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Report

A Novel Pollen-Pistil Interaction Conferring High-Temperature Tolerance during Reproduction via CLE45 Signaling

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Summary

Flowering plants in the reproductive stage are particularly vulnerable to ambient temperature fluctuations [1–6]. Nevertheless, they maintain seed production under certain levels of exposure to temperature change. The mechanisms underlying this temperature tolerance are largely unknown. Using an in vitro *Arabidopsis* pollen tube culture, we found that a synthetic CLV3/ESR-related peptide, CLE45, prolonged pollen tube growth. A subsequent screen of *Arabidopsis* mutants of leucine-rich repeat receptor-like kinase genes identified two candidate receptors for CLE45 peptide, *STERILITY-REGULATING KINASE MEMBER1* (*SKM1*) and *SKM2*. The double loss-of-function mutant was insensitive to CLE45 peptide in terms of pollen tube growth in vitro. The SKM1 protein actually interacted with CLE45 peptide. *CLE45* was preferentially expressed in the stigma in the pistil at 22°C, but upon temperature shift to 30°C, its expression expanded to the transmitting tract, along which pollen tubes elongated. In contrast, both *SKM1* and *SKM2* were expressed in pollen. Disturbance of CLE45-SKM1/SKM2 signaling transduction by either RNAi suppression of *CLE45* expression or introduction of a kinase-dead version of SKM1 into *skm1* plants reduced seed production at 30°C, but not at 22°C. Taken together with the finding that CLE45 peptide application alleviated mitochondrial decay during the in vitro pollen tube culture, these results strongly suggest that the pollen-pistil interaction via the CLE45-SKM1/SKM2 signaling pathway sustains pollen performance under higher temperatures, leading to successful seed production.

Results and Discussion

Involvement of *CLE45* in Successful Seed Production under Relatively High Temperature

Members of the CLV3/ESR-related (CLE) peptide family play crucial roles as developmental cues in the plant body plan [7–15]. The *Arabidopsis thaliana* genome contains 32 *CLE* genes, which encode 27 different CLE peptides composed of 12 or 13 amino acid residues. Jun et al. [16] and other public expression databases have shown the possibility that several *CLE* genes are involved in cell-to-cell communication between pollen and pistils. We set up an *Arabidopsis* in vitro pollen tube culture based on previously published methods with some modifications [17, 18] and examined the effect of synthetic CLE peptides on pollen tube growth at 30°C (see Figure S1A available online). We found that CLE45, CLE43, and CLV3 peptides markedly prolonged pollen tube growth, as shown by increases in the proportion of pollen tubes that reached ≥600 μm in length (Figures 1A–1C) without affecting pollen germination (Figure S1B). Among the three CLE genes, *CLE45* was expressed in the stigma as well as in the vascular tissues (Figures 1D–1H; Figure S1C). Temperature shift from 22°C to 30°C expanded the *CLE45* expression domain to the transmitting tract, through which pollen tubes grow, suggesting a high-temperature-dependent function of *CLE45*.

We produced *CLE45*-RNAi plants by overexpressing an inverted repeat sequence of a *CLE45*-specific fragment (Figure S1D–S1H). Because the preliminary experiments indicated that five independent T2 *CLE45*-RNAi lines exhibited similar phenotypes, two lines were randomly selected and further analyzed. In these two *CLE45*-RNAi lines, *CLE45* transcripts were present at approximately half the level of wild-type (WT; Figures S1E and S1F). The RNAi selectively affected *CLE45* expression (Figure S1G). As a control, we produced plants harboring an inverted repeat sequence of a lambda phage DNA fragment instead of the *CLE45* fragment (RNAi-control). When the *CLE45*-RNAi plants were grown at 22°C, no clear phenotype in vegetative or reproductive growth, including pollen tube growth, was observed. Next, we exposed the plants to 30°C for 3 days and observed the third-youngest fruits of stage 17 flowers [19], in which pollination, fertilization, and seed development had progressed during the 3-day high-temperature exposure (HTE) (Figures 1I–1M). In the *CLE45*-RNAi plants, HTE did not inhibit pollen tube growth in the pistil, in which pollen tubes reached almost all of the ovules (Figure S1I). However, HTE reduced significantly the number of seeds in fruits and fruit length in the *CLE45*-RNAi plants (Figures 1J and 1L), but not in the WT and RNAi-control plants. By contrast, there was no significant defect in the number of seeds or fruit size in the *CLE45*-RNAi plants grown at 22°C (Figures 1I and 1K). Such defects were not observed when the *CLE45*-RNAi plants were subjected to a drought stress (Figure S1J), suggesting that *CLE45* functions in tolerance specifically against high temperature. Loss of seeds occurred randomly within the fruits of the *CLE45*-RNAi plants (Figure 1M), suggesting a similarity of the position of seeds in the fertilization-defective mutant phenotypes rather than those of the pollen tube elongation-defective mutants in which pollen tubes can reach only ovules at the top of fruits [20–26].

