and β-Hex. One of the strategies to elucidate fruit softening is to identify and characterize proteins expressed during ripening and whose biochemical activities can be mechanistically related to the observed cell wall changes. Using *p*-nitrophenyl-α-D-mannopyranoside (*p*NP-Man, K_m 4.6 mM) and *p*-nitrophenyl-β-D-*N*-acetylglucosaminide (*p*NP-GlcNAc, K_m 0.225 mM) as substrates, we found the maximum activity of α-Man and β-Hex at the breaker and pink stages of tomato ripening, respectively (Figs. 1*A* and 2*A*). However, α-Man and β-Hex activities were not detected in other parts of the plant (e.g., stem, leaves, and roots). To correlate specific activity with protein accumulation patterns during ripening, immunoblot analysis was performed using polyclonal antibodies raised against β-Hex and α-Man. The analysis revealed maximum accumulation of α-Man and β-Hex proteins at the breaker and pink stages, respectively (Figs. 1*B* and 2*B*). Ripening-related changes like climacteric ethylene production, chlorophyll degradation, lycopene synthesis, and cell wall disassembly start at the breaker stage, and subsequently an increase in expression of ripening-related cell wall hydrolases is evident (13, 21). The accumulation of α-Man and β-Hex at the critical stage of tomato ripening strengthened our hypothesis that they are involved in ripening and/or softening. To address this issue,

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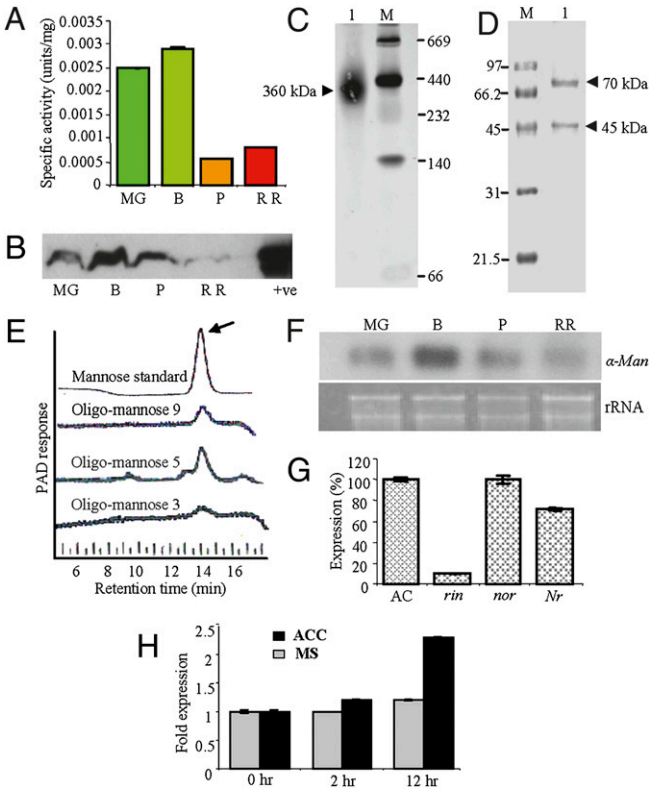

Fig. 1. Identification and isolation of ripening–specific tomato α-Man. (*A*) α-Man activity at ripening stage of tomato. MG, mature green; B, breaker; P, pink; RR, red ripe. Data are mean ± SEM, $n = 4$. (*B*) Immunoblot analysis of α-Man at different stages of ripening with purified enzyme as positive control. (*C*) Purified α-Man resolved on 6% nondenaturing PAGE (lane 1). M, marker (kDa). (*D*) Purified protein denatured and separated on 12.5% SDS/PAGE (lane 1). (*E*) High-performance anion exchange chromatograms show the *N*-glycan processing ability of α-Man. Arrow indicates the release of mannose residues. (*F*) Northern blot shows expression of α-Man gene in wild-type tomato at different stages of ripening. (*G*) qRT-PCR analysis showing the relative expression of α-Man in *rin* (ripening inhibitor), *nor* (nonripening), and *Nr* (never ripe) at the same chronological age of wild type (AC, Ailsa Craig). Data are mean ± SEM ($n = 3$). (*H*) Inducibility of α-Man by ACC, as revealed by qRT-PCR analysis. Data are mean ± SEM ($n = 4$).

