

CRISPR-Cas9-mediated mutagenesis of the flowering repressor gene *VcCENTRORADIALIS* (*VcCEN*) induces early flowering in tetraploid highbush blueberry

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Key Message

We achieved the first all-allelic mutant of tetraploid blueberry through genome editing. *VcCEN* mutagenesis led to flowering within 1 year of *Agrobacterium* infection, useful for breeding and functional analyses.

Abstract

Flowering marks the vegetative-to-reproductive growth transition and is the most important event in the plant life cycle. Unlike annual plants, perennial fruit trees flower and set fruits only after an extended juvenile phase, which is an impediment to efficient breeding and gene function analyses. In this study, we generated an early flowering blueberry line via the CRISPR-Cas9-mediated mutagenesis of *VcCENTRORADIALIS* (*VcCEN*). The expression of *VcCEN* in the apical bud was negatively correlated with flower bud formation. Moreover, in the cultivar that flowers in both autumn and spring, the *VcCEN* expression level was lower and decreased earlier than in the normal cultivar that flowers in only spring. The expression data suggested that *VcCEN* functions as a flowering repressor. The CRISPR-Cas9 vector harboring a gRNA targeting *VcCEN* was introduced into the blueberry genome via *Agrobacterium*-mediated transformation. Mutations (e.g., 1–10 bp indels) were detected in the stable transformants, with all *VcCEN* alleles of the tetraploid genome mutated in some lines. Compared with the wild-type (WT), the *cen* mutants exhibited repressed vegetative growth. Additionally, in the mutants, first flowering occurred within 1 year after the *Agrobacterium* infection, which was approximately 1–2 years earlier than in WT. The mutants set a single terminal flower without entering dormancy, whereas WT produced an apical flower and multiple axillary flowers. This early flowering trait is conducive to efficient breeding and gene functional analyses, especially in fruit crops with a long juvenile phase.

Introduction

Blueberries are perennial plants classified in the section *Cyanococcus* of the genus *Vaccinium*. Most *Vaccinium* species originated in Central and South America and were subsequently dispersed to North America (Luby et al, 1991). There are several types of blueberries, including highbush blueberry (*V. corymbosum*), rabbiteye blueberry (*V. virgatum*), lowbush blueberry (*V. angustifolium*), and half-high blueberry (highbush and lowbush hybrid), all of which originated in North America. Blueberry is one of the most economically important woody plant species because its fruits contain substantial amounts of antioxidants with beneficial effects on human health (Prior et al, 1998; Ehlenfeldt and Prior 2001). Thus, there has been a continuous increase in the demand for blueberry and for novel elite cultivars. Recent genomic and transcriptomic analyses identified many candidate genomic regions responsible for important agronomic traits, including fruit firmness, fruit weight, pH, soluble solids content, epicuticular wax composition, and volatile organic compound content (Cappai et al. 2018, 2020; Edger et al. 2022; Ferrão et al. 2018, 2020; Qi et al. 2019). It takes several years to validate the functions of the candidate genes related to these fruit traits using stable transformants because of their long juvenile phase. Artificially manipulating the flowering of fruit trees with a long juvenile phase enables us to shorten the period required before the transformants can be phenotyped and to accelerate breeding by decreasing the generation time.

Plants go through a series of phase transitions during their life cycle in response to internal and external factors. Flower initiation marks the switch from vegetative to reproductive growth and is the most important event in the plant life cycle. The differences in plant reproductive patterns reflect the diversity in adaptations to various ecological contexts. In annual herbaceous plants, flower initiation occurs only once, whereas flower initiation in perennial woody plants is an annual occurrence that ensures reproductive growth continues consistently over multiple years. The extended lifespan and polycarpic growth habits of perennial trees require a highly intricate system that coordinates responses to environmental signals and determines the appropriate flowering time (Albani and Coupland 2010). In fruit trees, there are perennial-specific mechanisms regulating flowering (e.g., phase transitions and bud dormancy) (Wang and Ding 2023). However, studies on the molecular mechanisms underlying flower development have mainly involved annual model plants. Accordingly, the corresponding mechanisms in perennial woody plants remain uncharacterized.

Genetic research on flowering mechanisms has primarily focused on the phosphatidyl ethanolamine-binding protein (PEBP) gene family (Karlgrén et al. 2011), which consists of *FLOWERING LOCUS T (FT)*, *TERMINAL FLOWER1/CENTRORADIALIS (TFL1/CEN)*, and *MOTHER OF FT AND TFL1 (MFT)*. In angiosperms, PEBPs function as promoters and suppressors of flowering, while also controlling the plant architecture. Earlier research showed *FT* and its orthologs in other plant species promote flowering, whereas *TFL1/CEN* and its orthologs encode a flowering repressor in many plant species (Karlgrén et al. 2011). In *Arabidopsis*, *FT* is expressed mainly in leaves

and the encoded protein is translocated to buds. Additionally, FT along with the transcription factor FLOWERING LOCUS D (FD) promotes the expression of downstream genes, including *APETALA1* (*API*), thereby inducing flower differentiation (Abe et al. 2005). Arabidopsis *TFL1/CEN* is expressed in the shoot apical meristem and promotes vegetative growth along with *FD* (Huang et al. 2012). Loss-of-function mutations to *TFL1/CEN* genes can lead to precocious and continuous flowering in perennial fruit crops, such as kiwifruit (*Actinidia chinensis*) (Varkonyi-Gasic et al. 2019), apple (*Malus domestica*) (Charrier et al. 2019), and pear (*Pyrus communis* L.) (Charrier et al. 2019). Although researchers have attempted to knock out the tetraploid highbush blueberry *CEN*-like gene (*VcCEN*), a mutant with mutations to all *VcCEN* alleles was not obtained and promotion of flowering was not observed in the mutant (Omori et al. 2021). Therefore, *VcCEN* function has not been fully characterized in blueberry. Furthermore, expression patterns of *VcCEN* have yet to be thoroughly investigated. Compared with diploids, it is generally more difficult to confer phenotype mutation in polyploid crops because usually phenotype change could be achieved when all of the alleles are mutated in a specific gene and there are more target sequences. Furthermore, the accumulation of mutations during crossing is not feasible for fruit crops that have a long juvenile phase and a heterozygous genome. In previous genome editing studies involving highbush blueberry (*Vaccinium corymbosum*), the mutated allele frequency was 0%–37.5% (Omori et al. 2021), 0%–23.8% (Vaia et al. 2022), and 0%–28.8% (Han et al. 2022); none of these studies generated an all-allelic mutant, reflecting the need for methods that can increase the genome editing efficiency in blueberry.