Identification of Putative Pollen Receptors Responsible for the CLE45 Peptide Perception

Next, we tried to identify the receptor (or receptors) for CLE45 peptide. The known receptors for CLE peptides belong to the class XI leucine-rich repeat receptor-like kinase (LRR-RLK XI) family [27]. We selected candidates in silico by searching LRR-RLK XI genes that can be expressed in pollen [28–30]. Although BAM3 had previously been implicated in CLE45 peptide perception in roots [31], we excluded it because of its lack of gene expression in pollen. Pollen grains of the corresponding putative knockout mutant lines were subjected

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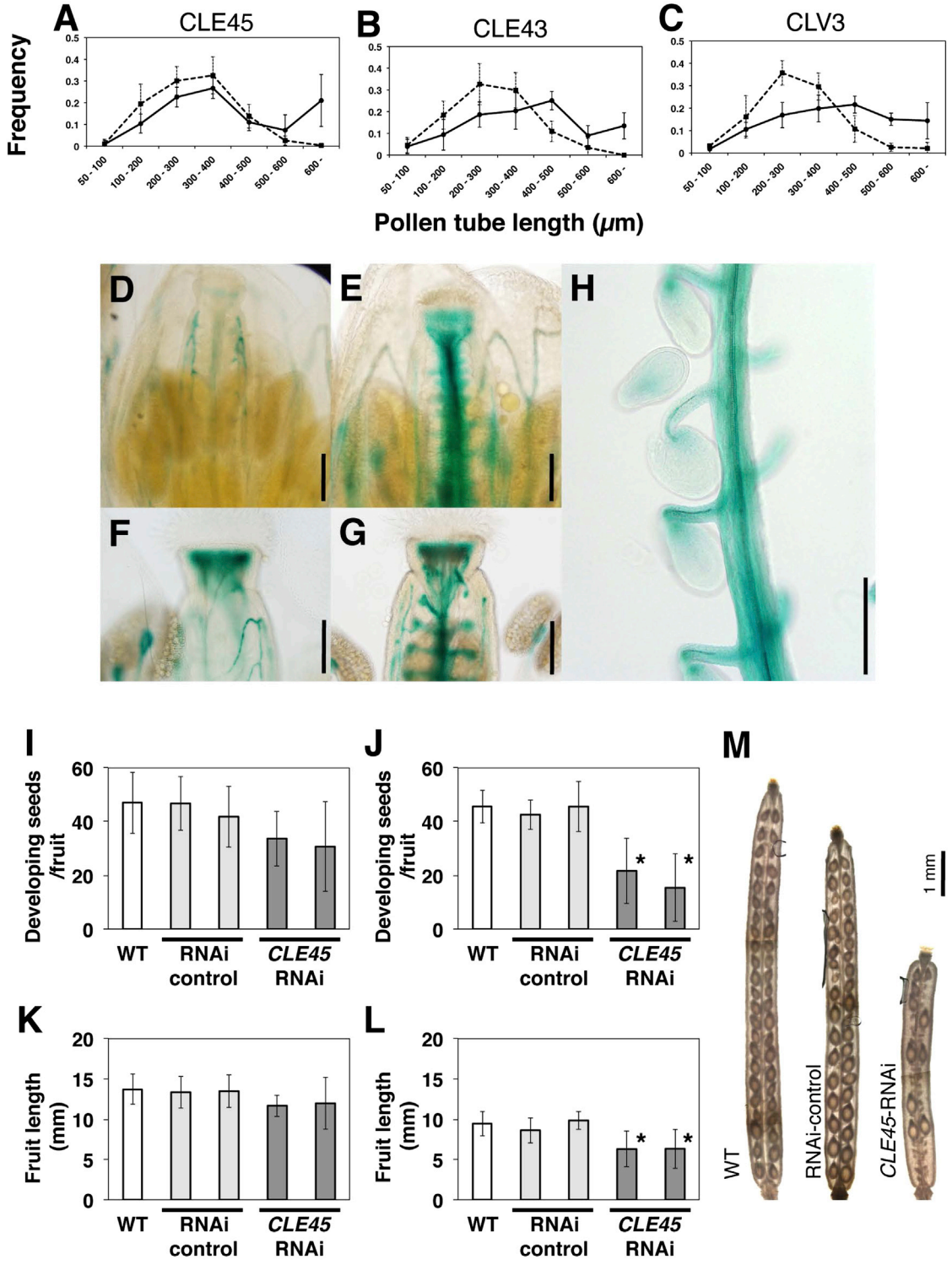