we purified (Table S1) and characterized both α-Man and β-Hex from tomato pericarp. Purified α-Man and β-Hex constituted ≈360- and ≈300-kDa proteins, respectively, on nondenaturing PAGE (Figs. 1*C* and 2*C*). However, the molecular masses of α-Man and β-Hex on superdex 200 analytical gel filtration column were found to be ≈290 and ≈206 kDa, respectively. This discrepancy in molecular mass determined by gel filtration and nondenaturing PAGE analyses could be due to the glycoprotein nature, which was confirmed by periodic acid-Schiff (PAS) staining and deglycosylation with endoglycosidase H (Fig. S1 *A–D*). Further, when separated on SDS/PAGE, α-Man and β-Hex resolved into two (70 and 45 kDa) and a single (80 kDa) polypeptide(s), respectively, suggesting that they function as oligomeric proteins in tomato cells (Figs. 1*D* and 2*D*).

On SDS/PAGE, purified α-Man was resolved into two subunits of 70 and 45 kDa, under reducing (with β-mercaptoethanol; Fig. 1*D*) as well as nonreducing conditions (without β-mercaptoethanol; Fig. S1*E*). This indicated that the subunits are associated with the hydrophobic interaction rather than the interdisulfide bonds. These two subunits are probably derived by cleavage of the 114-kDa precursor polypeptide (Fig. S2*A*), which is encoded by α-Man mRNA (3,090 nt). This assumption was made on the basis of the fact that the amino acid sequences of 70- and 45-kDa polypeptides were actually matching with the N-terminal and C-terminal, respectively, of the encoded polypeptide (mass spectrometry analysis). Northern blot analysis using ^{32}P-labeled 3′ or 5′ end sequence (≈500 bp) of α-Man identified a single mRNA species of ≈3 kb (Fig. 1*F*). This suggests that the subunits are the consequence of posttranslational protease cleavage rather than posttranscriptional modification.

The roles of α-Man and β-Hex in ripening and/or softening were examined by cloning the genes from tomato using degenerate primers and then systematically testing their functions. *In silico* analysis of the α-Man sequence revealed the coding region to be 3,090 bp long, encoding a polypeptide of 1,029 aa, with a calculated molecular mass of 114 kDa (Fig. S2*A*). This is comparable to the combined molecular mass of the α-Man subunits (115 kDa), as determined by SDS/PAGE (Fig. 1*D*). The protein has three glycosyl hydrolase domains (Fig. S2*B*) and showed 69% identity with *Vitis vinifera* α-Man (XP_002276092.1). The coding region of β-Hex is 1,728 bp long, encoding a polypeptide of 575 aa with a calculated molecular mass of 64 kDa (Fig. S2*C*), which is less than the molecular mass determined by SDS/PAGE (80 kDa). This was attributed to posttranslational modifications because it has eight probable *N*-glycosylation sites (Fig. S2*C*), and the glycoproteic nature of the protein was confirmed by PAS staining (Fig. S1*A*). However, deglycosylation of the purified protein with EndoH revealed a ≈5-kDa glycans moiety (Fig. S1*C*). This suggests that there could be an involvement of other kinds of posttranslational modification(s) in addition to the glycosylation. β-Hex has two domains related to glycosyl hydrolase 20 (Fig. S2*D*) and showed 68% identity with the *V. vinifera* ortholog (XP_002266897.1). To verify gene expression patterns and to corroborate earlier results, Northern blot analysis was performed, which revealed that α-Man and β-Hex transcripts were most abundant at the breaker and pink stages of ripening, respectively (Figs. 1*F* and 2*F*).

α-Man and β-Hex Are the Cell Wall Proteins Involved in *N*-glycan Processing. During ripening, many fruits, including tomato, disassemble the components of the cell wall, resulting in changes in the cell wall rheologic properties and softening of the ripe fruit (3, 4). The subcellular localization revealed both α-Man and β-Hex to be cell wall proteins (Fig. S3 *A* and *B*). Free *N*-glycans in the pericarp account for >1 μg/g of the fresh weight of tomato, which further increases during the ripening process (15). Moreover, blocking of *N*-glycosylation with tunicamycin delays fruit ripening. Further, when injected in fruits, *N*-glycans are known to stimulate red coloration and ethylene production (14). To determine the *N*-glycan processing ability of α-Man and β-Hex, the purified enzymes were incubated with different *N*-glycans commonly found in fruit pericarp. Further, release of mannose or GlcNAc was determined by high-performance anion exchange chromatography (Figs. 1*E* and 2*E*). Cell wall localization and *N*-glycan processing abilities of α-Man and β-Hex suggest their participation in the degradation of cell wall *N*-glycoproteins and the generation of free *N*-glycans, which further stimulate ripening, possibly by interacting with the protein(s) to transduce the potential ripening signal.