In this study, we characterized the *VcCEN* expression profile and generated early flowering blueberry via the CRISPR-Cas9-mediated mutagenesis of *VcCEN*. This is the first report of obtaining all-allelic mutant in tetraploid blueberry. The phenotyping of the mutant lines provides new insights into the effects of *VcCEN* on vegetative growth and flowering in blueberry. Additionally, the mutants may be useful for shortening the generation time of fruit trees with a long juvenile phase, with potential implications for enhancing traditional breeding methods.

Materials and Methods

Plant materials

For the gene expression analysis of highbush blueberry cultivars ‘Blue Muffin’ (‘BM’) and ‘Bluecrop’ (‘BC’), 3- to 5-year-old plants were grown in pots at the experimental farm of Kyoto University, Kyoto, Japan (34°N; 135°E).

For genetic transformations, ‘BM’ was maintained and cultured under *in vitro* conditions. The *in vitro* shoots were propagated in shoot proliferation medium [MW basal medium (Tetsumura et al, 2008) supplemented with 20 g/L sucrose, 2.2 mg/L zeatin, and 6 g/L agar]. The MW medium consisted of equal volumes of MS (Murashige and Skoog 1962) and WPM (McCown and Lloyd 1981) media.

Construction of a phylogenetic tree

To identify the blueberry *CEN*-like gene, *Arabidopsis thaliana* CENTRORADIALIS homolog (AT2G27550.1) protein sequence was used as a query to perform BLAST search against highbush blueberry genome ‘Draper’ (Colle et al. 2019). A phylogenetic tree was constructed using publicly available protein sequences from NCBI (<https://www.ncbi.nlm.nih.gov/>) and MEGA X. Protein sequences encoded by PEBP gene family members were collected to identify the *CEN* clade.

Analysis of *VcCEN* expression in blueberry

Published transcriptome data for highbush blueberry ‘Draper’ (Colle et al. 2019) were analyzed using the Genome Database for Vaccinium (GDV; <https://www.vaccinium.org/>) to examine *VcCEN* expression in different organs.

To thoroughly investigate the *VcCEN* expression pattern, a quantitative real-time PCR (qPCR) analysis was performed. Shoot tips were collected from ‘BC’ and ‘BM’ plants after the expanding leaves were removed. All samples were immediately frozen in liquid nitrogen and stored at -80°C until used. To obtain sufficient amounts of RNA, 3–4 buds were pooled as one biological replicate for the RNA extraction. Samples were collected monthly from May to October in 2017. Total RNA was isolated from the frozen samples using the PureLink™ Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA). A total of 1 μg RNA was used as the template for synthesizing full-length cDNA using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). The qPCR analysis was completed using the LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) and the Thunderbird SYBR mix (Toyobo). PCR was performed using the following protocol: initial denaturation at 95°C for 5 min, followed by 45 cycles at 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s. The relative expression levels of *VcCEN* were calculated according to the $2^{-\Delta\Delta\text{Ct}}$ method, with the blueberry *UBIQUITIN CONJUGATING ENZYME 28* (*VcUBC28*) gene selected as the internal reference. Significant differences in gene expression among the months were determined on the basis of Tukey’s honest significant difference test ($P < 0.05$). Three biological replicates and two technical replicates were analyzed.

CRISPR-Cas9 vector construction

Two CRISPR-Cas9 vectors with different promoters were used. One of the vectors contains the *Arabidopsis U6* (*AtU6*) promoter for gRNA expression and the cauliflower mosaic virus (CaMV) 35S promoter for *Cas9* expression, whereas the other vector contains blueberry gene promoters (*VcU6-7* and *VcUBQ3b* promoters for gRNA and *Cas9* expression, respectively) that are useful for generating high gRNA and *Cas9* expression levels in blueberry (Omori et al, 2023). The Focas software (Osakabe et al. 2016) and DeepHF (Wang et al, 2019) was used to identify target sequences for the efficient and specific editing of *VcCEN*. Two target sites were selected and the gRNA sequences were inserted into

the CRISPR-Cas9 vectors (Fig. 1) according to the Golden Gate Cloning method. The resulting plasmids were verified by Sanger sequencing using the 3730xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) and then inserted into *Agrobacterium tumefaciens* strain EHA105 cells via electroporation (MicroPulser Electroporator; Bio-Rad, Hercules, CA, USA).

Genetic transformation of blueberry

Agrobacterium cells containing CRISPR-Cas9 vectors targeting *CEN* were grown in liquid LB medium with 50 mg/L kanamycin and 20 mg/L rifampicin for 24-48 h at 28°C. After a centrifugation, the pellet was resuspended in liquid co-cultivation medium (MW basal medium supplemented with 20 g/L sucrose, 20 mg/L acetosyringone). The suspended *Agrobacterium* solution was further diluted to an optical density at 600 nm (OD₆₀₀) of 0.3-0.4 and incubated at 28°C for 4 h. Leaf explants from 1- to 2-month-old 'BM' stocks were incubated in 30 ml suspended cells with shaking at 100 rpm for 1 h at 28°C. After blotting dry with a filter paper, the leaf explants were co-cultivated with *Agrobacterium* for 6 days on the solid co-cultivation medium in darkness. The explants were placed on selection medium (MW supplemented with 20 g/L sucrose, 1.0 mg/L thidiazuron, 0.5 mg/L α -naphthaleneacetic acid, 15 mg/L kanamycin, 250 mg/L meropenem, and 6 g/L agar). After a 2-week incubation in darkness, the explants were transplanted to fresh selection medium. The transplanting was repeated every 3 weeks for 2 months to induce callus and adventitious bud formation. The transgenic shoots from separate explants were considered to have resulted from independent transgenic events. Regenerated shoots were transferred to the shoot proliferation medium (MW supplemented with 20 g/L sucrose, 2.2 mg/L zeatin, 30 mg/L kanamycin, 250 mg/L meropenem, and 6 g/L agar). For each vector, 2 to 4 independent transformation experiments were performed, using approximately 100 explants per experiment. For all media, the pH was adjusted to 5.2 before they were sterilized in an autoclave at 121 °C for 20 min. All tissue-cultured plants were maintained at 25 °C with a 16-h photoperiod (30 mE/m²/s from cool white fluorescent tubes).

