The Delayed Terminal Flower Phenotype Is Caused by a Conditional Mutation in the CENTRORADIALIS Gene of Snapdragon

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The snapdragon (Antirrhinum majus) centroradialis mutant (cen) is characterized by the development of a terminal flower, thereby replacing the normally open inflorescence by a closed inflorescence. In contrast to its Arabidopsis counterpart, terminal flower1, the cen-null mutant displays an almost constant number of lateral flowers below the terminal flower. Some partial revertants of an X-radiation-induced cen mutant showed a delayed formation of the terminal flower, resulting in a variable number of lateral flowers. The number of lateral flowers formed was shown to be environmentally controlled, with the fewer flowers formed under the stronger flower-inducing conditions. Plants displaying this “Delayed terminal flower” phenotype were found to be heterozygous for a mutant allele carrying a transposon in the coding region and an allele from which the transposon excised, leaving behind a 3-bp duplication as footprint. As a consequence, an iso-leucine is inserted between Asp148 and Gly149 in the CENTRORADIALIS protein. It is proposed that this mutation results in a low level of functional CEN activity, generating a phenotype that is more similar to the Arabidopsis Terminal flower phenotype.

Regarding the influence of the PEBP proteins on inflorescence structure, the Cen and Tfl1 mutants do not show fully identical phenotypes. In snapdragon, the CEN gene seems to control only the indeterminate fate of the inflorescence, whereas in Arabidopsis TFL1 has an additional effect on flowering time. Furthermore, the number of lateral flowers seems to be more or less constant in centroradialis (F. Cremer, H. Saedler, and P. Huijser , unpublished data), but it is affected by the environment in terminal flower1. High temperature (Alvarez et al., 1992) and long days (Shannon and Meeks-Wagner, 1991) both reduce the number of lateral flowers formed by different mutant alleles of TFL1, from which at least tfl1-2 represents a null allele (Ohshima et al., 1997).

In this paper, we describe a new allele of CENTRORADIALIS resulting in a mutant phenotype displaying one of the additional features of the tfl1 mutant, which is a variable number of lateral flowers in response to environmental changes. The reduced activity of this conditional allele is correlated with the three-dimensional structure of the CENTRORADIALIS protein (Banfield and Brady, 2000) and suggests that different genes are involved in the control of determinacy in the Arabidopsis and snapdragon inflorescences.

RESULTS

The Dtf Phenotype Is Due to a Mutation in the CEN Gene

A plant with a Cen mutant phenotype appeared in progeny raised after an x-ray mutagenesis of F1 seeds obtained from a cross between the commercial snap-
dragon cv Snowman and the inbred line T53 (Harrison and Carpenter, 1973). This plant was shown to be homozygous for a new cen mutant allele that we named cen-2 after a complementation test with the traditional Gatersleben mutant allele.

Among several hundreds of plants in progenies of self-pollinated cen-2 plants, four wild-type-looking plants appeared, suggesting that the cen-2 allele was unstable. Genetic instability due to active endogenous transposons residing at mutated loci is well documented in snapdragon (Harrison and Fincham, 1964; Kunze et al., 1997). The progenies of these four putative revertants gave similar results and will not be referred to separately later on. As expected for true revertant plants, Cen and wild-type plants segregated in these progenies. However, puzzling observations were made later when quite a large proportion of the plants that had been initially scored as having either a mutant or a wild-type phenotype based on their main inflorescence did show the opposite phenotype for their secondary inflorescences. Furthermore, inflorescence development in some wild-type-looking plants ceased prematurely after some 20 to 50 flowers. Moreover, one plant formed a fully developed terminal flower after 37 lateral flowers. This phenotype, designated Delayed terminal flower (Dtf), is presented in Figure 1A. From this time onward, we decided to check every plant repeatedly and to dissect the apex of every inflorescence arrested in its development. This allowed us to show that a large proportion of the plants with an arrested inflorescence initiated a terminal flower that aborted early in development (Fig. 1B). In a second generation obtained by selfing, the plants that had consistently displayed a Cen mutant phenotype gave only mutant progenies with developed terminal flowers, plants that had consistently presented a wild-type phenotype without an aborted terminal flower gave only wild-type progenies, and progeny of the plants that had displayed the Dtf phenotype segregated in the same way as the progeny of the original revertants. These segregation ratios indicated that the Dtf phenotype was caused by heterozygosity at the locus involved and that the four putative revertant plants should have already been Dtf plants. We did not observe this initially because we sampled the plants before they developed secondary inflorescences.

