Insights into transcriptional regulation of β-D-N-acetylhexosaminidase, an N-glycan-processing enzyme involved in ripening-associated fruit softening

Mohammad Irfan*, Sumit Ghosh*,†, Vinay Kumar, Niranjan Chakraborty, Subhra Chakraborty and Asis Datta‡

National Institute of Plant Genome Research, New Delhi 110067, India

* These authors contributed equally to this work.
† Present address: CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow 226015, India
‡ To whom correspondence should be addressed. E-mail: asis_datta@rediffmail.com, asis_datta@nipgr.res.in

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Abstract

Tomato (Solanum lycopersicum) fruit ripening-specific N-glycan processing enzyme, β-D-N-acetylhexosaminidase (β-Hex), plays an important role in the ripening-associated fruit-softening process. However, the regulation of fruit ripening-specific expression of β-Hex is not well understood. We have identified and functionally characterized the fruit ripening-specific promoter of β-Hex and provided insights into its transcriptional regulation during fruit ripening. Our results demonstrate that RIPENING INHIBITOR (RIN), a global fruit ripening regulator, and ABSCISIC ACID STRESS RIPENING 1 (SlASR1), a poorly characterized ripening-related protein, are the transcriptional regulators of β-Hex. Both RIN and SlASR1 directly bound to the β-Hex promoter fragments containing CArG and C2-3 cis-acting elements, the binding sites for RIN and SlASR1, respectively. Moreover, β-Hex expression/promoter activity in tomato fruits was downregulated once expression of either RIN or SlASR1 was suppressed; indicating that RIN and SlASR1 positively regulate the transcription of β-Hex during fruit ripening. Interestingly, RIN could also bind to the SlASR1 promoter, which contains several CArG cis-acting elements, and SlASR1 expression was suppressed in rin mutant fruits, indicating that RIN also acts as a positive regulator of SlASR1 expression during fruit ripening. Taken together, these results suggest that RIN, both directly and indirectly, through SlASR1, regulates the transcription of β-Hex during fruit ripening. The fruit ripening-specific promoter of β-Hex could be a useful tool in regulating gene expression during fruit ripening.

Key words: Fruit ripening; fruit ripening-specific promoter; β-Hex; RIN; SlASR1; transcriptional regulation.

Introduction

Fleshy fruit ripening is a coordinated developmental process that imparts nutritional and sensory qualities to fruits while altering their colour, texture, aroma, and flavour (Klee and Giovannoni, 2011; Osorio et al., 2013; Seymour et al., 2013a, b). Therefore, understanding the regulatory mechanisms of fruit ripening may be useful in improving fruit sensory and nutritional qualities, as well as post-harvest stability. Tomato (Solanum lycopersicum) is a model plant for the biochemical and genetic analysis of fleshy fruit development and ripening processes. Previous studies on tomato have provided insights into N-glycans that are present in the pericarp of fruits and accumulate during the ripening process (Priem and Gross, 1992; Priem et al., 1993), and how the N-glycosylation process influences fruit ripening (Handa et al., 1985; Meli et al., 2010). The ripening-stimulating activity of free N-glycans has previously been noted by injecting them into mature green tomato fruits, which resulted in induction of red colouration and ethylene production (Priem and Gross, 1992). Moreover, blocking of N-glycosylation delayed fruit ripening (Handa et al., 1985). In this context, the role of a particular enzyme
during the ripening process was elucidated by molecular and biochemical characterization of the fruits either suppressing or overexpressing the ripening-specific N-glycan-processing enzymes β-D-N-acetylhexosaminidase (β-Hex) and α-mannosidase (Meli et al., 2010; Ghosh et al., 2011).

β-Hex is a member of the glycosyl hydrolase family 20 (GH20). The homologues of this enzyme are present in a wide range of organisms from prokaryotes to eukaryotes (Slamaova et al., 2010). GH20 family members catalyse cleavage of the terminal N-acetyl-β-D-glucosamine (β-D-GlcNAc) and N-acetyl-β-D-galactosamine (β-D-GalNAc) residues present in N-acetyl-β-D-hexosaminides. In plants, β-Hex-mediated removal of the β-D-GlcNAc residues from N-glycans results in generation of the paucimannosidic N-glycans that are present in most plant glycoproteins (Gutternigg et al., 2007; Strasser et al., 2007; Liebminger et al., 2011). β-Hex activity increases during ripening of many fruits (Jagadeesh and Prabha, 2002; Jagadeesh et al., 2004) and RNA interference (RNAi)-mediated suppression of β-Hex expression resulted in a reduced rate of fruit softening in climacteric tomato and non-climacteric capsicum (Capsicum annuum) (Meli et al., 2010; Ghosh et al., 2011). Transgenic analysis revealed that β-Hex-mediated trimming of N-glycans attached to cell wall glycoproteins is a regulatory mechanism in controlling the fruit softening process (Meli et al., 2010). Interestingly, suppression of β-Hex expression resulted in reduced transcript levels of the ethylene response factor (ERF) family transcription factor and several ripening-related enzymes involved in degradation of the cell wall cellulose, hemicellulose, and pectin polysaccharides. Besides, ethylene positively regulates β-Hex and its expression is suppressed in ripening-impaired mutants such as ripening-inhibitor (rin), non-ripening (nor), and never-ripe (Nr), which are deficient in ripening-associated ethylene biosynthesis or ethylene perception (Tigchelaar et al., 1978; Wilkinson et al., 1995; Vrebalov et al., 2002; Meli et al., 2010). Moreover, the involvement of β-Hex in ripening-assosiated softening of peach fruit (Prunus persica) has recently been reported (Cao et al., 2014). Inhibition of β-Hex activity in peach fruits resulted in delayed fruit softening, a reduced level of ethylene production, and reduction in the transcript level of β-Hex as well as genes involved in ethylene biosynthesis and cell wall degradation (Cao et al., 2014). Therefore, downregulation of ethylene and cell wall degradation-related transcripts in β-Hex-suppressed fruits suggested the existence of a feedback mechanism in the fruit-softening process.

Genetic and biochemical analysis of the tomato MADS box transcription factor RIPENING INHIBITOR (RIN) provided key insights into transcriptional control of the fruit ripening process (Vrebalov et al., 2002; Giovannoni, 2007; Fujisawa et al., 2013; Zhong et al., 2013). RIN is considered a master regulator of ripening, since mutation hindered almost the entire fruit-ripening process, including colour development, softening, and the climacteric rise in respiration rate and ethylene production (Vrebalov et al., 2002). The RIN-mediated transcriptional regulation of fruit ripening has been well studied using chromatin immunoprecipitation and transcriptome and proteome analyses, which revealed several direct and indirect RIN target genes (Ito et al., 2008; Fujisawa et al., 2011; Martel et al., 2011; Fujisawa et al., 2012; Kumar et al., 2012; Qin et al., 2012; Fujisawa et al., 2013; Zhong et al., 2013). These included ripening-related genes involved in the ethylene biosynthesis and signalling pathway, cell wall modification, and carotenoid and aroma biosynthesis, and also some key transcriptional regulators such as NON-RIPENING, COLORLESS NON-RIPENING, and FRUITFULL1. Taken together, these studies suggested that RIN constitutes an upstream component of the ethylene-dependent and -independent ripening pathways.

