Mutagenesis of kiwifruit CENTRORADIALIS-like genes transforms a climbing woody perennial with long juvenility and axillary flowering into a compact plant with rapid terminal flowering

Erika Varkonyi-Gasic1,*, Tianchi Wang1, Charlotte Voogd1, Subin Jeon1, Revel S. M. Drummond1, Andrew P. Gleave1 and Andrew C. Allan1,2

1The New Zealand Institute for Plant & Food Research Limited (Plant & Food Research), Auckland, New Zealand
2School of Biological Sciences, University of Auckland, Auckland, New Zealand

Summary

Annualization of woody perennials has the potential to revolutionize the breeding and production of fruit crops and rapidly improve horticultural species. Kiwifruit (Actinidia chinensis) is a recently domesticated fruit crop with a short history of breeding and tremendous potential for improvement. Previously, multiple kiwifruit CENTRORADIALIS (CEN)-like genes have been identified as potential repressors of flowering. In this study, CRISPR/Cas9-mediated manipulation enabled functional analysis of kiwifruit CEN-like genes. Mutation of these genes transformed a climbing woody perennial, which develops axillary inflorescences after many years of juvenility, into a compact plant with rapid terminal flower and fruit development. The number of affected genes and alleles and severity of detected mutations correlated with the precocity and change in plant stature, suggesting that a bi-allelic mutation of either AcCEN4 or AcCEN may be sufficient for early flowering, whereas mutations affecting both genes further contributed to precocity and enhanced the compact growth habit. CRISPR/Cas9-mediated mutagenesis of AcCEN4 and AcCEN may be a valuable means to engineer Actinidia amenable for accelerated breeding, indoor farming and cultivation as an annual crop.

Introduction

Flowering plants have adopted different life-history strategies to survive adverse environmental conditions, and time their flowering for optimal reproduction. Annual and perennial growth habits are a sequence of different fates that can be acquired by meristems during the plant life cycle. Upon floral induction, vegetative shoot meristems undergo a transition to indeterminate inflorescence meristems, which give rise to determinate floral meristems. Unlike annuals, where all meristems are consumed within the same growing season, perennials maintain indeterminate meristems for the next season (Thomas et al., 2000). Fluctuations between annuality or perenniality have been observed (Bena et al., 1998; Hu et al., 2003; Tank and Olmstead, 2008) and selection for faster flowering and determinate growth habit underpins domestication of tender perennial crops such as tomato, which is commonly grown for production as an annual. However, many horticultural crop species are woody perennials, characterized by extended juvenile periods and large plant size, adapted to environments unlikely to favour natural mutations causing dramatic growth habit changes.

Actinidia are woody perennial species characterized by a relatively large genome with the basic chromosome number x = 29, ploidy variation, large plant size, climbing growth habit and excessive vigour. A genus comprising more than 50 species, Actinidia belong to the order Ericales, with all members being deciduous and dioecious with a temperate flowering phenology (Ferguson, 2016). A dichasium inflorescence or single flowers develop in the lower leaf axils of the shoots that emerge after winter dormancy and a juvenile unproductive period. The genus Actinidia includes several economically important horticultural species, commonly known as kiwifruit and widely recognized for health benefits. As a recently domesticated crop, few kiwifruit cultivars are the result of deliberate breeding programmes, being only one or two generations removed from the wild (Datson and Ferguson, 2011). Consequently, orchard management has many issues including functional dioecy, expensive management practices required to tame the long-lived woody perennial climbers, inadequate crop yield in warmer climates, and susceptibility to diseases such as the devastating kiwifruit bacterial canker (Scortichini et al., 2012). Extended juvenility and long crossing cycles in particular are considerable constraints for genetic analysis and for creating improved cultivars. Newly established plants may take up to 5 years to grow a full canopy in the field (Ferguson, 2016), whereas A. chinensis cultivars commonly used for gene functional studies typically take 5 years to first flowering in glasshouse conditions.

Alteration of kiwifruit growth habit from a woody perennial climber into a short determinate plant with precocious flowering would be desirable to enable indoor growth, increase productivity and accelerate the breeding process. Genes involved in meristem transitions have been identified as good candidates for a switch in plant growth habit (Melzer, 2008), particularly PEBP (phosphatidylethanolamine-binding protein) genes, homologs of Arabidopsis FLOWERING LOCUS T (FT) and TERMINAL FLOWER1 (TFL1) (Wickland Daniel and Hanzawa, 2015). FT-like genes are the key determinants of flowering time, and their antagonists

*Correspondence (Tel +64 9 925 7258; fax +64 9 925 7001; email erika.varkony-gasic@plantandfood.co.nz)

Keywords: Actinidia, CEN, CENTRORADIALIS, CRISPR/Cas9, flowering, kiwifruit.
**TLI-like and CENTORADIALIS (CEN)-like are conserved repressors of flowering, acting in the shoot tip to maintain the indeterminate fate** (Bradley et al., 1996, 1997; Lifschitz et al., 2014). Manipulations of FT and CEN availability and dosage provide means to manipulate developmental mechanisms; overexpression of FT genes (Klocko et al., 2016; Srinivasan et al., 2012; Wenzel et al., 2013) and down-regulation of TLI/CEN genes (Freeman et al., 2011; Kotoda et al., 2006; Yamagishi et al., 2016) have been advanced as tools for accelerated breeding. These approaches have often proven to be unreliable (Zhang et al., 2010) or failed to promote flowering in fruit crops, affecting other aspects of development instead (Freeman et al., 2015; Varkonyi-Gasic et al., 2013). In contrast, stable mutations in these genes have underpinned domestication of crops such as legumes, sugar beet, sunflower and strawberry (Blackman et al., 2010; Iwata et al., 2012; Kwak et al., 2012; Liu et al., 2010; Pin et al., 2010), or in case of a natural mutation in the CEN homolog SELF PRUNING (Pnuel et al., 1998), revolutionized production and industrial processing of tomato.

