Mutation in the gene encoding 1-aminocyclopropane-1-carboxylate synthase 4 (CitACS4) led to andromonoecy in watermelon

Summary Although it has been reported previously that ethylene plays a critical role in sex determination in cucurbit species, how the andromonoecy that carries both the male and hermaphroditic flowers is determined in watermelon is still unknown. Here we showed that the watermelon gene 1-aminocyclopropane-1-carboxylate synthase 4 (CitACS4), expressed specifically in carpel primordia, determines the andromonoecy in watermelon. Among four single nucleotide polymorphism (SNPs) and one InDel identified in the coding region of CitACS4, the C364W mutation located in the conserved box 6 was co-segregated with andromonoecy. Enzymatic analyses showed that the C364W mutation caused a reduced activity in CitACS4. We believe that the reduced CitACS4 activity may hamper the programmed cell death in stamen primordia, leading to the formation of hermaphroditic flowers.

It has been reported that 1-aminocyclopropane-1-carboxylic synthase (ACS), a key enzyme for ethylene biosynthesis, is important for sex determination in several Cucurbitaceae species (Boualem et al. 2008, 2009). In cucumber and melon, mutations that cause reduced ACS activities lead to andromonoecy (Boualem et al. 2008, 2009). Modern watermelon (Citrus lanatus) varieties have three common sex forms: monoecious (carrying both male and female flowers), andromonoecious (carrying both male and hermaphroditic flowers) and gynoecious (carrying female flowers only) (Ji et al. 2015), and a recessive locus (a) is associated with the andromonoecy (Ji et al. 2015). Eight ACS genes, Cla014652 (CitACS1), Cla014557 (CitACS2), Cla006634 (CitACS3), Cla001230 (CitACS4), Cla000483 (CitACS9), Cla015122 (CitACS10), Cla022653 (CitACS11), and Cla006245 (CitACS12) are present in the watermelon genome (Guo et al. 2013, 2015). Four genes in ACS family, CitACS1, 2, 3, and 4, were speculated to associate with sex determination (Salman-Minkov et al. 2008; Prothro et al. 2013; Guo et al. 2015). Homology analyses showed that the CitACS4 shared 94% sequence identity with the CmACS-7 in melon at the amino acid level (Figure 1A). The 1,720 bp genomic region of CitACS4, containing three exons and two introns (Figure S1), encodes a polypeptide with 444 amino acids (Figure 1A). To determine if the C364W mutation had compromised the enzymatic activity of ACS, three constructs, His6-CitACS4, His6-CitACS4 C364W and His6-CmACS-7, were made and transformed into Escherichia coli. Fusion proteins were affinity-purified using Ni columns. The ACS enzymatic activity was measured using a buffer containing 10 μM pyridoxal 5'-phosphate (PLP) and 200 μM S-adenosyl methionine (SAM) (Boualem et al. 2008, 2009). Results obtained showed that the ACS activity of His6-CitACS4 was similar to that of His6-CmACS-7, while the ACS activity of His6-CitACS4 C364W was significantly reduced, suggesting that the C364W mutation has compromised the enzymatic activity of CitACS4 (Figure 2A). The 3-dimensional modeling for CitACS4, which referred to the LeACS8 structure in tomato (Huai et al. 2001), showed that the C364 residue is located in the α-carboxylate backbone (Figure S3A, B). In this model, the cysteine residue possesses a mercapto group that can potentially form a disulfide bond to maintain the protein stability (Figure S3B). The C364W substitution may have disrupted the stability of CitACS4, and subsequently damaged the activity of the enzyme.

To examine the expression pattern of CitACS4, total RNA was extracted from different parts of watermelon plant. Quantitative real-time PCR assays were performed and results showed that CitACS4 was expressed specifically in female and Herhap flowers (Figure 2B). According to the stages defined for flower development in cucumber (Bai et al. 2004), CitACS4...
was expressed primarily in stage 6 female and hermaphroditic flower buds, and lower levels of expression were detected in stage 10 and 14 flowers (Figures S4, S5). RNA in situ hybridization analysis revealed that CitACS4 was expressed specifically in the carpel primordia of female and hermaphroditic flower buds at stages 5 and 6 (Figure 2C, D), while no expression was detected in male flower buds (Figure 2E). The CitACS4 expression pattern is similar to that of the CsACS2 in cucumber, and the CmACS-7 in melon (Boualem et al. 2008, 2009). All primers used in this study were listed in Table S1.