The regenerated non-infected explants served as the wild-type (WT) controls. Non-transgenic and transgenic shoots were immersed in 3 g/L indole-3-butyric acid to induce rooting. They were then transferred to the rooting medium, which comprised half-strength MW basal medium supplemented with 20 g/L sucrose and 2 g/L gellan gum, but no plant growth regulators (Tetsumura et al. 2008). All rooted shoots were transferred to a plant box containing peat moss and vermiculite (3:1, v/v). The plantlets were watered and fertilized every month with 0.1% Hyponex.

Analysis of the CRISPR/Cas9-targeted sequence

Genomic DNA was extracted from the regenerated kanamycin-resistant shoots using Genomic-tip (Qiagen, Venlo, The Netherlands). The genomic integration of transgenes was confirmed by PCR using a primer set designed for the *Cas9* sequence. The *VcCEN* target sequences in the transgenic lines

were amplified by PCR, purified, and analyzed by Sanger sequencing. Details regarding the primer sequences are provided in Supplementary Table 1. To estimate the mutated allele frequency in the pooled DNA samples, sequence chromatograms were analyzed using the online tool DECODR (<https://decodr.org/>; Bloh et al. 2021). Some of the amplified fragments were inserted into the pGEM-T Easy vector (Promega, Madison, WI, USA). Sixteen clones were sequenced to estimate the mutated allele frequency.

Phenotypic assessment of the *CEN*-mutated lines

Six WT plants and three plants from two mutated ‘BM’ lines with all-allelic mutation (12 plants in total) were included in the phenotypic analysis. The target region of the *gRNA2* in the two lines was sequenced 2 months after potting to confirm the absence of chimerism, with no antibiotic selection applied. At 2 months after potting, in August 2021, the potted plants were transferred to a greenhouse with no heating conditions. The plant height and the length of the longest shoot of each line were measured at 3, 6, and 9 months after potting. The date of first flowering was also recorded for each line. The significance of the differences between the WT and mutated lines was assessed on the basis of Student’s *t*-test ($P < 0.05$). A pollen germination test was conducted to determine pollen viability. Briefly, the collected pollen grains were placed on germination medium (15% sucrose, 1.5% agarose, and 0.01% boric acid) and incubated overnight at room temperature. Pollen germination was examined using a VH-S30 microscope (Keyence, Osaka, Japan).

Results

***VcCEN* expression in blueberry**

Three *VcCEN* alleles were identified in the highbush blueberry ‘Draper’ genome (Fig. 2A). An *in silico* analysis revealed that *VcCEN* was expressed only in the shoot (Fig. 2B). The analysis of seasonal changes in *VcCEN* expression in the ‘BC’ apical bud indicated that *VcCEN* expression peaked in June and gradually decreased until October (Fig. 2C). Flower bud initiation in highbush blueberry in Japan starts in July to September depending on cultivars (Ogiwara et al. 2010), suggesting that *VcCEN* expression may be negatively correlated with flower bud formation. Most highbush blueberry cultivars, including ‘BC’, bloom in spring (typically at the end of March to April), whereas ‘BM’ blooms from July to December in addition to the spring bloom. The *VcCEN* expression level in ‘BM’ was lower than that in ‘BC’ and decreased in June, which was earlier than the decrease in ‘BC’ (Fig. 2D). The comparison of these two cultivars suggested that the low *VcCEN* expression level in ‘BM’ may have resulted in unusual flowering. Overall, these findings imply *VcCEN* may encode a flowering repressor in blueberry, making it a potential candidate gene for manipulating blueberry flowering.

Detection of CRISPR-Cas9-induced *CEN* mutations in genetic transformants

Transgenic blueberry plants carrying the CRISPR-Cas9 vector targeting *VcCEN* were generated via Agrobacterium-mediated transformation. Mutations were detected in nine of the 11 transgenic lines (mutation rate of 82%) (Fig. 3A). The highest mutated allele frequency was 100%, which means all alleles were edited. The mutated allele frequency was higher for gRNA2 than for gRNA1, indicative of the importance of selecting an appropriate gRNA for efficient genome editing. The mutation types in one of the edited plants are shown in Fig. 3B–D. Specifically, DECODR and clone sequencing revealed 1- to 10-bp insertions/deletions a few bases upstream of the PAM sequence in the transgenic line (Fig. 3C, D).

Phenotypic observations of *cen* mutants

At 4 months after rooting, approximately 90% of the rooted shoots were successfully acclimated to the ambient conditions. The average plant height data revealed that the three individuals from two selected mutant lines (AS_gRNA2#1 and VV_gRNA2#1, Fig. 3A) with no remaining intact allele were significantly shorter than the non-transgenic control at 6 and 9 months after potting (Fig. 4A). In addition, shoot lengths of *cen* mutants tended to be shorter than WT at 9 months after potting (Fig. 4B). These results suggested that a mutation to *VcCEN* may induce suppressed vegetative growth in blueberry.

First flowering of the *cen* mutants occurred earlier (8–11 months after potting) than the WT plants (20–28 months after potting) (Fig. 5). The mutant plants exhibited a compact growth habit and terminal flowering. The *cen* mutants produced a single or double flower at the top of the shoot, whereas the WT plants produced a florescence with several flowers (Supplementary Fig. 2). The terminal bud of the mutants did not enter dormancy and bloomed within 1 month after bud formation. In summer, the shoot apex of the mutant lines occasionally transformed directly into a flower without growth cessation and a typical bud set (Supplementary Fig. 3). The vegetative growth continued until just before the formation of the terminal flower. This flowering pattern in the mutants was reproduced under high temperature (28 °C) and long photoperiod (16 h) conditions. Accordingly, the exposure to a high temperature and a long photoperiod appeared to enhance vegetative growth. Moreover, in the mutant, there was a spontaneous transition from vegetative growth to reproductive growth, resulting in the rapid formation of floral organs. In the *cen* mutant grown in the greenhouse without heating, flower buds formed throughout the year and they bloomed continuously from March to December without entering dormancy. In January and February, flower bud development was suppressed and no blooming was observed, probably because of the low temperatures during winter. When the mutants were grown in small pots and the longest shoot was shorter than 40 cm, they did not flower, similar to the WT control. Flower buds only developed on shoots longer than approximately 60 cm for both the WT and mutant plants. There were no obvious morphological differences in the flowers and pollen germination between the WT and *cen* mutant plants (Supplementary Fig. 4).