Figure 1. Identification and Characterization of a Novel *CLE* Gene in Regulating Pollen Performance and Seed Production under Relatively High Temperature

(A–C) Novel CLE peptide activities in promoting WT *Arabidopsis* pollen tube growth in vitro. Distributions of pollen tubes of the indicated tube lengths were measured in the presence (solid lines with filled circles) or absence (dashed lines with filled squares) of synthetic CLE45 (A), CLE43 (B), or CLV3 (C) peptide. Pollen grains of WT *Arabidopsis* were cultured for 20 hr at 30°C. Each data set represents the mean of at least four independent cultures ± SD; 30–40 pollen tubes were measured in each culture.

(D–H) *CLE45* expression in pistils of *Arabidopsis* flowers. GUS expression in *pCLE45::GUS* transgenic *Arabidopsis* flowers was examined before (D and E) and after (F–H) anthesis. Pistils of 7-week-old plants without (D and F) or with (E, G, and H) incubation for 24 hr at 30°C are shown. A dissected transmitting tract is shown in (H). Scale bars represent 200 μm.

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to an in vitro pollen tube growth assay with CLE45 or CLE43 peptide (Figures 2A–2D; Figure S2A). Of the selected T-DNA insertion lines (Table S1), pollen tube growth of mutants defective in At2g25790 (SALK_087435) and At5g56040 (SALK_052069) was significantly insensitive to CLE43 peptide and partly to CLE45, but not to CLV3 (Figures 2B and 2C; Figure S2B). Thus, At2g25790 and At5g56040 were named *STERILITY-REGULATING KINASE MEMBER1* (*SKM1*) and *SKM2*, respectively, based on their function described below. The CLE43 peptide-insensitive phenotype was complemented by introduction of *pSKM1::SKM1-YFP* and *pSKM2::SKM2-YFP* into *skm1* and *skm2*, respectively (Figures 2B and 2C). Pollen tube growth of the *skm1 skm2* double mutant showed complete insensitivity to CLE45 (Figure 2D). These results suggest that SKM1 and SKM2 are components downstream from both CLE45 and CLE43 and may be their receptors.

Although all of the published data indicated preferential expression of *SKM1* in pollen [28–30], *SKM2* expression in pollen was not clear. Fluorescence from the *pSKM2::SKM2-YFP* construct used for complementation was too low to detect in any tissues. Therefore, we produced *pSKM2::GUS* plants, which showed preferential GUS activity in stipules and pollen grains (Figure 2E; Figure S2C). *pSKM2::GUS* plants subjected to HTE showed weakly enhanced GUS expression in pollen (Figure 2F). In *skm1* plants complemented with *pSKM1::SKM1-YFP*, SKM1-YFP was expressed in vascular tissues of roots (Figure S2D). In flowers, SKM1-YFP was detected in pollen at both 22°C and 30°C but was not observed in pistils (Figures 2G and 2H).

We expected that the *skm1 skm2* double mutant would phenocopy the *CLE45*-RNAi plants. However, the mutant produced a normal number of seeds even after HTE (Figure 2J). This result might be due to redundancy of CLE45 receptors, as seen in CLV3 receptors [32]. To overcome such redundancy, we introduced a kinase-dead SKM1 (KDSKM1) into *skm1* plants, which was expected to cause a dominant-negative effect on downstream signal transduction [33, 34]. The KDSKM1 construct was generated by substituting the conserved lysine for ATP binding with glutamic acid in the kinase domain of *pSKM1::SKM1-YFP* (Figure S2E). As a result, five independent T2 *skm1* lines harboring KDSKM1 exhibited a reduction in seed number by HTE. Typical results from two T2 lines are shown in Figures 2I and 2J. This phenotype was quite similar to that of the *CLE45*-RNAi plants (Figures 1I and 1J). However, they showed no significant reduction in fruit length (Figures 2K and 2L).

