

Gene network associates with CsCRC regulating fruit elongation in cucumber

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Abstract

In cucumber (*Cucumis sativus* L.), fruit length is a crucial agronomic trait that affects appearance quality and crop yield. Different cucumber germplasms exhibit a large variation in fruit length, and the underlying candidate genes and regulatory mechanisms need to be further characterized. Recently we showed that an essential gene in modulating the cucumber fruit length, the YABBY transcription factor CRABS CLAW (CsCRC), activates its downstream target gene auxin responsive protein *CsARP1* to promote cell expansion and fruit elongation. Here, we show that auxin activity and three fruit development-related genes are involved in the CsCRC-regulated gene network in cucumber. We found that a PHYTOCHROME INTERACTING FACTOR (CsPIF1) protein elevates CsCRC transcription by directly binding to its promoter. The protein interaction assays showed that a reproductive YABBY transcription factor INNER NO OUTER (CsINO) and a bHLH protein SPATULA (CsSPT1) have direct interaction with CsCRC, suggesting their redundant role in cucumber fruit development.

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Introduction

Cucumber is an agronomically important vegetable crop cultivated worldwide. The cucumber fruit belongs to the pepo fruit that can be eaten fresh or processed into pickles, in which the fruit length is a predominant trait that directly affects crop yield and product appearance quality^[1]. Fruit length is also an important index to distinguish cucumbers from different market types. There were significant differences in fruit length (5–60 cm) among different cucumber germplasm, with multiple genetic factors participating^[2]. In a recent study, we elucidated that the natural variation in *CRABS CLAW* (CsCRC) contributes to fruit length divergence in cucumber^[3].

CsCRC gene is a positive regulator conferring the allelic variations in the cucumber germplasms with different fruit length. CRC protein belongs to the YABBY family, which contains a conserved C2C2 zinc-finger and a DNA-binding YABBY domain^[4–8]. In *Arabidopsis*, CRC signals were detected in the floral tissues, the nectary, the abaxial region of the carpel, the receptacles, sepals, and petals^[9]. In contrast, we found CsCRC expression displayed a more concentrated signal in the cucumber carpel, developing ovary and nectary^[3]. CRC has an active role in carpel development, nectary formation, floral meristem determinacy, and sex determinance^[4,10,11]. *Arabidopsis crc* null mutants showed wider and shorter siliques with unfused carpels, reduced ovules, and lack of nectary^[9,12]. Overexpression of CRC resulted in sepals developing some carpel characters and gynoecium transforming into cylindrical structures comprised of stigma and style in *Arabidopsis*^[4]. To explore whether CsCRC has a redundant role with CRC in *Arabidopsis*, we first investigated the ectopic expression of CsCRC in wild

type Landsberg *erecta* (Ler) and found an intense staining of *proCsCRC:GUS* in the developing stigma (Fig. 1a). We then transformed the CsCRC driven by the 35S promoter into the *crc-1* mutant and selected three transgenic lines for phenotype observation (Fig. 1b). Overexpression of CsCRC rescued the unfused-stigma phenotype in *crc-1* (Fig. 1b, c), but was unable to rescue the short siliques phenotype, suggesting that CsCRC has a partially conserved role in gynoecium development similar to CRC gene in *Arabidopsis*, while displaying a unique function in fruit length regulation in cucumber.

The gynoecia defects in the *crc* mutant can also be partially restored by auxin activity^[13]. CRC participates in auxin homeostasis by inhibiting *TORNADO* (*TRN*) transcription, which modulates auxin maxima in *Arabidopsis*^[14]. In cucumber, CsCRC acts as a positive regulator in fruit length elongation through activating the downstream auxin responsive protein *CsARP1* and promotes cell expansion. To investigate the auxin role in cucumber fruit length regulation, we performed exogenous IAA and polar auxin-transport inhibitor N-1-naphthylphthalamic acid (NPA) treatments in cucumber inbred line R1461. IAA treatment resulted in elongated fruit length and enlarged cell size, whereas NPA treatment gave rise to the opposite effects, suggesting that auxin promotes fruit elongation *via* cell expansion in cucumber (Fig. 2a–c). Interestingly, the expression of *CsARP1* and *CsCRC* were significantly induced by IAA and repressed by NPA (Fig. 2d). These data suggests that auxin promotes fruit elongation through elevated cell expansion and enhanced CsCRC and *CsARP1* expression in cucumber.

