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Repeated Inactivation of the First Committed Enzyme Underlies the Loss of Benzaldehyde Emission after the Selfing Transition in Capsella

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Report

Current Biology

Repeated Inactivation of the First Committed Enzyme Underlies the Loss of Benzaldehyde Emission after the Selfing Transition in *Capsella*

Highlights

- Benzaldehyde as a major scent compound was lost in selfing Red Shepherd's Purse
- This is due to inactivation of the cinnamate:CoA ligase *CNL1*
- *CNL1* has been inactivated twice by novel mutations in the selfing lineage
- Modulation of CNL activity also underlies scent evolution in other flowering plants

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In Brief

Sas et al. show that a key gene required for synthesizing the major scent compound benzaldehyde has been inactivated twice independently in the selfing Red Shepherd's Purse compared to its outbreeding ancestor. Together with parallel work in *Petunia*, this suggests the gene as an evolutionary hotspot for changing chemical plant-insect communication.

Accession Numbers

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Report

Repeated Inactivation of the First Committed Enzyme Underlies the Loss of Benzaldehyde Emission after the Selfing Transition in *Capsella*

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SUMMARY

The enormous species richness of flowering plants is at least partly due to floral diversification driven by interactions between plants and their animal pollinators [1, 2]. Specific pollinator attraction relies on visual and olfactory floral cues [3–5]; floral scent can not only attract pollinators but also attract or repel herbivorous insects [6–8]. However, despite its central role for plant-animal interactions, the genetic control of floral scent production and its evolutionary modification remain incompletely understood [9–13]. Benzenoids are an important class of floral scent compounds that are generated from phenylalanine via several enzymatic pathways [14–17]. Here we address the genetic basis of the loss of floral scent associated with the transition from outbreeding to selfing in the genus *Capsella*. While the outbreeding *C. grandiflora* emits benzaldehyde as a major constituent of its floral scent, this has been lost in the selfing *C. rubella*. We identify the *Capsella CNL1* gene encoding cinnamate:CoA ligase as responsible for this variation. Population genetic analysis indicates that *CNL1* has been inactivated twice independently in *C. rubella* via different novel mutations to its coding sequence. Together with a recent study in *Petunia* [18], this identifies cinnamate:CoA ligase as an evolutionary hotspot for mutations causing the loss of benzenoid scent compounds in association with a shift in the reproductive strategy of *Capsella* from pollination by insects to self-fertilization.

RESULTS

Flowers of *C. grandiflora* Emit Benzaldehyde with a Diurnal Rhythm, whereas the Selfing Species *C. rubella* Does Not

The transition from outbreeding to selfing represents one of the most frequent evolutionary changes in flowering plants [19], and it is often accompanied by a suite of morphological and functional changes to the flowers, including a strong reduction in flower size and in some cases a virtually complete loss of floral scent [20, 21]. Such reduction in attractive signals is likely to contribute to pre-zygotic reproductive isolation between the outbreeding ancestor and the derived selfer. However, the contribution of different trait changes, such as scent loss, to such pre-zygotic isolation remains poorly understood [20]. The genus *Capsella* provides a promising model to study the genetic basis of the "selfing syndrome," with the self-compatible *C. rubella* having diverged from a self-incompatible *C. grandiflora*-like ancestor between 50,000 and 100,000 years ago, as demonstrated by phylogenetic and population genetic analyses [22–25]. While *C. grandiflora* is largely restricted to northern Greece, after the transition to selfing *C. rubella* has spread throughout the Mediterranean region and also to America.

In contrast to *C. rubella*, *C. grandiflora* plants emit a strong, marzipan-like scent. To chemically analyze this difference, we collected volatiles emitted from inflorescences by headspace sampling and analyzed these by coupled gas chromatography-mass spectrometry (GC-MS). We identified benzaldehyde (BAld), phenylethyl alcohol, and phenylacetaldehyde as the major benzenoids emitted by *C. grandiflora*, all of which were not detectable in the headspace of *C. rubella*. By contrast, *C. rubella* emitted detectable amounts of benzoic acid (BA), which was present only in very low amounts in the *C. grandiflora* headspace (Figure 1A; Figure S1). In addition, *C. grandiflora* flowers emitted low amounts of the terpenoid *trans*-beta-ocimene and traces of *cis*-beta-ocimene, both of which were undetectable in the *C. rubella* samples. As BAld was the most prominent benzenoid in the *C. grandiflora* floral scent, our further analysis focused mainly on this compound. While all of several *C. grandiflora* accessions emitted BAld, none could be detected for any tested accessions of the selfing species (Figure 1A), indicating that the loss of BAld emission is fixed in the latter relative to the outbreeding ancestor.

Scent emission often varies throughout the day, in accordance with the activity patterns of pollinators [26–28]. To test this for *Capsella*, we measured benzenoid emission and internal pools in the inflorescence from 4 a.m. (1 hr before the start of exposure to light) until 9 p.m. (1 hr after the end of exposure to light). To minimize the effect of genetic variation within the outbreeding *C. grandiflora*, we used an inbred, self-compatible *C. grandiflora*-like line that had been generated by introgression of the non-functional *C. rubella*