Interactions of Pistil CLE45 and Pollen SKM1

To confirm that *CLE45* and *SKM1* contribute to seed production as pistil and pollen factors, respectively, we performed artificial crosses between WT pollen and *CLE45*-RNAi pistils and between WT pistils and *skm1* KDSKM1 pollen at 30°C. The plants were incubated for 18 hr at 30°C, immediately after artificial pollination. Under this condition, crossing of WT with either *CLE45*-RNAi pistils or *skm1* KDSKM1 pollen resulted in a reduction in seed numbers, although crossing of WT with either RNAi-control pistils or *skm1* SKM1 pollen did not affect seed production (Figure 3A). The artificial cross between WT pollen and *CLE45*-RNAi pistils also reduced fruit length, which is consistent with the result obtained from self-pollination (Figure 1L). Crossing of *skm1* KDSKM1 pollen and *CLE45*-RNAi pistils did not enhance the phenotype of WT pollen and *CLE45*-RNAi pistils (Figure 3B). Crossing of *skm1 skm2* pollen with WT pistils caused a decrease in seed production under more severe conditions, i.e., at 33°C, but not at 30°C (Figure 3C). These results strongly support that *CLE45* and *SKM1/SKM2* function in pistils and pollen, respectively, as components of the same signaling pathway.

We further tested whether CLE45 peptide bound directly to SKM1 protein. For this purpose, we synthesized [(4-azidosalicyl)Lys2]CLE45 peptide (ASA-CLE45) for photoaffinity labeling as described previously by Ogawa et al. [12]. ASA-CLE45 showed the same activity as nonlabeled CLE45 peptide (Figure 3D). A recombinant SKM1 protein was expressed in tobacco BY-2 cells as an SKM1-HaloTag (HT) form, in which the SKM1 kinase domain had been swapped with HaloTag (Figure 3E). Photoaffinity labeling of microsomal fractions overexpressing SKM1-HT by [^{125}I]ASA-CLE45, followed by immunoprecipitation with anti-HaloTag antibodies, showed labeling of a 130 kDa band after SDS-PAGE and autoradiography (Figure 3F). The binding was outcompeted by a 1,000-fold excess of nonlabeled CLE45 peptide, but not by root meristem growth factor 1 (RGF1; Matsuzaki et al. [35]) or CLE2 peptide (Figure 3F). These results suggest a direct and specific binding of CLE45 peptide to SKM1 protein.

Role of CLE45 Signaling in Pollen Tube Growth

CLE45 signaling prolonged pollen tube growth at 30°C in vitro, but not in situ. On the other hand, in situ, the CLE45 signaling protected seed production at 30°C. To elucidate a common mechanism between the in vitro and in situ events, we performed time-lapse observation of the in vitro growth of individual pollen tubes. CLE45 peptide did not accelerate the growth rate but rather delayed it by 36% during the first 20 min (Figure 4A). Nevertheless, CLE45 prolonged the growing period for >3 hr, at which time the pollen tubes without CLE45 peptide almost stopped growing. In contrast, CLE45 peptide did not prolong the pollen tube growth at 22°C (Figure 4B), consistent with normal seed production in CLE45 signaling-defective plants at 22°C. Next, we examined pollen tube viability with thiazolyl blue tetrazolium bromide (MTT), which is generally used as a colorimetric indicator of mitochondrial dehydrogenase activity. In the in vitro culture at 30°C, 30%–40% of pollen tubes were strongly stained deep purple by MTT at 4 hr (Figures 4C–4G). The proportion of MTT-positive cells per total cells (both MTT-positive and -negative cells) decreased dramatically during the culture (Figure 4F). The decrease was alleviated greatly by the addition of CLE45 peptide and only slightly by CLE43 peptide, but it was not alleviated by CLV3 (Figure 4F). The alleviation effect of CLE45 peptide was suppressed in the *skm1 skm2* double mutant (Figure 4G). In contrast, introduction of either