We speculated that the Dtf phenotype could be due to: (a) a new allele of CEN, (b) another gene that would mask the Cen mutant phenotype by delaying the formation of the terminal flower, or (c) another terminal flower-causing mutation that would be unmasked by cen reversion. Therefore, Dtf plants were crossed to the wild-type inbred line Sippe 50 and the segregating F2 progeny were analyzed. The absence of Dtf plants in the F2 suggested that the Dtf phenotype was due to heterozygosity involving the cen-2 mutant allele and a new mutant allele derived from it as a result of partial reversion, or that the second locus would be tightly linked to the CEN locus.
Using the CEN cDNA as a probe, it could be confirmed molecularly that the Dtf phenotype is due to heterozygosity of the cen-2 allele and a partially reverted allele we will refer to as cen-2r(dtf). In addition, phenotypically wild-type plants proved to be homozygous for cen-2r(dtf) as can be seen on Figure 2.

To check if the strong cen-2 allele has a specific role in the Dtf phenotype, we crossed homozygous cen-2r(dtf) plants with the traditional Gatersleben cen mutant, homozygous for the cen-594 allele (Bradley et al., 1996). The presence of Dtf plants in the F1 indicated that the strong allele has no specificity of itself and that the Dtf phenotype only depends on specific features of the cen-2r(dtf) allele.

**cen/cen-2r(dtf) Confers a Conditional *centroradialis* Phenotype**

The variable number of lateral flowers observed on Dtf plants suggested that changes in the environment influenced the phenotype. Therefore, we compared the flowering behavior of cen-2/cen-2, cen-2/cen-2r(dtf) and cen-2r(dtf)/cen-2r(dtf) genotypes under different growth conditions. cen-2/cen-2 plants were compared with cen-2/cen-2r(dtf) at different temperatures, day lengths, light intensities, and light qualities. Figure 3A shows that flowering time behaved as expected from earlier experiments with the Sippe 50 inbred line (Cremer et al., 1998). Under all conditions, cen-2/cen-2r(dtf) tended to flower slightly earlier than cen-2/cen-2, with statistically significant differences observed for conditions 3, 5, 6, and 7 as described in the legend of Figure 3. Under the same conditions, the cen-2/cen-2 plants had a constant LFN (Fig. 3B), with the exception of condition 7 (Fig. 3).

![Figure 2.](image) Dtf plants are heterozygous for the CEN gene. Autoradiograph of a Southern blot probed with the 700-bp 5' end of CEN cDNA. 1, cen-2 plants; 2, dtf plants; 3, homozygous revertants. DNA was cut with EcoRI.

where a statistically significant reduction in LFN was observed. Under the weak inductive conditions 1, 2, and 3 (Fig. 3) cen-2/cen-2r(dtf) plants did not produce a terminal flower before they died or ceased further development of their inflorescence. A terminal flower was made under all other conditions, with LFN being notably reduced under condition 7 (Fig. 3) to only 11 lateral flowers. Under all conditions, the LFN of cen-2/cen-2 was significantly smaller than that of cen-2r(dtf). Because the LFN of cen-2r/den-2r(dtf) was only affected under very good growing conditions, a second experiment with cen-2r/cen-2r(dtf) was carried out at 25°C. The effect of light intensity, light quality, and day length on flowering time, TLN, and LFN are presented in Figure 3C. A parallel decrease in all three variables was observed with improving growth conditions, with LFN decreasing from 56 to 11. These experiments clearly demonstrated that the cen-2/cen-2r(dtf) plants show a conditional *centroradialis* phenotype, appearing wild type under weak flower-inducing conditions, like the Cen mutant under strongly inductive conditions, and like Dtf under intermediate conditions. We also checked the effect of cen-2r(dtf) homozygosity on the phenotype of plants when grown under different conditions. None of the cen-2r(dtf)/cen-2r(dtf) plants formed a terminal flower under conditions 5 and 6 described in the legend of Figure 3. Under conditions identical to conditions 10 and 11 of Figure 3, one out of 15 plants made a terminal flower after 40 and 35 lateral flowers, respectively; four out of 15 under conditions 13 (LFN = 40.5); and nine out of 15 under condition 7 (LFN = 44.3). Thus, under strong flower-inducing conditions, some cen-2r/dtf/cen-2r(dtf) plants may develop a Dtf phenotype. Concerning their flowering time, cen-2r(dtf)/cen-2r(dtf) plants flowered sometimes earlier, sometimes later than the cen-2/cen-2r(dtf) plants, without a significant difference.

