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Brief Communication

Targeted disruption of tomato chromoplast-specific lycopene β-cyclase (*CYC-B*) gene promotes early accumulation of lycopene in fruits and enhanced postharvest cold tolerance

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Lycopene is a potent antioxidant carotenoid pigment found in tomatoes and other red fruits. In green tissues, lycopene acts as a free-radical scavenger conferring protection during photosynthesis (Khan *et al.*, 2021). High-lycopene tomato genotypes containing knockout mutations within the lycopene catabolic *CYC-B* gene include the spontaneous *old-gold* (*og*) or *old-gold crimson* (og^c) mutations, and the chemically-induced mutation A949G (Ronen *et al.*, 2000; Silletti *et al.*, 2012). These mutations only exist in determinate tomatoes harbouring mutant *sp* alleles in the *SELF-PRUNING* (*SP*) locus (Solyc06g074350), which controls plant architecture (Pnueli *et al.*, 1998). Most production systems destined for the fresh market, use indeterminate growth habit tomatoes (Peet and Welles, 2005). Genetic variants in the *SP* and *CYC-B* loci are difficult to separate by conventional breeding because of their tight genetic linkage (Figure S1a) (Pnueli *et al.*, 1998). Because of this, tomato breeders have failed to use *og*, og^c, and A949G mutants to obtain high-lycopene indeterminate tomatoes.

Here we used CRISPR/Cas9 to generate knockout variants of *CYC-B* (Solyc06g074240) in two indeterminate elite tomato genotypes (LT16 and LT46) harbouring wild-type (WT) *SP* alleles (Appendix S1). The resulting *cyc-b* knockout lines exhibited indeterminate growth and increased lycopene content in the fruit, overcoming the existing genetic linkage between these traits.

To disrupt *CYC-B*, two sgRNAs (1 & 2) were designed to target positions 44–63 or 274–293 bp downstream from the ATG codon, respectively (Figures 1a and S1b). SgRNA activity was pre-validated by *in vitro* DNA-cleavage assays using Cas9/gRNAs ribonucleoprotein complexes and *CYC-B* DNA template (Figure S1c). A binary vector containing a polycistronic sgRNA construct and a Cas9 expression cassette was used to transform LT16 and LT46 genotypes. Five knockout alleles for *CYC-B*, harbouring small deletions (1, 2, 4, or 7 bp), or a large deletion (229 bp), were obtained (Figures 1a and S2). All the edited alleles encoded truncated nonfunctional CYC-B proteins (Figure 1b). T-DNA-free homozygous mutants of both genotypes were obtained within the first two generations after plant transformation (Figure 1c; Table S1). LT16-*cycb*Δ7 line, containing a 7 bp (6 + 1) deletion affecting both sgRNAs targets, lacked off-target mutations in 3 predicted target genes (Figure S3).

Fruit quality parameters (acidity, soluble solids content, and firmness) were similar between LT16-*cycb*Δ7 and the WT (Table S3), and no developmental alterations were observed in LT16-*cycb*Δ7. In contrast, the colour of flowers and fruits from the edited line exhibited substantial differences with the WT (Figure 1d). LT16-*cycb*Δ7 flowers showed tawny orange petals and anthers in contrast to the bright yellow flowers of the WT. External and internal colour indexes (CI) of the fruit were higher in LT16-*cycb*Δ7, both at Breaker and Red-Ripe stages. The differences in red colour development were more evident in the columella, placenta, and locular tissue.

Interestingly, chromoplasts of the locular tissue of LT16-*cycb*Δ7 differed substantially from WT chromoplasts. Edited plants contained abundant lycopene-accumulating crystalloid chromoplasts, with more and larger lycopene crystals than the WT. In contrast, WT locular tissue contained mainly round-shaped plastoglobuli-like structures accumulating β-carotene (Figures 1e and S4).