In both tomato and capsicum fruits, β-Hex transcripts showed ripening-specific accumulation that can be correlated with the increase in protein level and enzyme activity during fruit ripening (Meli et al., 2010; Ghosh et al., 2011). This temporal relationship between the transcript and protein levels with the enzyme activity indicated that the rate of β-Hex transcription might be the controlling factor in determining its protein level and enzyme activity during ripening. However, how β-Hex transcription is regulated during fruit ripening is currently unknown. Therefore, we have identified and functionally characterized the fruit ripening-specific promoter of β-Hex to understand how its transcription is regulated during fruit ripening. The results of the present study demonstrate that RIN acts as a positive transcriptional regulator of β-Hex. In rin fruits, the transcript level of β-Hex was downregulated and β-Hex promoter-driven expression of GUS reporter was significantly reduced. Moreover, DNA–protein interaction analysis by electrophoretic mobility shift assay (EMSA) confirmed binding of RIN to the β-Hex promoter sequence. Further, yeast one-hybrid (Y1H) screening and EMSA analysis led to the identification of ABSCISIC ACID STRESS RIPENING 1 (SIASR1) as another β-Hex promoter-interacting protein. Virus-induced gene silencing (VIGS)-mediated suppression of SIASR1 in fruits caused transcriptional downregulation of β-Hex. However, the transcript level of SIASR1 was upregulated during ripening of wild-type tomato and inhibited in the rin mutant. Moreover, RIN also interacted with the SIASR1 promoter in EMSA. Thus, RIN could both directly and through SIASR1 regulate the expression of β-Hex during fruit ripening.

Materials and methods

Plant materials and growth conditions

Tomato (cv. Pusa Ruby) and capsicum (cv. California Wonder) seeds were obtained from the National Seeds Corporation Ltd, New Delhi. Tomato mutants used in the study were procured from the Tomato Genetics Resource Center, University of California at Davis and were in an Ailsa Craig background. Seeds were germinated in pre-sterilized soil and later transplanted into pots containing soil, agropeat and vermiculite (2:1:1). Plants were grown in a growth chamber with 25/22°C day/night temperature, 65% relative humidity and 16/8h light/dark regime. For the analysis, fruits were harvested at 3, 5, 10, and 15, and 20 days after anthesis (AA) and at the mature green (MG), breaker (BR), pink (P), and red ripe (RR) stages after tagging the flowers at anthesis. Fruits after ~40 days of anthesis were considered MG (the surface of the tomato was completely green; the shade of colour varied from light to dark), MG + 4 days as BR stage, B + 2 days as P stage, and P + 3 days as RR stage.
**Isolation and analysis of β-Hex promoter**

β-Hex promoters from tomato and capsicum were isolated using the Universal GenomeWalker™ Kit (Clontech, USA). Genomic DNA was extracted from leaves following the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987), and digested separately with PvuII, XmnI, MscI, DraI, and Spel enzymes, providing five genome walking libraries. In capsicum, instead of PvuII, Smal was used. PCR was carried out separately for each library with a GenomeWalker adapter-specific primer (AP1) and a gene-specific primer (GSP1).

Primary PCR product was used as a template to perform nested amplification using AP2 and GSP2 primers. The amplified PCR product was cloned into pGEM-T Easy vector and sequenced. Tomato and capsicum β-Hex promoter sequences (GenBank accession nos. KJ494862 and KJ494863, respectively) were analysed in silico to find out putative cis-acting elements using NewPLACE (Higo et al., 1999), PlantCARE (Lescot et al., 2002), and MatInspector (Cartharius et al., 2005) servers, and the FUZZNUC program (EMBOSS package; Rice et al., 2000).

**Construction of promoter::GUS fusion**

Tomato and capsicum β-Hex promoters::GUS fusion constructs were prepared in binary vector pBI121 after replacing the CaMV 3SS promoter with the β-Hex promoter. Tomato and capsicum β-Hex promoters were PCR amplified using high fidelity pfX DNA polymerase to incorporate appropriate restriction sites and cloned into pBI121 following standard restriction digestion and ligation methods. Positive clones were transformed into Agrobacterium tumefaciens (strain EHA105) following electroporation.

**Agrobacterium-based transient and stable transformation**

Agroinjection was performed into the pericarp of tomato fruits at MG stage, as described previously (Orzaez et al., 2006) but with some modifications. Primary culture was raised at 28°C in YEP medium containing rifampicin (50 mg ml⁻¹) and kanamycin (100 mg ml⁻¹) using a single isolated colony of Agrobacterium transformed with an appropriate construct. Further, a part of the culture (200 μl) was used to inoculate 50 ml induction medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 2 mM MgSO₄, 20 mM acetosyringone, and 10 mM MES, pH 5.6) with antibiotics and grown at 28°C until the optical density at 600 nm (OD600) attained 0.8–1.0. Cells were then recovered and resuspended in 50 ml of infiltration medium (10 mM MgCl₂, 10 mM MES, and 200 mM acetosyringone, pH 5.6) and further incubated at room temperature with gentle agitation (20 rpm) for 2 h. Culture was then injected into the fruits at 4–5 spots with the help of a syringe (1 ml; needle size: 0.33×13 mm). The needle was introduced up to 3–4 mm in depth into the fruit tissue and infiltration solution was gently injected.

To generate transgenic tomato plants, cotyledons from 2-week-old seedlings were used as described previously (Fillati et al., 1987) but with some modifications. Briefly, tomato seedlings were sterilized using 4% commercial bleach and germinated on Murashige and Skoog (MS) medium. After 2 weeks of germination, cotyledons were cut and co-cultivated for 30 min with Agrobacterium in MS medium containing acetosyringone (0.1 μM). Cotyledons were then collected for selection on MS plates containing kanamycin (50 mg 1⁻¹), cefotaxime (250 mg 1⁻¹), and zeatin (1 ng 1⁻¹). When plantlets regenerated, they were transferred to rooting medium (MS containing kanamycin (50 mg 1⁻¹), cefotaxime (250 mg 1⁻¹), and IAA (1 ng 1⁻¹)). Transgenic seeds were germinated in MS medium containing kanamycin (50 mg 1⁻¹) to get progeny plants.

**RNA isolation and quantitative RT-PCR**

RNA was isolated (Menke et al., 1999) and purified using an RNeasy Mini Kit (Qiagen). Five micrograms of total RNA were quantified using a nanodrop (ND 1000) and reverse transcribed to cDNA using superscript II RT (Invitrogen). qRT-PCR was performed using One Step Real Time RT-PCR (Applied Biosystems) with SYBR Green, as described previously (Ghosh et al., 2013). The analysis was done in triplicate from cDNA derived from at least two independent experiments. Using the 2⁻ΔΔCT method (Bovy et al., 2002), data are presented as the fold change in gene expression or percentage of expression, normalized to the endogenous reference gene (actin) and relative to control. The oligonucleotide primers used for qRT-PCR are listed in Supplementary Table S1 (at JXB online).

**GUS histochemical and fluorometric assay**

The GUS assays were performed as described previously (Jefferson et al., 1987) but with some modifications. For histochemical GUS analysis, 20-day-old seedlings, roots, leaves, flowers, and fruits at different stages of development and ripening were collected from wild-type and promoter::GUS fusion transgenic plants. The seedlings, roots, leaves, and flowers were immersed whole in GUS-staining solution (100 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, 3H₂O, 0.1% Triton-X100, 20% methanol, and 1 mM X-Gluc), while fruits were cut into transverse sections, dipped in GUS-staining solution, vacuum infiltrated, and incubated overnight at 37°C in darkness following destaining in 75% ethanol; they were photographed using a Canon G6 powershot with 4X zoom or Canon EOS 400D DIGITAL (10.1 megapixel) and Nikon AZ100 5X microscope.

**Quantitative GUS activity was determined by measuring the production of 4-methylumbelliferone (4-MU) as pmol 4-MU mg⁻¹ min⁻¹.** For this, samples from three individual plants of single copy transgenic lines were pooled and homogenized in 400 μl GUS extraction buffer (50 mM sodium phosphate buffer, pH 7.0, 10 mM DTT, 10 mM EDTA, 0.1% sodium lauryl sarcosine, and 0.1% Triton X100); supernatant was collected after centrifugation. Further, 50 μl of supernatant was added to 450 μl MUG assay buffer (GUS extraction buffer containing 10 mM MUG) and incubated at 37°C. After 1 h incubation, 100 μl of aliquots were removed and mixed with 900 μl 0.2 M Na₂CO₃ to terminate the reaction. GUS activity was measured using a fluorometer (Cary Eclipse, Varian) with excitation at 380 nm and emission at 454 nm.