**Changing Growing Conditions Can Trigger the Formation of the Terminal Flower**

To check at what time the decision is made to form the terminal flower, environmental shift experiments have been carried out with the cen-2/cen-2r(dtf) plants. In a first set of experiments, plants were shifted from less inductive low-light conditions (16-h light, 25°C, VHO + 2I, and 9,000 lux; see “Materials and Methods”) to more inductive high-light conditions (idem, 30,000 lux) after 0, 5, 10, or 15 d following the macroscopic appearance of the first flower buds or, alternatively, 0, 10, or 20 d following anthesis. Control plants were not shifted and remained under 9,000 lux. According to the Kruskal-Wallis statistical test, none of the series showed a significant difference for flowering time, TLN, and LFN. The values varied respectively from 64.3 ± 4.3 to 67.4 ± 2.1 d, 28.8 ± 5.6 to 32.3 ± 5.6 leaves, and 28.8 ± 12.4
to 36.4 ± 6.8 lateral flowers. These results indicated that the LFN was determined prior to the macroscopic appearance of the flower buds.

In a second experiment, starting conditions less favorable for flowering were chosen (16 h, 20°C, 9,000 lux, and VHO). Starting at the third week after sowing, every week until week 17, a batch of plants was shifted to more inductive conditions (16 h, 25°C, 30,000 lux, and VHO). The influence of the transfer date on flowering time, TLN, and LFN is presented in Figure 4. The evolution of TLN indicated that plants became determined to flower before week 13 under the less inductive conditions. Flowering time increased until the 14th week, indicating an effect of the growing conditions on flower development. Plants that were shifted before week 8 developed a Cen mutant phenotype, and those shifted thereafter developed a Dtf phenotype. One-way analysis of variance-on-ranks according to Kruskal-Wallis, followed by pair-wise comparisons with the Mann-Whitney test (Weber, 1986), indicated that LFN increased until week 16, when it reaches the level observed in control plants. These results indicate that the formation of the terminal flower can be induced by a shift to more inductive conditions, even when the plant is already determined to flower.

The cen-2r(dtf) Allele Contains a Footprint Due to a Transposon Excision

Based on a 4.4-kb preliminary sequence of the CEN promoter, kindly provided by Dr. Desmond Bradley (The Sainsbury Laboratory, Norwich, UK), and the published CEN cDNA sequence, we were able to design primers and to sequence 6 kb of the CEN wild-type allele of line 164, a derivative of the 165E line from which the preliminary sequence originated. Based on the definitive sequence we obtained, we amplified, sequenced, and compared the corresponding region of the cen-2r(dtf) mutant allele. It was unexpected that many differences were detected in the form of deletions, insertions, and base changes, most differences appearing in intron sequences. Base changes in the coding region of the cen-2r(dtf) allele result in modification of the CEN protein at positions 5 (Val → Ile), 28 (Lys → Gln), and 35 (Ser → Ala). Furthermore, a 3-bp insertion in frame leads to the addition of an iso-Leu between Asp148 and Gly149. Because of the large number of differences between the 164 wild type and the cen-2r(dtf) allele, we sequenced the CEN allele from Snowman, which was one of the parents of the mutagenized line and whose F1 with cen-2 never formed a terminal flower. This allele was identical to the cen-2r(dtf) allele except for the 3-bp insertion noted earlier. Therefore, we assumed that this insertion was responsible for the Dtf phenotype and was due to the excision of a transpo-
son leaving a 3-bp footprint. To obtain sequence from this transposon, DNA of cen-2 homozygous plants was used as template for a thermal asymmetric interlaced-PCR reaction (Liu et al., 1995), with three primers located 5’ to the 3-bp insertion identified in the revertant plants. The fragment obtained contained part of the left terminus of a new member of the CACTA transposable element family (Kunze et al., 1997; Fig. 5). This element, inserted in the cen-2 allele at the same position as the 3-bp insertion in the cen-2r(dtf) allele, was named Tam10. It is responsible for the cen-2 mutation and its insertion could be due to the x-ray mutagenesis treatment. Its sequence was most similar to the right end of the Tam9 element, with which it shares 77% of identity in the first 120 bp (Tröbner et al., 1992). Using primers designed from the left terminal sequence of Tam9 and a primer located 3’ of the insertion site in the cen-2 allele, we were able to amplify and determine the 69-bp terminal sequence of the right end of Tam10. Only one mismatch and one base insertion compared with the Tam9 sequence were found.