Lycopene accounts for 90% of total carotenoids in Red-Ripe tomatoes, followed by β-carotene, lutein, and phytoene/phytofluene (Ronen *et al.*, 2000). Similar to og^c mutants, we found that LT16-*cycb*Δ7 had higher lycopene and lower β-carotene contents in fruit than the WT, especially in the Breaker stage (Figure 1d; Table S2). These differences were less evident in the pericarp of Red-Ripe tomatoes (Table S2). In addition, fruits from LT16-*cycb*Δ7 developed a more uniform external and internal red colour and higher CI values compared to the WT during postharvest maturation at 25 °C (Figure 1f), representing a commercial advantage for edited plants because the fresh-market supply chain usually uses fruit harvested at Breaker stage.

Tomato fruit is highly sensitive to chilling injury (ChI) disorder when exposed to low non-freezing temperatures (Rai *et al.*, 2022). ChI is associated with loss of membrane permeability and accumulation of reactive oxygen species (ROS). The protective role of lycopene during chilling stress has been reported in grapefruit peel associated with higher ROS scavenging properties (Lado *et al.*, 2016). Here, we demonstrated that CYC-B-edited plants were more tolerant to ChI during postharvest cold storage. When edited and WT fruits were harvested at Breaker stage and stored for 13 days at 5 °C, the percentage of fruits with visible symptoms of ChI was remarkably reduced in LT16-*cycb*Δ7 (20%), compared to

the WT (63%) (Figure 1g). Moreover, levels of lipid peroxidation of fruit epidermis induced by low-temperature (revealed by Schiff's staining), were evidently lower in LT16-*cycb*Δ7 than in the WT (Figure 1h). These results suggest that early lycopene accumulation during fruit ripening prevents low-temperature-induced damage to fruits. To our knowledge, this is the first report establishing a direct association between lycopene content and chilling tolerance in tomato.