The production of ethylene in female floral primordia, mediated by the ACS activity, triggers the programmed cell death (PCD) in male floral organs (Bai et al. 2004). In this study we showed that the andromonoecious sex form in watermelon was caused by a recessive mutation in CitACS4. The compromised enzymatic activity of CitACS4 in andromonoecious watermelon varieties may have caused a reduced ethylene production in carpel primordia, leading to the formation of flowers with both male and female organs. This result may potentially be used in breeding and genetic improvement of watermelon in the future.

ACKNOWLEDGEMENTS

We thank Professor Ningning Wang at Nankai University for helps in biochemical analyses, and Professor Chun-Ming Liu at the Institute of Botany, the Chinese Academy of Sciences, for critical reading of the manuscript. This work was supported by grants from National Natural Science Foundation (3136110355, 31401893, 31272184), National Key Development Plan Pilot Projects (Functional genomics research and application of wheat and other crops), Beijing Scholar Program and Beijing Excellent Talents Program (2015BAD12B02, 2014BAD12B08) and Ministry of Agriculture of China (CARS-26).

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Figure 1. A mutation in a conserved site of CitACS4 is associated with andromonoecy
(A) Alignment of CmACS-7 and CitACS4 proteins. The A57V and C364W indicate the amino acid changes associated with the andromonoecious genotype in melon and watermelon, respectively. (B) Alignments of CitACS4, CmACS-7, CsACS2 and homologous proteins from AmACS3 (Antirrhinum majus, AAC70353), AtACS3 (Arabidopsis thaliana, AF322390), LeACS8 (Lycopersicon esculentum, AF179247), MtACS (Medicago truncatula, AAL35745), PgACS1 (Picea glauca, ABM60747), VvACS7 (Vitis vinifera, CAN66901) and ZmACS65 (Zea mays, AAR25560). G33C, P209S and S399L are mutations in CsACS2, A57V is the mutation in CmACS-7, and C364W is the mutation in CitACS4. All these mutations lead to andromonoecy.
Figure 2. Enzyme activity and expression analyses of CitACS4

(A) Enzyme activities of His6-CitACS4, His6-CitACS4C364W and His6-CmACS-7 produced in Escherichia coli. Note that amounts of ethylene produced by CitACS4C364W is significantly lower (indicated with **) than those produced by His6-CitACS4 and His6-CmACS-7. AA: His6-CitACS4; aa: His6-CitACS4C364W; CmACS-7: His6-CmACS-7. (B) Expression analyses of CitACS4 expressions in different organs. Note that, in stage 6 floral buds, CitACS4 expressions in female (Ff) and hermaphroditic (Hf) flower buds are significantly higher (indicated with **) than those in male flower buds (Mf) and any other organs tested. (C) to (E) RNA in situ hybridization analyses showing female flower in stage 6 (C), hermaphroditic flower in stage 5 (D) and male flower in stage 4 (E). S, sepal; P, petal; St, stamen; C, carpel. Bar = 100 μm.

Keywords: 1-aminocyclopropane-1-carboxylic acid synthase; Citrullus lanatus; sex determination


Edited by: Chun-Ming Liu, Institute of Botany, CAS, China

Received Oct. 22, 2015; Accepted Jan. 28, 2016
Available online on Feb. 3, 2016 at www.wileyonlinelibrary.com/journal/jipb

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AUTHOR CONTRIBUTIONS

G. J. and J. Z. drafted the manuscript. G. J., H. Z., J. Z. and J. S. performed the experiments. G. J., H. S., S. T., S. G. and Y. R. analyzed the data. G. G., H. S. and J. G. contributed materials. Y. X. designed the experiment, supervised the study, and revised the manuscript.
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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web-site.

Figure S1. Sequence analysis of CitACS4

Figure S2. Analysis of AKKZW × XHB F2 population using the dCAPs_FspBI marker

Figure S3. Superposition of the ACS structure

Figure S4. Expression analysis of CitACS4 in female flowers

Figure S5. Expression analysis of CitACS4 in hermaphroditic flowers

Table S1. Primers used in this study