Cucumber *ECERIFERUM1* (*CsCER1*) plays a vital role in pollen fertility

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Summary

Pollen is critical for pollination and fertilization in sexual production of flowering plants. Alkanes and their derivatives are major components in the tryphine (i.e., pollen coat) and they are essential for pollen hydration. *ECERIFERUM1* (*CER1*) played a key role in alkanes biosynthesis of tryphine, however, its role in pollen fertility is still largely unknown. In order to explore the effect of abnormal expression of *CsCER1* on pollen fertility, this study was conducted to analyze pollen activity, germination percentage of pollen grain and pollen tube length in *CsCER1*-interfering (*CsCER1i*) and *CsCER1*-overexpressing (*CsCER1ox*) cucumber lines. The results showed that compared to wild-type plants, fruit fresh weight, seed number and percentage of vigorous pollen were notably decreased in *CsCER1i* lines, seed number, percentage of vigorous pollen and germination percentage of pollen grain were significantly reduced in *CsCER1ox* lines. Our results indicated that abnormal expression of *CsCER1* obviously reduced pollen fertility by inhibiting pollen activity and germination percentage of pollen grain in cucumber. In conclusion, *CsCER1* played a vital role in pollen fertility of cucumber, which provided a sound theoretical foundation for genetic improvement of cucumber.

Keywords

cucumber, *CsCER1*, pollen activity, pollen grain germination, pollen tube length

Significance of this study

What is already known on this subject?

- Pollen is critical for pollination and fertilization in sexual production of flowering plants. Since alkanes are major components of tryphine (i.e., pollen coat), abnormal expression of *CER* genes in alkanes biosynthesis induced unhealthy tryphine and exine of pollen.

What are the new findings?

- The abnormal expression of *CsCER1* obviously reduced pollen fertility by inhibiting pollen activity and pollen grain germination in cucumber.

What is the expected impact on horticulture?

- *CsCER1* played a vital role in pollen fertility, which provided a sound theoretical foundation for genetic improvement of cucumber.

Introduction

Pollination is a critical process for sexual production in flowering plants (Higashiyama and Yang, 2017; Lord and Russell, 2002). Pollen, a male gametophyte, is essential for pollination and fertilization. When pollens mature, they leave from anther, then adhere, hydrate and germinate on the stigma (Dresselhaus and Franklin-Tong, 2013). Pollen hydration is the key step in pollination and also a highly regulated and complex process (Hiscock and Allen, 2008; Li et al., 2017). In pollen hydration, identification and screening are conducted between pollens and stigma; only suitable pollens hydrate on the stigma. This process is closely related to tryphine (i.e., pollen coat) and lipids of stigma surface (Elleman et al., 1992; Rejón et al., 2016; Wilson and Zhang, 2009). In the tryphine, alkanes and their derivatives were major components and they were necessary for pollen hydration (Murphy, 2006; Yu et al., 2019).

Wax is a major component of cuticle, which allows a plant to adapt itself to environmental changes (Shi et al., 2015). Alkanes, a major component of waxes, contribute 50%–80% of waxes in the most plant (Bourdenx et al., 2011). In alkane-forming pathway, *ECERIFERUM1* (*CER1*) and *ECERIFERUM3* (*WAX2*) participant in the production of very long chain (VLC) alkanes and their derivatives (Bernard et al., 2012). In *Arabidopsis thaliana cer1* and *wax2* mutants, both alkanes and their derivatives were significantly decreased (Bourdenx et al., 2011; McNevin et al., 1993). Abnormal wax synthesis induced defective formation of pollen exine in rice and *Arabidopsis thaliana* mutants, contributing to reduced pollen fertility (Aarts et al., 1995; Ariizumi et al., 2003). Pollen fertility of *Arabidopsis thaliana wax2* mutant was significantly lower than that of wild-type owing to absence of tryphine and sporopollenin (Wolters-Arts et al., 1998). Moreover, hydration and fertility of these pollens recovered under high humidity or additional exogenous lipids. In *Arabidopsis thaliana cer1* mutant, pollen hydrated and produced seed in vitro or under high humidity, not in vivo (Hülkamp et al., 1995b). However, the regulation mechanisms of *CER1* on cucumber pollination still remain to be clarified.

Cucumber (*Cucumis sativus* L.) is one of the major greenhouse vegetable crops (Miao et al., 2019). In the previous study, our group has successfully transformed cucumber by constructing *CsCER1* interference and over-expression vectors and employing an agrobacterium-mediated method (Wang et al., 2015). It was demonstrated that abnormal expression of *CsCER1* affects epidermis wax content in cucumber, especially for alkanes. In this study, pollen activ-

ity, germination percentage of pollen tube and pollen tube length in transgenic cucumber were investigated to explore effect of abnormal expression of *CsCER1* on pollen fertility and provide a theoretical basis for genetic improvement of cucumber.