Expression of α-Man and β-Hex Is Inhibited in Tomato Ripening–Impaired Mutants and Regulated by Ethylene. The expression of α-Man and β-Hex particularly during ripening led us to examine the ripening-impaired mutants *rin*, *nor*, and *Nr*. Expression analyses revealed that α-Man transcript levels were ≈10% and 70% of wild type in *rin* and *Nr* mutants, respectively, whereas, α-Man transcript level was similar to wild type in the *nor* mutant (Fig. 1*G*). The transcript levels of β-Hex were ≈20% and 10% of wild type in *rin* and *nor* fruits, respectively. However, in the case of *Nr* fruits, β-Hex transcript levels were ≈40% and ≈10% at pink and red ripe stages, respectively (Fig. 2*G*). These mutants are deficient in ripening-associated ethylene biosynthesis or ethylene perception, and they exhibit delayed fruit softening (22–24). The reduced expression of α-Man and β-Hex in these mutants strongly suggests their involvement in fruit softening and regulation by ethylene (25), through the

NEVER RIPE (NR) receptor. We show that 1-aminocyclopropane 1-carboxylic acid (ACC), the precursor of ethylene, induces α-Man and β-Hex in tomato seedlings ≈2.5- and ≈4-fold, respectively (Figs. 1*H* and 2*H*). These observations indicate that α-Man and β-Hex are regulated by ethylene and act downstream of ripening regulators.

Silencing of α-Man or β-Hex Resulted in Firmer Fruits with Reduced Softening and Enhanced Shelf Life. For functional characterizations of α-Man and β-Hex, we resorted to the "knockdown" approach to demonstrate their roles in ripening and/or softening. Endogenous expression of α-Man and β-Hex was silenced in tomato by expression of gene-specific hairpin RNAs under the control of CaMV 35S promoter (26) (Fig. S2*E*). Stable RNAi along with the antisense and overexpression lines were raised by *Agrobacterium*-mediated transformation of tomato cotyledons. To confirm and quantitate suppression of genes in RNAi fruits, quantitative real-time RT-PCR (qRT-PCR) was performed, which revealed up to 99% suppression of α-Man and β-Hex expression at the breaker and pink stages, respectively. However, antisense lines showed 55–80% suppression, and overexpression lines had up to 30-fold more transcript level than control (Fig. S4 *A–C*). Furthermore, we confirmed the generation of α-Man and β-Hex specific 21–23 mer siRNAs, which is the hallmark of RNAi-mediated silencing (Fig. 3*C*).