**Y1H assay**

Y1H assay was carried out using a Matchmaker One-Hybrid Library Construction and Screening Kit (Clontech). Tomato β-Hex promoter sequence was PCR amplified to append an EcoRI site at the 5’-end and a SpeI site at the 3’-end and cloned upstream of the His3 reporter in pHIS2.1 vector to make bait construct (pHIS2.1-HP).

For cDNA preparation, total RNA was isolated from P stage tomato fruits, roots, and seeds enriched for mRNA (Dynabeads® mRNA Purification Kit, Invitrogen) and first-strand cDNA synthesis was performed following the manufacturer’s protocol. First-strand cDNA thus generated was amplified using long-distance (LD)-PCR. Further, ds cDNA generated by LD-PCR was column purified using a CHROMA SPIN TE-400 column. pHIS 2.1/bait plasmid was transformed into competent yeast Y187 cells and transformed yeasts were selected on S/D-Trp medium. To suppress the basal level expression of His3 from the reporter-bait plasmid 3AT (3-amino-1,2,4-triazole) was used. Competent cells were prepared from bait plasmid-transformed yeast cells and used for library screening. The transformed cells were spread on S/D-Leu-Trp/Bis/+ 5 mM 3AT (TDO + 3-AT) plates. Yeast colonies, thus obtained, were streaked three times on TDO + 3-AT to segregate false prey plasmids and eliminate false positives.

**EMSA**

EMSA was performed as described previously (Shkolnik and Bar-Zvi, 2008) with minor modifications. Briefly, a 200 bp β-Hex promoter fragment upstream of ATG was PCR amplified using primers listed in Supplementary Table S1. HindIII/XbaI-digested

**Supplementary Table S1**

| Primer Name | Forward Primer Sequence | Reverse Primer Sequence |
|-------------|-------------------------|-------------------------|
| pBI121 | 5'-CTAGCTAGATGTAAGCAGGAACTCGG-3' | 5'-GAATTGTTTAGGAGACTTACCTG-3' |
| pHIS2.1-HP | 5'-CTAGCTAGATGTAAGCAGGAACTCGG-3' | 5'-GAATTGTTTAGGAGACTTACCTG-3' |

**References**

- Doyle, J.L., and Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemistry*, 24, 1115-1118.
- Ghosh, S., et al. (2013). *J. Exp. Bot.*
- Jefferson, T.A., et al. (1987). *Nature* 329, 530-534.
- Bovy, Y., et al. (2002). *Plant J.* 30, 625-634.
- Ghosh, S., et al. (2008). *J. Exp. Bot.*
β-Hex promoter fragment was end filled with [α-32P]CTP (3000 Ci mmol⁻¹, 50 μCi), using DNA polymerase I (Klenow) fragment (New England Biolabs) and purified using a Sephadex G-50 column. EMSA was performed with [α-32P]CTP labelled β-Hex promoter fragments incubated with purified GST-SIASR1 protein in gel-shift assay binding buffer (20 mM HEPES, pH 7.5, 20% glycerol, 0.05 μg poly(dIdC):poly(dIdC), 0.8 mM ZnCl₂, 2.5 mM EDTA, 2.5 mM DTT, and 25 mM NaCl) at 25 °C for 30 min. For competition assays, unlabelled promoter fragments were used as specific competitive inhibitors and an unrelated DNA [200 bp region downstream of ATG of tomato actin (FJ532351.1)] was used as a non-specific competitor. After incubation for 30 min, the reaction was loaded onto 6% native PAGE. The gel was run at room temperature at a constant current of 10 mA using 0.5X TBE running buffer. The protein–DNA complexes and free probes were visualized by autoradiography. In vitro binding of RIN protein (AF448522.1) with the CaRG box present within the promoters of β-Hex and SIASR1 (obtained from the solgenomics tomato genome database) was carried out with both normal and mutated double-strand radiolabelled probe containing the CaRG box and its flanking sequences. 5′-end radiolabelling of probes was carried out with T4 polynucleotide kinase enzyme using [γ-32P]ATP (3000 Ci mmol⁻¹, 50 μCi). The RIN protein was incubated with radiolabelled probe in gel-shift assay binding buffer (20 mM HEPES, pH 7.5, 20% glycerol, 0.05 μg poly(dIdC):poly(dIdC), 10 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, and 25 mM NaCl) for 30 min at 25 °C. The assay was also performed using a 200-bp promoter fragment of β-Hex and a 142-bp region of the SIASR1 promoter containing the CaRG box. The specificity of binding was further determined using cold competitor DNAs (unlabelled probe).

VIGS

pTRV1 and pTRV2 were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA) and used as VIGS vectors for silencing of the SIASR1 gene as described previously (Liu et al., 2002). In brief, 330 bp coding sequence of SIASR1 (Solyc04g071610.2.1) was PCR amplified using Advantage 2 DNA polymerase (Clontech) and cloned into pTRV2 at the EcoRI/XbaI site. The empty pTRV2 and S/PDS cloned into pTRV2 (pTRV2-S/PDS) were used as controls. All the vectors (pTRV1, pTRV2, pTRV2-S/PDS, and pTRV2-SIASR1) were mobilized into Agrobacterium (strain GV3101). For agroinjection, a mixture of Agrobacterium suspension containing pTRV1 and pTRV2 vectors at a ration of 1:1 were prepared. Fruits at 30 DAA were injected with 1 ml Agrobacterium culture through the carpodip with the help of a syringe to initiate VIGS.

ACC and ABA treatment

Tomato seeds were germinated on MS media. 15-day-old seedlings were transferred to liquid MS medium containing 1 mM ACC or 0.1 mM ABA, harvested at different time points, and frozen immediately in liquid nitrogen. Seedlings transferred to MS liquid medium without ACC/ABA were used as a control.

Statistical analysis

All values are presented as the means (± SE) of biological replicates. Three technical replicates were considered for each biological sample and used to calculate one average value per biological sample.

Results

Isolation and transgenic analysis of β-Hex promoter

A comparison of the genomic (EU244856) and cDNA (EU244854) sequences revealed that tomato β-Hex gene consists of two exons (778 bp and 950 bp, respectively) that are interrupted by a 782-bp intron (Supplementary Figure S1 at JXB online). To isolate the promoter sequence, directional genome walking PCR was carried out using a set of adapter and gene-specific primers that were designed based on the genomic sequence (Supplementary Figure S2 at JXB online). Comparison of the sequences obtained from the genome walking PCR with the β-Hex gene revealed a 1001-bp sequence (KJ494862) upstream of the translation start site. Following random amplification of cDNA ends (5′-RACE, Clontech), the putative transcription start site (TSS) was determined to be 9 bp upstream of the translation start site (Supplementary Figure S1). All the sequences were verified with the recently published tomato genome sequence (solgenomics.net).

