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Short catkin1, a novel mutant of *Castanea mollissima*, is associated with programmed cell death during chestnut staminate flower differentiation

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ABSTRACT

The fact that male flowers far outnumber female flowers is a factor that limits nut yield in Chinese chestnut. A naturally occurring mutation of male catkins was found on a single branch of a Chinese chestnut tree in the mountains near Beijing, China. The mutation was named *short catkin1* (*sck1*). The catkin length of *sck1* was only 1/6 to 1/8 that of the wild-type male catkins on the same tree. The mutation was associated with a greater number of female flowers and increased yield. Observations on the development of male catkins with the *sck1* phenotype showed that the distal part of the catkins aborted at the stage of chestnut staminate flower differentiation. Further research using transmission electron microscope analysis showed that the process of cell death in *sck1* catkins had the typical characteristics of programmed cell death at the subcellular level, such as condensed chromatin, dissolved nucleolus, degraded karyoplasm, burst karyotheca, and disintegrated chloroplasts or mitochondria. Significantly, DNA laddering was detected in tissues of *sck1* catkins. In conclusion, the results showed that *sck1* was associated with PCD.

1. Introduction

Chinese chestnut (*Castanea mollissima* Bl.) is an important tree species both for its ecological and economic value. However, the fact that male flowers far outnumber female flowers results in relatively low nut yield. Accordingly, improving nut yields has been a focus of research (Shi and Stösser, 2005; Zhang and Su, 2007).

As a monoecious plant, chestnut produces two types of catkins, unisexual catkins with only male flowers and bisexual catkins with functional male and female flowers. When female flowers begin to differentiate in the spring, they undergo a stage of nutrition competition because of rapid vegetative and reproductive growth. Nearly 40% of the intrinsic nutrition from a chestnut tree is consumed by development of the male inflorescences (Feng et al., 1995). Therefore the abundant male flower development can largely limit the female-flower differentiation. The proportion of male to female flowers in each shoot is about 1000–3000:1 (Shi and Stösser, 2005). As a result, thinning 90–95% of the male inflorescences by hand or by a chemical agent could significantly increase nut yield (Liu et al., 1999; Zhao and Liu, 1999). But thinning male inflorescences is time-consuming and labor intensive.

Recently, a natural mutant of chestnut with extremely short catkins was found on a single branch of a chestnut tree in a mountainous area near Beijing, China. The mutation was named *short catkin1* (*sck1*). In the present study, we provided that both morphological and molecular evidence to demonstrate *sck1* was associated with programmed cell death (PCD). *Sck1* markedly decreases the number of flowers in the male catkins, and at the same time promotes more female-flower differentiation, thereby increasing nut yield.

2. Materials and methods

2.1. Plant material

Phenotypic characters were investigated and buds were sampled on current-year shoot once a week from mid-June of one year to mid-May of the next year except for the dormant period in the fall and winter. The experiment used ten 3-year-old grafted trees with the mutant catkin phenotype.

2.2. Interior morphological analysis

2.2.1. Paraffin-sectioning

The sample buds and catkins were fixed in formalin–acetic acid–alcohol (FAA) for 24–48 h at room temperature and then stored at 4 °C. Paraffin embedding was done as follows: 70%