Prolonging the desirable texture during ripening is the key to increasing fruit shelf life (27). For texture analysis and shelf life determination, transgenic and control (only vector transformed/nontransformed) fruits were harvested at the pink stage and stored at room temperature (23–25 °C and 55–60% relative humidity). To quantify texture, we analyzed the firmness of the fruit (*Materials and Methods*), which revealed enhancement of firmness in α-Man and β-Hex RNAi fruits. Ten days after the pink stage, α-Man and β-Hex RNAi fruits were ≈2.5-fold and ≈2-fold firmer than control, respectively (Fig. 3*B*), and showed no signs of deterioration up to 45 days (Fig. 3*A*). The α-Man and β-Hex antisense fruits retained the texture for 25–30 days and were ≈1.5-fold firmer than control. Overexpression lines showed early signs of fruit deterioration and were ≈30% softer than their counterpart at the pink stage (Fig. 3*B*). The firmness of T_1 and T_2 fruits of α-Man and β-Hex RNAi plants was compared with that of T_0 fruits, which revealed a stable and heritable transfer of the character (Fig. S5 *A* and *B*). The RNAi lines had no negative effect on vegetative growth, fruit development, days to maturity, seed production, and yield (Table S2). Moreover, RNAi fruits essentially underwent normal climacteric ripening and color development while attached to the plant but held their texture and showed longer vine life. Further, time-lapse photography revealed that RNAi fruits, harvested at the pink stage, retained their texture and firmness up to 45 days, whereas control started shrinking and losing their texture after 15 days (Fig. 3*A*). We then investigated the cell wall changes during ripening of transgenic fruits and found that the cell wall of α-Man RNAi fruits was much more compact with more polysaccharide deposition on the wall than control (Fig. 3*D*). However, reduced cell separation was observed in β-Hex RNAi fruits as compared with control (Fig. 3*E*). The enzymes α-Man and β-Hex target glycoproteins and cleave the terminal α-mannose and GlcNAc residues, respectively, present in *N*-linked glycans. Therefore, to know the status of the α-mannose- and GlcNAc-containing glycoproteins in RNAi fruits, we performed lectin blotting using *Galanthus nivalis* agglutinin and wheat germ agglutinin. The analysis revealed enhanced levels of α-mannose- and GlcNAc-containing glycoproteins in the fruit of α-man and β-Hex RNAi lines, respectively (Fig. S6 *A* and *B*). To substantiate these results, we isolated the *N*-glycans linked to glycoproteins and found increased levels in RNAi fruits compared with control (Fig. S6*C*). These results suggest that the intact cell wall polysaccharides are broken down to a lesser extent in RNAi fruits as compared with control.

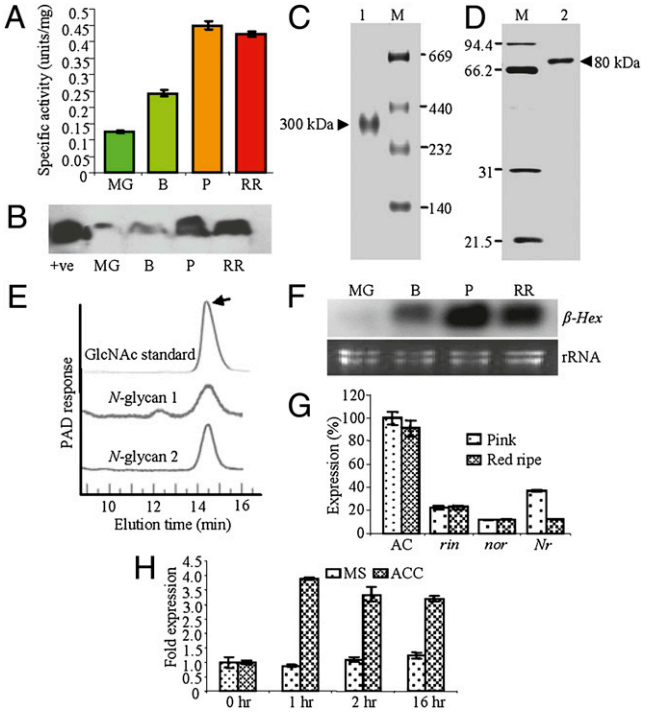

Fig. 2. Identification and isolation of ripening-specific tomato β-Hex. (*A*) β-Hex enzyme activity measured during tomato ripening. MG, mature green; B, breaker; P, pink; RR, red ripe. Data are mean ± SEM ($n = 4$). (*B*) Immunoblot shows β-Hex protein level during ripening stages of tomato. (*C*) Six percent nondenaturing PAGE analysis of purified protein (lane 1). (*D*) Purified β-Hex resolved on 12.5% SDS/PAGE (lane 2). M, marker (kDa). (*E*) *N*-glycan processing function of β-Hex was determined by high-performance anion exchange chromatography. *N*-glycan 1 and *N*-glycan 2 are the biantennary *N*-linked core pentasaccharide and asialo, agalacto, biantennary, respectively. (F) Expression of β-Hex gene in wild-type tomato, at ripening stages, determined by Northern blot analysis. (*G*) Relative expression of β-Hex in *rin*, *nor*, and *Nr* mutants compared with wild type (AC, Ailsa Craig). (*H*) ACC inducibility of β-Hex, determined by qRT-PCR analysis. Data are mean ± SEM ($n = 3$).