To establish the nature of the footprints that are able to generate a Dtf phenotype, two other independent revertant alleles were sequenced between primers F14 and B14b. Both revealed a footprint identical to the cen-2r(dtf) allele footprint (Fig. 5). It is interesting that according to the excision mechanism proposed by Saedler and Nevers (1985), alternative 3-bp addition footprints could also be expected, resulting in the incorporation of an Asp or a Tyr instead of an iso-Leu. Because none of these alternative events occurred in the three revertants analyzed, they are either less common or result in a null allele that therefore would not be detected as revertant. Furthermore, from all the plants we have grown, no excision reestablishing the correct sequence and giving a true wild-type plant has been observed.

As mentioned above, the Snowman allele strongly differs from the 164 allele. It is interesting that the most notable difference between these two wild-type alleles is the presence of a 1.9-kb insertion in the Snowman allele 580 bp upstream of the ATG start codon (Fig. 5). This insertion is flanked by a 17-bp direct repeat likely to be the result of a target site duplication upon insertion of a transposon. The left end of this transposon contains 9 bp that are an inverted repeat of the target site, followed by a 6-bp inverted repeat from base 12 to 23. No long terminal repeat, additional inverted repeat, poly-A tail, nor candidate open reading frame is found in the transposon sequence. On the right end, the sequence did not reveal any notable features. These characteristics did not allow us to assign this transposon to any of the known classes of transposable elements. A genomic blot with DNA from different lines of snapdragon probed with this fragment gave a complex pattern, indicating that the element represents a family with a high number of heterogeneously sized transposons (data not shown). This element has been named Tam12, according to the Antirrhinum majus community usage.
Three-Dimensional Structure of the CEN Protein

The recently published structure of the CENTRO-RADIALIS protein (Banfield and Brady, 2000) shows that the Asp148–Gly149 region is in close contact and forming H bonds with the Glu89–His90 region. Figure 6A shows this region of the protein coded by the Snowman allele. This region has a sequence identical to the published protein and has a very similar three-dimensional structure (data not shown). The corresponding region in the bovine protein has been shown to contact phosphorylethanolamine, the polar head group of phosphatidylethanolamine (Serre et al., 1998), and is the putative ligand-binding site in the bovine and human proteins (Banfield et al., 1998; Serre et al., 1998). In the CEN protein, Glu89 forms a cis-peptide bond with Arg88 and the Glu89 side chain forms H bonds with Thr152 (Banfield and Brady, 2000). As suggested by structure prediction using the SWISS-MODEL program (Guex and Peitsch, 1997), the iso-Leu insertion between Asp148 and Gly149 will modify the intramolecular bonds in this region (Fig. 6C). As a result, the affinity to the ligand is probably modified. The inserted iso-Leu also modifies the protein surface (Fig. 6, B and D) and could influence putative protein-protein interactions (Banfield and Brady, 2000).

DISCUSSION

Inflorescence Architecture

Determinacy/indeterminacy of the inflorescence is usually a stable character and is often used in species identification. In several species, occasional conversions of an indeterminate into a determinate inflorescence have been reported (Penzig, 1922), but the heritability of these transformations has rarely been studied. Only in a few species have these transformations been shown to be due to mutations (Bradley et al., 1996, 1997). On the other hand, to our knowledge, the transformation from a determinate to an indeterminate inflorescence has not been described. This is in support of Troll’s typological classification, with indeterminate inflorescences derived from determinate inflorescences (Troll, 1964; Troll, 1969), due to the gain of a new function preventing the central part of the inflorescence apical meristem from differentiating into a floral meristem. Yet, from a strictly empirical point of view and in line with Troll’s model, there is also the possibility that at least some naturally occurring determinate inflorescences could be derived from indeterminate ones, due to a subsequent loss of the indeterminacy function, as demonstrated by all known mutants. Many examples for losses of functions in the wild are given by Fong et al. (1995), Kunze et al. (1997), and Lönnig and Saedler (1997).