Through northern hybridization, β-Hex transcript level was found to accumulate maximally during tomato ripening with a peak at the P stage (Meli et al., 2010). In order to corroborate the earlier results, quantitative RT-PCR (qRT-PCR) analysis was carried out to examine the transcript level of β-Hex throughout fruit development and ripening of tomato. The analysis revealed maximum transcript levels at the P stage of ripening as compared to the developing and unripe fruits (Fig. 1). Further, to investigate the fruit-ripening-specific activation of the promoter, tomato transgenic plants were generated by constructing tomato β-Hex promoter::β-gluconuronidase (HP::GUS) gene fusions (Figs 2A–C, and 3A and B). We included the 1001-bp sequence upstream of the translation start site as the tomato β-Hex promoter. 25 independent transgenic lines were generated for the HP::GUS construct and 10 lines were initially analysed by histochemical GUS staining to determine the ripening-specificity of the promoter. Finally, two independent T2 transgenic lines (L30 and L39) were selected for detailed examination of the tissue and organ-specific activation of the β-Hex promoter. For this, fruits of different developmental and ripening stages and roots, stems, leaves, and flowers were taken for histochemical and fluorometric GUS activity assays and for determining GUS transcript levels (Figs 2B–C, and 3A and B). The analysis revealed maximum transcript levels and activity of GUS in ripening fruit, quite similar to the β-Hex expression pattern during tomato ripening (Fig. 1). Consistent with the role of β-Hex in fruit pericarp softening, prominent GUS activity was recorded in the fruit pericarp tissue (Fig. 2B). However, GUS activity was not detected in seedlings, roots, stems, or leaves. In the case of flowers, slight GUS activity was noticed in sepals; however, no GUS staining was observed in petals (Fig. 3A and B). In contrast, constitutive expression of GUS was noticed in seedlings, roots, stems, leaves, flowers, and fruits when the gene was expressed under the control of the CaMV 35S promoter. β-Hex expression is regulated by the plant hormone ethylene, which plays a pivotal role in tomato ripening (Meli et al., 2010). Therefore, the transcript level and activity of GUS was determined after treatment of HP::GUS transgenic seedlings with 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene. The upregulation of GUS transcript and activity indicated the activation of the β-Hex promoter in response to ethylene (Fig. 4A). Taken together, these results substantiate the fruit ripening-specific
and ethylene-inducible expression of $\beta$-Hex which was undetectable in other plant parts (Meli et al., 2010). These analyses also suggest that the 1001-bp upstream sequence of $\beta$-Hex does contain the cis-acting elements involved in fruit-ripening specific expression of the gene, and thus can be regarded as the full-length $\beta$-Hex promoter.

In order to get an overview of the putative cis-acting regulatory elements that may control the transcription of $\beta$-Hex,
the promoter sequence was analysed through NewPLACE (https://sogo.dna.affrc.go.jp), PlantCARE (bioinformatics.psb.ugent.be), and MatInspector (www.genomatix.de) for the identification of the putative transcription factor binding sites. The analysis revealed an array of cis-acting elements that can be recognized by the transcription factors involved in plant hormone signalling such as ethylene, auxin, gibberellin, abscisic acid, and brassinosteroids (Supplementary Table S2 at JXB online). Crosstalk of these hormones in fruit ripening is discussed elsewhere (Osorio et al., 2013; Seymour et al., 2013a). Among the cis-acting elements, MADS-box transcription factor RIN binding sites (CArG box; Fujisawa et al., 2013) were of particular interest, because β-Hex expression was downregulated in rin mutant fruit (Fig. 6D; Meli et al., 2010).

β-Hex exhibits similar expression profiles during the ripening of both climacteric tomato (Meli et al., 2010) and non-climacteric capsicum (Ghosh et al., 2011). This prompted us to test activation of the capsicum β-Hex promoter (KJ494863) in tomato fruit. Capsicum β-Hex promoter sequence (951 bp) was isolated following the directional genome walking PCR approach and used to drive the expression of the GUS reporter (Fig. 5A and Supplementary Figure S3, at JXB online). Functional analysis of the capsicum β-Hex promoter was carried out by Agrobacterium-mediated transient expression (agroinjection) in tomato fruits as described in the materials and methods section. Fruits were agroinjected at the MG stage and harvested at BR, P, and RR stages for measuring GUS activity by histochemical and fluorometric assays (Fig. 5B). Capsicum β-Hex promoter-driven expression of GUS in tomato fruit indicated activation of the capsicum promoter in tomato fruit. This result suggests the existence of a conserved transcriptional mechanism for the expression of β-Hex in these Solanaceae fruits which have different ripening behaviour. In silico analysis of the capsicum and tomato β-Hex promoters identified several conserved cis-acting elements, including CArG boxes that may be involved in controlling the transcription of β-Hex in both climacteric and non-climacteric fruits (Supplementary Table S2).
Transcriptional regulation of β-Hex by RIN

The MADS-box transcription factor RIN modulates gene expression during fruit ripening by binding to the CArG box [C(T/A/C)(A/T/G)G] present within the promoter region of the fruit ripening-related genes (Ito et al., 2008; Martel et al., 2011; Fujisawa et al., 2013). In silico analysis revealed six CArG boxes within the β-Hex promoter sequence (Supplementary Figure S4 at JXB online). Moreover, β-Hex expression was suppressed up to 80% in rin mutants as compared to wild-type fruit (Fig. 6D; Meli et al., 2010). These results suggest RIN-mediated transcriptional control of β-Hex expression during fruit ripening. To substantiate these results and test direct regulation by RIN, the ability of RIN protein to bind the β-Hex promoter was determined by EMSA (Fig. 6A, B). For this, the N-terminal region (1–139aa) of RIN (AF448522.1), including the DNA-binding domain and dimerization interface (CD search, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), was expressed in E. coli and purified (Supplementary Figure S5B, at JXB online), given that a C-terminal truncated version of RIN produced clear signal as compared to the full-length protein in EMSA (Ito et al., 2008). For the DNA bait, we initially used two β-Hex promoter fragments of different lengths (26 and 200bp) containing the same CArG box (Box 6), present at −62bp to −71bp, close to the TSS in comparison to the other five CArG boxes (Fig. 6A; Supplementary Figure S4). In EMSA, DNA–protein complex was detected when wild-type CArG box was used. However, DNA–protein complex was not formed in the case of mutated CArG box (C and G were replaced with T and A, respectively). Moreover, the specificity of the DNA–protein complex was determined by using unlabelled specific (CArG probe sequence) and non-specific (tomato actin sequence) competitor DNAs (Fig. 6A). These results suggest that RIN specifically interacts with the CArG box sequence present within the β-Hex promoter. We next tested whether RIN could also interact with five additional CArG boxes identified within the β-Hex promoter sequence and whether these six CArG boxes have different binding specificities with the RIN protein (Fig 6B and Supplementary Figure S4). For this, 26-bp β-Hex promoter fragments including the CArG box along with their flanking sequences were used as DNA baits. Although DNA–protein complexes were noticed for all the six CArG boxes, EMSA gave intense signals in CArG boxes (box 5 and 6) close to the TSS; suggesting efficient binding of RIN to these two CArG boxes compared to the other four boxes. In order to further confirm the transcriptional regulation of β-Hex expression by RIN, β-Hex promoter-driven expression of GUS was compared in wild-type and rin mutant fruits, followed Agrobacterium-mediated transient expression (Fig. 6C). Histochemical staining and fluorometric assay revealed significantly reduced activity of GUS in rin mutants as compared to wild-type fruits when GUS expression was driven by the β-Hex promoter. In contrast, similar levels of GUS activity were detected in wild-type and rin mutant fruits under the control of the CaMV 35S constitutive promoter. Altogether, these results demonstrate that the transcription factor RIN positively regulates the expression of β-Hex.

Y1H assay identified SIASR1 as a β-Hex promoter interacting protein

Besides the CArG box, other putative transcription factor binding sites were also identified within the β-Hex promoter (Supplementary Table S2), suggesting that, in addition to RIN, β-Hex expression might be regulated by other transcription factors as well. To test this possibility,
**β-Hex** promoter-interacting proteins were identified by Y1H screening using a cDNA library generated from PolyA+ RNAs isolated from P stage tomatoes as described in the materials and methods section. Y1H screening revealed ABSCISIC ACID STRESS RIPENING 1 (SlASR1; Solyc04g071610.2.1), ERF6 (Solyc01g065980.2.1), calmodulin (Solyc02g094000.1.1), and a putative calmodulin-binding protein (Solyc10g081040.1.1) as the candidate transcriptional regulators of **β-Hex**. In order to test whether these promoter-interacting proteins are expressed during fruit ripening, their transcripts levels were determined during tomato ripening.