Suppression of α-Man or β-Hex Leads to Down-regulation of Ripening-Related Genes. Overall, the results demonstrate a substantial improvement in fruit shelf life by targeting *N*-glycan-processing enzymes. Furthermore, *N*-glycan processing significantly affects ripening-associated changes in the cell wall: transgenic fruits showed reduced cell separation and compact cell wall compared with control (Fig. 3 *D* and *E*). Therefore, we were interested in knowing whether *N*-glycans, generated by α-Man and β-Hex activities, could play a physiologic role in the regulation of gene expression patterns, related to fruit ripening processes. For this, we performed comparative transcriptomic studies of pink-stage β-Hex RNAi fruits and breaker-stage α-Man RNAi fruits vs. control (only vector transformed). The analyses revealed down-regulation of many genes that are associated with fruit ripening and cell wall degradation in β-Hex and α-Man RNAi fruits (Table S3). Further, to validate these results, qRT-PCR was performed for a few genes related to fruit ripening and/or softening (Fig. 4 *A* and *B*). The RNAi fruits showed down-regulation of genes that encode certain cell wall–degrading proteins, such as pectin methylesterase, glucan endo-1,3-β-D-glucosidase, β-1,3-glucanase, endo-xyloglucan transferase, pectinesterase, expansin, pectinacetylesterase, α-galactosidase, pectate lyase, (1-4)-β-mannan endohydrolase, and β-galactosidase. Therefore, suppression of α-Man or β-Hex activity in transgenic fruits not only inhibited *N*-glycoprotein degradation but also affected cellulose, hemicellulose, and pectin degradation. This effect on the other cell wall–degrading