In snapdragon and Arabidopsis, the gain of indeterminate inflorescence involved the acquisition of the CEN and TFL1 function (Bradley et al., 1996, 1997). Putative null alleles of these genes result in the transformation of the apical meristem into a terminal flower (Bradley et al., 1996, 1997; Ohshima et al., 1997). However, there should be a slight difference in their mode of action because certain aspects of the mutant phenotypes differ in the two species. Further-
more, when the two genes are transformed into an identical background in tobacco, only CEN is able to affect the phenotype of the transgenic plants (Amaya et al., 1999). In addition to its effect on flowering time, not observed with CEN (Cremer et al., 1998), the loss of TFL1 function results in the development of a variable number of lateral flowers in response to different growth conditions (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992). Null alleles of cen are known to form their terminal flower after a more or less constant number of lateral flowers (Bradley et al., 1996) irrespective of the environment (F. Cremer, H. Saudler, and P. Huijser, unpublished data). In this paper, we describe a conditional allele of CEN, cen-2r(dtfr), that also results, when in a heterozygous combination with a null allele, in a variable number of lateral flowers in response to different growth conditions, like the loss of TFL1 function (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992). Under strong flower-inducing conditions, this heterozygous combination results in a Cen-null mutant phenotype. Under weak flower-inducing conditions, a wild-type phenotype is obtained. Grown in intermediate conditions, a new phenotype is observed, displaying a determinate inflorescence with a variable number of lateral flowers. The number of lateral flowers varies from 10 to more than 50 and is negatively correlated with the flower-inducing strength of the growing conditions. Therefore, we refer to this phenotype as Delayed terminal flower. It is surprising that the Dtf plants also flower a little bit earlier than the Cen mutant plants, but this character is not always found in the plants homozygous for the revertant allele. This erratic behavior could be due to unknown genes segregating in the background and originating in the Snowman and T53 lines used for the mutagenesis.

Partial Reversion of the cen-2 Allele Resulted in a Conditional Allele

Molecular analysis of cen-2r(dtfr) revealed that it arose from a transposon excision leaving behind a 3-bp insertion footprint. As a result, an iso-Leu is added to the encoded CEN protein between Asp148 and Gly149. This presumably modifies the affinity for the ligand, e.g. phosphatidyylethanolamine, another phospholipid or a kinase, as proposed by Banfield and Brady (2000), by comparison to the direct interaction of the human PEBP and the Raf-1 kinase (Yeung et al., 1999). Because the modification involves part of the outer surface of the CEN protein, it cannot be ruled out that a yet unknown protein-protein interaction would be affected. The transposon excision and the 3-bp addition thus appears to partially restore CEN function. Moreover, the better the growing conditions the less the cen-2r(dtfr) allele seems to be functional, and a terminal flower may be formed as quickly as in a genuine null mutant. It is interesting that the decline in functionality depends not only on temperature but also on day length and light intensity. Therefore, we believe that the conditional phenotype is not due to a modified thermostability of the mutant protein. Instead, we propose that the conditional phenotype is subject to the strength of the flower promoting activity represented by floral meristem identity genes like FLORICAULA (FLO; Coen et al., 1990) and SQUAMOSA (SQUA; Huijser et al., 1992). In Arabidopsis, constitutive expression of the respective orthologs, LEAFY (LFY) and APETALA1 (API), leads to the formation of a terminal flower (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). Furthermore, constitutive expression of LFY or API has been shown to repress TFL1 expression (Libregen et al., 1999; Ratcliffe et al., 1999), demonstrating the antagonistic roles of the TFL1 and the LFY/API1 functions. The transition to flowering has been shown to be at least partly controlled by the level of LFY expression and this level has been shown to be increased under stronger flower-inducing conditions (Blazquez et al., 1997). We may assume that a similar mechanism acts in snapdragon. Due to a reduced level or function of the CEN-2R(DTF) protein in cen-2/cen-2r(dtfr) plants, FLO and SQUA would be able to overcome their inhibition by CEN in the central part of the apical meristem under stronger flower promoting conditions. In cen-2r(dtfr) homozygous plants, the function of the CEN protein [CEN-2R(DTF)] is still reduced compared with wild type, but its level may be twice as high compared with cen-2/cen-2r(dtfr) plants. It is only under extremely good inductive conditions, and only in a few plants, that the CEN inhibition of FLO and SQUA activity can be overcome in these homozygous plants. Moreover, this is achieved very late in inflorescence development, when the COPS activity is believed to reach a low level according to the model of Schultz and Haughn (1993). According to this model, COPS activity decreases with node production and, at critical levels, morphological programs associated with a phase change are activated, in this case the formation of a terminal flower. It is unfortunate that the large variability in the timing of the terminal flower formation in Dtf plants under controlled growth conditions precluded testing the above hypothesis by quantifying the level of squa and flo expression in the shoot apical meristem.