(I–M) Functional analysis of *CLE45* in *Arabidopsis*. Numbers of developing seeds (I and J) and fruit lengths (K and L) were measured in WT, RNAi-control (light gray column), and *CLE45*-RNAi (dark gray column) lines grown at 22°C (I and K) and after incubation for 3 days at 30°C (J and L). The third-youngest fruits of stage 17 flowers as described by Smyth et al. [19] were examined. Results were obtained from two independent T2 lines for RNAi-control and *CLE45*-RNAi. Each data point represents the mean of 12 fruits from a compilation of three experiments ± SD. Asterisks indicate significant differences ($p < 0.05$ by two-sample t test) between *CLE45*-RNAi lines and the other WT and RNAi-control lines. Representative fruits after incubation for 3 days at 30°C are shown in (M). See also Figure S1.

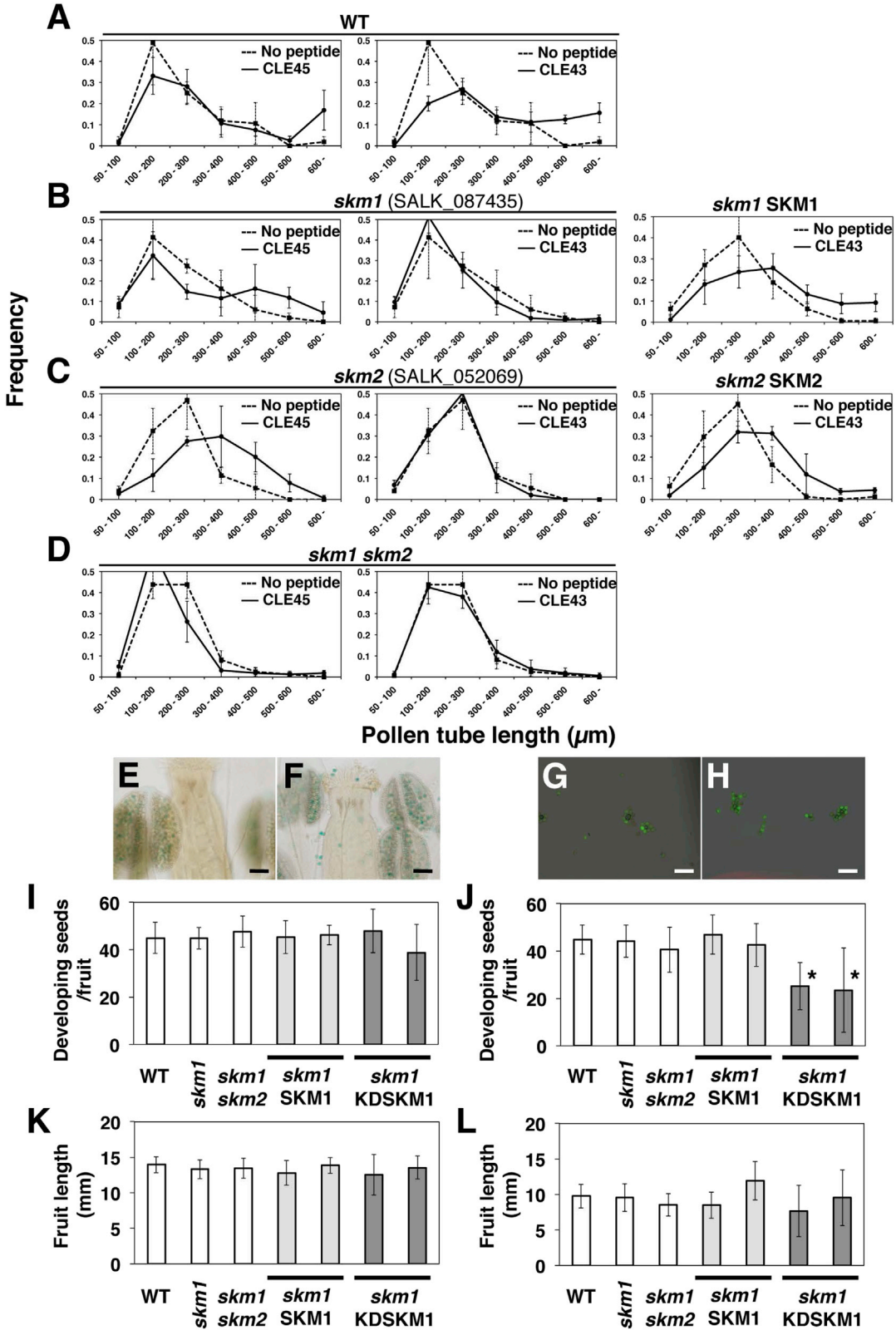

Figure 2. Identification and Characterization of Putative Receptors for CLE45 Peptide