Discussion

Genome editing in polyploid plants presents additional challenging to implement due to the presence of multiple alleles for each gene (Schaart et al., 2021). In previous studies involving genome editing of highbush blueberry, the mutated allele frequencies ranged from 0 to 37.5% (Omori et al., 2021; Vaia et al., 2022; Han et al., 2022). None of these efforts successfully produced an all-allelic mutant, highlighting the need for improved techniques to enhance genome editing efficiency in tetraploid blueberry. Our study demonstrates the first successful creation of an all-allelic mutant in tetraploid blueberry through genome editing. We observed that higher mutated allele frequencies when using gRNA2 compared with gRNA1, suggesting that selecting a suitable gRNA is crucial for increasing genome editing efficiency. Recent advancements deep learning-based selection of gRNA, such as DeepHF used in this study, may help enhance editing efficiency (Wang et al, 2019; Gerashchenkov et al, 2020). Although the vector harboring blueberry endogenous promoters also slightly enhanced editing efficiency over the conventional vector with *Arabidopsis U6* and 35S promoter, further investigation with more transgenic lines will be required. Genome editing technology has been utilized to improve crop yield, nutrient, stress tolerance (Atia et al. 2024; Ben-Amar 2024) and many countries adapted guidelines that permit the cultivation of non-transgenic gene-edited lines similar to conventionally bred lines (Buchholzer and Frommer 2023). This is the first instance of editing a practical gene in blueberry, and it is expected that various gene-edited blueberries will be developed for practical use in the future.

The *TFL1/CEN* gene family plays a crucial role in the regulation of flowering and architecture in various plant species. Earlier research demonstrated that *TFL1/CEN* genes encode flowering repressors in different plant species (Jin et al. 2021). The *CEN* gene was first identified as the gene responsible for controlling the inflorescence architecture in *Antirrhinum* (Bradley et al. 1996). The *cen* mutation changes inflorescence style from indeterminate to determinate single flower in *Antirrhinum*. Although extensive research has been conducted to characterize the function of *CEN* in model annual plants, the importance of its role in perennial plants has not been thoroughly investigated. In fruit trees, flowering comprises of perennial-specific several complex processes such as vegetative to reproductive phase transitions and bud dormancy (Wang and Ding, 2023). In the current study, the results of our analyses of *VcCEN* expression and function collectively provide new insights into the molecular mechanism underlying blueberry flowering. More specifically, our findings indicate *VcCEN* functions as a flowering repressor in blueberry. Additionally, the pleiotropic effects of *VcCEN* were revealed; the mutation to *VcCEN* resulted in changes to vegetative growth, photoperiod sensitivity, juvenile phase duration, flowering architecture, and flower bud dormancy. Moreover, *VcCEN* was expressed at high levels in actively growing shoots in the summer, but the *cen* mutants were shorter than the WT control, indicative of the positive effects of *VcCEN* on vegetative growth. In blueberry,

flower initiation is induced by short-day conditions, with a critical day length threshold of approximately 12 h (Hall 1963; Phatak and Austin 1990). Increasing the day length (e.g., 16-h photoperiod) reportedly leads to the lack of flower bud formation in three highbush blueberry cultivars (Bañados and Strik, 2006). In the present study, the WT plants did not bloom under long-day conditions, whereas the shoot apex of the mutant lines occasionally transformed directly into a flower without growth cessation and a typical bud set, suggesting a mutation to *VcCEN* can alter photoperiod sensitivity. A similar phenomenon was often observed in rabbiteye blueberry plants grown in a warm subtropical climate (Omori et al. 2022). The growing shoots of some rabbiteye blueberry cultivars directly turn into inflorescences, with decreases in *VcCEN* expression as the floral meristem forms (Omori et al. 2022). These two phenomena may be mediated by a similar mechanism. Although *VcCEN* mutagenesis induced early flowering, small plants with shoots shorter than 40 cm did not flower even in the mutants, suggesting there is a minimum shoot length threshold for plants to bear flower buds. A mutated *VcCEN* also substantially changed the inflorescence architecture in blueberry. This phenotype is a typical consequence of mutations to *TFL1/CEN* genes in various plant species (Bradley et al. 1996; Charrier et al. 2019; Varkonyi-Gasic et al. 2019; Shannon and Meeks-Wagner 1991). Similar to other studies, the mutagenesis of *VcCEN* limited the development of the normally indeterminate inflorescence and led to the production of a terminal flower. Thus, this study and reported studies collectively suggested that mutating *VcCEN* is useful for promoting flowering and overcoming several traits typical of woody plants that restrict flowering, including a long juvenile phase and dormancy.

The annualization of fruit trees may revolutionize the breeding of fruit tree crops with a long juvenile phase. Most perennial plants have a long juvenile phase and lack flowers for a certain period. This characteristic is detrimental to efficient breeding and genetic analyses. For example, introducing disease resistance genes via backcrossing involving closely related wild species and pyramiding useful genes are time-consuming processes. ‘Fast-track breeding’ has been developed as a novel strategy for efficiently breeding stress-resistant fruit trees. This approach is based on the overexpression of genes that promote flowering, including *FT* orthologs. It has been used to introduce fire blight and apple scab resistance genes from wild apple into an elite cultivar, with five generations produced within 7 years (Schlathölter et al. 2018). Similarly, the citrus tristeza virus resistance gene from trifoliate orange (*Poncirus trifoliata*; Endo et al. 2020) was introduced into other citrus germplasm. A similar approach may be applied to incorporate some important traits from wild *Vaccinium* plants into blueberry cultivars. Some wild *Vaccinium* species that may be used for the cross-pollination of blueberry have valuable traits, including vigor, unique fruit volatile compositions, fruit firmness, waxy foliage, low chilling requirements, cold hardiness, and drought tolerance (Edger et al. 2022).

