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Brief Communication

CRISPR/Cas9-mediated genome editing of *MaACO1* (aminocyclopropane-1-carboxylate oxidase 1) promotes the shelf life of banana fruit

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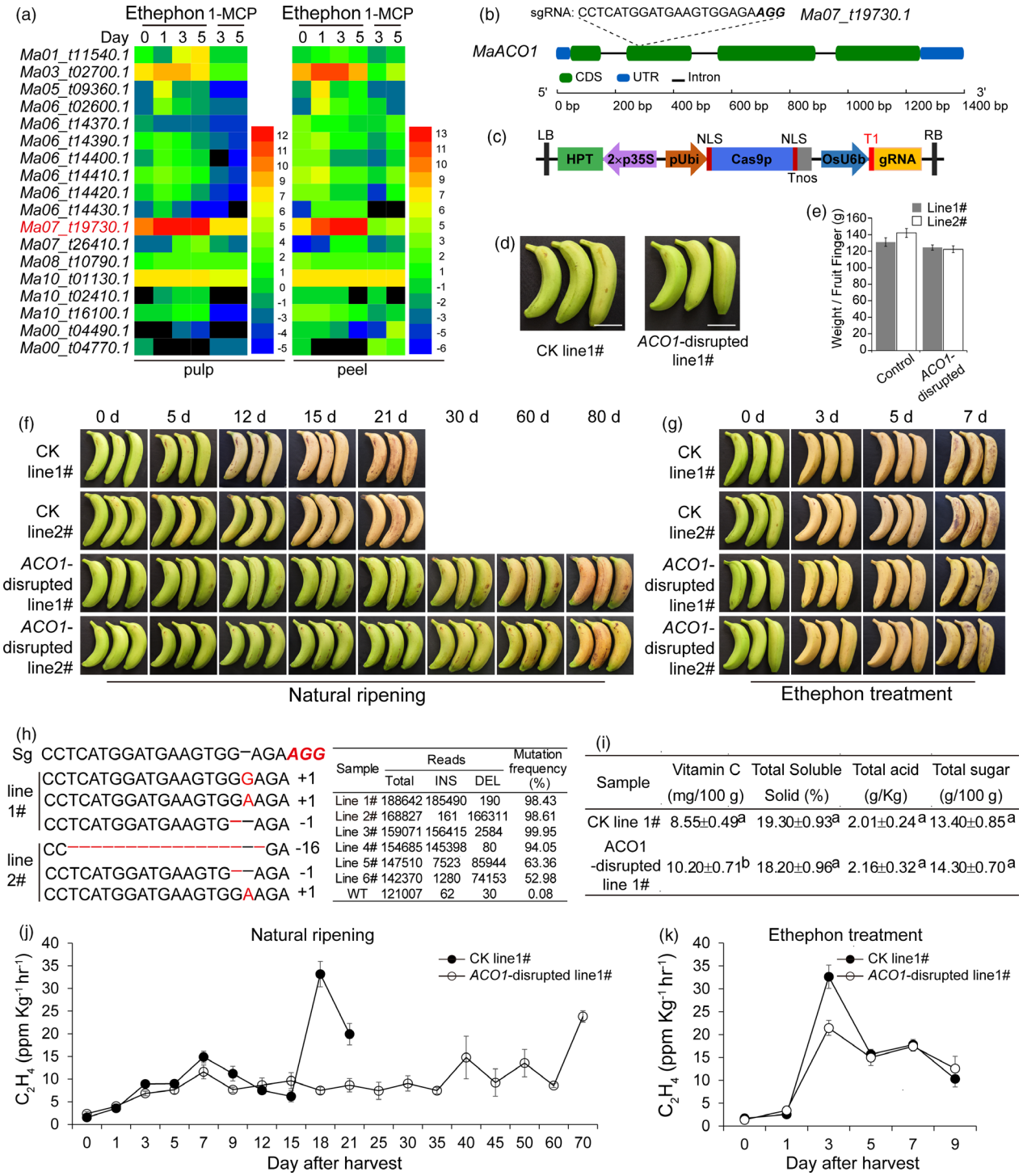
Banana is a fruit with high nutrient content and high economic importance. It is regarded as a main staple food in developing countries. As a typical climacteric fruit, banana will ripen and decay in one week after exogenous ethylene induction. The short shelf life of banana largely limits its storage, transportation and marketing and causes great postharvest loss (Krishnakumar and Thirupathi, 2014). The shelf life of banana is closely related to ethylene production, which is the first factor considered for developing postharvest preservation technology. A reduction of endogenous ethylene production or impaired ethylene signal transduction by genetic modification might be highly efficient methods to delay the ripening process (Elitzur *et al*., 2016).

In climacteric fruit, ethylene synthesis is controlled by the transcription of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) genes. ACO is responsible for converting ACC into ethylene by a reduction reaction. In banana, Inaba *et al*.(2007) showed that *MA-ACO1* (GenBank No. X91076) might play a more important role in banana fruit ripening than *MA-ACO2* (GenBank No. X95599) according to expression analysis. Another two ACO homologs, named *Mh-ACO1* (GenBank No. AF030411) and *Mh-ACO2* (GenBank No. U86045), show high induction by ethylene and specific expression in flower and fruit tissues and also imply their close involvement on banana fruit ripening (Do *et al*., 2005; Huang *et al*., 1997; Liu *et al*., 1999). A recent report indicated that there are 18 ACC oxidase homologous genes on *Musa acuminata* genome (Xia *et al*., 2016). Further analysis indicated that the encoded sequences of *MA-ACO2 and Mh-ACO1* partially matched the annotated sequence of *Ma00_t04770.1* and *Ma06_t14370.1*, respectively. *MA-ACO1* and *Mh-ACO2* are *Ma07_t19730.1* paralogues; their nucleotide sequences exhibit greater than 98% similarity with *Ma07_t19730.1*. To determine the role of *MaACOs* in fruit ripening, we performed RNA-seq of mature green fruit under ethephon or 1-methylcyclopropene (1-MCP) treatment. The results showed that among the *MaACO* gene family, *Ma07_t19730.1* and *Ma03_t02700.1* showed high transcript abundance under natural conditions in both pulp and peel tissues (day 0), and the highest transcript abundance of *Ma07_t19730.1* and *Ma03_t02700.1* occurred in pulp and peel tissues, respectively, in all of the examined *MaACOs* (Figure 1a, day 0). Moreover, the transcripts of these ACO genes were strongly induced by ethephon and were inhibited by 1-MCP to a greater extent in *Ma07_t19730.1* in both the pulp and peel (Figure 1a), which suggests that *Ma07_t19730.1* might play a more important role than *Ma03_t02700.1* in banana fruit ripening. Xia *et al*.(2016) reported that RNAi of *Mh-ACO2, the Ma07_t19730.1* paralogue, in *Musa acuminata* (AAA group, cv. Grand Nain) has strong effects on the expression of genes involved in ethylene signalling in ripening banana fruits.

To investigate whether *Ma07_t19730.1* is responsible for ethylene synthesis in *Musa acuminata* (AAA group, cv. Brazilian) and affects fruit ripening postharvest, *MaACO1* (*Ma07_t19730.1*)-disrupted mutants were developed using the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated endonuclease 9 (CRISPR/Cas9) system of banana that our group recently established (Hu *et al*., 2017). We selected one sgRNA '-5'-CCTCATGGATGAAGTGGAGAAGG-3'-' that specifically targets the second exon of *MaACO1* (Figure 1b). The sgRNA driven by an OsU6b promoter was introduced into the pYLCRISPR/Cas9Pubi-H vector (Ma *et al*., 2015) to obtain a pCRISPR/Cas9-ACO1T vector (Figure 1c). The embryogenic cell suspension (ECS) induced from *Musa acuminata* (AAA group, cv. Brazilian) was used to perform the genetic transformation (Hu *et al*., 2013). Six independent lines were regenerated from rooting medium with hygromycin B. After the integration of the vector cassette fragment was checked using PCR, healthy plantlets were transplanted and cultivated in a greenhouse until fruit maturation.

During the whole growth cycle, the *MaACO1*-disrupted lines exhibited similar growth and development as the wild type except for slightly lower height. For fruit traits, the length and weight of a single fruit finger in *MaACO1*-disrupted lines were about 15% shorter and 5%−14% lighter, respectively, than those of the wild type, and results in a little bit lower yield in *MaACO1*-disrupted lines (Figure 1d,e). To determine the impact of *MaACO1* on fruit ripening, two of six mutant lines were evaluated in the field. We compared the ripening process of the wild-type and two *MaACO1*-disrupted lines during postharvest storage at room temperature. Wild-type fruit completely ripened 21 days after harvest, whereas the ripening process of *MaACO1*-disrupted fruit



Sample	Reads			Mutation frequency
	Total	INS	DEL	(%)
Line 1#	188642	185490	190	98.43
Line 2#	168827	161	166311	98.61
Line 3#	159071	156415	2584	99.95
Line 4#	154685	145398	80	94.05
Line 5#	147510	7523	85944	63.36
Line 6#	142370	1280	74153	52.98
WT	121007	62	30	0.08