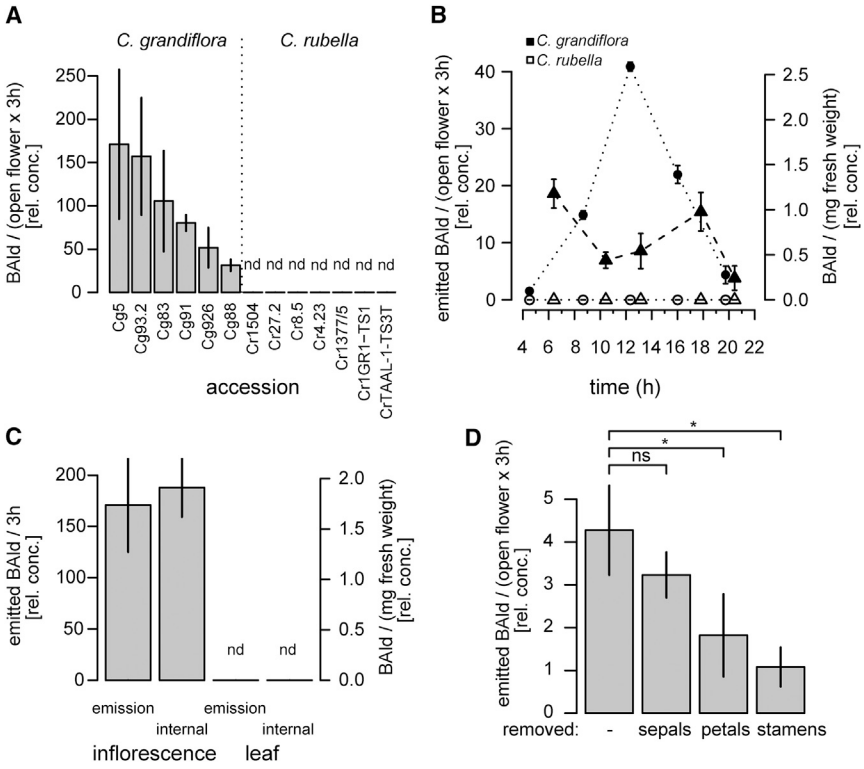

Figure 1. Determination of Benzaldehyde Emission Rates from the Inflorescences of *Capsella* Species

(A) Quantification of emitted benzaldehyde (BAld) in the headspace of the indicated *Capsella* accessions. Values are mean ± SD from five biological replicates per accession. Nd, not detected; rel. conc., relative concentration of BAld normalized to 10 ng tridecane as internal standard in the samples.

(B and C) Quantification of emitted BAld (headspace, circles, left y axis) and of BAld in the internal pool (dichloromethane extracts, triangles, right y axis) from an inbred, self-compatible *C. grandiflora*-like line and *C. rubella* over the course of a day (B) and in a comparison of leaves and inflorescences from the inbred, self-compatible *C. grandiflora*-like line (C). Values are mean ± SD from four biological replicates per accession.

(D) Quantification of emitted BAld in the headspace of flowers of the self-compatible *C. grandiflora*-like line from which the indicated floral organs had been removed. Values are mean ± SD from four biological replicates per treatment (significant differences at *$p < 0.05$ based on Student's t test).

See also Figure S1.

S locus into *C. grandiflora* [29]. Starting from a very low level at the end of the night, BAld emission showed a sharp peak around noon, before declining again to very low levels toward the end of the day (Figure 1B). Similar patterns were seen for phenylethyl alcohol and phenylacetaldehyde (Figure S1). Internal pools showed a roughly inverse pattern, especially for BAld, with relatively high levels in the morning and in the afternoon, yet lower levels around noon and at the end of the day. This pattern is consistent with the notion that *C. grandiflora* is mainly pollinated by insects active during the day. The diurnal pattern of BA emission in *C. rubella* was very similar to that of BAld emission in *C. grandiflora* (Figure S1).

BAld is only emitted from and detectable in extracts of *C. grandiflora* inflorescences, but not of rosette leaves (Figure 1C). In the related *Brassica rapa*, BAld is mainly emitted from stamen filaments and petals [30]. We removed petals or stamens from opened flowers of the self-compatible *C. grandiflora* line and measured BAld emission from the remaining organs. As a control for the wounding treatment, sepals were removed from a third group of plants. Plants lacking either petals or stamens emitted significantly ($p < 0.05$, one-tailed t test) less BAld than control plants (Figure 1D). Thus, we conclude that stamens and petals are the main source of BAld emission in *C. grandiflora*, which contribute additively to the total BAld emission from the flower (Figure 1D), resembling the situation in *B. rapa*.

A Major QTL Underlies the Absence of BAld Emission in *C. rubella*

To determine the genetic basis for the loss of BAld emission in *C. rubella*, we performed quantitative trait locus (QTL) mapping on the *C. grandiflora Cg926* × *C. rubella Cr1504* recombinant-inbred line (RIL) population [29]. BAld was undetectable in the headspace of 34 tested RILs (33%) versus 69 RILs showing emission (Figure S2A); this pattern is suggestive of one major locus determining BAld emission. Consistent with this notion, only one major QTL was detected in the middle of chromosome 2 (confidence interval from 16 to 50 cM), explaining 40.3% of the variation in BAld emission (Figure 2A). No additional QTL influencing the variation in the amounts of emitted BAld could be detected, possibly reflecting the limited power of the experiment. On average RILs without detectable BAld emission formed smaller flowers than ones with emitted BAld, reflecting the presence of a linked petal-size QTL on chromosome 2 [29]; importantly, however, several of the lines without detectable BAld emission formed larger petals than ones with high levels of emitted BAld, indicating that the absence of detectable BAld emission is not merely a consequence of smaller flowers.