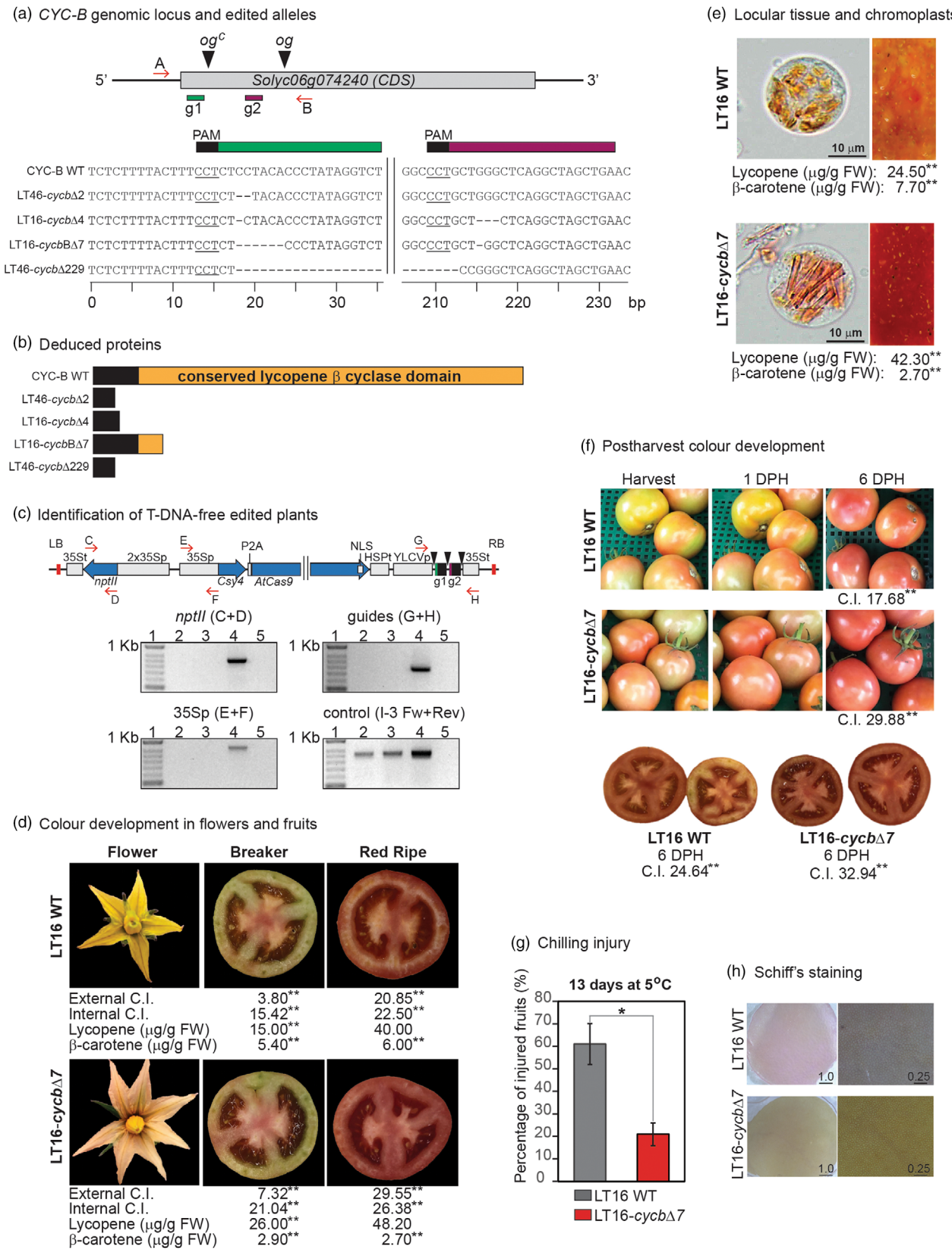

Figure 1 (a) Illustration of *CYC-B* gene and sgRNA target sequences of WT and edited lines. Location of sgRNAs targets (Guide1 green, Guide2 purple), og^c and *og* mutations, and primers (A and B) used for mutation screening, are shown. (b) Predicted CYC-B proteins from WT and edited alleles. (c) Identification of T-DNA-free edited plants. Primers for PCR amplification of different parts of the gene construct are shown in the T-DNA schematic representation. PCR amplification of *nptII* (primers C + D), 35S promoter (E + F), Guide cassettes (G + H), and tomato *I-3* endogenous control gene (I-3 Fw + Rev), were separated by electrophoresis in agarose gels. Lanes: (1) Hyperladder 50 bp (Meridian), (2) WT, (3) LT16-*cycb*Δ7, (4) LT16-1 T0 transgenic, (5) DNA-free control. (d) Flowers and fruits of LT16-WT and LT16-*cycb*Δ7. Internal and external colour index (C.I.) and lycopene and β-carotene content (μg/g FW) of whole fruit are indicated. (e) Locular tissue and individual cells showing characteristic chromoplasts and lycopene and b-carotene content of LT16-WT and LT16-*cycb*Δ7. (f) Postharvest colour development of Breaker fruit from WT and LT16-*cycb*Δ7 stored at 25 °C. DPH: days postharvest. (g) Percentage of Breaker fruit from LT16-WT and LT16-*cycb*Δ7 with chilling injury symptoms after 13 days of storage at 5 °C (*$P = 0.0869$). (h) Schiff's staining of epidermis discs from LT16-WT and LT16-*cycb*Δ7 fruit stored at 5 °C for 19 days. Bars are in millimetres. Analysis of variance (ANOVA) followed by a Tukey's test (*$P < 0.1$; **$P < 0.05$) was used for mean comparison between WT and edited line for each developmental stage (d, e, and f). GLMM analysis was used in (g).

Combining genetically linked traits remains a challenge in plant breeding. We used CRISPR/Cas9 to combine, indeterminate growth habit and high lycopene traits without affecting other traits of the elite germplasm. Additionally, we provide direct evidence of the protective role of lycopene during fruit cold storage, reducing ChI symptoms and yielding fresh market tomatoes with higher commercial value.

Author contributions

AA, JL, MGA and SV designed the experiments. AA performed the experiments and wrote the manuscript with contributions from all authors. All authors read and approved the manuscript's final version.

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Competing interests

Authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1 Experimental procedures used in this study.
Appendix S2 Supplementary Figures S1–S4.
Appendix S3 Supplementary Tables.