Studies in diploid kiwifruit A. chinensis revealed three FT genes and five CEN-like genes, with overexpression of FT genes resulting in extreme precocity, repression of vegetative growth and development of poorly differentiated in vitro flowers; in contrast, overexpression of all Actinidia CEN-like genes delayed flowering in Arabidopsis and overexpression of AcCEN fully repressed flowering in Actinidia eriantha (Moss et al., 2018; Varkonyi-Gasic et al., 2013; Voogd et al., 2017). We hypothesized that the multiple CEN homologs evolved to control the balance of vegetative growth and flowering in kiwifruit, to regulate the first onset of flowering, and maturity and flowering time. For the selection of TFL1-like genes as prime candidates for regulation of kiwifruit architecture and flowering time and generate plants amenable for breeding. These approaches have often proven to be unreliable (Zhang et al., 2010) or failed to promote flowering in fruit crops, affecting other aspects of development instead (Freeman et al., 2015; Varkonyi-Gasic et al., 2013). In contrast, stable mutations in these genes have underpinned domestication of crops such as legumes, sugar beet, sunflower and strawberry (Blackman et al., 2010; Iwata et al., 2012; Kwak et al., 2012; Liu et al., 2010; Pin et al., 2010), or in case of a natural mutation in the CEN homolog SELF PRUNING (Pnuel et al., 1998), revolutionized production and industrial processing of tomato.

Studies in diploid kiwifruit A. chinensis revealed three FT genes and five CEN-like genes, with overexpression of FT genes resulting in extreme precocity, repression of vegetative growth and development of poorly differentiated in vitro flowers; in contrast, overexpression of all Actinidia CEN-like genes delayed flowering in Arabidopsis and overexpression of AcCEN fully repressed flowering in Actinidia eriantha (Moss et al., 2018; Varkonyi-Gasic et al., 2013; Voogd et al., 2017). We hypothesized that the multiple CEN homologs evolved to control the balance of vegetative growth and flowering in kiwifruit, to regulate the first onset of flowering, and terminal and axillary meristem identity. Genome editing using the CRISPR (clustered regularly interspaced short palindromic repeat)-associated endonuclease Cas9 has proven a powerful and efficient tool for targeted mutagenesis, with the potential for editing multiple genomic loci and generating a range of mutations, affecting one or more alleles and gene family members (reviewed in (Komor et al., 2017). In this study, we utilized this approach to simultaneously target two kiwifruit CEN-like genes AcCEN4 and AcCEN. We demonstrate the role of these two genes in maturity, architecture and flowering time and generate plants amenable for rapid breeding cycles, urban farming and production as annuals. This study reveals the potential of fast-tracked targeted improvement of a large, long-lived woody perennial, with implications in the domestication and rapid improvement of non-cultivated Actinidia and other long-lived perennials, expanding horticultural crop diversity, and increasing sustainability of fruit production and food security.

**Results**

**Identification of kiwifruit CEN-like gene targets**

Kiwifruit has multiple CEN-like genes which can act as conserved floral repressors (Voogd et al., 2017). Relatively high expression of AcCEN4 and AcCEN in actively growing shoot tips and in latent axillary buds, respectively (Figure 1a), identified these two kiwi- fruit CEN-like genes as prime candidates for regulation of kiwifruit architecture, maturity and flowering time. For the selection of CRISPR/Cas9 target sequences, alignment of A. chinensis CEN gene family cDNA sequences was used to identify homologous regions between AcCEN4 and AcCEN. Two target sequences designated E1 and E4, in exon 1 and exon 4 of AcCEN4, respectively, were chosen based on full or almost full sequence identity between AcCEN and AcCEN4, but insufficient homology for targeting in the related PEBP genes (Figure S1). Two constructs which contained target sequences E1 and E4, each fused to generate target-specific sgRNA sequences, were placed either under the control of Arabidopsis U6-26 and U6-29 promoters (U6-CEN4), or Arabidopsis U3-b and U3-d promoters (U3-CEN4), and introduced into a binary vector between the cauliflower mosaic virus (CaMV) 35S promoter-driven gene for kanamycin resistance and the Cas9 gene driven by the parsley Ubiquitin promoter (Figure 1b–d, Figure S1). A construct containing the conserved sequence of Actinidia FT1 gene driven by the CaMV 35S promoter (35S:AcFT1) was used to compare the effect of ectopic overexpression of a strong activator of flowering and mutagenesis of CEN-like repressors of flowering.

**Differential effects of ectopic overexpression of Actinidia FT1 and mutagenesis of CEN-like genes on growth and flowering**

The U6-CEN4, U3-CEN4 and 35S:AcFT1 constructs were introduced into kiwifruit A. chinensis using standard regeneration and transformation protocols and kanamycin selection (Wang et al., 2007). This resulted in adventitious buds initiating 8 weeks post Agrobacterium co-cultivation, giving rise to undifferentiated in vitro flowers with 35S:AcFT1 construct (Figure 2a). In contrast, all U6-CEN4 and U3-CEN4 lines demonstrated normal leaf development during in vitro growth (Figure 2b), giving rise to kanamycin-resistant lines. PCR amplification and sequencing of AcCEN4 gene fragments identified potential mutations in 75% (6/8) T0 U6-CEN4 lines chosen for initial analysis (Figure S2) and a total of 25 lines were maintained for phenotypic evaluation. Possible mutations were also identified in 30% (3/10) U3- CEN4 lines. Three U6-CEN4 lines (lines 14, 17 and 18) developed a single terminal flower 8–9 months after co-cultivation with Agrobacterium, when plants had a minimum of six expanding leaves and established roots (Figure 2c). The flowers showed a mostly normal morphology, apart from a subtending leaf-like bract and a leaf-like appearance of one or two of the sepals (Figure 2d). These vegetative features of terminal flowers suggested an abrupt termination of vegetative growth, indicating a potential absence of an FT activator of flowering during the early stage of in vitro growth. To test this, expression analysis of tissue culture-grown wild-type A. chinensis was performed. Little or no expression of AcFT was detected in the early stage after regeneration, but the expression increased with shoot differentiation and appearance of the AcCEN4 transcripts, whereas AcFT1 was detected both in the early and later stage and AcFT2 was absent (Figure 2e).