**Fig. 6.** RIN-mediated transcriptional regulation of **β-Hex.** (A) EMSA demonstrates the formation of DNA–protein complex between RIN and tomato **β-Hex** promoter fragments. EMSA was performed using double-stranded radiolabelled probes containing either 26-bp or 200-bp promoter fragments with normal (CArG and HP-200) or mutated (mCArG and mHP-200) CArG box elements with their flanking sequences. Cytosine (C) and guanine (G) were replaced with thymine (T) and adenine (A), respectively, to create mutated CArG probes. Specificity of the DNA–protein complex was analysed by using competitor DNAs. Arrows indicate DNA–protein complex. (B) EMSA shows binding of RIN protein to six different CArG boxes identified within the tomato **β-Hex** promoter. (C) Activation of the **β-Hex** promoter in the wild type (cv. Ailsa Craig) and *rin* mutant was determined by transient expression of GUS reporter in fruits. Mature green tomato fruits were agroinjected with HP::GUS construct. Agroinjected fruits were harvested at R stage and histochemical and fluorometric GUS assays were performed. Control fruits were agroinjected with 35S::GUS construct for constitutive expression of GUS reporter driven by CaMV 35S promoter. (D) Relative expression of **β-Hex** in wild type (cv. Ailsa Craig) and *rin* mutant fruits was determined by qRT-PCR using actin as an endogenous control. Data are presented as the mean (±SE) of two biological replicates.
RIN-dependent transcriptional regulation of β-Hex

The expression patterns of these promoter-interacting proteins were quite similar to β-Hex expression during fruit ripening (Fig. 1; Meli et al., 2010), suggesting their involvement in transcriptional regulation of β-Hex. We selected SIASR1 for further characterization because three putative SIASR1-binding sites [C2(C/G)A, Kalifa et al., 2004a; Shkolnik and Bar-Zvi, 2008] could be recognized within the β-Hex promoter region. ASR1-family proteins probably have dual functions, as chaperones in cytosol and transcriptional regulators in the nucleus (Kalifa et al., 2004a; Ricardi et al., 2012). Although the exact physiological role of SIASR1 was not elucidated, SIASR1 abolished abscisic acid (ABA) and glucose responses, and improved stress tolerance when overexpressed in Arabidopsis and tobacco, respectively (Kalifa et al., 2004b; Shkolnik and Bar-Zvi, 2008). In grapefruit, an ASR1 homologue (VvMSA) was proposed to be involved in sugar and ABA signalling (Cakir et al., 2003). Recently, candidate transcriptional targets of SIASR1 under water-stressed conditions, including cell wall-related and aquaporin genes, were identified (Ricardi et al., 2014). The transcript level of SIASR1 was upregulated during tomato ripening (Fig. 8A and Iusem et al., 1993); however, its role in fruit ripening is yet to be examined.

Transcriptional regulation of β-Hex by SIASR1

In order to substantiate the result of the Y1H assay (Fig. 7A), the ability of SIASR1 to interact with the β-Hex promoter was confirmed through EMSA using bacterially expressed recombinant protein, GST-SIASR1 (Fig. 7B and Supplementary Fig. S5C). A part of the β-Hex promoter (~1 to ~200 bp) which contains a tandem repeat of the putative SIASR1 binding site [C2(C/G)A] was used as a probe in EMSA, which revealed specific interaction of SIASR1...
with the β-Hex promoter sequence. GST alone did not show any binding activity. Although, the excess unlabelled 200-bp promoter DNA fragment was able to compete for the binding activity of GST-SIASR1, nonspecific DNA fragments (tomato actin sequence) were unable to compete (Fig. 7B). These results demonstrate specific binding of SIASR1 to the β-Hex promoter DNA fragment. Further, to confirm a role of SIASR1 in regulation of β-Hex transcription in planta, VIGS-mediated suppression of SIASR1 was carried out in tomato fruits using tobacco rattle virus (TRV)-based vectors (Liu et al., 2002). A mixture of Agrobacterium suspension containing pTRV1 and pTRV2 vectors in 1:1 ratio was agroinjected into tomato fruits at 30 DAA to initiate VIGS (described in the materials and methods section). For silencing of SIASR1, pTRV1 and pTRV2-SIASR1 (SIASR1 cloned into pTRV2) vectors were used. However, the empty pTRV2 vector and pTRV2-SIPDS (tomato phytoene desaturase cloned into pTRV2) along with pTRV1 were used as controls in VIGS. Around 17 days after agroinjection, when pTRV1/pTRV2 vector-injected control fruits had progressed to the RR stage, pTRV1/pTRV2-SIPDS vector-injected fruits developed a yellowish phenotype due to low lycopene values because of the suppression of SIPS, confirming the efficacy of VIGS in tomato fruit (Fig. 7C). To test the silencing of SIASR1 in VIGS fruits, the transcript level of SIASR1 was determined through qRT-PCR analysis (Fig. 7D). The analysis revealed up to 75% suppression of SIASR1 expression in VIGS fruits. Interestingly, β-Hex expression was significantly downregulated (~50%) in SIASR1-suppressed fruits as compared to the vector control (Fig. 7D). Moreover, β-Hex and SIASR1 exhibited similar expression patterns during tomato fruit ripening, ACC treatment, and in ripening-impaired mutants (Figs 1, 4A, 6C, and 8A; and Supplementary Figure S7, at JXB online). Altogether, these results demonstrate that SIASR1 acts as a positive transcriptional regulator of β-Hex during fruit ripening.

ABA positively regulates the expression of SIASR1 (Amitai-Zeigerson et al., 1995). Moreover, the level of ABA in tomato fruits increases just prior to the induction of ethylene biosynthesis during fruit ripening, and depletion of ABA levels led to delayed ripening in tomato (Zhang et al., 2009; Sun et al., 2011 and 2012). Therefore, we tested whether β-Hex expression is also regulated by ABA. qRT-PCR expression analysis of β-Hex in wild type and GUS in HP::GUS transgenics after ABA treatment revealed upregulation of β-Hex and GUS, respectively (Fig. 4B and C). These results confirmed the role of ABA in positive regulation of β-Hex expression.

Transcriptional regulation of SIASR1 by RIN

RIN and SIASR1 directly interacted with the β-Hex promoter and the transcript level of β-Hex was downregulated when expression of either RIN or SIASR1 was suppressed in tomato fruits (Figs 6A–D and 7B–D). Altogether, these results suggest that both RIN and SIASR1 are the positive transcriptional regulators of β-Hex. RIN is considered an upstream component of the ethylene-dependent and -independent fruit ripening pathways (Vrebalov et al., 2002; Giovannoni, 2007; Fujisawa et al., 2013). Moreover, SIASR1 expression was upregulated during tomato ripening (Fig. 8A; Iusem et al., 1993). Thus, we explored the possibility that SIASR1 is under the transcriptional control of RIN. To address this issue, the expression level of SIASR1 was assessed in rin mutants through qRT-PCR analysis, which revealed up to 90% suppression of SIASR1 expression in rin mutants as compared to wild-type fruit (Fig. 8A). Further, to test whether RIN could directly interact with the SIASR1 promoter, EMSA was carried out using a radiolabelled 26-bp SIASR1 promoter DNA fragment with a CArG box element and its flanking sequences. We also used a radiolabelled 142-bp region of the SIASR1 promoter containing two overlapping CArG boxes for EMSA (Fig. 8B). These analyses revealed specific interaction of RIN with the SIASR1 promoter sequence, suggesting a direct role for RIN in transcriptional regulation of SIASR1. Therefore, RIN could modulate the expression of β-Hex during fruit ripening, both directly and indirectly through SIASR1.