Auxin signaling pathways has an interplay mechanism with light signal that antagonistically regulates plant growth^[15]. In a previous study, we revealed that the rare CsCRC^A allele only

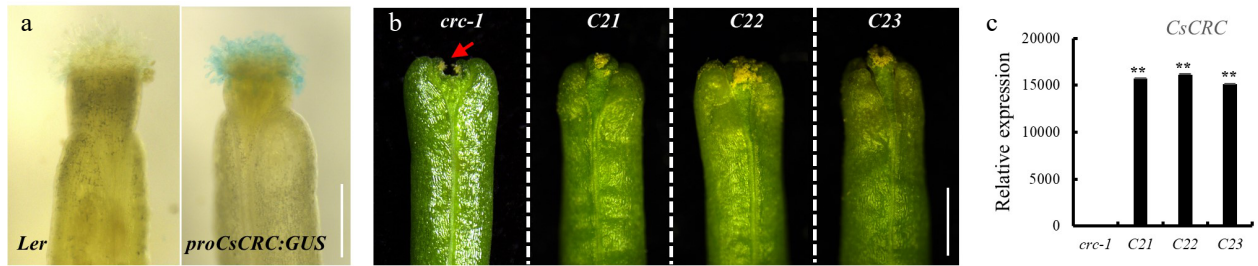


Fig. 1 Ectopic expression of *CsCRC* in *Arabidopsis*. (a) *proCsCRC:GUS* reporter activity in wild-type *Arabidopsis Ler* showing intense staining in stigma. (b) Ectopic expression of *CsCRC* rescued the unfused-stigma phenotype in *Arabidopsis crc-1* mutant. (c) RT-qPCR analysis of *CsCRC* overexpression in *cre-1* mutant. The arrow indicates the stigma defect in *cre-1*. **: significance at $P < 0.01$. Three biological replicates and three technique replicates were performed for each RT-qPCR analysis. Scale bar = 1 mm.

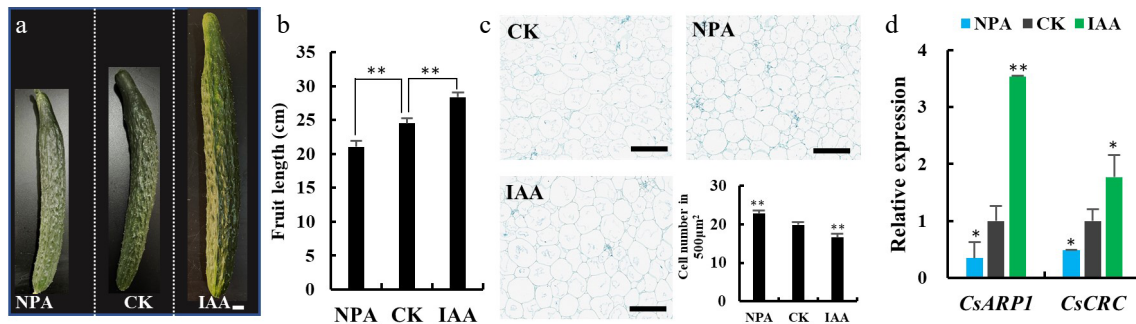


Fig. 2 Auxin promotes fruit elongation and activates the expression of *CsCRC* and *CsARPI*. (a) Cucumber fruits at commercial stage upon IAA and NPA treatments. (b) Quantification of fruit length at 10 DAA after treatment. (c) Longitudinal sections in the mesocarp fruits at 10 DAA. Cell numbers in the mesocarp of 10DAA fruit were counted. (d) The relative expression of *CsARPI* and *CsCRC* in cucumber ovaries upon treatment. Scale bars are (a) 1 cm and (c) 200 μm. *: significance at $P < 0.05$; **: significance at $P < 0.01$. Three biological replicates and three technical replicates were performed for each RT-qPCR analysis.