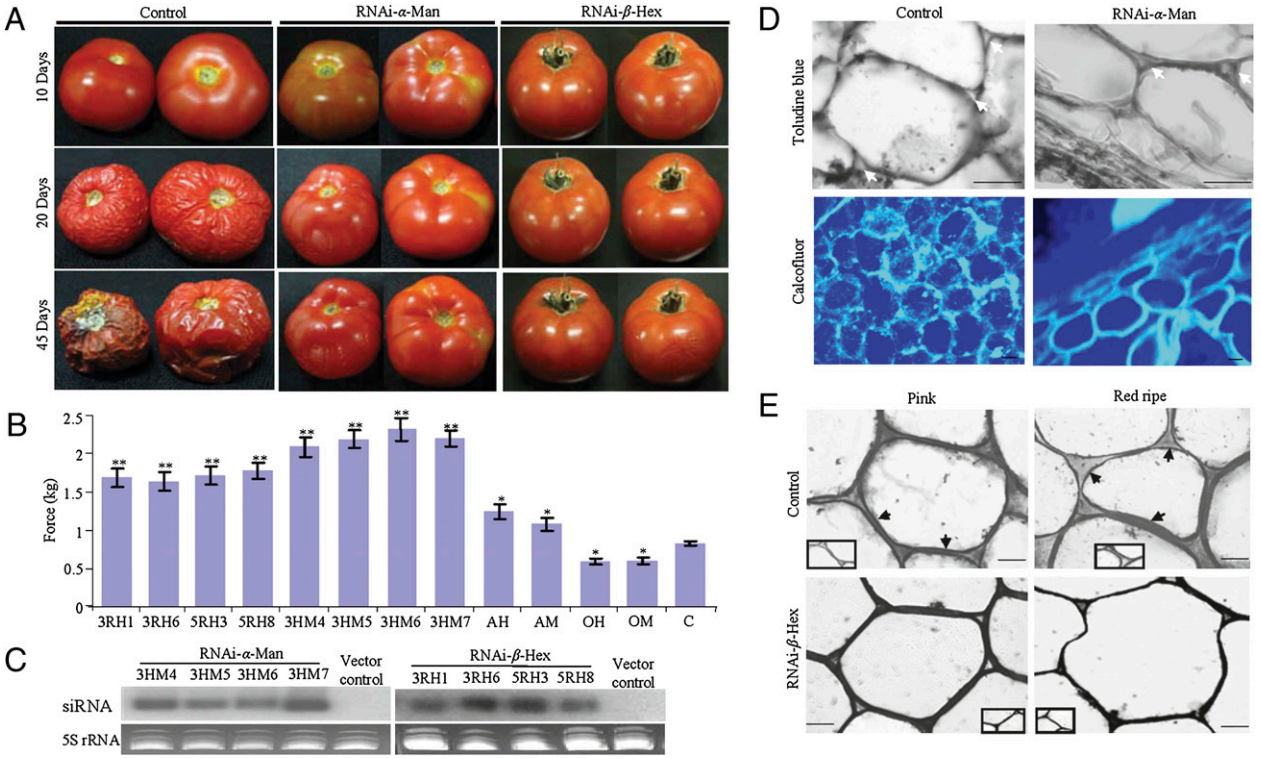

Fig. 3. Silencing of α-Man or β-Hex enhances tomato shelf life. (*A*) Transgenic (T_0) and wild-type (control) fruits were harvested at pink stage and stored at room temperature (22–24 °C in 55–60% relative humidity). The progression of fruit deterioration was recorded by time-lapse photography. Time after harvest is specified by days. (*B*) Texture analysis of α-Man and β-Hex suppressed or overexpressed T_0 generation transgenic fruits, done at 10 days after pink stage. 3RH1, 3RH6, 5RH3, and 5RH8, RNAi lines of β-Hex; 3HM4, 3HM5, 3HM6, and 3HM7, RNAi lines of α-Man; AH and AM, antisense lines of β-Hex and α-Man, respectively; OH and OM, overexpression lines of β-Hex and α-Man, respectively; C, wild-type control. Data are mean ± SEM ($n = 15$). $^{**}P < 0.0005$; $^{*}P < 0.005$. (*C*) Detection of gene-specific siRNAs by Northern blot analysis in RNAi fruits targeting either α-Man or β-Hex. (*D*) Light microscopy images of tomato pericarp to study cell wall at 20 days after pink stage. Images with toluidine blue and calcofluor are depicted for RNAi-α-Man and vector control. Arrows indicate intercellular spaces. (Scale bars, 20 μm.) (*E*) β-Hex RNAi fruits exhibited reduced cell separation as compared with control (wild type). Cell walls of pink and red ripe stage fruits from control and RNAi plants were visualized under bright-field microscopy with toluidine blue. Arrows indicate intercellular spaces. *Inset:* Cell wall region. (Scale bars, 10 μm.)

enzyme(s) may be due to inhibition of formation of biologically active free *N*-glycans in β-Hex/α-Man-RNAi fruits, which are known to induce ripening (15). Furthermore, reduced accumulation of free *N*-glycans in RNAi fruits compared with control suggests the possible involvement of free *N*-glycans during softening (Fig. S6*D*). Conversely, the degradation products of enzymes, which act on cellulose, hemicellulose, and pectin, are not reported to act as inducers of ripening. Therefore, suppressing the activities of α-Man and β-Hex has an added advantage over the others by not only blocking degradation of the cell wall *N*-glycoproteins but also by inhibiting possible ripening-inducing events activated by free *N*-glycans. Moreover, we cannot rule out the possibility of α-Man and β-Hex modulating the function of cell wall–modifying enzymes through their maturation process.

We also found that the expression of ethylene biosynthesis genes (S-adenosylmethionine synthetase, ACC synthase, and ACC oxidase) and transcription factors like ERF and SPL3/LeCNR were suppressed in RNAi fruits (Fig. 4 *A* and *B* and Table S3); ERF is involved in the expression of ethylene-inducible genes, and LeCNR, a MADS box protein, is essential for fruit ripening (28, 29). Although α-Man and β-Hex are induced by ethylene (Figs. 1*H* and 2*H*), they may also further act by promoting ethylene biosynthesis, possibly through free *N*-glycans, which when injected in the fruit demonstrated ethylene production (14). Altogether, our results provide evidence that α-Man and β-Hex function through degradation of cell wall glycoproteins, followed by an increase in the free *N*-glycans level. The results also address the occurrence of free *N*-glycans in tomato and their potential involvement in ripening and/or softening. A model presented in Fig. S6*E* illustrates the function and regulation of α-Man and β-Hex during climacteric fruit ripening.

Conclusion

This work identified two *N*-glycan processing enzymes and their cognate genes, contributing to ripening-associated softening in tomato. Our strategy allows the achievement of desired texture/firmness and enhancement of fruit shelf life by judicious manipulation of *N*-glycan processing without any ill effects. The study highlights the importance of free *N*-glycans and their processing in developmental processes like ripening. It also extends the scope of carbohydrate active enzymes (CaZymes). The high levels of α-Man and β-Hex activities in the fruits such as papaya, banana, and mango also suggest their potential involvement in the softening of other climacteric fruits (30). In conclusion, the engineering of plants with modified *N*-glycan processing ability provides a strategy for crop improvement that can be extended to other important fruit crops.