Discussion

Although the physiological function of β-Hex in the ripening-associated fruit softening process has been elucidated (Jagadeesh and Prabha, 2002; Jagadeesh et al., 2004; Meli et al., 2010; Ghosh et al., 2011; Cao et al., 2014), the transcriptional regulation of β-Hex during fruit ripening is currently unknown. To gain an insight into the transcriptional regulation of β-Hex during fruit ripening, we have identified and functionally characterized the fruit ripening-specific promoter of β-Hex. The spatial and temporal expression patterns of the GUS reporter gene under the control of the β-Hex promoter were determined in transgenic tomato plants. Consistent with the fruit ripening-specific expression of β-Hex (Fig. 1), upregulation of promoter activity during tomato ripening was noticed (Fig. 2B–C). In fruits, maximum β-Hex promoter activation was recorded in pericarp tissues. However, β-Hex promoter was not activated in seedlings, roots, stems, leaves, and flower parts except sepals, which showed very low GUS activity (Fig. 3A–B). These results corroborate the role of β-Hex in ripening-associated fruit softening (Meli et al., 2010; Ghosh et al., 2011; Cao et al., 2014). In silico analysis of the β-Hex promoter also revealed the presence of the cis-acting regulatory elements related to the fruit-ripening process, in addition to ubiquitous elements such as TATA and CAAT boxes (Supplementary Table S2). Moreover, a comparison of the expression profiles of β-Hex and GUS, in response to ACC and ABA treatment, in wild type and in HP::GUS transgenics, respectively, revealed similar patterns (Fig. 4A–C and Meli et al., 2010). These results indicated that the 1001-bp β-Hex promoter region used to drive the GUS expression contains all the cis-acting regulatory elements indispensable for the spatio-temporal regulation of the endogenous β-Hex gene.

Our results suggest that MADS-box transcription factor RIN and SIASR1 are the positive regulators of β-Hex expression during fruit ripening. Both these proteins showed
binding activities to the β-Hex promoter sequence (Figs 6A and B) and β-Hex expression/promoter activity was decreased when the expression of either RIN or SlASR1 was suppressed (Figs 6C and D, and 7C and D). In silico analysis of the β-Hex promoter also revealed the presence of CARG and [C2→3(C/G)A] boxes, the binding sites for RIN and SlASR1, respectively, thus, validating their role in transcriptional regulation of β-Hex. Interestingly, RIN was also found to act as a positive transcriptional regulator of SlASR1; because the expression of SlASR1 was downregulated in rin mutant and RIN protein directly bound to the SlASR1 promoter sequence that contains several CARG motifs (Fig. 8A and B). Thus, RIN may exert both direct and indirect control to positively regulate the expression of β-Hex during fruit ripening.

RIN is considered the global fruit ripening regulator (Vrebalov et al., 2002). Previous studies have identified direct and indirect targets of RIN, including those involved in ethylene biosynthesis and signalling, fruit textural changes, and pigment biosynthesis (Ito et al., 2008; Fujisawa et al., 2011; Martel et al., 2011; Fujisawa et al., 2012; Kumar et al., 2012; Qin et al., 2012; Fujisawa et al., 2013; Zhong et al., 2013). Two earlier genome-wide chromatin immunoprecipitation experiments (ChIP-chip) revealed a list of genes including both β-Hex (Solyc01g081610.2.1) and SlASR1 (Solyc04g071610.2.1) that are associated with the RIN binding sites (Fujisawa et al., 2013; Zhong et al., 2013); however, the precise role of RIN in regulating the expression of β-Hex and SlASR1 during fruit ripening was not studied. Our results demonstrate that β-Hex and SlASR1 are among the direct targets of RIN during fruit ripening. Conversely, the exact role of SlASR1 in fruit ripening is yet to be determined. Although the nuclear localization of SlASR1 has been fairly well investigated, its function as a transcriptional regulator is not well studied (Shkolnik and Bar-Zvi, 2008; Ricardi et al., 2012). SlASR1 showed induced expression during tomato ripening (Fig. 7E and Amitai-Zeigerson et al., 2012). Both these hormones play crucial functions in tomato ripening (Zhang et al., 2009; Sun et al., 2011; Klee and Giovannoni, 2011; Sun et al., 2012; Osorio et al., 2013; Seymour et al., 2013). Moreover, the expression of SlASR1

**Fig. 8.** RIN regulates the expression of SlASR1 during fruit ripening. (A) qRT PCR transcript analysis of SlASR1 during ripening stages of wild type (cv. Ailsa Craig) and corresponding ages of rin mutant fruits. Data are presented as the mean (±SE) of at least three biological replicates. (B) EMSA to determine the interaction of RIN with the promoter fragments of SlASR1. Radiolabelled double stranded 26-bp (left side gel image) and 142-bp (right side gel image) fragments of SlASR1 promoter were used as probes in EMSA. The specificity of the interaction was checked by using mutated CARG box (mCARG) and an excess of specific competitor DNAs (unlabelled probes) in EMSA. This figure is available in colour at JXB online.
was downregulated in mutant fruits in which the expression of either of the fruit-ripening regulators \textit{RIN, NOR}, and \textit{NR} was suppressed (Fig. 8A and Supplementary Figure S7). In \textit{nor} and \textit{Nr} mutant fruits, the suppression of \textit{SIASR1} expression may be due to the impaired biosynthesis and/or signalling of ethylene (Tigchelaar et al., 1978; Wilkinson et al., 1995; Vrebalov et al., 2002), because ethylene regulates the expression of \textit{SIASR1} (Fig. 7F). Therefore, a role of \textit{SIASR1} in tomato ripening cannot be ruled out. Earlier, ChIP-chip analysis using leaves from stressed tomato plants revealed several potential direct transcriptional targets of \textit{SIASR1}; however, \beta-Hex was not identified (Ricardi et al., 2014).

Although, we do not exclude the possibility that some of the targets of \textit{SIASR1} are yet to be found, it is also likely that \textit{SIASR1} does not activate the \beta-Hex promoter in leaves. This is quite possible because \beta-Hex promoter remained inactive in leaves (Fig. 3A, B). Moreover, the methylation status of the promoter is likely to determine binding of the transcription factors to the promoter and further activation of \beta-Hex transcription. The \beta-Hex promoter region was found to exhibit differential methylation patterns in leaves and fruits during maturation and ripening (Zhong et al., 2013). Interestingly, the differentially methylated region of the \beta-Hex promoter was hypomethylated during the progression of tomato ripening but hypermethylated in \textit{rin} and another ripening mutant, \textit{colorless non-ripening} (Zhong et al., 2013).

Both ethylene and ABA positively regulate the expression of \beta-Hex (Fig. 4A–C and Meli et al., 2010). \beta-Hex promoter was specifically activated in fruits during ripening (Fig. 2A–C) but remained inactive in seedlings, roots, stems, and leaves (Fig. 3A and B). Interestingly, \beta-Hex promoter-driven expression of \textit{GUS} and \beta-Hex genes was noticed when seedlings (HP:GUS transgenics and wild type, respectively) were treated with ethylene and ABA (Fig. 4A–C). These results indicate that although \beta-Hex promoter remained inactive in normal seedlings, it is responsive to ethylene and ABA treatments. This also supports the essential roles of ethylene and ABA in activating the \beta-Hex promoter during natural fruit ripening, when the endogenous levels of these hormones increase. During ripening, the early induction of \beta-Hex at the BR stage might be brought about by ABA because the level of ABA in tomato fruit increases just prior to the P stage when ethylene attains the maximum level (Fig. 1; Meli et al., 2010 and Sun et al., 2012). Although \beta-Hex transcript level started increasing from the BR stage, maximum transcript level was attained at the P stage (Fig. 1; Meli et al., 2010). Autocatalytic ethylene biosynthesis during the P stage might be responsible for high-level transcription of \beta-Hex at this stage. \textit{SIASR1} expression is positively regulated by ethylene and ABA (Fig. 7E; Supplementary Figure S7 at JXB online; Amitai-Zeigerson et al., 1995); thus, its involvement in both ethylene and ABA-regulated transcription of \beta-Hex cannot be excluded. Although ethylene is known to regulate \textit{RIN} expression positively, the role of ABA is yet to be elucidated (Fujisawa et al., 2013).