The early flowering trait of the *cen* mutant may also be useful for validating agronomically important genes in blueberry. Many recent genetic studies have identified candidate genes associated

with agronomically important traits of blueberry (Edger et al. 2022). The precise characterization of the functions and practical utility of genes requires experiments involving stable transformations or mutagenesis. However, even though many candidate genes have been identified, very few have been functionally validated, especially in fruit tree crops. Creating and phenotyping transgenic plants require a lot of time, area, and labor if the individual plants are large and have a long generation time. Shortening the juvenile phase can accelerate gene validation studies. For example, “rapid flowering” kiwifruit (*A. chinensis*) was previously used to functionally validate *Friendly boy* (*FrBy*), which is a Y chromosome-encoded sex-determinant gene (Akagi et al. 2019). In another study, an early flowering grape mutant ‘microvine’ that flowers continuously and has a shortened generation time was used to generate a segregating population and validate the contribution of the candidate gene *VviPLATZ1* to female flower morphology (Iocco-Corena et al. 2021).

Data availability

All data supporting the findings of this study are included within the article and its supplementary materials. Further inquiries can be directed to the corresponding author.

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Contributions

Hisayo Yamane and Masafumi Omori designed project and Masafumi Omori performed the experiments and wrote the manuscript. Yuriko Osakabe and Keishi Osakabe provided the CRISPR-Cas9 vector. Hisayo Yamane and Ryutaro Tao supervised all the work and revised the manuscript.

Conflict of interests

The authors declare no conflicts of interest.

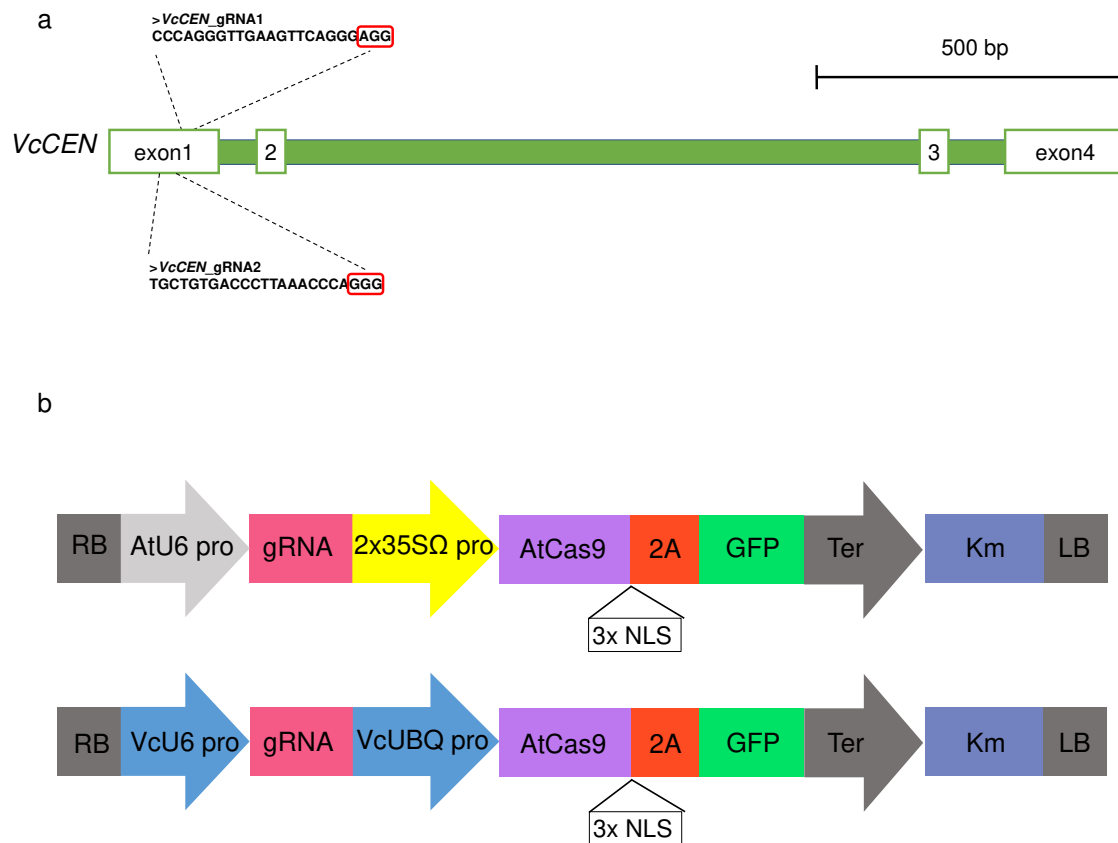


Figure 1. *VcCEN* sequences targeted by gRNA and schematic diagrams of the CRISPR-Cas9 vectors used in this study.

(a) *VcCEN* gene structure and gRNA sequences. Two *VcCEN* target sequences are presented and red boxes indicate the PAM sequence. The *VcCEN* gene has four exons (white box) and three introns (green line).

(b) Schematic diagrams of the CRISPR-Cas9 vectors with different promoters. AtU6: Arabidopsis *U6* promoter; VcU6-7: blueberry *U6* promoter; 2x35SΩ: 2× CaMV 35S promoter with the omega enhancer sequence; VcUBQ3b: blueberry ubiquitin gene promoter; gRNA: location of the gRNA insertion; AtCas9: *Arabidopsis thaliana* codon-optimized SpCas9; 2A: 2A self-cleaving peptide; Km: kanamycin resistance marker expression cassette; RB: T-DNA right border; LB: T-DNA left border.