Materials and Methods

Plant Material and Growth Conditions. Tomato (cv. Pusa ruby) seeds were obtained from the National Seeds Corporation Ltd., New Delhi. Mutants used in the study were procured from the Tomato Genetics Resource Center, University of California at Davis, and were in Ailsa Craig background. Seeds were germinated in presterilized soil and later transplanted to the greenhouse with at ≈25 °C, 70% humidity, and 14/10 h light/dark regimen. For analysis, fruits were harvested at mature green, breaker, pink, and red ripe stages after tagging of flowers at anthesis.

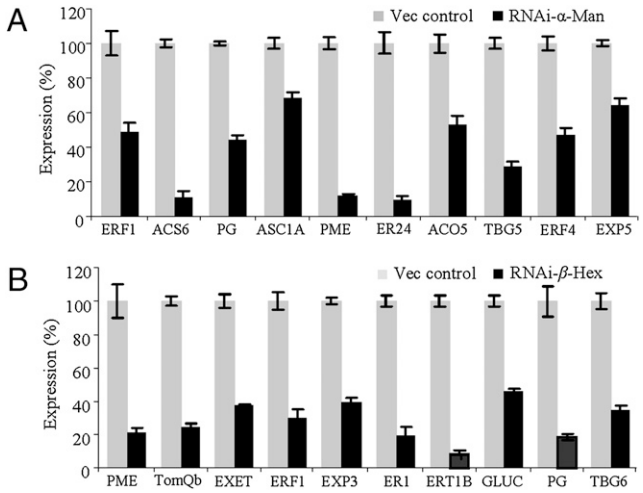

Fig. 4. Suppression of α-Man or β-Hex causes down-regulation of ripening-related genes. qRT-PCR analysis revealed suppression of some ripening-related genes in α-man (*A*) and β-Hex (*B*) RNAi fruits. Genes identified through microarray analysis were selected for qRT-PCR analysis. Tomato actin was used as endogenous control. Data are mean ± SEM (n = 3). ACO5, ACC oxidase (Q6A1K7); ACS6, ACC synthase (Q9SAZ4); ASC1A, ACC synthase (Q9FY02); GLUC, β-1,3-glucanase (Q01413); EXET, endo-xyloglucan transferase (Q40144); ER1, fruit-ripening protein (P20076); ER24, ethylene responsive transcriptional coactivator (Q9SWW1); ERF1, ethylene response factor 1 (Q8S9H4); ERF4, ethylene response factor 4 (Q84XB0); ERT1B, ripening-related mRNA (×72729.1); EXP3, expansin (Q9ZP33); EXP5, expansin (Q9ZP31); PG, polygalacturonase (Q9ZPI8); PME, pectin methylesterase (Q43143); TBG5, β-galactosidase (Q9LLT0); TBG6, β-galactosidase (Q9LLS9); TomQb, glucan endo-1,3-β-D-glucosidase (Q42890).

Protein Purification, Mass Spectrometry, and Gene Cloning. α-Man and β-Hex enzymes were purified from tomato pericarp by subjecting to ammonium sulfate precipitation, followed by anion exchange, ConA affinity (only for β-Hex), and gel filtration chromatography. Purified protein was characterized by 12% SDS/PAGE, 6% nondenaturing PAGE, glycoprotein staining, and EndoH treatment. The protein gel bands were cut from Coomassie-stained SDS/PAGE and analyzed by electrospray ion trap time-of-flight mass spectrometry (LC-MS/MS) (Q-Star Pulsar *i*; Applied Biosystems). The spectra were analyzed by Mascot sequence matching software (Matrix Science) against the Viridiplantae (green plants) database. Degenerate primers were used to clone α-Man and β-Hex through 3′ and 5′ RACE methods (*SI Materials and Methods*).