\beta-Hex exhibits similar expression patterns and plays a conserved physiological function during the ripening of both the climacteric fruit tomato, which requires ethylene for the ripening, and the non-climacteric fruit, capsicum, which does not require ethylene for ripening (Meli et al., 2010; Ghosh et al., 2011). In order to gain insight into the conserved transcriptional regulation of \beta-Hex during ripening of both climacteric and non-climacteric fruits, we tested capsicum \beta-Hex promoter-driven expression of \textit{GUS} in tomato fruit (Fig. 5B). The activation of \textit{capsicum} promoter in tomato fruits indicates that it contains all the essential \textit{cis}-acting regulatory elements required for its activation in tomato. \textit{In silico} analysis of tomato and capsicum \beta-Hex promoters revealed several common \textit{cis}-acting regulatory elements including recognition sites for the MADS box transcription factor \textit{RIN} and \textit{SIASR1}, which could be involved in conserved transcriptional regulation of \beta-Hex in climacteric and non-climacteric fruits. Capsicum and tomato share the fruit ripening-regulatory components including the genes for ethylene biosynthesis and signalling; however, their regulation during fruit ripening differs in these fruits (Lee et al., 2010; Osorio et al., 2012; Kim et al., 2014). Although the genes involved in ethylene biosynthesis do not exhibit induced expression during capsicum ripening, the expression pattern of the transcription factors (\textit{RIN, TAGL1}, and \textit{NOR}), ethylene signalling (\textit{NR, ETR4, EIN2}, and \textit{EILs}), and downstream ethylene target genes, such as those involved in cell wall metabolism and fruit pigment biosynthesis, was conserved during fruit ripening. A similar expression pattern of \textit{RIN} in these fruits and complementation of \textit{RIN} function by the capsicum orthologue suggest its possible involvement in the transcriptional regulation of \beta-Hex during ripening of capsicum fruit as well (Kim et al., 2014; Dong et al., 2014). In conclusion, this work led to the isolation of the fruit ripening-specific promoter of \beta-Hex and identification of \textit{RIN} and \textit{SIASR1} as the transcriptional regulators of \beta-Hex. Together, the results demonstrate that \textit{RIN} and \textit{SIASR1} directly bind to the \beta-Hex promoter and positively regulate the expression of \beta-Hex during tomato ripening.

**Supplementary material**

Supplementary data can be found at JXB online.

- **Supplementary Table S1.** List of primers used in the study.
- **Supplementary Table S2.** The putative \textit{cis}-acting regulatory elements identified within tomato and capsicum \beta-Hex promoters through \textit{in-silico} analysis (NewPLACE, PlantCARE, and MatInspector).
- **Supplementary Figure S1.** Genomic organization of tomato \beta-Hex.
- **Supplementary Figure S2.** Tomato \beta-Hex promoter isolation by the PCR-based genome walking method.
- **Supplementary Figure S3.** Isolation of the capsicum \beta-Hex gene promoter.
- **Supplementary Figure S4.** Sequences of \beta-Hex and \textit{SIASR1} promoters showing position of the CArG boxes.
- **Supplementary Figure S5.** Purification of recombinant GST-RIN and GST-SIASR1 protein from \textit{E. coli}.
- **Supplementary Figure S6.** The mRNA expression of putative \beta-Hex promoter-binding protein genes.
- **Supplementary Figure S7.** The mRNA expression of \textit{SIASR1} in wild-type and ripening mutants \textit{nor} and \textit{Nr}. 

**Fig. 8A**.

**Fig. 7F**.
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References
Amitäi-Zeigerson H, Scolnik PA, Bar-Zvi D. 1995. Tomato Asr1 mRNA and protein are transiently expressed following salt stress, osmotic stress and treatment with abscisic acid. Plant Science 110, 205–213.

Bovy A, de Vos R, Kemper M et al. 2002. High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor gene LC and C1. The Plant Cell 14, 2509–2526.

Cakir B, Agasse A, Gaillard C, Saumonneau A, Delrot S, Atanassova Y. 2003. A grape ASR protein involved in sugar and abscisic acid signaling. The Plant Cell 15, 2165–2180.

Cao L, Zhao C, Su S, Luo C, Han M. 2014. The role of β-hexosaminidase in peach (Prunus persica) fruit softening. Scientia Horticulturae 169, 226–233.

Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T. 2005. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 21, 2933–2942.

Dong T, Chen G, Tian S, Xie Q, Yin W, Zhang Y, Hu Z. 2014. A non-climacteric fruit gene CaMADS-RIN regulates fruit ripening and ethylene biosynthesis in climacteric fruit. PLoS One 9, e95559.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19, 11–15.

Fillati JJ, Kiser J, Rose R, Comai L. 1987. Efficient transfer of a C glycosylphosphate tolerance gene into tomato using a binary Agrobacterium tumefaciens vector. Biotechnology 5, 726–730.

Fujisawa M, Nakano T, Ito Y. 2011. Identification of potential target genes for the tomato fruit-ripening regulator RIN by chromatin immunoprecipitation. BMC Plant Biology 11, 26–40.

Fujisawa M, Nakano T, Shima Y, Ito Y. 2013. A large-scale identification of direct targets of the tomato MADS Box transcription factor RIPENING INHIBITOR reveals the regulation of fruit ripening. The Plant Cell 25, 371–386.

Fujisawa M, Shima Y, Higuchi N, Nakano T, Koyama Y, Kasumi T, Ito Y. 2012. Direct targets of the tomato-ripening regulator RIN identified by transcriptome and chromatin immunoprecipitation analyses. Planta 235, 1107–1122.

Ghosh S, Meli VS, Kumar A, Thakur A, Chakraborty N, Chakraborty S, Datta A. 2011. The N-glycan processing enzymes α-mannosidase and β-D-N-acetylhexosaminidase are involved in ripening-associated softening in the nonclimacteric fruits of capsicum. Journal of Experimental Botany 62, 571–582.

Ghosh S, Singh UK, Meli VS, Kumar V, Kumar A, Irfan M, Chakraborty N, Chakraborty S, Datta A. 2013. Induction of senescence and identification of differentially expressed genes in tomato in response to monoterpene. PLoS One 8, e67002.

Giovannoni JJ. 2007. Fruit ripening mutants yield insights into ripening control. Current Opinion in Plant Biology 10, 283–289.

Gutternigg M, Kretschmer-Lubicz D, Paschinger K, Rendić D, Hader J, Geier P, Ranftl R, Jantsch V, Lochnit G, Wilson IB. 2007. Biosynthesis of truncated N-linked oligosaccharides results from non-orthologous hexosaminidase-mediated mechanisms in nematodes, plants, and insects. Journal of Biological Chemistry 282, 27825–27840.

Handa AK, Singh NK, Biggs SM. 1985. Effect of tunicamycin on in vitro ripening of tomato pericarp tissue. Plant Physiology 63, 417–424.

Higo K, Ugawa Y, Iwamoto M, Korenaga T. 1999. Plant cis-acting regulatory DNA elements (PLACE) database. Nucleic Acids Research 27, 297–300.

Ito Y, Kitagawa M, Ihashi N, Yabe K, Kimbara J, Yasuda J, Ito H, Inakuma T, Hiroi S, Kasumi T. 2008. DNA-binding specificity, transcriptional activation potential, and the n mutation effect for the tomato fruit-ripening regulator RIN. The Plant Journal 55, 212–223.

Iusem ND, Bartholomew DM, Hitz WD, Scolnik PA. 1993. Tomato (Lycopersicon esculentum) transcript induced by water deficit and ripening. Plant Physiology 102, 1353–1354.

Jagadeesh BH, Prabha TN. 2002. β-Hexosaminidase, an enzyme from ripening bell capsicum (Capsicum annum var. variata). Phytochemistry 61, 295–300.