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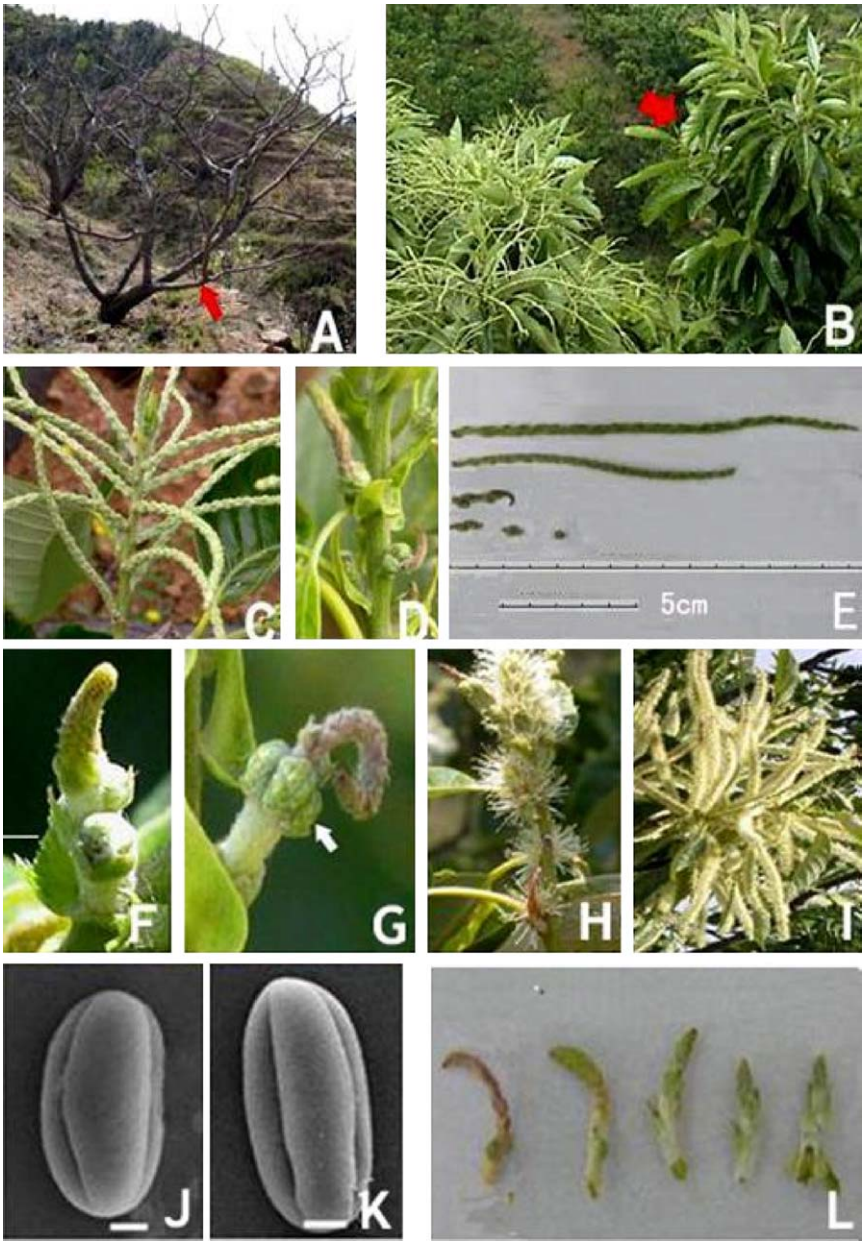

Fig. 1. The source and characteristics of the *sck1* mutant: (A) *Sck1* was found on a single branch of a wild chestnut tree in a mountainous area of Beijing, China (red arrow, before budding); (B) comparison of male catkins on a wild-type branch (left) with the *sck1* branch (right; red arrow); (C) and (D) are close-up photos of the wild-type (C) and *sck1* (D) immature male catkins; (E) comparison of wild-type catkins (top) with *sck1* catkins (bottom); (F) the male inflorescence of *sck1* grew normally to about 2 cm in length; (G) the male inflorescence of *sck1* stopped growing and gradually became yellow, finally dying and curving; the floret bundles at the proximal end of *sck1* catkins developed normally (arrow); (H) the floret bundles at the proximal end of *sck1* catkins flowered normally; (I) wild-type male catkins; (J) pollen of *sck1* (bar = 6 μm); (K) pollen of wild-type (bar = 5.7 μm); (L) excised male catkins from *sck1*, showing development from right (early) to left (late development). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

ethanol, 2 h; 85% ethanol, 2 h; 95% ethanol, 2 h; 100% ethanol, two changes, 2 h each; xylene:ethanol (1:1), 1 h; xylene, two changes, 1 h each; paraffin-saturated xylene solution, 37 °C, 48 h; paraffin wax (58–60 °C), two changes, 2 h each; embedding tissues into paraffin blocks. Paraffin blocks were trimmed to 10 μm by microtome. Paraffin ribbon was placed in slides and baked at 45–50 °C for 2 h. Safranin O and Fast Green-stained sectioned material, preserved by neutral balsam, was observed under the microscope (Reinoso et al., 2002).