(A–D) Effects of synthetic CLE45 or CLE43 peptide on the growth of *skm* pollen tubes in vitro. Pollen grains of WT (A), *skm1* and the complemented mutant *skm1* harboring *pSKM1::SKM1-YFP* (*skm1* SKM1) (B), *skm2* and the complemented mutant *skm2* harboring *pSKM2::SKM2-YFP* (*skm2* SKM2) (C), and the *skm1 skm2* double mutant (D) were cultured in the presence (solid lines with filled circles) or absence (dashed lines with filled squares) of synthetic CLE45 or CLE43 peptide for 20 hr at 30°C, and the number of pollen tubes with indicated length was measured. Each data set represents the mean of four independent cultures ± SD. Pollen mixtures of three independent T1 lines were used for *skm1* SKM1 and *skm2* SKM2; 30–40 pollen tubes were measured in each culture.

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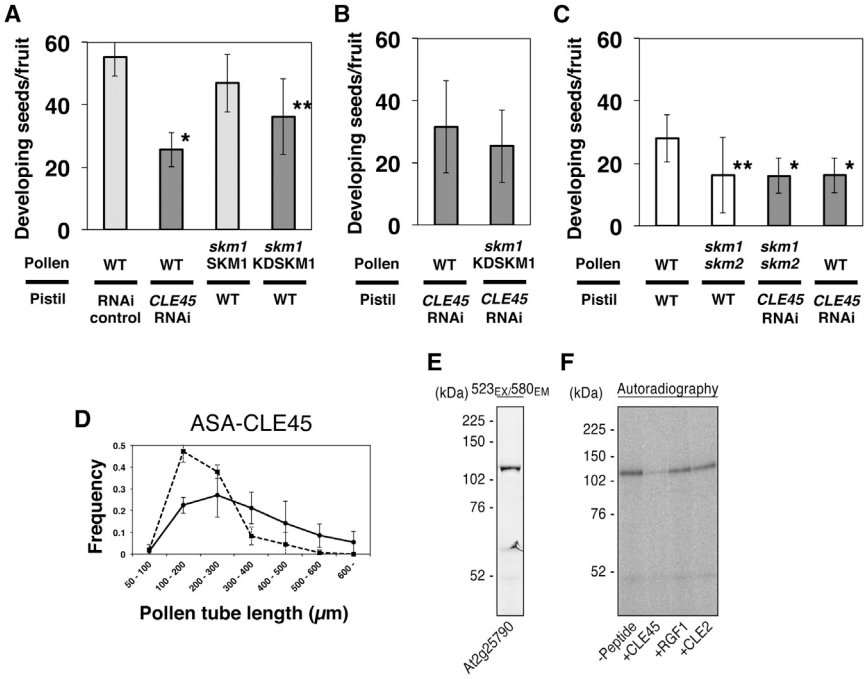

Figure 3. Interaction between Pistil CLE45 and Pollen SKM1

(A–C) Seed production in pistils artificially pollinated under high-temperature conditions. Numbers of developing seeds were measured in the indicated crosses. Plants were incubated for 18 hr at 30°C (A and B) or 33°C (C) after artificial pollination and then grown further for 3 days at 22°C. Results were obtained from each of the T2 lines used in Figures 1 and 2 for *CLE45*-RNAi, *skm1* SKM1, and *skm1* KDSKM1. Each data point represents the mean of five to eight crossed pistils ± SD. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.10$ by two-sample t test) between RNAi-control and *CLE45*-RNAi or between *skm1* SKM1 and *skm1* KDSKM1 in (A), and between the WT and the other crosses in (C).

(D–F) In vitro CLE45 peptide-SKM1 protein binding assay.

(D) Pollen grains of WT *Arabidopsis* were cultured for 20 hr at 30°C in the presence (solid lines with filled circles) or absence (dashed lines with filled squares) of ASA-CLE45 peptide. Each data set represents the mean of four independent cultures ± SD; 30–40 pollen tubes were measured in each culture.

(E) The SKM1-HaloTag form protein (At2g25790) visualized by HaloTag TMR reagent.

(F) Photoaffinity labeling of SKM1-HT by [^{125}I]ASA-CLE45 in the absence or presence of excess unlabeled CLE45, CLE2, or RGF1 peptides. The photoaffinity labeling experiment was repeated and gave the same result.

the *SKM1* or *SKM2* gene into the *skm1 skm2* double mutant recovered the alleviation effect of CLE45 peptide (Figure 4G).

The loss of CLE45-SKM1/SKM2 signaling did not significantly affect pollen tube growth but seemed to affect pollen-embryo sac interactions in situ, because the pollen tubes reached almost all of the ovules, although some ovules were later aborted (Figure 1M; Figures S1I and S2J). Mitochondrial decay under high temperature has also been observed in rice pollen after anthesis [36] and in cultured tomato pollen tubes [37]. Indeed, a loss of function of a mitochondrial ankyrin repeat protein leads to failure in male-female gamete recognition at fertilization [20]. Similarly, a defect in a nuclear-encoded mitochondrial RNA polymerase results in inhibition of both fertilization and in vitro pollen tube growth [38]. Taken together, our results strongly suggest that the CLE45-SKM1/SKM2 signaling pathway sustains pollen performance through the maintenance of mitochondrial activity under higher temperatures, leading to adequate fertilization.

Although extensive studies in pollen-pistil interactions under normal temperature conditions have revealed key factors [39], temperature-tolerance machineries in pollen-pistil interactions remain poorly understood [40]. In this study, we have shown that CLE45 signaling enhances high-temperature tolerance by reinforcing communication between pollen tubes and pistils. This system, composed of the heat-inducible signaling peptide in female tissues and its receptors in the male gametophyte, may contribute to stable seed production by protecting pollen performance against short-term exposure to high temperature, which is often caused by diurnal or seasonal temperature fluctuations.

Supplemental Information

Supplemental Information includes two figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.06.060.

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(E and F) *SKM2* expression by GUS reporter in *pSKM2::GUS* transgenic *Arabidopsis* flowers. Pollen grains of 7-week-old plants without (E) or with (F) incubation for 24 hr at 30°C are shown. Scale bars represent 100 μm.

(G and H) *SKM1* expression by YFP reporter in a *pSKM1::SKM1-YFP* transgenic *skm1* mutant. Isolated pollen grains from 7-week-old plants without (G) or with (H) incubation for 24 hr at 30°C are shown. Scale bars represent 100 μm.

(I–L) Functional analysis of *SKM1* and *SKM2* in *Arabidopsis* plants. Numbers of developing seeds (I and J) and fruit lengths (K and L) were measured in WT, *skm1*, *skm1 skm2* double mutant, and *skm1* harboring either *pSKM1::SKM1-YFP* (*skm1* SKM1; light gray column) or the kinase-dead version (*skm1* KDSKM1; dark gray column) grown at 22°C (I and K) and after incubation for 3 days at 30°C (J and L). The third-youngest fruits of the stage 17 flowers as described by Smyth et al. [19] were examined. Results were obtained from two independent T2 lines for *skm1* SKM1 and *skm1* KDSKM1. Each data point represents the mean of at least ten fruits for seed number and six for fruit length from a compilation of three experiments ± SD. Asterisks indicate significant differences ($p < 0.05$ by two-sample t test) between *skm1* KDSKM1 lines and the other plants, including WT, *skm1*, *skm1 skm2* double mutant, and *skm1* SKM1 lines.

See also Figure S2 and Table S1.

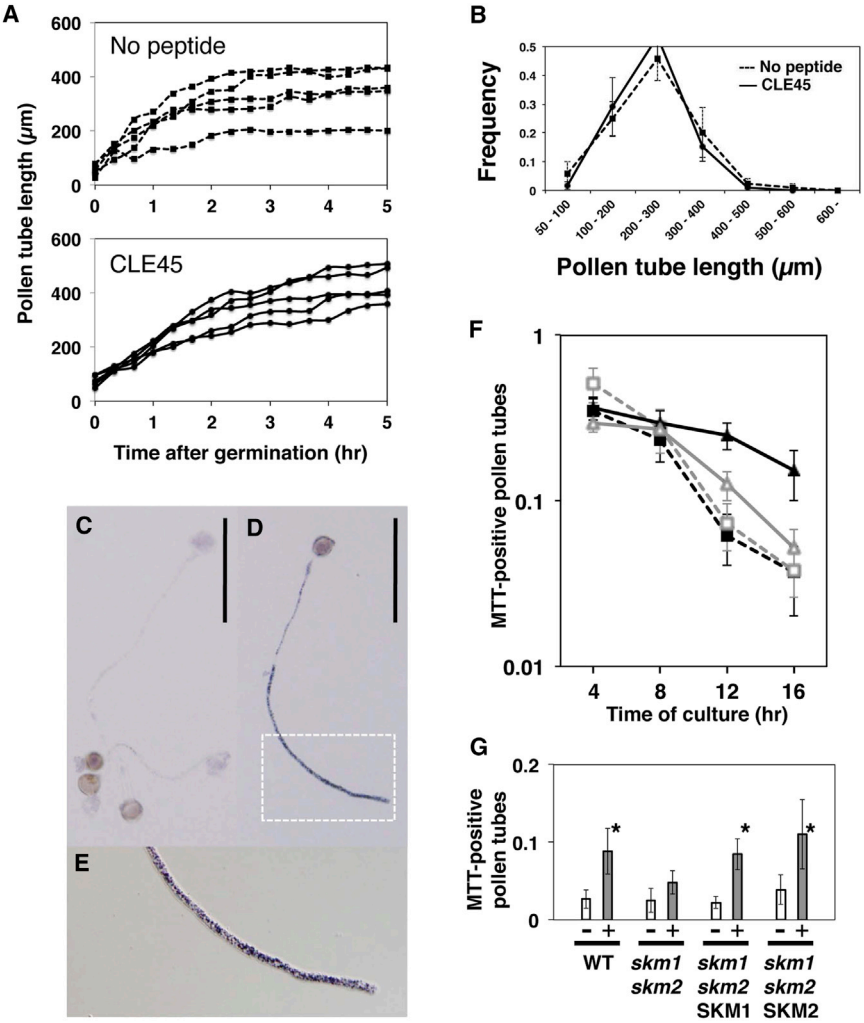

Figure 4. Characterization of CLE45 Peptide Activity in Alleviating Mitochondrial Decay during *Arabidopsis* Pollen Tube Culture In Vitro

(A) Time-lapse observation of WT *Arabidopsis* pollen tube growth with or without synthetic CLE45 peptide. Lengths of five pollen tubes at the indicated culture periods were measured in the presence (solid lines with filled circles, lower panel) or absence (dashed lines with filled squares, upper panel) of synthetic CLE45 peptide.

(B) Effect of synthetic CLE45 peptide in a culture condition at 22°C on WT *Arabidopsis* pollen tube growth. Pollen grains of WT *Arabidopsis* were cultured for 20 hr at 22°C in the presence (solid lines with filled circles) or absence (dashed lines with filled squares) of synthetic CLE45 peptide. Each data set represents the mean of four independent cultures ± SD; 30–40 pollen tubes were measured in each culture.

(C–G) Effect of synthetic CLE45 peptide on *Arabidopsis* pollen tube viability by MTT staining.

(C–E) MTT-negative (C) and -positive (D) pollen tubes at 4 hr of culture. The boxed area in (D) is magnified in (E).

(F) Proportion of MTT-positive pollen tubes at the indicated time points of in vitro culture with synthetic CLE45 (black solid lines with filled triangles), CLE43 (gray solid lines with open triangles), CLV3 (gray dashed lines with open squares), or no peptide (black dashed lines with filled squares). The y axis uses a logarithmic scale. Each data point represents the mean of four independent cultures ± SD; 200 pollen tubes were measured in each culture.

(G) Proportion of MTT-positive pollen tubes from WT, *skm1 skm2* double mutant, and its partially complemented lines. Pollen tubes were cultured for 20 hr at 30°C. Each data point represents the mean of at least four independent cultures ± SD; 100–200 pollen tubes were observed in each culture. Pollen mixtures of three independent T2 lines were used for the *skm1 skm2* double mutant harboring either *pSKM1::SKM1-YFP* (*skm1 skm2* SKM1) or *pSKM2::SKM2-YFP* (*skm1 skm2* SKM2). Asterisks indicate significant differences ($p < 0.05$ by two-sample t test) between the presence (+) and absence (–) of synthetic CLE45 peptide.

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