exists in a few semi-wild Xishuangbanna cucumber with short or round fruits. The Xishuangbanna area is close to the tropics and has more light intensity than northern China. The phytochromes, which are stimulated by light, promote the degradation of the PHYTOCHROME INTERACTING FACTOR (PIF) proteins and enhance the accumulation of auxin transcriptional repressors^[16]. We analyzed the 2038-bp promoter of *CsCRC*, and found one G-box and two E-boxes, which are the specific binding sites for PIFs (Fig. 3a). We then conducted Y1H assay and found that *CsPIF1* can directly bind to the promoter of *CsCRC* (Fig. 3b). To investigate whether *CsPIF1* regulates *CsCRC* expression *in vivo*, transient expression assays were performed in tobacco leaves, in which the 2038 bp upstream promoter region of *CsCRC* fused into the *luciferase* (*LUC*) gene was used as the reporter, and the coding sequence of *CsPIF1* driven by the 35S promoter was used as the effector (Fig. 3c). The LUC activity was significantly enhanced upon co-transformation of 35S:*CsPIF1* with *proCsCRC:LUC* (Fig. 3d). This data supports the fact that *CsPIF1* directly promotes the expression of *CsCRC*. Therefore, we presumed that the strong light intensity in Xishuangbanna reinforces *CsPIF1* degradation, thus inhibiting *CsCRC* expression and results in reduced fruit length.

Fruit development is fine-tuned by multiple genes involved in several aspects of the reproductive growth phase. YABBY gene family contains six members: *CRC* and *INNER NO OUTER* (*INO*), regulate reproductive growth, while other vegetative YABBY genes, *FILAMENTOUS FLOWER* (*FIL*), *YAB2*, *YAB3*, and *YAB5*, control leaf-derived organ development^[17]. In *Arabidopsis*, *CRC* can enhance *SPT* function in controlling carpel

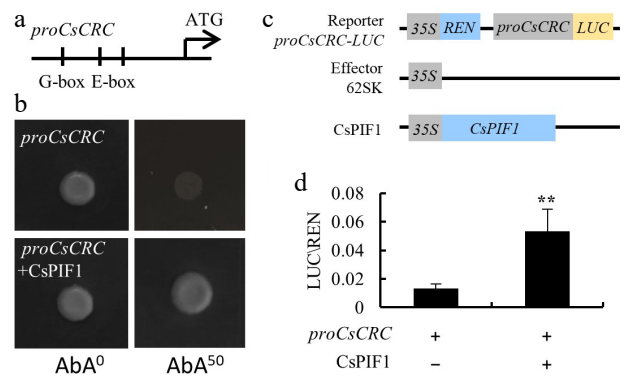


Fig. 3 *CsPIF1* is the upstream regulator of *CsCRC*. (a), (b) Y1H assay showed that *CsPIF1* bound to the G-box at the promoter of *CsCRC*. (c) Schematic diagram of the reporter and effector constructs used for LUC/REN assays. (d) LUC activity measurement after transient expression of *proCsCRC:LUC* with 35S:*CsPIF1* in tobacco leaves. Error bars represent standard errors. **: significance at $P < 0.01$.

development^[18]. Another reproductive YABBY gene *INO* is expressed in floral organs and has an important role in seed formation^[5,19]. To explore their potential role in the *CsCRC*-mediated cucumber fruit length regulation pathway, we performed protein interaction assays. Yeast two-hybrid analyses showed that *CsCRC* directly interacted with *CsSPT1* and *CsINO* *in vitro* (Fig. 4a). The interactions were confirmed by performing BiFC in tobacco leaves (Fig. 4b). Therefore, *CsSPT1* and *CsINO* may interplay with *CsCRC* to participate in cucumber fruit