We fine-mapped the causal gene underlying this QTL by phenotyping recombinants in the QTL region derived from two heterogeneous inbred families (HIFs) from the F8 RILs. This indicated that the *C. rubella* allele behaved recessively and localized the causal region to an interval of 9.5 kb between positions 7,536,516 and 7,546,052 (Figure 2B). To verify the causal role of this region, we crossed the two closest recombinants and selected plants homozygous for the *C. grandiflora* or for the *C. rubella* allele in this 9.5-kb interval from the F2 (Figure S2C); these two cohorts only differed systematically for the genotype in the focal region, with flanking regions fixed and any remaining unlinked regions of residual heterozygosity segregating equally in the two cohorts.

Plants homozygous for the *C. grandiflora* allele showed on average more than 5-fold higher BAld emission than those homozygous for the *C. rubella* allele (Figure 2C); while many of the latter showed no detectable emission, a few did so, possibly

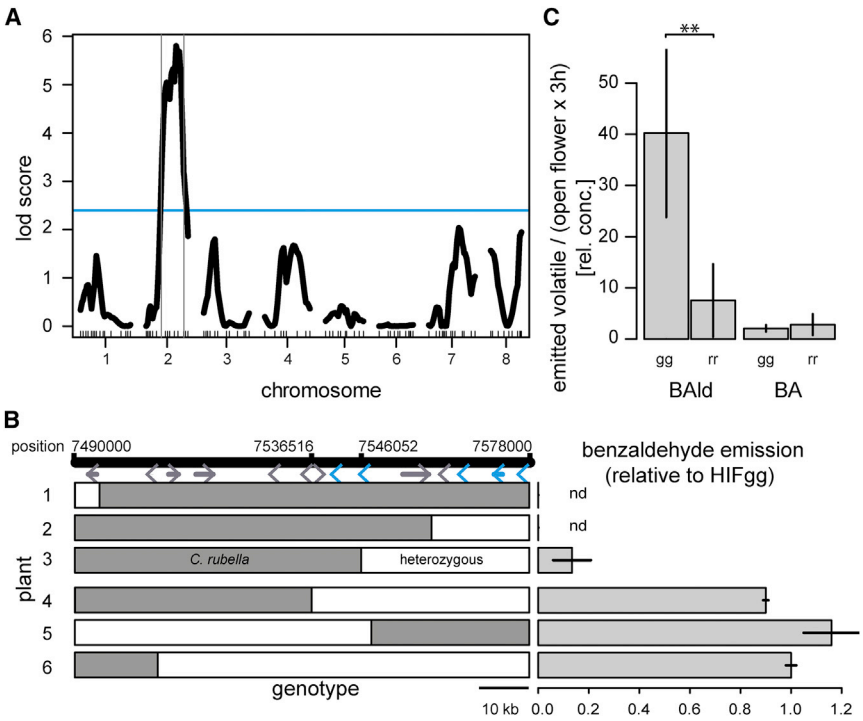

Figure 2. Mapping the Genetic Basis of the Loss of BAld Emission in *C. rubella*

(A) QTL mapping of BAld emission in the *C. grandiflora* × *C. rubella* RIL population. Horizontal line indicates the significance threshold based on 1,000 permutations. The vertical lines mark the 2-LOD confidence interval. Ticks on the x axis indicate the position of genetic markers used for genotyping the RIL population.

(B) Fine-mapping of the BAld emission QTL. Base pair positions on chromosome 2 are indicated. Blue arrows indicate *CNL* genes. Bars on the left show the genotype of informative recombinants (gray, homozygous for *C. rubella* allele; white, heterozygous). Bars on the right show BAld emission of the respective recombinants expressed relative to the emission of *C. grandiflora* homozygotes from the HIF measured in the same experiment. Values are mean ± SD from three measurements on consecutive days from the same individuals.

(C) BAld and BA emission in the headspace of F2 plants of the quasi-isogenic line homozygous for the *C. grandiflora* or the *C. rubella* allele in the 9.5-kb mapping interval. Values are mean ± SD from measurements of ten plants per genotype (significantly different at $^{**}p < 0.01$ based on Student's t test). See also Figure S2.

reflecting segregation of an additional minor locus influencing BAld emission. This is consistent with the low level of BAld emission detected in one of the parental recombinants used for the cross (plant 3 in Figure 2B). There was no difference between the two cohorts concerning BA emission. Plants with the *C. grandiflora* allele also emitted more phenylacetaldehyde than those with the *C. rubella* allele, yet the difference was less pronounced than for BAld. Thus, the mapped 9.5-kb interval contains the main determinant for the difference in BAld emission in our population.

Capsella Cinnamate:CoA Ligase *CNL1* Underlies the BAld Emission QTL

The mapped interval contains a predicted *DIRIGENT-LIKE* (*DIR*) gene, the complete predicted gene encoding a cinnamate:CoA ligase-like protein (termed *CNL1*), as well as the 3′ part of the coding sequence of a tandemly duplicated gene (termed *CNL2*). *Petunia* CNL catalyzes the first committed step in the peroxisomal CoA-dependent β-oxidative pathway for benzenoid biosynthesis [31, 32], making the *CNL* genes plausible candidates for the causal locus. Expression of the *DIR* gene was found at comparable levels in leaves and petals, whereas *CNL1* and *CNL2* expression from both alleles was only seen in petals, but not in leaves (Figure 3B). The *Capsella* CNL1 protein is more closely related to *Petunia* CNL and *A. thaliana* AAE11 than is *Capsella* CNL2 (Figure 3D), with both proteins containing the C-terminal SRL peroxisomal targeting sequence also found in *Petunia* CNL (Figure S3B).