**Growth habit and reproductive development in precocious lines**

When transplanted to soil, lines 14, 17 and 18 demonstrated a compact growth habit and terminal flowering after development of the minimum of six leaves (Figure 3a). Precocious flowering was further observed in U6-CEN4 line 7, 12 months after co-cultivation with Agrobacterium, with a terminal flower developing on a vine with 12 expanding leaves (Figure 3b,c). The morphology of the terminal flowers on all lines was similar, regardless of their precocity and growth habit. The number and appearance of floral organs was normal, with the exception of the subtending leaf and leaf-like sepals (Figure 3d–g). The flowers were marginally smaller but developed a multi-carpellate gynoe- cium comparable to wild-type. Pollination using A. chinensis male pollen resulted in fruit set and development (Figure 3h,i), except...
in line 14, which failed to set fruit after multiple pollination attempts. Two fruit each from lines 17 and 18 and one from line 7 were harvested to evaluate whether they produced functional seed. All harvested fruit contained >100 seed, which were able to germinate (Figure 3).

Induced mutations in early flowering lines

The initial PCR screen (Figure S2) identified mutations in AcCEN4 gene in 75% and 30% of U6-CEN4 and U3-CEN4 T0 lines, respectively, but only four U6-CEN4 T0 lines flowered early, three of which were extremely precocious and had a very compact growth habit (Figure 4a). To estimate the type and severity of mutations and the number of affected alleles, PCR amplification products were cloned and at least four clones were subjected to sequence analysis. Such genotyping identified mutations comprising small deletions or insertions in AcCEN4 and AcCEN alleles in one or both of the E1 and E4 sites, at positions indicative of sgRNA-directed Cas9 cleavage 3-nt upstream of the protoscaler adjacent motif (PAM) site (Figure 4b, Figures S2 and S3). A large deletion resulting from sgRNA-directed Cas9 cleavage at both E1 and E4 regions in AcCEN4 (E1-E4 deletion) was detected in all compact early flowering lines (lines 14, 17 and 18), giving rise to a truncated gene in which the fragments of exon 1 and exon 4 were brought together without an introduction of a frame-shift. In addition to this mutation, all compact lines had frame-shifts in E1 and/or E4 regions of AcCEN4. Mutations were also identified in AcCEN alleles. In the most severely affected line 14, a large insertion of 156 nt in the last exon was identified in all AcCEN sequences, sharing homology to 26S rRNA. In line 18, frame shift mutations were identified in all AcCEN sequences in either both E1 and E4 sites or just E4 sites, but both wild-type and mutant sequences were identified line 17, with a frame-shift in AcCEN exon 1. In line 7, a frame shift in exon 1 was seen in all AcCEN sequences and only in the last exon of some AcCEN sequences. A combination of mutant and wild-type sequences in AcCEN4 and AcCEN resulted in no visible alteration of growth and flowering time (Lines 19 and 22, Figure 4) and no mutations in related but very lowly expressed AcCEN3 were detected in early flowering lines (Figure S2).

Different mutant sequences or a combination of wild-type and mutant sequences identified in T0 lines suggested mutations occurring in single alleles, or genetic mosaics regenreated from cells of different genotypes in the same callus. Alternatively, additional mutagenesis was occurring through re-editing of the target gene, because of the stable integration of Cas9 gene and the sgRNA construct. The mosaicism or instability of mutations could affect phenotypes of plants multiplied by vegetative propagation, a common practice with woody perennials. To test if the appearance of plants remain stable with clonal propagation, one line (line 18) was re-established in tissue culture, and eight new sublines were generated from adventitious buds regenerated from excised leaf tissue or axillary shoot tissue. A uniform compact size was seen in all clonally propagated lines and all lines flowered and were capable of bearing fruit after developing 4-6 leaves, demonstrating that vegetative multiplication gives rise to comparable precocious plants (Figure 4c).

Bi-allelic mutation of either AcCEN4 or AcCEN may be sufficient for early flowering

The genotyping of lines 17 and 7 suggested that bi-allelic mutagenesis of AcCEN4 may be sufficient for early flowering. In line 7, a combination of wild-type sequences and a frame shift affecting only the C-terminal region of predicted AcCEN were detected (Figure 4b, Figure S3). Mutations in AcCEN potentially contributed to precocity and short plant stature seen in compact lines 14, 17 and 18, indicating that mutation of AcCEN may also result in early flowering. To address this hypothesis, additional gene-specific target sequences were identified (Figure 5a–d).
Two polycistronic tRNA-sgRNA (PTG) cassettes, each with four sgRNA sequences preferentially targeting AcCEN4 (PTG-CEN4) or AcCEN (PTG-CEN), were placed under the control of Arabidopsis U6-26 promoter, introduced into a binary vector between the 35S promoter-driven Cas9 gene and the gene for kanamycin resistance (Figure 5e) and transformed into kiwifruit A. chinensis. More than 30 lines were generated for each construct and grown in tissue culture, and 20 lines per construct were transplanted to soil and maintained for phenotypic evaluation. Precocious flowering was observed in a PTG-CEN4 and a PTG-CEN line on vines with 15 leaves, giving rise to fruit after pollination (Figure 5f). Genotyping of these lines identified small deletions or insertions in target sites, resulting in frame-shifts in AcCEN4 alleles in the precocious PTG-CEN4 line and frame-shifts in AcCEN alleles in the precocious PTG-CEN line (Figure 5g, Figure S3). Therefore, mutagenesis of both alleles of either of the genes resulted in a determinate phenotype with early flowering, yet not as early as seen with mutations in both genes.