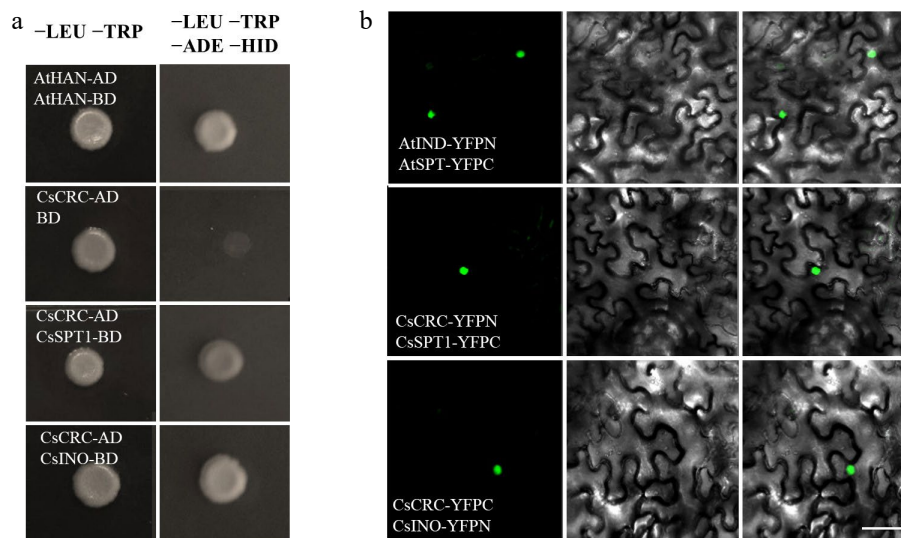


Fig. 4 Protein interactions detected with Y2H and BiFC assays. (a) Y2H assay showed the interaction of CsCRC with CsSPT1 and CsINO. The interaction of AtHAN-BD and AtHAN-AD was used as the positive control. (b) BiFC assay. The combination of AtIND-YFPC and AtSPT-YFPC was used as a positive control. Dark-field, bright-field and merged channels are shown sequentially from left to right. Scale bar = 50 μ m.

development. Further elucidation of the CsCRC-mediated gene network will shed a light on the molecular mechanisms affecting fruit development in cucumber.

MATERIALS AND METHODS

Plant materials and growth conditions

The Landsberg *erecta* (Ler) *Arabidopsis* and the *crc-1* mutant were obtained from Zhang's lab. The *Arabidopsis* were cultivated in a growth chamber under 16/8 h light/dark at 22 °C. Cucumber inbred line R1461 were grown in the glasshouse of Inner Mongolia University in Hohhot under standard environment control. The *GUS* reporter gene recombinant construct and *CsCRC* overexpression vector were transferred into *Agrobacterium* and transformed into Ler and *crc-1* mutant.

RT-qPCR

The young ovaries of R1461 were used for RNA extraction. The synthesized cDNA was used as the template for qPCR. The SYBR Premix Taq Mix (Takara) was used for RT-qPCR assay and an Applied Biosystems 7500 real-time PCR system was used for statistical analysis. *Arabidopsis ACTIN2* and cucumber *UBI* were used as reference genes, respectively. For each RT-qPCR analysis, three biological replicates and three technical replicates were performed.

Yeast One-Hybrid assay

CsPIF1 coding sequence was cloned into the pGADT7 vector (Clontech, CA, USA). The putative PIFs binding sites were found at the promoter of *CsCRC* and then cloned with the pAbAi vector (Clontech, CA, USA). The resultant constructs were introduced into the yeast Y1H Gold strain and selected by different AbA (Aureobasidin A) concentration on the SD/-LEU (Synthetic Dropout Medium/-Leucine) medium (Clontech, CA, USA).

LUC/REN assay

The 2038 bp upstream sequence of *CsCRC* was inserted into pGreenII 0800 vector and used as reporter. The coding

sequence of *CsPIF1* was cloned and constructed with pGreenII 62-SK vector. The constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 together with pSUPER and p19 plasmid^[20]. The bacterial fluid of effector: reporter (9:1) construct cell mixture was injected into young leaves of *Nicotiana benthamiana*. The LUC/REN ratio was measured by luciferin (Promega, USA). A strain of the *proCsCRC* and empty effector was used as a negative control.

Yeast Two-Hybrid assay

Coding sequence of *CsCRC*, *CsSPT1*, and *CsINO* genes were inserted into pGADT7 and pGBKT7 vectors. All constructs were transformed into yeast strain AH109 for preparation. Yeast two-hybrid assays were performed according to the description of Matchmaker TM GAL4 Two-Hybrid System 3 & Libraries (Clontech, CA, USA). Combination of each gene with blank vector pGADT7 and pGBKT7 were used as negative control. The primers used for Y2H vector construction are listed in [Supplemental Table S1](#).

Bimolecular Fluorescence Complementation (BiFC) assay

The coding sequence of *CsCRC*, *CsSPT1*, and *CsINO* which are lacking a stop codon were inserted into the fluorescence vectors pSPYCE-35S and pSPYNE-35S. Those constructs were transformed into *Agrobacterium* GV3101. Each combination of two proteins was injected into the young leaves of tobacco. The infected tobacco leaves were sampled and observed through Zeiss LSM 510 Meta confocal laser microscopy under 488 nm excitation wavelength. AtIND-YFPC and AtSPT-YFPC interaction was used as a positive control^[21].

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Conflict of interest

Xiaolan Zhang and Gen Che are the Editorial Board members of journal *Vegetable Research*. They were blinded from reviewing or making decisions on the manuscript. The article was subject to the journal's standard procedures, with peer-review handled independently of these Editorial Board members and their research groups.

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