2.2.2. Transmission electron microscopy (TEM)

During the catkin development period, freshly cut 3 mm^3 segments from the distal part of *sck1* catkins were fixed in 4% paraformaldehyde and 2% glutaric acid in 100 mM cacodylate buffer (pH 7.2) for 2 h at 4 °C, then eluted 3 times with 50 mM cacodylate buffer (pH 7.2), each time for 30 min. They were post-fixed in 1% OsO_4 in 50 mM cacodylate buffer for 2 h at 4 °C, and then eluted 3 times with cacodylate buffer. The samples were dehydrated in an ethanol series and acetone once, and then embedded in epoxy resin. 60 nm-ultra-thin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963), and observed under a Leica EMCU6 transmission electron microscope (Leica Microsystems GmbH, Wetzlar, Germany).

2.3. DNA ladder analysis

Genomic DNA was isolated from tissues of the distal part of *sck1* catkins using a CTAB method. Catkin tissues were ground

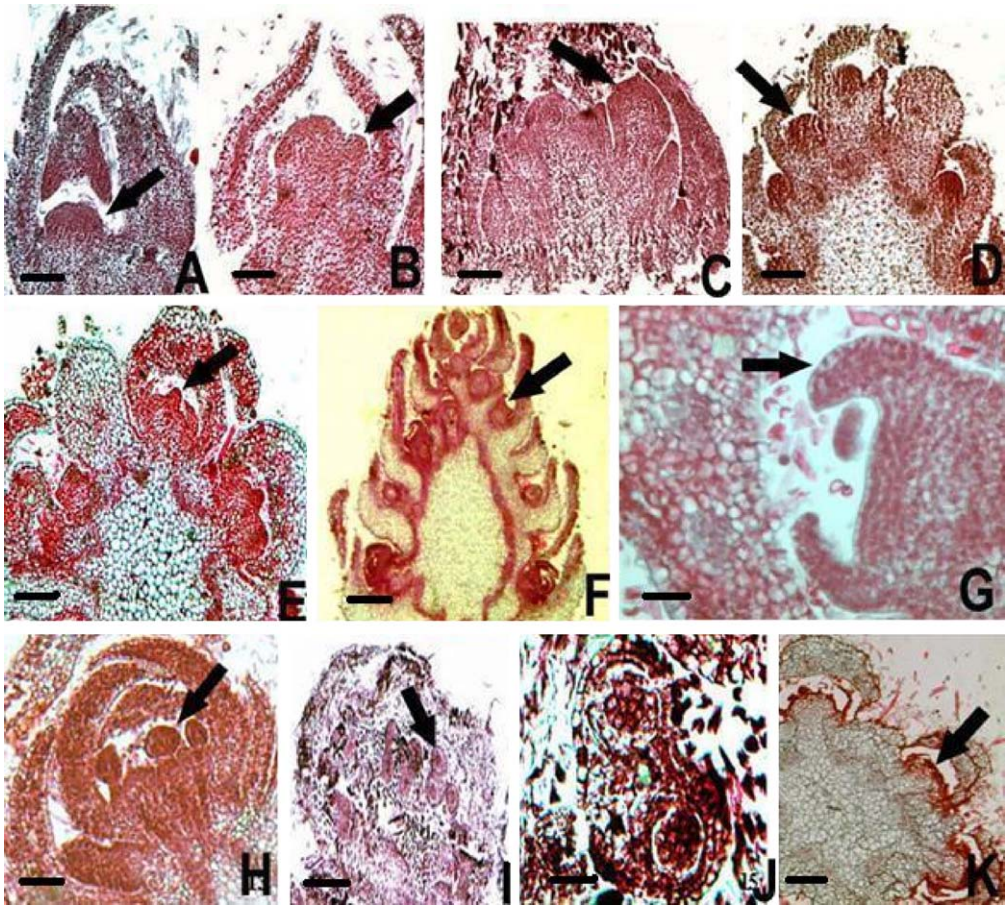

Fig. 2. Anatomical and morphological observations on developmental processes of wile-type and *sck1* staminate inflorescences. (A–C) Illustrated the developmental course of wild-type staminate inflorescence in the first growing season: (A) on new shoots at mid-June, axillary-bud apical meristem initiated floral differentiation, the flower primordium became wider and round, revealing a dome-like shape (bar = 200 μm); (B) In mid-July, the primordium of the staminate inflorescence was initiated (bar = 200 μm); (C) during the next 8–10 months, the apical meristem continually elongated and staminate inflorescences progressively increased in number and size (bar = 100 μm). As winter approaches, flower buds entered into a dormancy period. (D–J) Illustrated the developmental course of wild-type staminate inflorescence during the following spring: (D) with air temperature rising in the spring, flower buds became vigorous and active, quickly forming a staminate inflorescence axis with the dome-like primordia of a floral cluster (bar = 150 μm) on it at middle April; (E) floret primordia first appeared in the early days of May (bar=200 μm); (F and G) the staminate flower differentiation appeared in the first 10 days of May to form a horn-like shape (F, bar = 200 μm); (G) an enlarged photo of the arrowed part in F (bar = 10 μm); (H–J) showed the formation of the developmental course of the stamen in mid to late May; (H) the formation of stamen primordial (bar = 30 μm); (I) the formation of anthers (bar=100 μm); (J) an enlarged photo of the arrowed part in I (bar = 20 μm); (K) illustrated the early appearance of *sck1* anatomical morphology. Before the floral cluster differentiation, there was no difference in catkin development between the wild-type and *sck1*. After the period, the individual floret, the floral envelope, the stamen, and the anther of *sck1* stopped developing (bar = 50 μm), *i.e.* there were no normal floret primordia in the *sck1* staminate inflorescence.