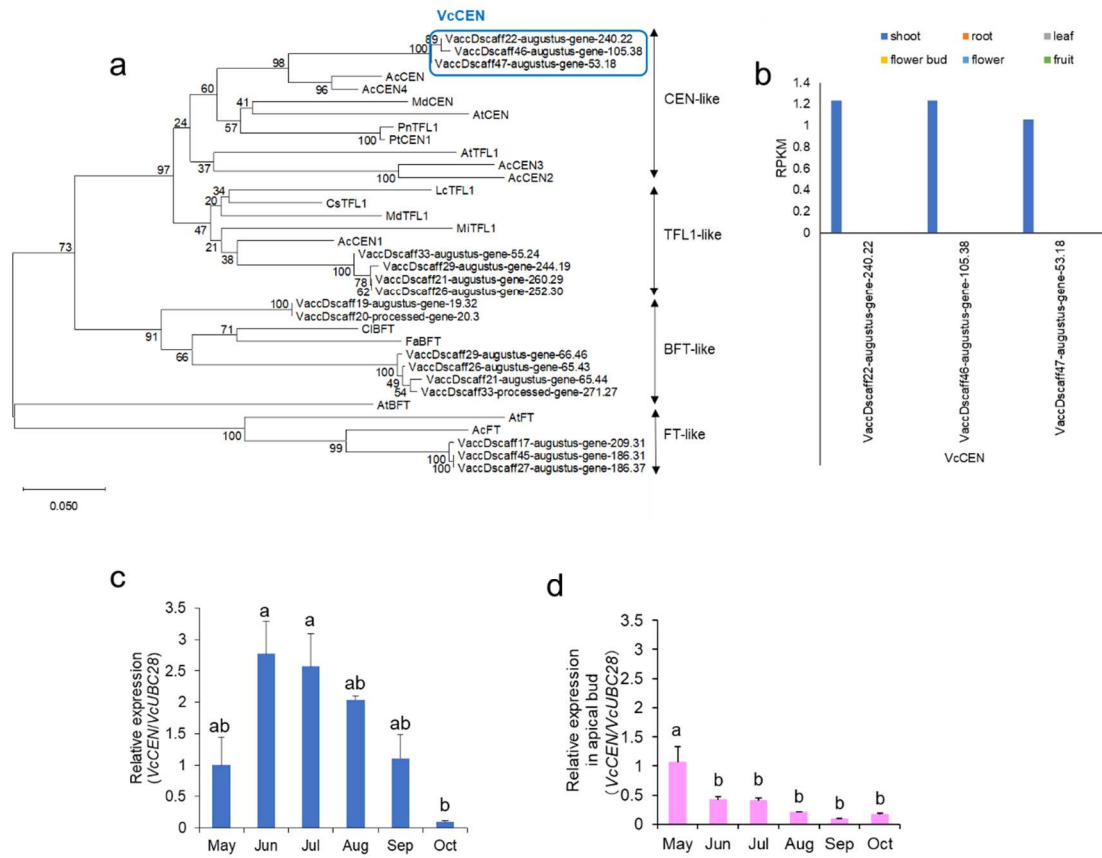


Figure 2. Phylogenetic and expression analyses of *VcCEN*.

(a) Phylogenetic tree of flowering-related PEBP family proteins constructed using the neighbor-joining method and the MEGA X program. The scale indicates the average substitutions per site. *VaccDscf*: gene ID from GDV; *AcCEN*: *Actinidia chinensis* CENTRORADIALIS (JX417424.1); *AcCEN4*: *Actinidia chinensis* CENTRORADIALIS4 (ARE72519.1); *MdCEN*: *Malus domestica* CENTRORADIALIS (BAG31958.1); *AtCEN*: *Arabidopsis thaliana* CENTRORADIALIS homolog (AT2G27550.1); *PnTFL1*: *Populus nigra* TERMINAL FLOWER 1 (BAG12897.1); *PtCEN1*: *Populus trichocarpa* CENTRORADIALIS (AAQ88444.1); *AtTFL1*: *Arabidopsis thaliana* TERMINAL FLOWER 1 (AAM27956.1); *AcCEN3*: *Actinidia chinensis* CENTRORADIALIS3 (ARE72518.1); *AcCEN2*: *Actinidia chinensis* CENTRORADIALIS2 (ARE72517.1); *AcCEN1*: *Actinidia chinensis* CENTRORADIALIS1 (ARE72516.1); *MiTFL1*: *Mangifera indica* TERMINAL FLOWER 1 (AGA19027.1); *LcTFL1*: *Litchi chinensis* TERMINAL FLOWER 1 (QNH85212.1); *CsTFL1*: *Citrus sinensis* TERMINAL FLOWER 1 (KAH9738276.1); *MdTFL1*: *Malus domestica* TERMINAL FLOWER 1 (NP_001280887.1); *CIBFT*: *Citrus limon* BROTHER of FT and TFL1 (AWW25017.1); *FaBFT*: *Fragaria × ananassa* BROTHER of FT and TFL1 (AMR34798.1); *AtBFT*: *Arabidopsis thaliana* BROTHER of FT and TFL1 (Q9FIT4.1); *AtFT*: *Arabidopsis thaliana* FLOWERING LOCUS T (AAF03936.1); *AcFT*: *Actinidia chinensis* FLOWERING LOCUS T (AGK89939.1).

(b) Expression levels of *VcCEN* in different organs. (c) Seasonal changes in *VcCEN* expression in the ‘BC’ apical bud. (d) Seasonal changes in *VcCEN* expression in the ‘BM’ apical bud.