To test directly for a role of the *CNL* genes in BAld biosynthesis, we transformed plants homozygous for the *C. rubella* QTL allele derived from one of the mapping HIFs with genomic constructs of the opposite *CNL* alleles from our RIL population. While neither allele of *CNL2* restored BAld emission, the *C. grandiflora* allele of *CNL1* resulted in BAld emission comparable to HIF plants homozygous for the *C. grandiflora* QTL allele (Figure 3A); by contrast, the *C. rubella*-derived allele had no effect. To test whether the causal difference between the opposite *CNL1* alleles was due to differences in the promoter region or the coding sequence, we transformed reciprocal chimeric constructs. The *C. grandiflora CNL1* coding sequence under the control of the *C. rubella* promoter resulted in the same BAld emission levels as the original *C. grandiflora* allele, whereas the reciprocal combination could not restore BAld biosynthesis. This result is consistent with the very similar expression levels of the two alleles (Figure 3B). Thus, a mutation in the transcribed region of *CNL1* is responsible for the loss of BAld emission in the *C. rubella* parent of our RIL population.

Expression of *CNL1* showed diurnal variation in transcript levels, with the peak of expression preceding the peak of BAld emission by several hours (Figure 3C). The much higher peak expression of the *C. grandiflora* allele than of the *C. rubella* allele in this experiment most likely reflects the use of the self-compatible *C. grandiflora* line here as compared to the mapping HIF in the above experiment, and it correlates well with the much higher BAld emission seen in the former than in the latter (compare Figure 1B to Figure 3A). Within the flower, the highest *CNL1* expression was detected in petals and stamens, consistent with the major role of these organs in BAld emission (Figure S2D; cf. Figure 1D).

Sequence comparison of the transcribed *CNL1* region from the opposite alleles in our RIL population identified 60 SNPs, four deletions, and three insertions (in introns and 3′ UTR) in the *C. rubella* allele (Figure S3A). Sixteen of the 60 SNPs resulted in amino acid exchanges in the encoded proteins (Figure S3B).

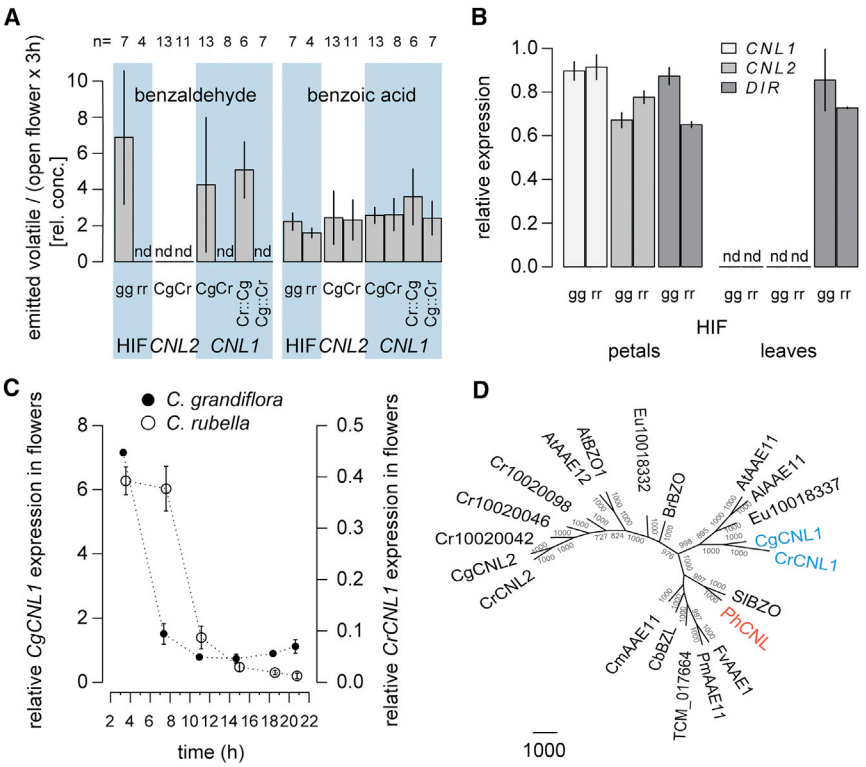

Figure 3. *Capsella* CNL1 Underlies the Loss of BAld Emission in *C. rubella*

(A) BAld and BA emission from transformants carrying the indicated constructs and the HIF background line used for transformation. *Cr*::*Cg* and *Cg*::*Cr* indicates promoter-swapping constructs. Values are mean ± SD from measurements of the indicated number of independent transformants per construct, with two measurements on consecutive days from the same individuals.

(B) qRT-PCR-based quantification of expression of *CNL1*, *CNL2*, and *DIR* genes in dissected petals and cauline leaves of plants homozygous for the *C. grandiflora*- or the *C. rubella*-derived QTL allele from the mapping HIF. Expression was normalized to the constitutively expressed *TUB* gene. Values are mean ± SD from three biological replicates. n.d., not detected.

(C) Expression of *CNL1* in the inbred, self-compatible *C. grandiflora*-like line (left y axis) and *C. rubella* (right y axis) over the course of a day, relative to the constitutively expressed *TUB* gene. Values are mean ± SD from four biological replicates.

(D) Phylogenetic analysis of *CNL* homologs from different angiosperm species. Bootstrap values (1,000 replicates) are indicated at branches. Species abbreviations are as follows: Al, *Arabidopsis lyrata*; At, *Arabidopsis thaliana*; Br, *Brassica rapa*; Cb, *Clarkia breweri*; Cg, *Capsella grandiflora*; Cm, *Cucumis melo*; Cr, *Capsella rubella*; Eu: *Eutrema salsugineum*; Fv, *Fragaria vesca*; Ph, *Petunia hybrida*; Pm, *Prunus mume*; Sl, *Solanum lycopersicum*; and TCM, *Theobroma cacao*.