Jagadeesh BH, Prabha TN, Srinivasan K. 2004. Activities of glycosidases during fruit development and ripening of tomato (Lycopersicon esculentum L.): Implication in fruit ripening. Plant Science 166, 1451–1459.

Jefferson RA, Kavanagh TA, Bevan MW. 1987. GUS fusions: -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. The EMBO Journal 6, 3901–3907.

Kalifa Y, Gilad A, Konrad Z, Zaccari M, Scolnik PA, Bar-Zvi D. 2004a. The water- and salt-stress-regulated Asr1 (abscisic acid stress ripening) gene encodes a zinc-dependent DNA-binding protein. Biochemical Journal 381, 373–378.

Kalifa Y, Perlson E, Gilad A, Konrad Z, Scolnik PA, Bar-Zvi D. 2004b. Over-expression of the water and salt stress-regulated Asr1 gene confers an increased salt tolerance. Plant, Cell and Environment 27, 1459–1468.

Kim S, Park M, Yeom SI et al. 2014. Genome sequence of the hot pepper provides insights into the evolution of pungency in Capsicum species. Nature Genetics 46, 270–278.

Klee HJ, Giovannoni JJ. 2011. Genetics and control of tomato fruit ripening and quality attributes. Annual Review of Genetics 45, 41–59.

Kumar R, Sharma MK, Kapoor S, Tyagi AK, Sharma AK. 2012. Transcriptome analysis of rip mutant fruit and in silico analysis of promoters of differentially regulated genes provides insight into LeMADS-RIN-regulated ethylene-dependent as well as ethylene-independent aspects of ripening in tomato. Molecular Genetics and Genomics 287, 189–203.

Lee S, Chung EJ, Joung YH, Choi D. 2010. Non-climacteric fruit ripening in pepper: increased transcription of EIL-like genes normally regulated by ethylene. Functional and Integrative Genetics 10, 135–146.

Lescot M, Déhais P, Moreau Y, De Moor B, Rouze P, Rombauts. 2002. PlantCARE: a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Research 30, 325–327.

Liebingger E, Veit C, Pabst M, Batoux M, Zipfel C, Altmann F, Mach L, Strasser R. 2011. β-N-Acetyllactosaminidases HEXO1 and HEXO3 are responsible for the formation of paucimannosidic N-glycans in Arabidopsis thaliana. Journal of Biological Chemistry 286, 10793–10802.

Liu Y, Schif M, Dinesh-Kumar SP. 2002. Virus-induced gene silencing in tomato. The Plant Journal 31, 777–786.

Martel C, Vrebalov J, Tafelmeyer P, Giovannoni JJ. 2011. The tomato MADS-box transcription factor RIPENING INHIBITOR interacts with promoters involved in numerous ripening processes in a COLORLESS NONRIPENING-dependent manner. Plant Physiology 157, 1568–1579.

Meli VS, Ghosh S, Prabha TN, Chakraborty N, Chakraborty S, Datta A. 2010. Enhancement of fruit shelf life by suppressing N-glycan processing enzymes. Proceedings of the National Academy of Sciences, USA 107, 2413–2418.

Menke FLH, Parchmann S, Mueller MJ, Kijne JW, Memelink J. 1999. Involvement of the octadecadienoid pathway and protein phosphorylation in fungal elicitor-induced expression of terpenoid indole alkaloid biosynthesis genes in Catharanthus roseus. Plant Physiology 119, 1289–1296.

Orzáez DF, Mirabel S, Wieland WH, Granell A. 2006. Agroinjection of tomato fruits, a tool for rapid functional analysis of transgenes directly in plant. Plant Physiology 140, 3–11.

Osorio S, Alba R, Nikoloski Z, Kochevenko A, Fernie AR, Giovannoni JJ. 2012. Integrative comparative analyses of transcript and
metabolite profiles from pepper and tomato ripening and development stages uncovers species-specific patterns of network regulatory behavior. *Plant Physiology* 158, 1713–1729.

Osorio S, Scossa F, Fernie AR. 2013. Molecular regulation of fruit ripening. *Frontiers in Plant Science* 4, 198. doi:10.3389/fpls.2013.00198.

Priem B, Gitti R, Bush CA, Gross KC. 1992. Mannosyl- and xylosyl-containing glycans promote tomato (*Lycopersicon esculentum* Mill.) fruit ripening. *Plant Physiology* 98, 399–401.

Qin G, Wang Y, Cao B, Wang W, Tian S. 2012. Unraveling the regulatory network of the MADS box transcription factor *RIN* in fruit ripening. *The Plant Journal* 70, 243–255.

Ricardi MM, Gonzale RM, Zhong S et al. 2014. Genome-wide data (ChIP-seq) enabled identification of cell wall-related and aquaporin genes as targets of tomato ASR1, a drought stress-responsive transcription factor. *BMC Plant Biology* 14, 29.

Ricardi MM, Guaimas FF, González RM, Burrieza HP, López-Fernández MP, Jares-Erijman EA, Estévez JM, Iusem ND. 2012. Nuclear import and dimerization of tomato ASR1, a water stress-inducible protein exclusive to plants. *PLoS One* 7, e41008.

Rice P, Longden I, Bleasby A. 2000. EMBOSS: the European Molecular Biology Open Software Suite. *Trends in Genetics* 16, 276–277.

Seymour GB, Chapman NH, Chew BL, Rose JKC. 2013a. Regulation of ripening and opportunities for control in tomato and other fruits. *Plant Biotechnology Journal* 11, 269–278.

Seymour GB, Ostergaard L, Chapman NH, Knapp S, Martin C. 2013b. Fruit development and ripening. *Annual Review of Plant Biology* 64, 219–241.

Shkolnik D, Bar-Zvi D. 2008. Tomato ASR1 abrogates the response to abscisic acid and glucose in Arabidopsis by competing with ABI4 for DNA binding. *Plant Biotechnology Journal* 6, 368–378.

Slamova K, Bojarova P, Petrasova L, Kren V. 2010. β-N-Acetylgalactosaminidase: What's in a name…? *Biotechnology Advances* 28, 682–693.

Strasser R, Bondili JS, Schoberer J, Svoboda B, Liebminger E, Glöss J, Altman F, Steinkellner H, Mach L. 2007. Enzymatic properties and subcellular localization of Arabidopsis β-N-acetylgalactosaminidases. *Plant Physiology* 145, 5–16.

Sun L, Sun Y, Zhang M et al. 2012. Suppression of 9-cis-epoxycarotenoid dioxygenase, which encodes a key enzyme in abscisic acid biosynthesis, alters fruit texture in transgenic tomato. *Plant Physiology* 158, 283–298.

Sun L, Wang YP, Chen P, Ren J, Ji K, Li Q, Li P, Dai SJ, Leng P. 2011. Transcriptional regulation of *SIPY1, SIP2C*, and *SISnRK2* gene families encoding ABA signal core components during tomato fruit development and drought stress. *Journal of Experimental Botany* 62, 5659–5669.

Tigchelaar EC, McGlasson WB, Buescher RW. 1978. Genetic regulation of tomato fruit ripening. *HortScience* 13, 508–513.

Vrebalov J, Ruezinsky D, Padmanabhan V, White R, Medrano D, Drake R, Schuch W, Giovannoni J. 2002. A MADS-Box gene necessary for fruit ripening at the tomato ripening-inhibitor (*rin*) Locus. *Science* 296, 343–346.

Wilkinson JQ, Lanahan MB, Yen HC, Giovannoni JJ, Klee HJ. 1995. An ethylene-inducible component of signal transduction encoded by never-ripe. *Science* 270, 1807–1809.

Zhang M, Yuan B, Leng P. 2009. The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. *Journal of Experimental Botany* 60, 1579–1588.

Zhong S, Fei Z, Chen YR et al. 2013. Single-base resolution methylomes of tomato fruit development reveal epigenome modifications associated with ripening. *Nature Biotechnology* 31, 154–159.