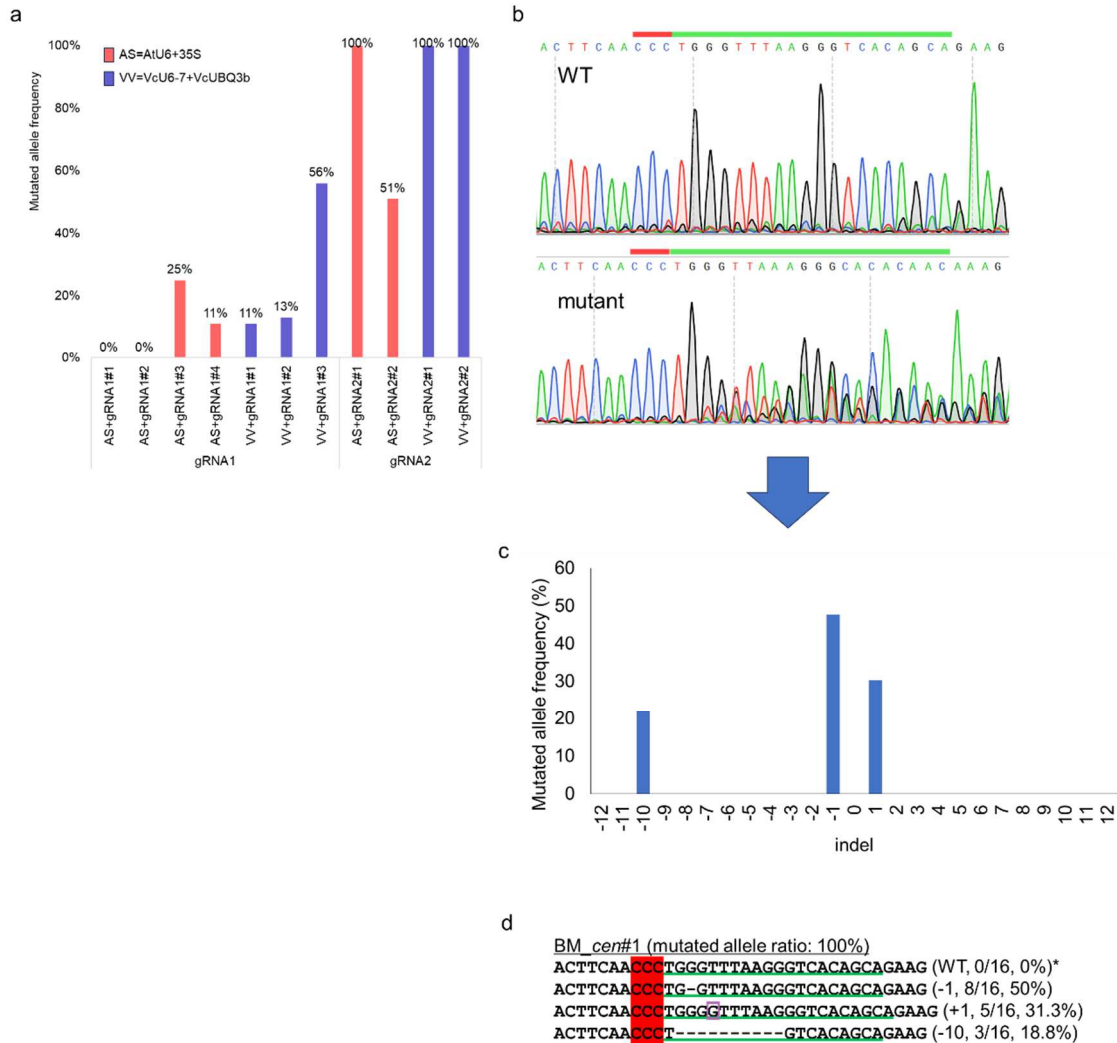


Figure 3. CRISPR-Cas9-induced *VcCEN* mutations.

(a) Mutated allele frequency of the transgenic lines transformed with different CRISPR-Cas9 vectors. The conventional vector (*AtU6* and 35S promoters) is indicated in red. The vector containing blueberry promoters (*VcU6-7* and *VcUBQ3b* promoters) is indicated in blue. To estimate the mutated allele frequency of each transformant, sequence chromatograms were analyzed using the online tool DECODR. (b) Direct sequencing of WT and the mutant. The PAM and 20 bp target sequences are indicated in red and green, respectively. (c) Sequence chromatograms were used to predict mutations. In this transgenic line, three kinds of mutations (–1, +1, and –10) were detected, which resulted in a

frame shift. (d) Clone sequencing results. WT (upper) and mutated (lower) sequences were aligned. An intact WT sequence was not detected in the transgenic line. The PAM sequence is highlighted in red. The gRNA target sequences are indicated by a green underline. The mutation types and mutated allele ratios of each line are presented to the right of the sequences. *(mutation type, number of clones with the mutation/total number of clones).

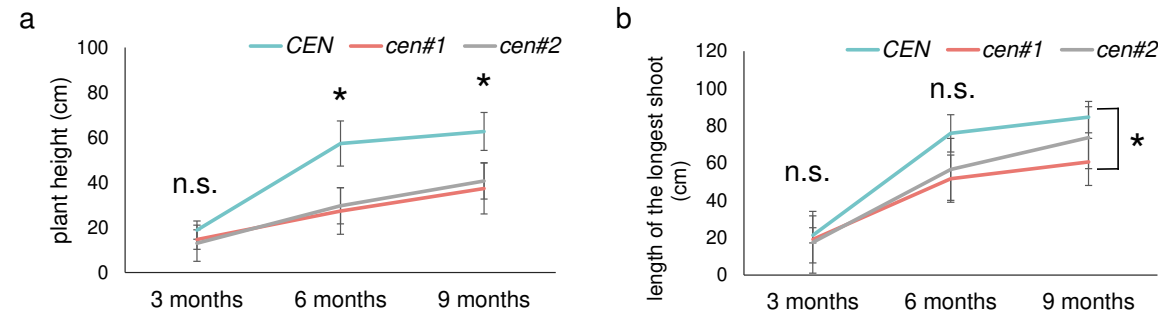


Figure 4. Comparison of the vegetative growth between the WT control (*CEN*) and the mutated lines (*cen*).

(a) Plant height of six individuals from two selected lines. Asterisks indicate significant differences was detected between WT and the two mutant lines. Data are presented as the mean $\pm$ standard error. Significant differences ($P < 0.05$) were determined according to Student's *t*-test. (b) Length of the longest shoot of the six individuals.

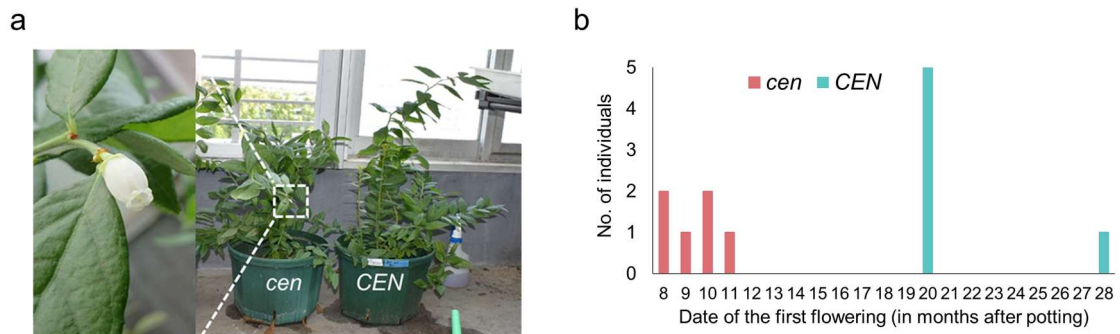


Figure 5. Early flowering in the *cen* mutant.