Altered architecture, continuous flowering and dormancy

In addition to premature flowering, the lines exhibited pleiotropic phenotypes including altered architecture and dormancy requirement. The developing terminal flower or fruit on compact early flowering lines was sufficient to prevent axillary bud outgrowth (Figure 6a), but their senescence or removal resulted in outgrowth of axillary shoots with terminal flowers, enabling simultaneous flower and fruit development on the stunted, bushy plant (Figure 6b). The U6-CEN line 7 and the precocious PTG lines were capable of developing axillary flowers in addition to the terminal flower (Figures 5f and 6c), giving rise to multiple fruit on a single shoot (Figure 6d). In these lines, shortening of day length induced bud set and leaf senescence comparable to control plants, whereas the compact plants remained insensitive to changes in day length and did not enter short-day induced dormancy. Instead, compact lines 14, 17 and 18 remained green and continued to develop new shoots with terminal flowers (Figure 6d, e). Later development in initially compact and vine lines was similar, with repeated cycles of lateral branch extension and flowering. The newly emerging lateral branches arising from vegetative buds at the base of the trunk bore longer internodes and commonly developed axillary flowers, producing multiple fruit after pollination (Figure 6f). Hard pruning resulted in emergence of new short shoots with terminal flowers (Figure 6g), followed by repeated cycles of lateral branch growth and flowering, ensuring continual production.

Analysis of the heterozygous T1 lines

Because of the dioecious nature of Actinidia, pollination was performed using pollen from a wild-type male diploid A. chinensis, carrying the wild-type alleles of AcCEN4 and AcCEN and giving rise to heterozygous progeny. To determine whether the early flowering trait could be efficiently transferred to the next generation through editing in the Cas9-sgRNA background after fertilization, seeds from a fruit of line 17 were germinated. A total of 80 germinated seedlings were scored for flowering for 4 months following germination, at which stage they produced between six and 15 leaves, reaching the flowering stage of compact and vine-like lines respectively. None of the lines flowered at this stage (Figure 7a), despite of the presence of Cas9 and the AcCEN4 E1-E4 deletion in the genome (Figure 7b). Therefore, the progeny in an outcrossing perennial kiwifruit remains largely heterozygous and flowers late when U6-CEN4 construct is used.

Discussion

AcCEN4 and AcCEN regulate the life cycle, height and architecture of kiwifruit

Previously, five kiwifruit CEN-like genes have been identified (Voogd et al., 2017). This gene duplication and divergence would
be expected to contribute to the regulation of kiwifruit architecture and flowering, but it has been highly problematic to differentiate their endogenous roles, functionally distinguish paralogous genes and generate mutations specifically affecting one or more alleles and members of this gene family. Genome editing can now circumvent some of these problems. Here, we combined the potential for editing single and multiple genomic loci by CRISPR/Cas9 and the ability to transform the diploid Actinidia chinensis, to address the hypothesis that AcCEN4 and AcCEN regulate the flowering time, life cycle and architecture of kiwifruit.

The number of affected genes and alleles generally correlated with the precocity and change in plant stature. Mutagenesis of both alleles of either AcCEN4 or AcCEN gave rise to a determinate phenotype with early flowering, whereas mutations in both genes resulted in even earlier flowering, resembling the highly compact habit and very early yields in double-determinate tomato, a consequence of mutations in two PEBP genes with flower-repressing activities (Fridman et al., 2002; Soyk et al., 2016). The type of mutations and position of frame shifts were likely to impact on the phenotypes. Small changes in FT and TFL1/CEN-like sequences can reverse their activating or repressing roles in regulation of flowering (Ahn et al., 2006; Ho and Weigel, 2014) and can interfere with flower-activating capacity of other members of the PEBP family (Blackman et al., 2010; Pin et al., 2010). All compact lines had the AcCEN4 E1-E4 deletion and a large insertion was seen in AcCEN that may have rendered line 14 infertile, although it produced terminal flowers indistinguishable from the fertile terminal flowers developing on other early flowering lines. Architecture ranging from compact plants to

Figure 3 Accelerated flowering and normal reproductive development in Actinidia chinensis transgenic lines. (a) Terminal flower bud (white arrow) on U6-CEN4 line 14 (right), compared to the vegetative terminal bud (black arrowhead) and axillary flower bud (black arrow) on an expanding shoot excised from a wild-type (WT) A. chinensis plant (left). The age of the plant at first flowering is indicated; Y, year; M, month. (b, c) Appearance of U6-CEN4 line 7 and a control 35S:GUS transgenic A. chinensis. The inserts show close-up images of a terminal flower bud and a vegetative shoot tip in line 7 and 35S:GUS control respectively. (d, e) Normal appearance of the gynecium. (f, g) The subtending leaves (arrows) and leaf-like sepals (arrowheads) on terminal flowers. (h) Developing fruit (30 days after pollination). (i) Mature fruit (120 days after pollination). (j) Seed germination.
vines is most likely the outcome of the rate of termination. The very early flowering lines were stunted, although more vigorous growth and internode expansion was seen in lateral shoots emerging later, giving rise to bushy plants with terminal and axillary flowers. The altered response to dormancy-inducing conditions was prominent in very early flowering lines, which