See also Figure S3.

We exploited the variation in *CNL1* haplotypes in the outbreeding *C. grandiflora* to narrow down the list of potentially causal polymorphisms (Figure 4A). To this end, we sequenced the *CNL1* locus from different *C. grandiflora* individuals phenotyped for BAld emission. For all but seven of the polymorphic sites between the two haplotypes from the RIL population, we found at least one *C. grandiflora* individual homozygous for the *C. rubella*-like allele, yet emitting BAld; this ruled out these polymorphisms as causal for the loss of scent in *C. rubella* (gray font in Figure 4A). Two of the remaining seven polymorphisms caused an amino acid exchange, one a leucine-to-isoleucine exchange and the other a serine-to-arginine exchange. Of these, the serine-to-arginine exchange at position 453 (T-to-A nucleotide exchange at genomic position 7,539,424) is the most plausible causal mutation, as it is located immediately next to highly conserved amino acids predicted to be involved in adenosine monophosphate and coenzyme A binding; it involves two biochemically very dissimilar amino acids; and the serine at this position is invariant across the proteins shown in Figure 3D.

CNL1 Was Inactivated Twice Independently by De Novo Mutations in *C. rubella*

To determine the likely evolutionary origin of the presumed causal mutation in *C. rubella*, we compared *CNL1* sequences from an additional 201 *C. grandiflora* and six *C. rubella* accessions, focusing on the polymorphisms found between the two haplotypes in our RILs. Essentially all of the *C. grandiflora* accessions were from northern Greece [33]; by contrast, the six *C. rubella* accessions were from around the Mediterranean region and included one accession from Argentina. Four of the *C. rubella* accessions carried the identical haplotype to our RIL population, while the fifth differed in one synonymous SNP at the 3′ end; however, one accession from Algeria had a different haplotype that clustered more closely with other *C. grandiflora* haplotypes, yet it carried a 4 bp deletion resulting in a frameshift 795 bp downstream of the start codon, causing a premature stop codon (Figure S4A). Consistent with an inactive *CNL1* allele, this accession did not emit detectable levels of BAld (Figure 1A). Thus, at least two independent loss-of-function *CNL1* alleles are present in *C. rubella*, suggesting that BAld synthesis has been lost at least twice independently by mutations to the same gene.

We next asked whether the two *CNL1* loss-of-function mutations had been captured from standing genetic variation in the ancestral population or arisen de novo in the *C. rubella* lineage. Haplotype analysis in 201 *C. grandiflora* accessions did not find a single occurrence of the 4-bp deletion causing the premature stop codon. Six *C. grandiflora* individuals were heterozygous for the S-to-R exchange (Figure S4A); however, all of them carried this allele on long *C. rubella*-like haplotypes at *CNL1* (Figure S4B), with identity to *C. rubella* extending even into flanking genes. Three of these individuals showed complete sequence identity to the *C. rubella* sequence across one haplotype of the entire locus.

To investigate the possibility that these shared haplotypes between the six *C. grandiflora* individuals and *C. rubella* reflect the maintenance of an ancestral polymorphism rather than more