Materials and methods

Plant materials and growth conditions

The T2 generation of transgenic cucumber lines obtained previously by our group was employed as the plant material in this study (Wang et al., 2015). Cucumber seeds were germinated at 28°C, then planted in plastic pots (10 cm diameter and 10 cm depth, one seedling per pot) filled with a mixed nutritious soil (nutritious floral soil: vermiculite = 2:1), and cultured in an incubator. The photoperiod was 12 h d⁻¹, light intensity was 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, day/night temperature was 25°C/18°C. When the second leaf fully expanded, cucumber seedlings were transplanted to a greenhouse. Agronomic managements were taken after transplanting. All the treatments were repeated at least three times and plants were measured with the methods described below.

Determination of fruit quality and fertility

Cucumber fruits were harvested and weighed when seeds fully matured. Cucumber seeds were taken out and washed carefully, then fully dried at room temperature, seed number

and weight were recorded. Cucumber fruits were sampled and fruit quality was analyzed at 12 d after flowering. The hardness and total soluble solid were determined by using a rigiscan and a refractometer, respectively. According to the method described by Li (2000), soluble sugar content, soluble protein, vitamin C, nitrate were analyzed with anthrone ethyl acetate colorimetric method, coomassie brilliant blue staining method, 2,6-dichloro-indophenol titration method and sulfuric acid method, respectively.

Determination of pollen activity

Pollen activity was measured by a 2,3,5-triphenyltetrazoliumchloride (TTC) staining method (Valadares et al., 2019). The flowering male flowers were selected and pollens were soaked in TTC solution at 28°C for 5–8 minutes. Colored pollens were observed with an Olympus BX53 microscope (Olympus Corporation, Japan).

Determination of in vitro pollen grain germination

The pollens on the flowering day were sown in a cucumber pollen medium (15% sucrose, 0.01% boric acid, 1 mM $\text{Ca}(\text{NO}_3)_2$ and 0.5% agar) and cultured at 25°C. Half an hour later, the image of pollen grain germination were obtained by an Olympus BX53 microscope. Germination percentage of pollen grain was calculated by dividing numbers of germinated pollens by numbers of all pollens. Pollen tube length was analyzed with ImageJ software.

FIGURE 1. Image (A) and fertility parameters (B–D) of WT, *CsCER1i* and *CsCER1ox* lines. Scale bar = 3 cm (A). Different letters in the same column indicate significant differences and bars indicate standard deviations ($P < 0.01$, $n = 10$, B–D).

Statistical analysis

All statistical analyses were conducted using SPSS 20.0 software (IBM, USA). Duncan's test was carried out to make comparisons among treatment mean values from significant one-way ANOVA ($P < 0.01$).

Results

Fruit quality and fertility of wild-type and transgenic cucumber

Compared to wild-type (WT) plants, seed number was significantly declined by 72.4% and 37.1% respectively in

TABLE 1. Fruits quality of WT, *CsCER1i* and *CsCER1ox* lines.

Plant	Hardness ($\times 105$ Pa)	Soluble solids (%)	Soluble sugar content (mg g ⁻¹ FW)	Soluble protein content (mg g ⁻¹ FW)	Vitamin C content (mg 100 g ⁻¹ FW)	Nitrate content (mg g ⁻¹ FW)
WT	13.09 $\pm$ 0.96 A	3.04 $\pm$ 0.10 A	40.79 $\pm$ 4.78 A	1.12 $\pm$ 0.14 B	4.76 $\pm$ 0.26 A	76.43 $\pm$ 52.05 A
<i>CsCER1i</i>	13.68 $\pm$ 0.59 A	3.06 $\pm$ 0.07 A	35.23 $\pm$ 9.71 A	0.80 $\pm$ 0.15 B	4.90 $\pm$ 0.55 A	48.79 $\pm$ 22.08 A
<i>CsCER1ox</i>	13.59 $\pm$ 0.89 A	3.09 $\pm$ 0.08 A	52.16 $\pm$ 19.35 A	1.54 $\pm$ 0.37 A	4.39 $\pm$ 0.63 A	43.77 $\pm$ 1.42 A

Different capital and small letters in the same column indicated significant differences ($P < 0.01$, $n = 3$).

FIGURE 2. Images of colored pollen grains stained with TTC in WT, *CsCER1i* and *CsCER1ox* lines. The dark red, light red and colorless pollens indicate vigorous, inactive, and aberrant pollens, respectively. Black arrows indicate aberrant pollen. Bars = 200 μ m.

FIGURE 3. Percentage of pollen with different activities in WT, *CsCER1i* and *CsCER1ox* lines. Different letters indicate significant differences and bars indicate standard deviations ($P < 0.01$, $n = 5$).

FIGURE 4. In vitro pollen grain germination of WT, *CsCER1i* and *CsCER1ox* lines. Black arrows indicate short pollen tubes. Scale bars = 200 μ m.