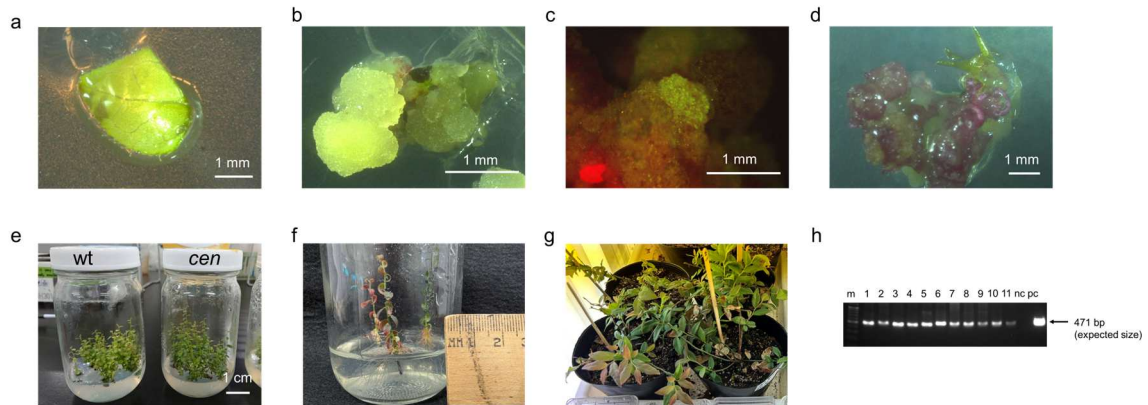
(a) Flowering was observed in the *cen* mutant at 8 months after potting. (b) Number of individuals for specific first flowering dates (i.e., months after potting). At 2 months after potting, in August 2021, the potted plants were transferred to a greenhouse with no heating conditions.

552 **Supplementary Table 1. Sequence information for the primers used in this study**

Name	Sequence	Applications
VcCEN_expression_F	5'-CATAGGAATCCACAGGTATGTG-3'	For expression analysis
VcCEN_expression_R	5'-AAGACAGCAGCAACAGGGAG-3'	For expression analysis
VcUBC28_expression_F	5'-CCATCCACTTCCCTCCAGATTATCC-3'	For expression analysis
VcUBC28_expression_R	5'-ACAGATTGAGAGCAGCACCTTGG-3'	For expression analysis
VcCEN_gRNA1F	5'-GATTGTCACCCTTCCAACAACAAGG-3'	For construction of the CRISPR/Cas9 vector
VcCEN_gRNA1R	5'-AAACCCTTGTTGTTGGAAGGGTGAC-3'	For construction of the CRISPR/Cas9 vector
VcCEN_gRNA2F	5'-GATTGTATCACCCTTCCAACAACAA-3'	For construction of the CRISPR/Cas9 vector
VcCEN_gRNA2R	5'-AAACTTGTTGTTGGAAGGGTGATAC-3'	For construction of the CRISPR/Cas9 vector
Cas9_F	5'-ACTAAGGCTGAGAGGGGAGG -3'	For PCR analysis to confirm transformation
Cas9_R	5'-GATCTCACCGTTAGCGAGGG -3'	For PCR analysis to confirm transformation
VcCEN_F	5'-TGTCCATGTCTGTGTCCGTG-3'	For sequence analysis
VcCEN_R	5'-TCCTATGTTTGGCCTTGGA-3'	For sequence analysis

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Supplementary Figure 1. The transformation process and generation of transgenic plants

(a) A leaf explant on co-cultivation medium. (b) Callus formation on selection medium three weeks after infection with Agrobacterium. (c) GFP expression in transgenic callus. (d) Shoot regeneration 3 months after Agrobacterium infection. (e) Shoot propagation of WT and transgenic *cen* mutant. (f) Rooting induction in the mutant 1 month after IBA treatment. (g) Transgenic plants in pots 4 months after rooting. (h) PCR amplification of *Cas9* from transgenic lines. M: marker; lanes 1 to 4: AS_gRNA1#1-4(Fig. 3A); lanes 5 to 7 : VV_gRNA1#1-3; lanes 8,9: AS_gRNA2#1, 2; lanes 10, 11: VV_gRNA2#1, 2. NC: negative control. Wild type DNA was used as the template.; PC : positive control CRISPR-Cas9 vector was used as the template.

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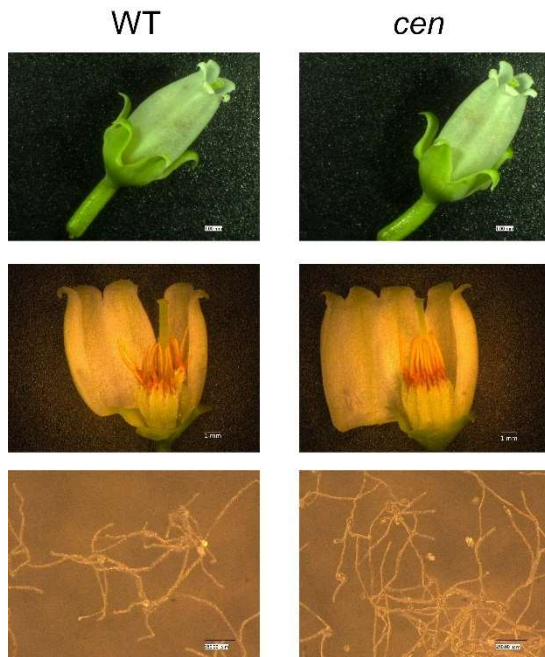


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569 **Supplementary Figure 2. Comparison of the flower architecture between the mutant (*cen*) and**
570 **WT (*CEN*).** The *cen* mutants produced a single or double flower at the top of the shoot and bloomed
571 within 1 month after bud formation without entering dormancy. In contrast, WT produced an apical
572 bud and multiple axillary buds with several flowers.
573



Supplementary Figure 3. Direct transformation into a flower without growth cessation and typical bud set. Bar = 2 cm. The plant was photographed every 6 days in July.



Supplementary Figure 4. Morphological comparison of the flower and pollen germination between WT and the *cen* mutant.