Staining and Microscopy. Fruit sections were cut with the help of a cryostat microtome (Leica CM1510S) and were allowed to dry on the slides. The slides were then dipped in aqueous solution of 0.05% toluidine blue (31) in 0.1 M phosphate buffer at pH 6.8 for 2 min and were washed with water for 2 min. To examine the cell wall structure, the sections were immersed in 0.05% calcofluor (32) and washed with distilled water. The stained sections were mounted in water under a coverslip and photographed using a Nikon 80i epifluorescent/phase contrast/bright field microscope. The subcellular localization methods are described in *SI Materials and Methods*.

qRT PCR Analysis. For gene expression analysis, 5μg total RNA was extracted by the LiCl precipitation method (33) and was reverse transcribed and used for qRT PCR analysis (34) with SYBR green dye (*SI Materials and Methods*).

High-Performance Anion Exchange Chromatography, Immunoblotting, and Detection of siRNA. One microgram of enzyme was incubated with 100 ng of the *N*-linked oligosaccharide (Dextra) at 37 °C overnight. After passing through the PVDF membrane, 10 μL of the sample was analyzed with an HPAE-PAD system (Dionex DX 500 BioLC) using a Carbopac PA-1, 4 × 250-mm column. The standard (mannose or GlcNAc) was injected at a concentration of 100 nmol before and after each analysis. For immunoblot analysis, proteins were resolved on 12.5% SDS/PAGE and later electro-transferred to nitrocellulose membrane. After blocking the nonspecific sites, the membrane was incubated with the primary antibody specific to β-Hex or 70-kDa subunit of α-Man (1:2,500). Immunodetection was done with HRP-conjugated secondary antibody (Thermo Scientific). Total RNA was enriched for the small-molecular-weight RNA as described previously (35). Small-molecular-weight RNA was resolved on 15% urea PAGE and electro-transferred to nylon membrane. The nylon membrane was UV cross-linked, and Northern blotting was carried out (*SI Materials and Methods*).

RNAi/Antisense/Overexpression Plasmids Construction and Tomato Transformation. α-Man and β-Hex were silenced using the pHANNIBAL vector as described previously (26). Antisense constructs were prepared in pBI121 by replacing the GUS gene and cloning the α-Man or β-Hex full-length sequence in reverse orientation. Overexpression constructs consisted of full-length sequence of α-Man or β-Hex gene cloned in pBI121. To generate transgenic tomato plants, cotyledons from 2-week-old seedlings were used as described previously (36). Briefly, tomato seeds were sterilized using 4% commercial bleach and germinated on Murashige and Skoog (MS) medium. After 2 weeks of germination, the cotyledons were cut and cocultivated for 30 min with *Agrobacterium tumefaciens* strain EHA105 harboring different constructs. The cotyledons were then collected for selection on MS plates containing 50 mg/L kanamycin. When the plantlets regenerated, they were transferred to rooting medium. Transgenic seeds were germinated in MS medium containing 50 mg/L kanamycin to get the progeny plants.

Textural Analysis. Fruit firmness was determined using TA-XT Plus (Stable Microsystems). Each fruit was analyzed with a 75-mm-wide P75 compression plate and compressed to a vertical displacement of 5 mm with the test speed of 1 mm s^{-1}. The firmness was defined as the response force to a 5 × g applied force. The significance was calculated by unpaired t test.

ACC Treatment. Fifteen-day-old tomato seedlings, germinated on MS media, were transferred to liquid MS media containing 1 mM ACC, harvested at the different time points, and frozen immediately in liquid nitrogen.

Microarray. To know any changes in gene expression profile due to silencing of α-Man and β-Hex genes, we compared the transcriptomes of RNAi and control fruits using Affymetrix GeneChip tomato genome array. This comprehensive array consists of more than 10,000 *Solanum lycopersicum* probe sets to interrogate more than 9,200 *S. lycopersicum* transcripts. Duplicate biologic samples from transgenic and vector control were used for microarray analysis. Fruits from breaker (α-Man) or pink (β-Hex) stages of ripening were taken for analysis. Detailed methods are described in *SI Materials and Methods*.

Lectin Blotting and *N*-glycans Isolation. Proteins were resolved on 12.5% SDS/PAGE and electro-transferred to the nitrocellulose membrane. Lectin blotting (*SI Materials and Methods*) was performed using wheat germ agglutinin (binds to GlcNAc; Sigma) or *G. nivalis* agglutinin (binds to mannose; Roche). Glycoprotein-linked *N*-glycans and free *N*-glycans were isolated as described previously (37, 38), with few modifications (*SI Materials and Methods*).

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