CsCER1-interfering (*CsCER1i*) and *CsCER1*-overexpressing (*CsCER1ox*) lines (Figure 1A and 1C). Seed number in *CsCER1i* was barely a half of that in *CsCER1ox* lines. Fruit fresh weight was notably decreased only in *CsCER1i* lines as compared to WT (Figure 1B). No significant differences in hundred-grain weight (Figure 1D) and most characteristics of fruit quality were found among treatments (Table 1). The results suggested that fertility of both *CsCER1i* and *CsCER1ox* lines was significantly lower than that of WT.

Pollen activity of wild-type and transgenic cucumber

Pollens were stained with TTC and pollen activity was analyzed. In abnormal expression of *CsCER1* (*CsCER1i* and *CsCER1ox*), percentage of vigorous pollen was significantly lower, while percentage of aberrant pollen and inactive pollen were notably higher than WT (Figures 2 and 3).

In vitro pollen grain germination of wild-type, *CsCER1i* and *CsCER1ox* lines

Compared to WT, germination percentage of pollen grain was significantly reduced by 51.9% in *CsCER1ox* lines (Figures 4 and 5A). Pollen tube length was also decreased by 43.8% in *CsCER1i* when compared to WT (Figure 5B). These results showed that abnormal expression of *CsCER1* affects pollen grain germination.

FIGURE 5. Germination percentage of pollen grain (A) and pollen tube length (B) in WT, *CsCER1i* and *CsCER1ox* lines. Different letters in the same column indicated significant differences and bars indicate standard deviations ($P < 0.01$, $n = 3$).

Discussion

The abnormal expression of *CsCER1* influences alkane biosynthesis, which is closely related to pollen fertility (Wang et al., 2015; Yu et al., 2019). So far more than 20 kinds of CER family mutants have been found. Mutants in CER family genes, including *cer1*, *cer3*, *cer6*, three *CER2-LIKE* and *CER3/FLP1* genes exhibited high sterility (Aarts et al., 1995; Ariizumi et al., 2003; Haslam et al., 2015). Since alkanes are major components of tryphine, abnormal expression of CER genes in alkanes biosynthesis induced unhealthy tryphine and exine (Jiang et al., 2013; Preuss et al., 1993; Xu et al., 2019). Our previous studies demonstrated that *CsCER1* was expressed in pollen. *CsCER1* encoded an enzyme which catalyzed formation of alkane and produced VLC alkanes (Wang et al., 2015). In *CsCER1i* and *CsCER1ox* lines, the abnormal expression of *CsCER1* inhibited alkane biosynthesis, so that tryphine structure would be largely affected and defects would be induced. This could result in decreased protection ability and pollen activity. In order to verify this hypothesis, pollen activity was analyzed with TTC staining method. The decreased percentage of vigorous pollens, increased percentage of aberrant and inactive pollens verified this hypothesis in *CsCER1* transgenic cucumber lines (Figures 2 and 3).

In pollen, alkanes and their derivatives in the tryphine were necessary for pollen hydration. Compared to wild-type plants, *Arabidopsis thaliana cer1* mutants generated more but smaller lipid droplets in the tryphine, thus impaired the process of pollen hydration and displayed male sterility at low humidity (Aarts et al., 1995; Preuss et al., 1993). Moreover, *cer6-2* mutants were defective in the tryphine, which contributed to disordered pollen-stigma interaction and male sterility. According to our previous study, decreased alkanes content in *CsCER1* transgenic cucumber lines was detrimental to biosynthesis of tryphine, which could induce abnormal pollen hydration (Hülkamp et al., 1995a; Wang et al., 2015). Successful pollen hydration was conducive to pollen germination and pollen tube growth, however, abnormal pollen contributed to decreased pollen fertility. The germination percentage of pollen grain was obviously lower in *CsCER1* transgenic cucumber lines than that of WT (Figures 4 and 5A).

During pollen hydration, pollen's water content enhances, energy transformation accelerates, components of cell wall accumulates, and all these changes contribute to formation of pollen tube (Wang et al., 2008; Zheng et al., 2018). Pollen tube growth also consumes a lot of energy, accompanied with the rapid increase in cell volume and accumulation of cell wall components (Hou et al., 2016). The initial growth of pollen tube in vivo is slow and autotrophic (Rounds et al., 2011). A number of pollen components serve as metabolic substrates to support pollen tube elongation (Schoenaers et al., 2017). In this study, pollen tube length of *CsCER1i-2* was obviously shorter than that of WT (Figure 5B). This could result from differences in metabolic substrates of pollen tube elongation, however, the regulation mechanism still needs to be explored.

Conclusions

The abnormal expression of *CsCER1* obviously reduced pollen activity and inhibited pollen grain germination. *CsCER1* played a vital role in pollen fertility and this provided a sound theoretical foundation for genetic improvement of cucumber.

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