| U6-CEN4#14* | E1 | E4 | wt | insertion (+156) | cen4cen4/cencen |
|--------------|----|----|----|-----------------|----------------|
| U6-CEN4#18** | E1-E4 del | indel | wt | indel | cen4cen4/cencen |
| U6-CEN4#17** | indel | indel | wt | indel | cen4cen4/CENcen |
| U6-CEN4#7*** | indel | wt | wt | wt | cen4cen4/CENCEN |
| U6-CEN4#22**** | wt/indel | wt | wt | wt | CEN4cen1/CENCEN |
| U6-CEN4#19**** | wt/indel | wt/indel | wt | wt | CEN4/CEN_ |

Figure 4 CRISPR/Cas9-mediated editing of AcCEN4 and AcCEN genes. (a) The height and architecture of U6-CEN4 plants. Arrows and arrowheads indicate fruit and flowers respectively. The insert shows a close-up image of the vegetative shoot tip in line 22. The asterisks denote the phenotypes: *, compact plant, early flowering, no fruit set; **, compact plant, early flowering; ***, vine habit, early flowering; ****, vine habit, no flowering. (b) Mutations identified in E1 and E4 sites in AcCEN4 and AcCEN alleles. (c) Rapid flowering and fruit development in U6-CEN4#18 lines after re-establishment and propagation in tissue culture.
remained green and continued to produce new shoots, with a brief pause in growth during fruit development. These phenotypes, particularly the altered architecture with increased lateral branching and insensitivity to environmental conditions initiating bud-set and dormancy, were in common with trees overexpressing FT genes (Endo et al., 2005; Klocko et al., 2016; Srinivasan et al., 2012), demonstrating common outcomes of altered FT/CEN balances. While ectopic overexpression of kiwifruit FT genes gave rise to in vitro flowers (Moss et al., 2018; Voogd et al., 2017), the terminating effect of mutations in AcCEN4 and AcCEN became evident in the stage when the flowering activator AcFT expression was detectable, suggesting that the mutations affecting CEN genes are only relevant in the presence of a functional FT, as previously emphasized in tomato (Lischitz et al., 2014).

The shoot character of terminal flowers was indicative of an abrupt arrest in growth and insufficient activation of floral fate. It is possible that mutations in CEN-like genes may not fully activate floral meristem identity genes, particularly if an FT activator of flowering is not sufficiently expressed or perhaps not translated before the vascular connections and sink-source relationship are firmly established. In contrast, the axillary flowers were bractless (no subtending leaf) and with normal sepal, suggesting a fully acquired floral fate, despite their unusual position in the upper leaf axis. In wild-type A. chinensis these positions are occupied by latent buds, which have the capacity to develop into inflorescence-bearing shoots in the following spring, after sufficient winter chilling is perceived during winter dormancy (Walton et al., 1997). Development of single flowers in these positions suggests that mutations in CEN-like genes drive determinacy and offset the need for dormancy and winter chilling in axillary meristems, and the fully acquired floral fate in these flowers imply that additional mechanisms may contribute to activate floral meristem identity genes.

**Engineering determinacy, altered architecture and rapid flowering for accelerated breeding and urban farming**

The ability to convert perennial crops into annual production and grow plants in confined spaces is becoming increasingly important with predicted effects of climate change and population growth. Indoor farming is being adopted for annual production and grow plants in confined spaces is becoming increasingly important with predicted effects of climate change and population growth. Indoor farming is being adopted for annual production and growth for accelerated breeding and urban farming.
Figure 6 Pleiotropic phenotypes of precocious kiwifruit. (a) Single terminal fruit on a compact plant (arrow). (b) Simultaneous flower (arrowhead) and fruit (arrows) development on a compact plant. (c) Vine growth habit with terminal (arrow) and axillary (arrowheads) flower development on line 7. The insert shows the normal sepals on an axillary flower. (d, e) Differential responses to dormancy-inducing conditions. Developing fruit and the short shoot with terminal flower bud are indicated by arrows and an arrowhead respectively. (f) Internode elongation and multiple fruit developing in terminal and axillary positions on lateral shoots emerging from the base of the trunk. (g) Basal shoots with terminal flowers (arrowheads) emerging after hard pruning (arrow denotes where the shoot was removed).
The late flowering in the heterozygous progeny suggests no or very low frequency of heritable gene targeting in kiwifruit, at least when using the U6-CEN4 editing construct, in which the Arabidopsis U6 and the parsley Ubiquitin promoters drive the expression of sgRNAs and Cas9 respectively. A different set of promoters better suited for specific expression in Actinidia may facilitate genome editing after pollination and increase the heritability of the early flowering trait. In Arabidopsis, the expression of Cas9 from germline-specific promoters increased the efficiency of CRISPR/Cas9-mediated gene editing and the frequency of heritable gene targeting (Miki et al., 2018; Yanfei et al., 2016), hence a similar approach could be used for kiwifruit. Alternatively, generation of early flowering male and female parents using CRISPR/Cas9 mutagenesis of CEN genes could overcome the prolonged T1 generation time in perennials that require cross-pollination to produce fruit, typical of kiwifruit and many other horticultural crops.

Kiwifruit domestication started in the early 20th century. Increasingly popular, well known for high nutritional value and as a rich source of healthy compounds (Ampomah-Dwamena et al., 2009; Bulley and Laing, 2016; Montefiori et al., 2005; Park et al., 2013), kiwifruit has a tremendous potential for further improvement. The approach developed in this study shows a promising pathway to quickly generate new varieties, either by accelerated breeding with rapid flowering parents or targeted gene editing in already available selections. The plants generated in this study can be used as model plants in furthering detailed understanding of mechanisms and processes in kiwifruit, including fruit development and plant-pathogen studies. In addition, CRISPR/Cas9-mediated mutagenesis of AcCEN4 and AcCEN homologs paves the way to domesticate and improve non-cultivated Actinidia.

In conclusion, editing of Actinidia CEN4 and CEN genes converts kiwifruit into a compact plant with determinate growth habit and terminal flowering, amenable to growth in confined spaces, and providing the opportunity to cultivate kiwifruit as an annual crop. The fast life cycle holds the potential to dramatically accelerate targeted breeding, whereas optimization of growing conditions for faster growth and indoor farming could increase productivity and enable cropping in any geographical and climatic conditions. This approach could be a useful tool to accelerate the development of new varieties across the horticultural sector, whereas the precise adjustment of plant architecture offers possibilities for changes in the way in which new varieties are bred and cultivated.

Materials and methods

Plant material

Plant material from a female kiwifruit cultivar ‘Hort16A’ (A. chinensis Planch. var. chinensis) was used for transformation and expression studies. The media were described before (Wang et al., 2007). Pollination of flowers was performed using pollen collected from male diploid A. chinensis. The male plants were container-raised in a containment greenhouse at Plant & Food Research, Auckland, New Zealand. The pollen was collected in spring 2016 and stored in the freezer for 1 year.

Expression analysis

The RNaseq experiment was described before (Voogd et al., 2017). Briefly, A. chinensis axillary and terminal buds were collected at regular intervals over the season, subjected to RNA-seq, and uniquely mapped reads with no mismatches per paired
alignment were used to determine gene expression. The data were interrogated for expression of *Actinidia CEN*-like genes in axillary and terminal buds during active growth and presented as average fragments per kilobase of transcript per million reads (FPKM) ± SE of three (axillary buds) and two (terminal buds) biological replicates.

For RT-qPCR studies, total RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich). An aliquot of 1 μg RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen) and quantification using real-time PCR were performed with the FastStart DNA Master SYBR Green I mix (Roche Diagnostics) using the LightCycler 1.5 instrument and the LightCycler Software version 4 (Roche Diagnostics). Amplification was carried out using a 10⁻³ dilution of the cDNA template, with an initial denaturing step at 95 °C for 5 min, then 40 cycles of 95 °C for 5 s, 60 °C for 5 s, and 72 °C for 10 s. A non-template control was included in each run. Oligonucleotide primers (Table S1) were designed to produce amplification products of 100–150 nucleotides. The specificity of primer pairs was confirmed by melting curve analysis of PCR products and agarose gel electrophoresis followed by sequence analysis. The expression was normalized to kiwifruit ACTIN (GenBank accession FG403300) and presented as a mean ± SE of three biological replicates.

Target identification and vector construction

*Actinidia chinensis* PEBP gene family coding region sequences were aligned using Geneious ClustalW Alignment (Geneious, Biomatters Ltd, version 8.1.2) (www.geneious.com). Two target sequences designated E1 and E4, in exon 1 and exon 4 of ActCEN4, respectively, were chosen based on full or almost full sequence identity between *AcCEN* and *ActCEN4*, but insufficient homology for targeting in the related PEBP genes (Figure S1). BlastN alignment using the *A. chinensis* genome (Huang et al., 2013; Pilkington et al., 2018) was used to confirm absence of other highly homologous sequences, minimizing the potential for off-target editing. Two constructs were designed which contained target sequences E1 and E4, each fused to generate target-specific sgRNA sequences, and placed either under the control of *Arabidopsis* U6-26 and U6-29 promoters, or *Arabidopsis* U3-b and U3-d promoters. When required, additional nucleotides were added to ensure correct initiation of target sequence transcription from U6 and U3 promoters (Figure S1). Gateway recombination sites were added on each end and the resulting 1150 nt and 868 nt DNA fragments designated U6-CEN4 and U3-CEN4, respectively, were synthesized (Geneviz, South Plainfield, NJ; www.geneviz.com). The synthetic DNA was recombined between the CaMV 35S promoter-driven gene for kanamycin resistance and the Cas9 gene driven by the parsley Ubiquitin promoter, in vector pDE-Cas9(KanR) modified from pDE-Cas9 by replacing the HindIII fragment carrying the plant selection cassette with that from pDE-Cas9(D10A) (Fauser et al., 2014). Specific targeting of *AcCEN4* and *ActCEN* genes was achieved using the tandemly arrayed trnaRNA-gRNA structure described by Xie et al. (2015), using an optimized sgRNA design (Dang et al., 2015) and target selection criteria based on Doench et al. (2014). The constructs containing the polycistronic trnaRNA-sgRNA cassette placed downstream of the *Arabidopsis thaliana* U6-26 promoter, with Gateway recombination sites added on each end, were synthesized (Geneviz) and recombined with the destination vector pDE-KRS, derived from pHEX2, which expresses Cas9 from the 35S promoter and contains the plant kanamycin resistance gene. The 35S:AcFT1 construct and the 35S:GUS vector control were described previously (Vooogd et al., 2017). All resulting plasmids were transformed into Agrobacterium tumefaciens strain EHA105 by electroporation.