The Dtf Phenotype Mimics Aspects of the Arabidopsis tfl1 Mutation

In Arabidopsis, the tfl1 mutants, including the tfl1-2-null mutant (Ohshima et al., 1997), are early flowering and form a variable number of lateral flowers under different growth conditions (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992). In snapdragon, the flowering time of Dtf, like that of cen-null mutants, remains largely unaffected. This difference
Figure 6. (Legend appears on facing page.)

A

B

C

D
in flowering behavior can be easily explained at the level of transcription, with CEN becoming expressed in the shoot apical meristem only after its commitment to produce flowers, whereas TFL1 is already expressed during vegetative growth (Bradley et al., 1996, 1997).

With respect to the variable number of lateral flowers, the Dtf phenotype of snapdragon resembles more the Tfl1-null mutant phenotype than does the Cen-null mutant phenotype. As in tfl1, the number of lateral flowers made before the terminal flower is variable in Dtf, and this seems to be due to a low level of functional CEN activity.

This suggests that in Arabidopsis additional genes may act in parallel to TFL1 to repress LFY and AP1 expression in the apical meristem. A candidate for this function is the TFL2 gene. Like tfl1, tfl2 mutants form a terminal flower and are early flowering, but they also display a dwarfed phenotype and a reduced sensitivity to the photoperiod (Larsson et al., 1998). They generate a larger number of lateral flowers compared with tfl1 but the double mutant tfl1/tfl2 does not develop any lateral flowers. This observation supports the hypothesis that the tfl1 null mutant can still develop a variable number of lateral flowers due to partial functional redundancy of (an) other gene(s). The difference in phenotype between tfl1 and cen could be explained by this mechanism, but the mutant phenotype of the Arabidopsis ARABIDOPSIS THALIANA CENTORADIALIS (ATC) gene, which currently seems to be the best candidate to represent the true CEN orthologue (GenBank accession no. AB024715), would be helpful in clarifying this model. If TFL1 and TFL2 act in two parallel pathways, it is possible that ATC would be downstream of one or both of these genes and that a mutation in ATC would also result in the production of a terminal flower. On the other hand, other members of the PEBP family are known in Arabidopsis that are not involved in the control of the inflorescence meristem identity, like FLOWERING LOCUS T (FT; Araki et al., 1998; Kardailsky et al., 1999; Kobayashi et al., 1999) and TWIN SISTER OF FT (TSF; Kardailsky et al., 1999; Kobayashi et al., 1999).

To further characterize the differences and similarities between CEN and TFL1, it would be interesting to find out which aspects of the Tfl1 mutant phenotype can be complemented by the cen-2r (dtf) and the wild type CEN alleles of snapdragon in transgenic Arabidopsis.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