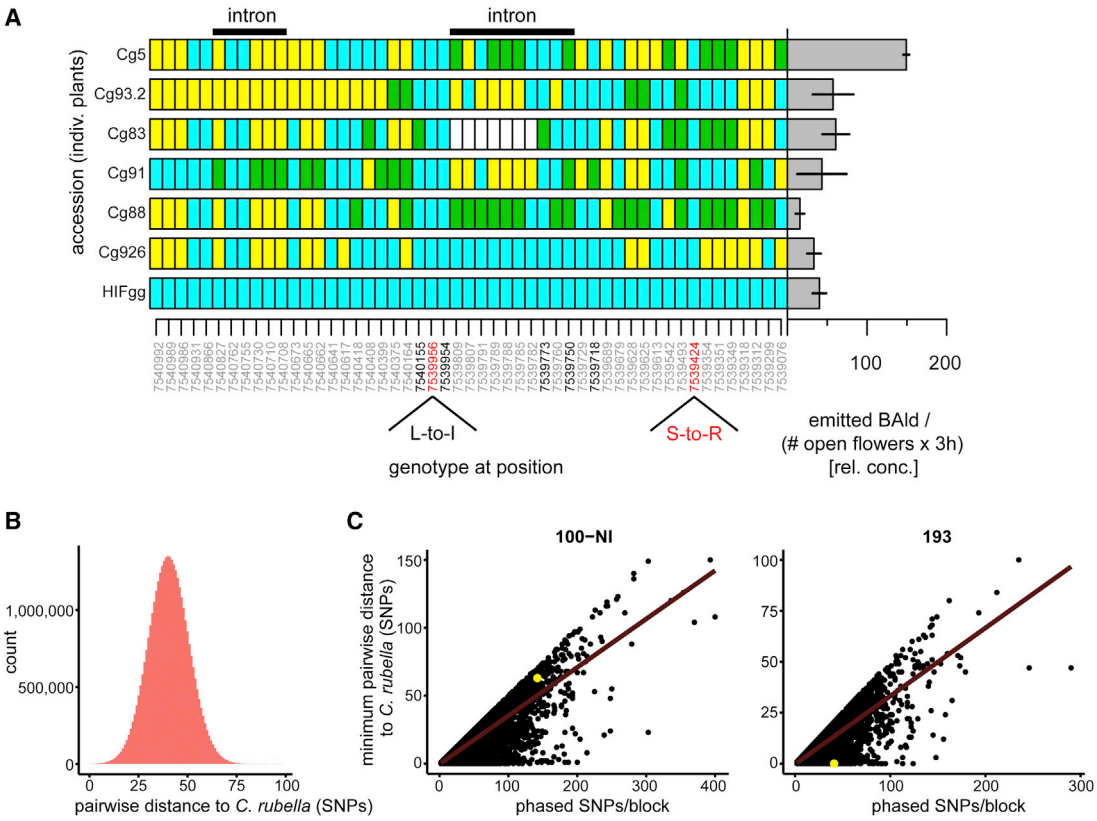

Figure 4. Population Genetic Analysis of *CNL1*

(A) Base pair positions on chromosome 2 are indicated for the 51 *CNL1* polymorphisms in the coding region and introns between Cr1504 and the segregating Cg926 RIL allele. The genotype of the phenotyped *C. grandiflora* individuals are shown (yellow, homozygous for *C. rubella* allele; blue, homozygous for *C. grandiflora* allele; green, heterozygous; white, not determined). Bars on the right show BAld emission of the respective individuals. Values are mean ± SD from three technical replicates per individual. Polymorphisms at gray marked positions are found as homozygous for the *C. rubella* allele. Positions of synonymous SNPs found to be either homozygous for the *C. grandiflora* allele or heterozygous are black. The two non-synonymous SNPs that are homozygous for the *C. grandilfora* allele in all BAld-emitting accessions are red. L, leucine; I, isoleucine; S, serine; R, arginine.

(B) Frequency distribution of simulated *C. grandiflora* haplotypes, with the indicated divergence from the *C. rubella* reference *CNL1* haplotype.

(C) Divergence of genome-wide phased haplotype blocks of the indicated *C. grandiflora* individuals from the *C. rubella* reference. Only the minimum-diverged haplotype per locus is shown. *CNL1* haplotypes are shown in yellow. The left panel shows an individual without, and the right panel shows an individual with the putatively causal S-to-R mutant *CNL1* allele. Solid lines show regression lines.

See also Figures S3 and S4.

recent introgression, we performed coalescent simulations. In particular, we simulated a demographic model with no gene flow based on [23], assuming a divergence time of 100 kya, a mutation rate of 7×10^{-9}, and a conservative recombination rate of ~2 cM/Mb. Based on the divergence of the simulated *C. grandiflora* haplotypes to the *C. rubella* reference, this indicated that the probability of finding three or more haplotypes identical to the *C. rubella* reference in our *C. grandiflora* sample was essentially zero (Figure 4B). To account for the lower effective recombination rate in the selfing *C. rubella*, we repeated the simulations with a very low ancestral population recombination rate (population recombination rate rho = $4N_e r$ = 5 for the entire region). This again indicated a <3% probability of finding three or more haplotypes identical to the *C. rubella* reference in our *C. grandiflora* sample. When comparing the divergence of genome-wide phased haplotype blocks of *C. grandiflora* individuals to the *C. rubella* reference, the *CNL1* haplotypes in individuals with suspected introgression showed high identity to *C. rubella* (Figure 4C). However, compared to individuals without the *C. rubella CNL1* haplotype, these samples did not show unusually high haplotype sharing across the genome, suggesting that haplotype sharing at CNL1 reflects a past history of gene flow rather than a very recent hybridization.

Overall, these results strongly support the notion that the S-to-R exchange allele in these individuals is derived from introgression of a *C. rubella*-like haplotype back into *C. grandiflora*, rather than maintenance of an ancestral polymorphism. Therefore, we conclude that the two loss-of-function alleles are likely to have arisen as de novo mutations in the *C. rubella* lineage.

DISCUSSION

Efficient communication between flowering plants and pollinators is crucial for successful plant reproduction, particularly for outbreeding species. Besides visual cues, chemical signals are used by plants to attract pollinating insects. Here we demonstrate

that plants of the outbreeding species *C. grandiflora* emit substantial amounts of BAld as the major scent compound. By contrast, the derived selfing species *C. rubella* no longer produces BAld, and this loss of scent forms part of its wider selfing syndrome. BAld emitted by other plant species contributes to attracting both pollinators (e.g., bees and syrphid flies) and herbivores [34]. Loss of BAld emission is due to inactivation of the *CNL1* gene coding for cinnamate:CoA ligase. Surprisingly, by surveying a number of *C. rubella* accessions sampled from a large geographical area, we have detected two independent loss-of-function alleles of *CNL1* in *C. rubella*, suggesting that floral scent has been lost at least twice independently by parallel de novo mutations to the same gene after the transition from outbreeding to selfing.

The CNL enzyme had been identified in *Petunia* as catalyzing the first committed step for BA synthesis via the peroxisomal CoA-dependent β-oxidative pathway (Figure S1B). The importance of *Petunia* CNL for the synthesis of BAld is unclear, however, with contradictory results from independent studies [31, 32]. In vivo labeling and flux modeling have suggested that the bulk of BAld in *Petunia* flowers is derived from the CoA-independent, non-β-oxidative pathway [14]. By contrast, our results argue for a major role of the CoA-dependent, but non-β-oxidative hybrid pathway in *Capsella*; this would be dependent on a cinnamoyl-CoA intermediate formed by CNL1 but then proceed via a non-β-oxidative route, similar to what has been described for *Hypericum androsaemum* [17] (Figure S1B). Theoretically, a β-oxidative pathway is possible, but as this would result in BA as an intermediate, BAld would need to be generated from BA via the reverse reaction of BAld dehydrogenase or via a carboxylic acid reductase. Previously characterized BAld dehydrogenases catalyze an essentially irreversible reaction from BAld to BA [35, 36]; and while carboxylic acid reductases are found in bacteria and fungi [37], to our knowledge no such enzymes have been described in plants and none appears to be encoded by the *Capsella* genome. Thus, our results provide strong genetic evidence for the biological importance of the CoA-dependent, non-β-oxidative pathway for BAld synthesis in *Capsella*.