Plant transformation and growth

*Agrobacterium tumefaciens* mediated transformation of *A. chinensis* was performed as previously described (Wang et al., 2007). Briefly, leaf strips excised from in vitro-grown shoots were co-cultivated with *Agrobacterium* suspension culture and transferred to regeneration and selection medium. Individual calli were excised from the leaf strips for further selection and bud induction, and adventitious buds regenerated from the calli were excised and transferred to shoot elongation medium. When shoots had grown to 1–2 cm high, they were transplanted onto rooting medium. Rooted transgenic plants were then potted and grown in a containment greenhouse at ambient conditions (temperature min 18 °C/ max 30 °C night/day, 14 h/10 h light/dark in summer, with gradual day length shortening to 12 h/12 h light/dark at the beginning of autumn). For clonal propagation, leaf tissue was surface-sterilized and allowed to regenerate and develop roots as described, then grown in a containment greenhouse as described.

Seed germination

Seed was collected from immature fruit 60 days after pollination or mature fruit 120 days after pollination. The fruit was surface-sterilized by soaking in 1.5% (v/v) sodium hypochlorite and 0.1% (v/v) Tween 20 for 15 min before seed extraction, after which the seed was further sterilized for 10 min. Seeds were germinated on a medium supplemented with coconut milk and casein hydrolysate (Table S2), before transplanting into soil for growth in the glasshouse (18 °C night, 28 °C day) at 18 h light/6 h dark cycles.

Genotyping

Genomic DNA was extracted from kanamycin resistant kiwifruit explants and glasshouse-grown plants using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer’s instructions. For fast evaluation of lines for editing events, PCR amplification was performed using the iProof High-Fidelity DNA Polymerase (Bio-Rad) using gene-specific oligonucleotide primers (Table S1), with an initial denaturing step at 95 °C for 5 min, then 35 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 1 min. Amplification products were purified using the DNA Clean & Concentrator-5 kit (Zymo Research) and sequenced using gene-specific oligonucleotide primers. For subsequent verification, amplification products were cloned into pGEM-T Easy (Promega) and at least four clones of each line were subjected to sequence analysis. Oligonucleotide primer synthesis and sequencing were performed by Macrogen (www.macrogen.com).

Acknowledgements

We thank Monica Dragulescu for maintenance of transgenic plants, Kui Lin-Wang and Sakuntala Karunairetnam for assistance with vector design, Cara Norling and Keith Funnel for help with seed germination, Geeta Chhiba for media preparation, Dinum Herath for assistance with DNA extraction and genotyping of T1 lines and Anne Gunson and Zac Hanley for critically reading the manuscript. The pDE-Cas9 vectors were kindly provided by Holger Puchtia and Simon Schiml. This work was funded by the New Zealand Foundation for Research, Science and Technology (contract C13X0806) and the New Zealand Foundation for Research, Science and Technology (contract C13X0806).
Doench, J.G., Hartenian, E., Graham, D.B., Tothova, Z., Hegde, M., Smith, I., Endo, T., Shimada, T., Fujii, H., Kobayashi, Y., Araki, T. and Omura, M. (2005) Actinidia

Dang, Y., Jia, G., Choi, J., Ma, H., Anaya, E., Ye, C., Shankar, P.

Bradley, D., Carpenter, R., Copsey, L., Vincent, C., Rothstein, S. and Coen, E.

Bena, G., Lejeune, B., Prosperi, J.M. and Olivieri, I. (1998) Molecular

Fauser, F., Schiml, S. and Puchta, H. (2014) Both CRISPR/Cas-based nucleases

Freiman, A., Golobovitch, S., Yablovitz, Z., Belausov, E., Dahan, Y., Peer, R., Aavrham, L. et al. (2015) Expression of FLOWERING LOCUS T2 transgene from Pyrus communis L. delays dormancy and leaf senescence in Malus x domestica Borkh, and causes early flowering in tobacco. Plant Sci 241, 164–176.

Fridman, E., Liu, Y., Carmel-Goren, L., Gur, A., Shores, M., Peban, T., Eshed, Y. et al. (2002) Two tightly linked QTLs modify tomato sugar content via different physiological pathways. Mol Genet Genomics 266, 821–826.

Ho, W.W.H. and Weigel, D. (2014) Structural features determining flower-promoting activity of Arabidopsis FLOWERING LOCUS T. Plant Cell 26, 552–564.

Hu, F.Y., Tao, D.Y., Sacks, E., Fu, B.Y., Xu, P., Li, J., Yang, Y. et al. (2003) Convergent evolution of perennility in rice and sorghum. Proc Natl Acad Sci USA 100, 4050-4054.

Huang, S., Ding, J., Deng, D., Tang, W., Sun, H., Liu, D., Zhang, L. et al. (2013) Draft genome of the kiwifruit Actinidia chinensis. Nat Commun 4, 2640.

Iwata, H., Gaston, A., Remay, A., Thouroude, T., Jeafure, J., Kawamura, K., Oyant, L.H.-S. et al. (2012) The TFL1 homologue KSN is a regulator of continuous flowering in rose and strawberry. Plant J 69, 116-125.

Klocko, A.L., Ma, C., Robertson, S., Esfandiar, E., Nilsson, O. and Strauss, S.H. (2016) FT overexpression induces precocious flowering and normal reproductive development in Eucalyptus. Plant Biotechnol J 14, 808-819.

Komer, A.C., Badran, A.H. and Liu, D.R. (2017) CRISPR-based technologies for the manipulation of eukaryotic genomes. Cell 168, 20–36.

Kotoda, N., Iwanami, H., Takahashi, S. and Abe, K. (2006) Antisense Expression of MdFTL1, a TFL1-like Gene, Reduces the Juvenile Phase in Apple. J Am Soc Hortic Sci 131, 74-81.

Kwak, M., Toro, O., Debouck, D.G. and Gepts, P. (2012) Multiple origins of the determinate growth habit in domesticated common bean (Phaseolus vulgaris). Ann Bot 110, 1573–1580.

