The expression of a candidate cucumber fruit sugar starvation marker gene *CsSEF1* is enhanced in malformed fruit induced by salinity

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Abstract The cucumber (*Cucumis sativus* L.) gene *Cucumis sativus Somatic Embryogenesis Zinc Finger 1* (*CsSEF1*) was suggested to be a good marker gene for sugar starvation in fruit. The expression of this gene in fruits is dramatically upregulated in plants that have suffered either complete defoliation or prolonged darkness. *CsSEF1* was initially discovered as a gene that was upregulated during somatic embryogenesis. We examined the difference in fruit parts and the effect of pollination on the upregulation of *CsSEF1* induced by defoliation treatment. The results indicated that the upregulation of *CsSEF1* in fruit by defoliation is not dependent on the presence of developing embryos. The expression of *CsSEF1* was upregulated in malformed fruit induced by salinity in which the development of placenta was arrested. Partial cutting of the distal part of the fruit showed that if placenta tissue remained there was no upregulation of *CsSEF1*, whereas when placenta tissue did not remain there was a marked upregulation of *CsSEF1*. These results could be consistently interpreted as showing that placenta tissue induced the transport of photoassimilates to the fruit and that without developing placenta tissue, pericarp tissue suffers from severe sugar starvation. This interpretation, in turn, enforces the view that *CsSEF1* is a good marker gene of fruit sugar starvation.

Keywords Cucumber · *Cucumis sativus* Somatic Embryogenesis Zinc Finger 1 · Growth arrest · Malformed fruit · Sugar starvation

Introduction

The formation of malformed fruit should be avoided in the commercial production of cucumber. Although it has long been assumed that the formation of malformed fruit is related to limited photoassimilate supply (Kato and Oda 1977), this has not been proven unambiguously. To examine this assumption at the cellular level, we sought marker genes that are upregulated in cucumber fruit upon complete defoliation (Tazuke and Asayama 2013; Tazuke et al. 2015). A potential marker gene was *Cucumis sativus Somatic Embryogenesis Zinc Finger 1* (*CsSEF1*), which was originally discovered as a gene that was upregulated during somatic embryogenesis (Grabowska et al. 2009), and whose expression was upregulated approximately 100 times in fruit upon complete defoliation. Although it is standard practice to use parthenocarpic fruit in cucumber production (Badgery-Parker et al. 2010), we pollinated female flowers to ensure fruit set (Tazuke and Asayama 2013; Tazuke et al. 2015); therefore, the effect of the existence of developing embryos should be examined. In solution culture, 60 mM NaCl salinity combined with low aeration rate of the solution induced malformed fruit in which the growth of the distal half, containing placenta tissue, was suppressed (Tazuke 2001). To assess Kato and Oda's proposal, the expression of *CsSEF1* in the malformed fruit induced as above was analyzed. Varga and Bruinsma (1990) reported that the formation of malformed fruit with sectioned growth is related to the poor development of seed or empty seed coat in the placenta tissue.

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To evaluate the significance of the placenta tissue, the effect of removal of the distal part of a fruit that contains placenta tissue on the expression of *CsSEF1* was also examined. The expression of two other genes that were upregulated by defoliation, namely, asparagine synthetase gene (*AS*), which is regarded as being an especially good marker of sugar starvation (Blasing et al. 2005; Contento et al. 2004; Lam et al. 1998; Price et al. 2004; Thum et al. 2004), and *Cucumis sativus* Fruit Defoliation Induced 1 (*CsFDII*), which we discovered (Tazuke et al. 2015), were also examined.

Materials and methods

Growth conditions and treatments

From May to July 2013, July to October 2014, and May to July 2015, cucumber (*Cucumis sativus* L. pure line cultivar Tokiwa) plants were grown in a glasshouse in 15 L pots filled with vermiculite and irrigated with half-strength Hoagland No. 2 solution (KNO₃ 6 mM, Ca(NO₃)₂ 4 mM, MgSO₄ 2 mM, and NH₄H₂PO₄ 1 mM as major elements, and Fe 3 ppm (as Fe-EDTA), B 0.5 ppm, Mn 0.5 ppm, Zn 0.05 ppm, Cu 0.02 ppm, and Mo 0.01 ppm as minor elements). The plants were pinched, which left about 10 leaves. Fruit on the primary node of the lateral shoots was used. For the pollination regime, female flowers were pollinated with pollen from a male flower of the same plant in the morning of the day of anthesis. In May 2013, pollinated fruits were harvested at 09:00 at 0, 2, 4, and 6 days after anthesis (DAA), weighed, cut into proximal and distal halves, and subjected to total RNA extraction. In August 2014, leaves of plants bearing pollinated and parthenocarpic fruits were completely defoliated at 09:00 at five DAA. At the same time, pollinated and parthenocarpic fruits were harvested as controls. At 09:00 the next day, treated pollinated and parthenocarpic fruits were harvested. The harvested fruits were immediately weighed and cut into proximal and distal halves and subjected to total RNA extraction. In July 2015, at 09:00, the distal part of the fruit was removed using a razor, which left 0.5–6 cm of fruit tissue at five DAA. The cut surface of the fruit was wrapped with polyethylene film. At the time of the treatment and at 09:00 the next day, fruits were harvested and subjected to total RNA extraction. From March to May 2016, cucumber plants of commercially used F₁ cultivar Tokiwa Hikari 3A were grown hydroponically. Each plant was grown in a container containing 6 L of half-strength Hoagland No. 1 solution (KNO₃ 5 mM, Ca(NO₃)₂ 5 mM, MgSO₄ 2 mM, and KH₂PO₄ 1 mM as major elements, and Fe 3 ppm (as Fe-EDTA), B 0.5 ppm, Mn 0.5 ppm, Zn 0.05 ppm, Cu 0.02 ppm, and Mo 0.01 ppm as minor elements). Tap water was used for the preparation of the solution. The solution was aerated at 3 L min⁻¹. The pH of the solution was adjusted to 6.0 each day.

Plants were pinched, which left about 10 leaves. Female flowers on the lateral shoots were used. When plants began to flower, 60 mM NaCl was added to the solution and the aeration frequency was reduced to 5 min h⁻¹, as reported by Tazuke (1997, 2001). At 09:00, control fruits and treated fruits at seven DAA were harvested, weighed, and subjected to total RNA extraction. The reason for the use of the cultivar was that Tokiwa is extremely susceptible to salinity; the plants died at 60 mM NaCl without reduction of aeration frequency.

Extraction of total RNA and reverse transcription (RT)

Tissues of harvested fruit were frozen with liquid nitrogen immediately, and then powdered with a mortar and pestle. Total RNA was extracted by using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA in the extract was removed with DNase (Qiagen) as described by Tazuke et al. (2015). The extracted total RNA was reverse transcribed with the PrimeScript RT Reagent Kit (TaKaRa, Otsu, Japan).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The transcription level of genes was examined by qRT-PCR using SYBR Premix Ex TaqTM II (TaKaRa) as reagent and the Thermal Cycler Dice Real Time System (TaKaRa). Actin gene was used as the internal control. The primers used were actin gene: 5'-CTCGAGACTGCAAAGAGCAG-3' and 5'-ATTCCTGCAGCTTCCATACC-3', *AS*: 5'-TGAGGGTT CACCAGATTTGA-3' and 5'-AATGGCATCAATCC CATCTT-3', *CsSEF1*: 5'-AGACCGATACCGGACTCAAC-3' and 5'-TGTGAGCGAAGAAACAGACC-3', and *CsFDII*: 5'-AATGCCCTCTTCAGCACTCT-3' and 5'-GAGGAA ATGTTGCAGGGATT-3'.

Statistical analysis

Statistical analyses were conducted with R (R Development Core Team 2015). Raw data were log-transformed if necessary.

Results

Effects of pollination and defoliation on the expression of candidate marker genes of sugar starvation of fruit

For *CsSEF1*, three-way factorial analysis of log-transformed data showed that defoliation led to highly significant ($p = 2 \times 10^{-10}$) upregulated expression, and that other factors were all insignificant. Analysis of the raw data

for *AS* showed that the difference between fruit parts was slightly significant ($p = 0.048$), with other factors being insignificant. Analysis of the raw data for *CsFDII* showed that the effect of defoliation was highly significant ($p = 0.0004$); again, the effects of other factors were insignificant (Fig. 1).

Developmental change in gene expression

For *CsSEF1*, the transcript level remained very low during fruit development, but two-way factorial analysis of variance conducted with log-transformed data indicated that the effect of DAA was highly significant ($p = 0.0006$). Thus, there was a slight increase in the expression of *CsSEF1* as fruits grew. Analysis of the raw data for *AS* showed that DAA ($p = 3 \times 10^{-6}$), fruit part ($p = 2 \times 10^{-4}$), and DAA–fruit part interaction

($p = 0.01$) were all significant. For *CsFDII*, the effects of neither DAA nor fruit part was significant (Fig. 2).

Upregulation of the three genes in malformed fruit induced by salinity

Salinity treatment induced the formation of malformed fruit in which the growth of the distal half of a fruit that contains placenta tissue was severely suppressed (Fig. 3a). In such fruits, the expression of all three genes was upregulated. The upregulations were, on average, 7.3, 1.9, and 1.5 times in *CsSEF1*, *AS*, and *CSFDII*, respectively. The p values of significance, when analyzed by Welch two sample t test for log-transformed data, were 0.025, NS, and 0.029 in *CsSEF1*, *AS*, and *CSFDII*, respectively (Fig. 3b). Even in the salinity regime, some fruits grew normally. In such fruits, there was no upregulation of the three genes (data not shown).

Fig. 1 Effects of complete defoliation, pollination, and fruit parts on the transcript level of *CsSEF1*, the gene for asparagine synthetase (*AS*), and *CsFDII* in cucumber fruit. Open bars proximal half of a fruit; dotted bars distal half of a fruit. Levels of transcription are shown relative to the level of the distal half of a defoliation treated fruit, five DAA. Vertical bars are the SE of means. The results of the analyses of variance are shown in the text

Fig. 2 Developmental change in the expression of *CsSEF1*, *AS*, and *CsFDII* of cucumber fruit. Circles proximal half of a fruit. Triangles distal half of a fruit. Levels of transcription are shown relative to the level of the distal half of a defoliation treated fruit, five DAA. Vertical bars are SE of the means. The results of the analyses of variance are shown in the text

Fig. 3 Effect of the combination of 60 mM NaCl salinity and low (5 min in 1 h) aeration frequency on **a** the fruit morphology and **b** the expression of *CsSEF1*, *AS*, and *CsFDII* of cucumber fruit at 7 days after anthesis. Development of the distal half, containing placenta tissue, is suppressed markedly. Open bars control; dotted bars salinity treatment. Levels of transcription are shown relative to the level of the distal half of a defoliation treated fruit, five DAA. Vertical bars are SE of the means

Effect of removal of the distal fruit part

The longitudinal section of cucumber fruit at five DAA is shown in Fig. 4a. There was no placenta tissue up to 4 cm from the base of a fruit. Placenta tissue existed from 4 cm from the fruit base. Removal of the distal fruit part caused marked upregulation of *CsSEF1* expression even when the leaves were left intact. There was no upregulation when cutting was too deep (left tissue length ca. 1 cm). Furthermore, when left tissue was long enough to contain placenta tissue (i.e., longer than 4 cm) there was no upregulation. When left tissue was between 2 and 4 cm, there was a marked upregulation of *CsSEF1*. The response was similar for the other two genes (Fig. 4b).

Discussion

Pollination did not change the level of expression of *CsSEF1* upon defoliation. The proximal half of a fruit contains few seeds, but the distal half of a fruit contains many seeds or empty seed coats. However, there was no

Fig. 4 Effect of the removal of the distal part of a young cucumber fruit on the expression of *CsSEF1*, *AS*, and *CsFDII*. **a** Longitudinal section of a pollinated fruit, 5 days after anthesis. Placenta tissue is only seen from approximately 4 cm from the base of the fruit. A representative view of the remaining part of a fruit used for the experiment after the removal of the distal part is shown in the inset. **b** Expression of the three genes in relation to the length of the left fruit tissue 1 day after cutting of the fruit part of pollinated fruit, 5 days after anthesis (DAA). Levels of transcription are shown relative to the level of the distal half of a defoliation treated fruit, five DAA. Data obtained from 17 fruits are shown

difference between fruit parts in the expression of *CsSEF1*. In control fruits, the transcript level of *CsSEF1* remained very low, although there was a significant developmental change. These results indicate that the upregulation of *CsSEF1* upon defoliation does not depend on the presence of a developing embryo.

There seemed to be little enhancement of the expression of *CsFDII* in parthenocarpic fruits. It is possible the expression of *CsFDII* is dependent on pollination. Together with the much higher enhancement of *CsSEF1* expression by defoliation, this may suggest that *CsSEF1* is a better marker of sugar starvation. On the other hand, *CsFDII* may be used as a pollination specific marker of sugar starvation.

The upregulation of *CsSEF1* in malformed fruit induced by salinity is consistent at the molecular level with the view of Kato and Oda (1977), which suggests the occurrence of severe sugar starvation in the malformed fruit. *Arabidopsis* homologs of *CsSEF1* are reported to be involved in salinity resistance (D'Orso et al. 2016; Han et al. 2014). Thus, there remains a possibility that *CsSEF1* responded to salinity stress directly.

The relationship between the growth of the ovary and ovule of cucumber fruit has long been studied (Varga and Bruinsma 1990). The results of fruit-part removal experiments are in line with those results, and may be consistently interpreted as follows. When the cutting was too deep (left tissue length ca. 1 cm), the growth activity of the fruit was inhibited; thus, no sugar starvation occurred. When the amount of tissue left was too long (longer than 4 cm), the sample contained placenta tissue, which had sink activity and drew photoassimilates; in this case, no sugar starvation occurred. When the left tissue length was approximately 2 cm, the growth activity of the fruit was not inhibited, so there was a demand for photoassimilates. However, the left tissue contained no placenta tissue, so it did not have a sink activity. Thus, photoassimilates were not drawn to the fruit, which led to severe sugar starvation.

In the previous experiments, *CsSEF1* was suggested to be a good marker gene of sugar starvation because its expression was upregulated by total defoliation and prolonged darkness (Tazuke and Asayama 2013; Tazuke et al. 2015). In this experiment, the relationship between the existence of actively growing placenta tissue and the upregulation of *CsSEF1* enforces the view that *CsSEF1* is a good marker gene of fruit sugar starvation. Parallel responses of AS and *CsFDII* with *CsSEF1* seen in most experiments provide further support for the present hypothesis. To prove that *CsSEF1* is a good marker of sugar starvation of cucumber fruit, elucidation of the function and signal transduction pathway is needed. *CsSEF1* belongs to a plant-specific tandem cysteine, cysteine, histidine zinc finger (TZF) gene (Pomeranz et al. 2011). There have been several reports on the function of TZF genes (Bogamuwa and Jang 2013; Guo et al. 2009; Han et al. 2014; Huang et al. 2011; Jan et al. 2013; Kim et al. 2008; Kong et al. 2006; Lee et al. 2012; Li and Thomas 1998; Lin et al. 2011; Sun et al. 2007; Zhou et al. 2014), but their function in fruit is unknown. We are currently examining the function of *CsSEF1* in fruit.

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