Sample	Vitamin C (mg/100 g)	Total Soluble Solid (%)	Total acid (g/Kg)	Total sugar (g/100 g)
CK line 1#	8.55±0.49^{a}	19.30±0.93^{a}	2.01±0.24^{a}	13.40±0.85^{a}
ACO1 -disrupted line 1#	10.20±0.71^{b}	18.20±0.96^{a}	2.16±0.32^{a}	14.30±0.70^{a}

Figure 1 CRISPR/Cas9-mediated editing of *MaACO1* enhances the shelf life of banana fruit. (a) The heat map was generated with the log 2 FPKM value of the *MaACO* family based on fruit transcriptome analysis. (b) Illustration of *MaACO1* gene structure and editing target. (c) Schematic diagram of the *MaACO1*-editing vector. (d) Comparison of fruit fingers between *MaACO1*-disrupted and wild-type plants. Bar = 5 cm. (e) The average weight of a single fruit finger from the third hand of the fruit bunch. (f) Fruit ripening phenotype of two *MaACO1*-disrupted mutant lines and the wild type under natural ripening conditions. Fruits were stored at 22°C. (g) Fruit ripening phenotype of two *MaACO1*-disrupted mutant lines and the wild type induced by ethephon. (h) Illustration of the three most prevalent mutation types at *MaACO1* targeted sites and mutation frequency. (i) The vitamin C, TSS, total sugar and total acid content of fruits. Letters indicate significantly different values using t-test ($P < 0.05$). (j i and K) Ethylene production of *MaACO1*-disrupted mutant lines and the wild type under natural ripening conditions (j) or induced by ethephon (k).

was greatly delayed in the ripening transition period postharvest (Figure 1f). The wild-type fruit was yellow with brown speckles at day 21, whereas *MaACO1*-disrupted fruit remained yellow or green with no speckles (little damage), even at day 60 (Figure 1f). Importantly, when the banana fruit of the wild-type and *MaACO1*-disrupted plants was treated with ethephon, they exhibited a similar response to ethephon and ripened normally (Figure 1g). Moreover, the ripening process of the *MaACO1*-disrupted fruit was delayed for about 1–2 days compared to that of wild-type fruit, implying improved shelf life for the *MaACO1*-disrupted fruit after ethephon treatment. In addition, the TSS, total sugar and total acid contents of these two lines were also comparable for completely ripe fruits, and the *MaACO1*-disrupted fruit has more vitamin C than that of wild type (Figure 1i), suggesting that *MaACO1* strongly affects banana shelf life and is an ideal target gene to edit for improved shelf life.

To identify the mutation pattern, six generated lines were used for deep amplicon sequencing with PCR products of the target regions amplified from genomic DNA. Indels occurring at the Cas9 cleavage sites were considered mutations. The mutant lines exhibited different indel patterns at different cleavage sites (Figure 1h). Four mutant lines are three allelic genes disrupted mutants, and 98.43% and 98.61% of the reads had deletion mutations in the *MaACO1*-disrupted line 1# and line 2#, respectively (Figure 1h). These data suggested that the target site of *MaACO1* was efficiently edited.

As *MaACO1* is mainly responsible for ethylene synthesis, we monitored the ethylene production of one wild-type line and one *MaACO1*-disrupted line (Figure 1j,k). Under natural ripening conditions, high amounts of ethylene were produced 18–21 days postharvest in wild-type fruits (Figure 1j), whereas, in the *MaACO1*-disrupted line, ethylene production was strongly delayed and reduced (Figure 1j). Under ethephon treatment, ethylene production exhibited a similar trend between the wild-type fruits and *MaACO1*-disrupted lines, but in the wild-type line, more ethylene was produced at day 3 compared with the *MaACO1*-disrupted line (Figure 1k).

In summary, we created several *MaACO1*-disrupted plants with different editing patterns using the CRISPR/Cas9 system. The mutant fruits exhibited reduced ethylene synthesis and extended shelf life under natural ripening conditions. Moreover, *MaACO1*-disrupted fruit was also sensitive to ethephon and ripened normally after ethephon treatment. In addition, the vegetative growth, lifecycle and fruit quality of the *MaACO1*-disrupted line were comparable to those of wild-type plants except for slightly lower height and yield. These data suggest that *MaACO1* is an ideal target for creating fruit with a long shelf life using the CRISPR/Cas9-mediated editing system. The application of newly created germplasm will greatly reduce the postharvest losses and will increase the economic value of the banana industry by improving the shelf life of banana fruit.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

C.H, G.Y and F.B designed the experiments; Q.Y, C.L, F.B and T.D provided genomics and bioinformatics analysis; C.H, W.H and T-X.D performed the genetic transformation of banana; O.S and G.D are responsible for plant cultivation; C.H, S.L, F.B and H.G performed the physiological and molecular experiments; C.H, G.Y and F.B supervised the project and wrote the manuscript.

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