in liquid N_2 immediately after being collected from the plants, and the frozen samples were homogenized in an extraction buffer that contained 2% CTAB, 100 mM Tris–HCl (pH 8.0), 20 mM EDTA (pH 8.0) and 1.4 mM NaCl, and the mix incubated at 65 °C for 1 h. Equal volumes chloroform:isoamyl alcohol (24:1) were then added and the tubes inverted 30 min and the samples centrifuged at 12,000 × *g* for 10 min, repeated twice. The DNA was precipitated by adding isopropyl:alcohol (2:3) at −20 °C for 30 min. DNA samples were digested with DNA-free RNase for 1 h at 37 °C and the DNA content was determined. For DNA analysis, samples of 4 μg DNA were loaded per lane and run on a 1% agarose gel at constant 70 V. The DNA was visualized by staining with 0.5 μg/ml ethidium bromide.

3. Results

3.1. Developmental progression and characteristics of male flowers with sck1

The buds containing the primary inflorescence of *sck1* initiated budding and shooting at the same time as wild-type buds (Fig. 1A–E). However, when the catkins grew to about 2 cm in length (Fig. 1F), the distal section of the male inflorescence on *sck1* began to stop growing, turned yellow, gradually curled (Fig. 1G and L), and finally withered after 15 d or so. The proximal section of the male inflorescence of *sck1* continued to grow, bloomed and developed into mature pollen in the same manner as wild-type (Fig. 1H–K). As a result, there were enough mature pollen grains for *sck1* to carry out pollination in an orchard.

3.2. Bud differentiation stages of male catkins

Paraffin-section studies indicated that the development of male catkins contains six continuous stages at the tissue-cell level in wild-type chestnut: male flower initiation, the floral cluster, the individual floret, the floral envelope, the stamen, and the anther (Bai et al., 2000). This development occurs over a 10-month period of differentiation, from mid-June of one year to mid-May of the next year (Fig. 2A–J). The male inflorescences of *sck1* died abnormally during the sixth stage of development as described above (Fig. 2K), suggesting that abnormal death of the floral tissue is closely associated with the shortened catkins.