The above hypothesis also raises a conundrum, however, concerning the source of the remaining BA that is detected in *C. rubella*. Assuming that there is indeed little flux through a CoA-independent, non-β-oxidative pathway, as suggested by the absence of detectable BAld in *C. rubella* plants, yet at the same time the CoA-dependent branches of BA synthesis are compromised by the loss-of-function *CNL1* alleles, then where does BA in *C. rubella* come from? One possibility is via the *CNL2* gene. Although this does not influence variation in BAld synthesis, it is conceivable that it provides cinnamoyl-CoA for the canonical β-oxidative pathway, leading to BA synthesis. Analysis of *cnl1 cnl2* double mutants will be required to test this.

CNL1 has been inactivated at least twice independently by parallel novel mutations in *C. rubella*; similarly, loss of *CNL* function underlies the loss of scent in *P. exserta* compared to *P. axillaris* [18]. What makes *CNL1* a preferred target for the evolutionary loss of BAld from floral scent, and is this loss more than a degenerative process that serves an adaptive function? A plausible hypothesis to explain the repeated inactivation of CNL1 is based on its presumed role as the first committed enzyme step for the synthesis of BAld in *Capsella*. Interfering with this step appears to cause selective effects on BAld synthesis without affecting BA levels. Besides this limited pleiotropy, inactivating the first enzymatic step in a biosynthetic pathway also has the theoretical advantage that no potentially toxic intermediates accumulate.

Concerning the repeated loss of BAld emission in *C. rubella*, an attractive explanation would be provided by antagonistic selection on floral scent by pollinators and herbivores, which have been shown to be attracted by the same scent compounds in some species [8, 34]. In outbreeding species, where pollinator attraction is essential for reproduction, a reduction in scent emission that renders the plants less conspicuous to herbivores would incur a high fitness penalty, if it also reduces pollinator attraction. However, in selfers, pollinator attraction is no longer essential, reducing the fitness cost from being less attractive; any fitness cost imposed by herbivores would then select for reduced scent emission. Alternatively, loss of BAld emission due to inactivation of *CNL* in *Capsella* and *Petunia* may result in an unidentified metabolic benefit or simply reflect a loss of constraint. Our fine-scale HIFs, which differ only in a <10-kb region surrounding the *CNL1* locus, provide a unique resource to test these and other hypotheses about the evolution of scent and the ecological significance of scent variation by field trials in environments with relevant pollinators and herbivores.

ACCESSION NUMBERS

The accession numbers for the *C. rubella* and *C. grandiflora CNL1* alleles reported in this paper are GenBank: KX960913, KX960914, and KX960915.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.10.026.

AUTHOR CONTRIBUTIONS

Conceptualization, C.S. and M.L.; Investigation, C.S. and F.M.; Formal Analysis, C.S., C.K., T.V.K., and S.I.W.; Writing – Original Draft, C.S. and M.L.; Writing – Review & Editing, all authors; Supervision, S.I.W., M.H., and M.L.

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REFERENCES

1. Harder, L.D., and Johnson, S.D. (2009). Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. New Phytol. *183*, 530–545.