Lifschitz, E., Ayre, B.G. and Eshed, Y. (2014) Florigen and anti-florigen – a systemic mechanism for coordinating growth and termination in flowering plants. Front Plant Sci 5, 465.

Liu, B., Watanabe, S., Uchiyama, T., Kong, F., Kanazawa, A., Xia, Z., Nagamatsu, A. et al. (2010) The soybean stem growth habit gene Dt1 is an Ortholog of Arabidopsis TERMINAL FLOWER1. Plant Physiol 153, 198–210.

Melzer, S. (2008) Flowering-time genes modulate meristem determinacy and growth form in Arabidopsis thaliana. Nat Genet 40, 1489-1492.

Miki, D., Zhang, W., Zeng, W., Feng, Z. and Zhu, J.-K. (2018) CRISPR/Cas9-mediated gene targeting in Arabidopsis using sequential transformation. Nat Commun 9, 1967.

Montefiori, M., McGhee, T.K., Costa, G. and Ferguson, A.R. (2005) Pigments in the fruit of red-fleshed kiwifruit (Actinidia chinensis and Actinidia delicosa). J Agric Food Chem 53, 9526–9530.

Moiss, S.M., Wang, T., Voogd, C., Brian, L.A., Wu, R., Hellens, R.P. and Varsak-Gasic, E. (2018) AcFt promotes kiwifruit in vitro flowering when overexpressed and Arabidopsis flowering when expressed in the vasculature under its own promoter. Plant Direct 2, e00068.

Park, Y.S., Im, M.H., Ham, K.S., Kang, S.G., Park, Y.-K., Namiesnik, J., Leonotivcz, H. et al. (2013) Nutritional and pharmaceutical properties of bioactive compounds in organic and conventional growing kiwifruit. Plant Foods Hum Nutr 68, 57–64.

Pilkington, S.M., Cowhurst, R., Hilario, E., Nardozza, S., Fraser, L., Peng, Y., Gunaseelan, K. et al. (2018) A manually annotated Actinidia chinensis var. chinensis (kiwifruit) genome highlights the challenges associated with draft genomes and gene prediction in plants. BMC Genom 19, 257.

Pin, P.A., Benilch, R., Bonnet, D., Wremeth–Weisch, E., Kraft, T., Gielen, J.L.J. and Nilsson, O. (2010) An Antagonistic Pair of FT Homologs Mediates the Control of Flowering Time in Sugar Beet. Science 330, 1397–1400.

Pruell, L., Carmel-Goren, L., Hareven, D., Guttinger, T., Alvarez, J., Ganal, M., Zamir, D. et al. (1998) THE SELF-PRUNING gene of tomato regulates vegetative to reproductive switching of sympodial meristems and is the ortholog of CEN and TFL1. Development 125, 1979–1989.

Scortichini, M., Marcelletti, S., Ferrante, P., Petriccione, M. and Firrao, G. (2012) Pseudomonas syringae pv. actinidiae: a re-emerging, multi-faceted, pandemic pathogen. Mol Pathol 13, 631–640.

Soyk, S., Müller, N.A., Park, S.J., Schmalenbach, I., Jiang, K., Hayama, R., Zhang, L. et al. (2016) Variation in the flowering gene SELF PRUNING 5G promotes day-neutrality and early yield in tomato. Nat Genet 49, 162.
Srinivasan, C., Dardick, C., Callahan, A. and Scorza, R. (2012) Plum (Prunus domestica) trees transformed with poplar FTI result in altered architecture, dormancy requirement, and continuous flowering. PLoS ONE 7, e40715.

Tank, D.C. and Olmstead, R.G. (2008) From annuals to perennials: phylogeny of subtribe Castillejinae (Orobanchaceae). Am J Bot 95, 608–625.

Thomas, H., Thomas, H.M. and Ougham, H. (2000) Annularity, perenniality and cell death. J Exp Bot 51, 1781–1788.

Varkonyi-Gasic, E., Moss, S.M.A., Voogd, C., Wang, T., Putterill, J. and Hellens, R.P. (2015) Homologs of FT, CEN and FD respond to developmental and environmental signals affecting growth and flowering in the perennial vine kiwifruit. New Phytol 196, 732–746.

Vanksluis, S., Flachowsky, H. and Hanke, M.-V. (2013) The Fast-track breeding approach can be improved by heat-induced expression of the FLOWERING LOCUS T gene as a research and breeding tool in Populus. J Exp Bot 61, 2549–2560.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Selection of CRISPR/Cas9 target sequences and construct design. (a) Alignment of the corresponding target E1 and E4 regions in AcCEN1, AcCEN4 and other Actinidia chinensis CEN, BROTHER OF FT (BFT), MOTHER OF FT (MFT) and FT genes. PAM sequence is in red and mismatches are highlighted in yellow. (b) The sequence of constructs used for CRISPR/Cas9-mediated editing of AcCEN4 and AcCEN genes. Green, gene-specific target sequence (protospacer); red, guide RNA (crRNA, linker loop and tracrRNA); blue, end of the promoter sequence; underlined, +1 position (appropriate nucleotide added where necessary, G and A for pU6 and pU3 respectively).

Figure S2 Evaluation of CRISPR/Cas9-mediated gene editing in transgenic kiwifruit. (a) Example of amplification using gene-specific primers, which identified the E1-E4 deletion in AcCEN4 (a), PTG-CEN4 (b) and PTG-CEN (c) lines. At least four clones of gene-specific amplification products were subjected to sequence analysis. wt, wild-type; +, insertion; −, deletion.

Table S1 Oligonucleotide primer sequences used in this study.

Table S2 Medium for seed germination.