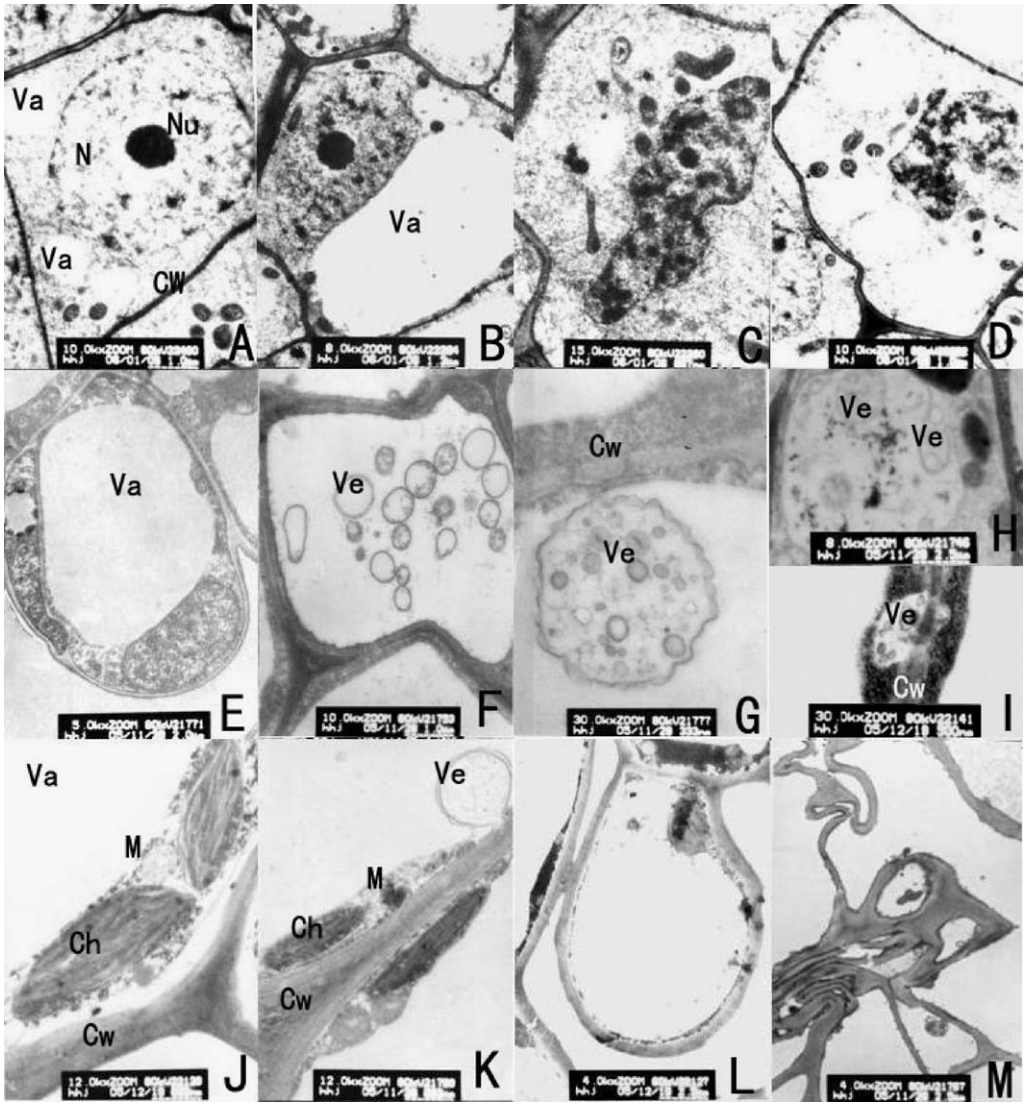

Fig. 3. Morphological analysis of PCD processes in the distal part of *sck1* during staminate inflorescence development. (A–D) The morphological change in the nucleus as PCD progresses: (A) Normal cell with a large nucleus, an obvious nucleolus and many small vacuoles (10k×); (B) PCD cell with the vacuole expanding at the initial phase (8k×); (C) PCD cell with the chromatin showing condensation, abutting the karyotheca (15k×); (D) PCD cell with chromatin showing high condensation, the nucleolus dissolved and the nucleus disaggregated (10k×); (E–I) the morphological change of the vacuole as PCD progresses: (E) PCD cell with the vacuole expanding (5k×); (F) PCD cell with many small vacuoles (10k×); (G) the vesicles with material approaching the cell wall (30k×); (H) the swelling vacuole engulfing the disaggregated inclusions via endocytosis (8k×); (I) the vesicles with material transported through the adjacent cells (×30k); (J–K) the morphological change of chloroplasts and mitochondria: (J) the chloroplasts and mitochondria compressed near the cell wall by the vacuole (12k×); (K) the chloroplasts bulged and the structure of its membrane broke; the faint membrane structure of the mitochondria (12k×); (L and M) the morphological change of PCD cells: (L) the cavitated cells, and some incomplete chloroplasts in the cells (4k×); (M) the extruded and folded cell walls (4k×).

3.3. Programmed cell death leads to the extremely short catkin

Programmed cell death (PCD) is a genetically controlled suicide that occurs as an integral part of life of most multicellular organisms (Rogers, 2006). In order to uncover the mechanism that causes cell death in the distal part of the male catkins of the *sck1* mutant, we focused on researching PCD using morphological and biochemical approaches. Further research by TEM analysis showed a progression of cellular change. The cell death of the abnormal tissue represented the typical characteristics of PCD at the sub-cellular level: the nucleus showed chromatin condensation, the nucleolus dissolved, the karyoplasm degraded, the karyotheca burst, the nucleus disintegrated, and the chloroplasts and mitochondria disintegrated gradually. The swollen vacuoles burst, shrank and fragmented (Fig. 3). Furthermore, analysis of floral cells of *sck1* at these stages revealed that DNA laddering could be detected using total DNA agarose gel electrophoresis (Fig. 4), suggesting that there was fragmented DNA in the nuclei. DNA laddering and fragmentation is feature of PCD (Kuriyama and Fukuda, 2002). Thus both morphological and biochemical assays have provided forceful evidence that the shorten catkin in *sck1* is associated with programmed cell death.

4. Discussion

One of the important observations here is that the symptoms of PCD in the male catkin with the *sck1* mutation appeared at an extremely early stage of staminate flower development. Deformation of the nuclei and gradual disappearance of chromatin were found to be relatively early symptoms of nuclear degradation. One of the earliest events observed was the fusion of smaller vacuoles, which culminated in the formation of a single central vacuole. This obviously occurred by rupture or degradation of the tonoplast coated septa between adjacent vacuolar spaces.

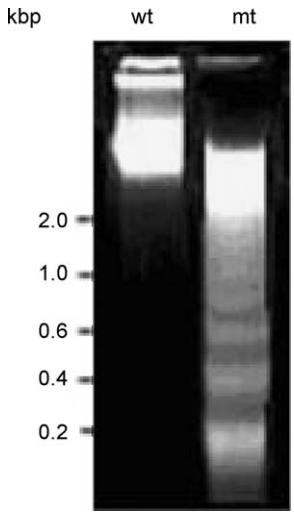

Fig. 4. DNA fragmentation in short-catkin cells undergoing death. 2% Agarose gel analysis of total DNA isolated from wild-type and *sck1* cells. The mutant *sck1* cells (mt) contained a DNA ladder not present in the cells from wild-type normal catkins (wt).

Some of the vacuolar inclusions seem to originate from these events. Further enlargement of the central vacuole is associated with the extension growth of the dying but still expanding nectariferous cells. Significant changes in the vacuome, usually in the form of increased vacuolation, have been widely reported in plant PCD (Rogers, 2005). Vacuolar inclusions also seem to originate from the pinching off of plasmatic evaginations into the vacuole. As the PCD process progresses, incorporation of plasmatic components into the vacuole increases in number. Degradation of vacuolar inclusions seems to occur through autophagy. In dying plant cells there is ample evidence for vacuolar autophagy (Krishnamurthy et al., 2000). Hallmarks of PCD were: increased vacuolation; increase in electron opacity of individual cells; progressive incorporation of plasmatic components into the vacuole reminiscent of autophagy; degradation of plastids starting with hydrolysis of starch; deformation of the nucleus and gradual disappearance of chromatin; loss of tonoplast integrity and subsequent autolysis of the rest of cellular debris; degeneration of the cells occurred against a background of increasing cell size (Gaffal et al., 2007). The strong evidence obtained from morphological studies showed that the short catkin mutant *sck1* might result from PCD.

The death of the distal part of the male catkin in *sck1* is beneficial for the nutrition distribution and balance between female and male flowers, which most likely results from short catkins saving more nutrition for female flower differentiation and development. PCD is only involved in the distal part of the male inflorescence in *sck1*; the lower male inflorescence developed normally as the wild type did. The viable pollen rate and pollination rate were tested by the TTC method (Beyhan and Serdar, 2008) and by artificial pollination (Feng et al., 2008) in the distal part of *sck1* and wild-type catkins. The viable pollen rate and pollination rate of *sck1* were 31% and 89.1%, respectively, which were not statistically significant from the wild-type 35% and 88.9%.

Feng et al. (2005) had observed no significant differences in vegetative characteristics such as shoot growth and leaf development between *sck1* and the wild-type chestnut trees, except that the trees with *sck1* had shorter catkins and more female flowers. The average number of burs per fruit-bearing branch in a grafted tree having *sck1* was 3.5 versus 2.1 burs in the wild type. This novel resource is not only of great significance for high nut production, but also is good material for research on programmed cell death and the mechanism underlying unisexual flower development. An intriguing question about the mutant is how the normal tissues in the proximal part of the male catkins protect themselves from the death signals in the distal part of the male catkins that are associated with withering and cell death.

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