2. Stebbins, G.L. (1970). Adaptive radiation of reproductive characteristics in angiosperms. I. Pollination mechanisms. Annu. Rev. Ecol. Syst. *1*, 307–326.
3. Raguso, R.A. (2008). Wake up and smell the roses: the ecology and evolution of floral scent. Annu. Rev. Ecol. Evol. Syst. *39*, 549–569.
4. Schiestl, F.P., and Johnson, S.D. (2013). Pollinator-mediated evolution of floral signals. Trends Ecol. Evol. *28*, 307–315.
5. Schiestl, F.P., and Schlüter, P.M. (2009). Floral isolation, specialized pollination, and pollinator behavior in orchids. Annu. Rev. Entomol. *54*, 425–446.
6. Kessler, D., Diezel, C., Clark, D.G., Colquhoun, T.A., and Baldwin, I.T. (2013). Petunia flowers solve the defence/apparency dilemma of pollinator attraction by deploying complex floral blends. Ecol. Lett. *16*, 299–306.
7. Galen, C., Kaczorowski, R., Todd, S.L., Geib, J., and Raguso, R.A. (2011). Dosage-dependent impacts of a floral volatile compound on pollinators, larcenists, and the potential for floral evolution in the alpine skypilot Polemonium viscosum. Am. Nat. *177*, 258–272.
8. Theis, N., and Adler, L.S. (2012). Advertising to the enemy: enhanced floral fragrance increases beetle attraction and reduces plant reproduction. Ecology *93*, 430–435.
9. Klahre, U., Gurba, A., Hermann, K., Saxenhofer, M., Bossolini, E., Guerin, P.M., and Kuhlemeier, C. (2011). Pollinator choice in Petunia depends on two major genetic loci for floral scent production. Curr. Biol. *21*, 730–739.
10. Schlüter, P.M., Xu, S., Gagliardini, V., Whittle, E., Shanklin, J., Grossniklaus, U., and Schiestl, F.P. (2011). Stearoyl-acyl carrier protein desaturases are associated with floral isolation in sexually deceptive orchids. Proc. Natl. Acad. Sci. USA *108*, 5696–5701.
11. Byers, K.J.R.P., Vela, J.P., Peng, F., Riffell, J.A., and Bradshaw, H.D., Jr. (2014). Floral volatile alleles can contribute to pollinator-mediated reproductive isolation in monkeyflowers (Mimulus). Plant J. *80*, 1031–1042.
12. Dudareva, N., Cseke, L., Blanc, V.M., and Pichersky, E. (1996). Evolution of floral scent in Clarkia: novel patterns of S-linalool synthase gene expression in the C. breweri flower. Plant Cell *8*, 1137–1148.
13. Nam, K.H., Dudareva, N., and Pichersky, E. (1999). Characterization of benzylalcohol acetyltransferases in scented and non-scented Clarkia species. Plant Cell Physiol. *40*, 916–923.
14. Boatright, J., Negre, F., Chen, X., Kish, C.M., Wood, B., Peel, G., Orlova, I., Gang, D., Rhodes, D., and Dudareva, N. (2004). Understanding in vivo benzenoid metabolism in petunia petal tissue. Plant Physiol. *135*, 1993–2011.
15. Dudareva, N., Klempien, A., Muhlemann, J.K., and Kaplan, I. (2013). Biosynthesis, function and metabolic engineering of plant volatile organic compounds. New Phytol. *198*, 16–32.
16. Sheehan, H., Hermann, K., and Kuhlemeier, C. (2012). Color and scent: how single genes influence pollinator attraction. Cold Spring Harb. Symp. Quant. Biol. *77*, 117–133.
17. Abd El-Mawla, A.M.A., and Beerhues, L. (2002). Benzoic acid biosynthesis in cell cultures of Hypericum androsaemum. Planta *214*, 727–733.
18. Amrad, A., Moser, M., Mandel, T., de Vries, M., Schuurink, R.C., Freitas, L., and Kuhlemeier, C. (2016). Gain and loss of floral scent production through changes in structural genes during pollinator-mediated speciation. Curr. Biol. Published online December 1, 2016. http://dx.doi.org/10.1016/j.cub.2016.10.023.
19. Barrett, S.C. (2002). The evolution of plant sexual diversity. Nat. Rev. Genet. *3*, 274–284.
20. Sicard, A., and Lenhard, M. (2011). The selfing syndrome: a model for studying the genetic and evolutionary basis of morphological adaptation in plants. Ann. Bot. (Lond.) *107*, 1433–1443.
21. Doubleday, L.A.D., Raguso, R.A., and Eckert, C.G. (2013). Dramatic vestigialization of floral fragrance across a transition from outcrossing to selfing in Abronia umbellata (Nyctaginaceae). Am. J. Bot. *100*, 2280–2292.
22. Hurka, H., Friesen, N., German, D.A., Franzke, A., and Neuffer, B. (2012). 'Missing link' species Capsella orientalis and Capsella thracica elucidate evolution of model plant genus Capsella (Brassicaceae). Mol. Ecol. *21*, 1223–1238.
23. Brandvain, Y., Slotte, T., Hazzouri, K.M., Wright, S.I., and Coop, G. (2013). Genomic identification of founding haplotypes reveals the history of the selfing species Capsella rubella. PLoS Genet. *9*, e1003754.
24. Foxe, J.P., Slotte, T., Stahl, E.A., Neuffer, B., Hurka, H., and Wright, S.I. (2009). Recent speciation associated with the evolution of selfing in *Capsella*. Proc. Natl. Acad. Sci. USA *106*, 5241–5245.
25. Guo, Y.L., Bechsgaard, J.S., Slotte, T., Neuffer, B., Lascoux, M., Weigel, D., and Schierup, M.H. (2009). Recent speciation of Capsella rubella from Capsella grandiflora, associated with loss of self-incompatibility and an extreme bottleneck. Proc. Natl. Acad. Sci. USA *106*, 5246–5251.
26. Dudareva, N., Murfitt, L.M., Mann, C.J., Gorenstein, N., Kolosova, N., Kish, C.M., Bonham, C., and Wood, K. (2000). Developmental regulation of methyl benzoate biosynthesis and emission in snapdragon flowers. Plant Cell *12*, 949–961.
27. Loughrin, J.N., Hamilton-Kemp, T.R., Andersen, R.A., and Hildebrand, D.F. (1990). Volatiles from flowers of *Nicotiana sylvestris*, *N. otophora* and *Malus* × *domestica*: headspace components and day/night changes in their relative concentrations. Phytochemistry *29*, 2473–2477.
28. Schiestl, F.P., Ayasse, M., Paulus, H.F., Erdmann, D., and Francke, W. (1997). Variation of floral scent emission and postpollination changes in individual flowers of *Ophrys sphegodes* subsp. *sphegodes*. J. Chem. Ecol. *23*, 2881–2895.
29. Sicard, A., Stacey, N., Hermann, K., Dessoly, J., Neuffer, B., Bäurle, I., and Lenhard, M. (2011). Genetics, evolution, and adaptive significance of the selfing syndrome in the genus Capsella. Plant Cell *23*, 3156–3171.
30. Knauer, A.C., and Schiestl, F.P. (2015). Bees use honest floral signals as indicators of reward when visiting flowers. Ecol. Lett. *18*, 135–143.
31. Colquhoun, T.A., Marciniak, D.M., