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Supplemental information

The abscisic acid-responsive transcriptional regulatory module CsERF110–CsERF53 orchestrates citrus fruit coloration

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Running Title: ABA promotes citrus fruit coloration

The Title: An abscisic acid-responsive transcriptional regulatory module CsERF110-CsERF53 orchestrates citrus fruit coloration

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One-sentence summary: Abscisic acid activates a novel transcriptional regulatory module CsERF110-CsERF53 that positively regulates citrus carotenoid accumulation.

Detailed description of methods

Subcellular localization assay

The fusion vectors CsERF110-GFP or *CsERF53*-GFP were co-transformed with the nuclear marker NF-YA4-mCherry into *Nicotiana benthamiana* (*N. benthamiana*) leaves through *Agrobacterium tumefaciens* (*A. tumefaciens*) GV3101 strain-mediated transformation, respectively. The florescence images were captured with the confocal microscope (TCS SP8, Leica, Germany) followed by 3 d incubation.

Extraction and determination of carotenoids

Carotenoid extraction and analysis of citrus fruit and calli were performed as described previously (Sun *et al.*, 2023, 2024). At least three biological replicates from independent extractions were performed.

Transcriptional activation analysis

The CDSs of *CsERF110* and *CsERF53* were cloned into the PGBKT7 vector containing a GAL4 DNA binding domain (BD) to generate the PGBKT7-CsERF110/CsERF53 vectors. PGBKT7-CsERF110 or PGBKT7-CsERF53 was transformed into the yeast strain AH109, and then were cultured on SD/-Trp and SD/-Trp/-His/-Ade medium. The growth status of yeast cells was used to evaluate the transcriptional activation of CsERF110 and CsERF53.

To further clarify whether CsERF110 and CsERF53 have transcriptional activation activity, we constructed the effector vectors pBD-CsERF110 and pBD-CsERF53. The reporter vector consists of GAL4-LUC and REN driven by the 35S promoter. The effector vector pBD-CsERF110 or pBD-CsERF53 were co-transformed with the reporter vector into *N. benthamiana* leaves through *A. tumefaciens* GV3101 strain-mediated transformation, respectively. The pBD-VP16 plasmid and empty vector pBD were used as positive control and negative control, respectively. The transcriptional activation activity of CsERF110 or CsERF53 was calculated by LUC/REN ratio followed by 3 d incubation.

Yeast two-hybrid (Y2H) assay

The full-length CDS of *CsERF110* and the truncated CDS (1-846) of *CsERF53* were ligated into the PGBKT7 vector to yield BD-CsERF110 and BD-CsERF53$^{1-846}$ vectors. The full-length CDSs of *CsERF110* and *CsERF53* were cloned into the PGADT7 vector to obtain AD-CsERF110 and AD-CsERF53 vectors. The following combinations AD+BD, BD-CsERF110+AD, BD-CsERF110+AD-CsERF53, BD+AD-CsERF53, AD+BD-CsERF53$^{1-846}$, AD-CsERF110+BD-CsERF53$^{1-846}$, BD+AD-CsERF110, and PGBKT7-53+PGADT7-RecT (positive control) were transformed into yeast strain AH109, and then were cultured on SD/-Trp/-Leu medium and SD/-Leu/-Trp/-His/-Ade medium. The interaction between CsERF110 and CsERF53 is indicated by yeast growth and 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) staining.

Dual luciferase reporter assay

See supplemental information for detail.

The overexpression constructs PK7-CsERF110 or PK7-CsERF53 as effector. The promoter of target genes was cloned into the pGreenII 0800-LUC vector to obtain the reporters (pGreenII 0800-ProCsCBGs-LUC and pGreenII 0800-ProCsERF53-LUC). The PK7-CsERF110 effector or PK7-CsERF53 effector and the corresponding reporter were co-transformed into *N. benthamiana* leaves via *A. tumefaciens* GV3101 strain-mediated transformation. Luciferase activities were measured with Dual-Luciferase® Reporter Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instruction followed by 3 d incubation.

References

Sun Q, He Z, Wei R, Yin Y, Ye J, Chai L, Xie Z, Guo W, Xu J, Cheng Y, *et al.* 2023. Transcription factor CsTT8 promotes fruit coloration by positively regulating the methylerythritol 4-phosphate pathway and carotenoid biosynthesis pathway in citrus (*Citrus* spp.). *Horticulture*

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