A new allele of CENTORADIALIS was obtained in an M2 after x-ray mutagenesis of F1 seeds from a cross between the snapdragon (Antirrhinum majus) F1 commercial cv Snowman (wild type for the CEN gene and heterozygote for different flower color genes) and the inbred line T53 (Harrison and Carpenter, 1973). This new allele was designated cen-2 after a complementation test with the traditional Gatersleben mutant allele, referred to by Bradley et al. (1996) as cen-594. Seeds of the Sippe-50 wild-type inbred line were obtained from Gatersleben (Germany) and propagated by selfing for several generations. It is the background in which the cen-1 allele has been isolated. The 164 wild-type line is a revertant of the 165E (niv-98::Tam3) line (Sommer et al., 1985). Stocks were grown in the greenhouse. For physiological experiments, plants were grown in the phytotronic growth chambers of the University of Liege (Belgium) as previously described (Cremer et al., 1998). Day length varied from 8 to 20 h, temperature from 12°C to 25°C, light intensity from 9,000 lux (140 μmol m⁻² s⁻¹) to 30,000 lux (390 μmol m⁻² s⁻¹), and light quality was either VHO or VHO + 2I (Cremer et al., 1998). VHO light was provided by Cool White VHO fluorescent tubes (Sylvania S.A., Zaventem, Belgium). VHO + 2I light corresponded to the addition of incandescent light provided by two 40-W incandescent bulbs per pair of 36-W VHO tubes. Time to anthesis of the first flower (flowering time), the number of leaves below the first flower on the main stem (TLN), and the number of lateral flowers were scored. The LFN preceding the terminal flower includes fully developed flowers as well as every flower bud detectable under a dissecting microscope. All experiments were duplicated. Repeated measurements were pooled before analysis. The Kolmogorov-Smirnov test of normality (Weber, 1986) failed at the 5% level for some of the series and therefore we decided to use non-parametric tests for later analysis. One-way analysis of variance on ranks according to Kruskal-Wallis (Weber, 1986) has been used to check for the influence of growing conditions. When statistically significant differences were observed, pair-wise comparisons have been done using the Mann-Whitney test on ranks (Weber, 1986). All statistical tests were performed using the Sigmasat 2.0 software (SPSS ASC, Erkrath, Germany).

**Molecular Biology Techniques**

DNA extraction from young leaves, Southern blotting, probes labeling, and hybridization were performed as de-

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**Figure 6.** Stereo view of the ligand binding site in CENTORADIALIS as predicted by Swiss model. A and B, The Snowman protein; C and D, the Dtf protein. A and C, An iso-Leu (orange) is inserted in the Dtf protein between Asp148 and Gly149 (green) of the Snowman protein. In Snowman, the chain from Arg147 to Thr152 forms numerous H bonds (dotted red lines) with the amino acids forming the ligand pocket and their neighbors, as well as with the evolutionary conserved Glu89 that seems to control access to the ligand pocket (Banfield and Brady, 2000). The Ile insertion in Dtf is predicted to affect the H bonds. B and D, Molecular surface corresponding to the region seen in A and C. The surface seems only affected in the vicinity of the iso-Leu inserted in the Dtf protein.
scribed by Sommer et al. (1990). All oligonucleotides used for this work were synthesized by MWG-Biotech (Ebersberg, Germany) or GibcoBRL Life Technologies (Eggenstein, Germany). Standard PCR reactions started at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min and ended at 72°C for 5 min. Thermal asymmetric interlaced-PCR was performed according to published protocol (Liu et al., 1995). PCR fragments were purified using QIAQuick Spin columns (QIAGEN, Hilden, Germany) and sequenced on PE/Applied Biosystems 377 and 3700 sequencers using BigDye-terminator chemistry. DNA sequence analysis was performed using the MacVector 6.5 computer program package (Oxford Molecular, Oxford). Transposon excision footprint were checked by sequencing PCR fragments obtained with primers F14 5′-GAG CTA TGA GAT GCC AAG GCC GAA C-3′ and B14b 5′-CCA TTT CTC CAT CTT TTC CTT C-3′. The sequence of the CEN alleles of the 164 wild-type line and of the cv Snowman have been submitted to the EMBL database under the accession nos. AJ251993 and AJ251994.

Molecular Modeling

The three-dimensional models of the Snowman wild type and Df mutant CEN proteins have been generated by homology modeling. The three-dimensional template structure has been the CEN protein, Brookhaven protein database code 1QOUB (Banfield and Brady, 2000), and the calculations were done with the homology modeling package Swiss-model (Guex and Peitsch, 1997; http://www.expasy.ch/swissmod/). PDB Viewer 3.5 (Guex and Peitsch, 1997; http://www.expasy.ch/spdbv/) has been used to compute H bonds and for three-dimensional model visualization. Final graphics were prepared with POV Ray version 3.1g (http://www.povray.org/). Numbering of amino acids corresponds to the CEN sequence published by Bradley et al. (1996).

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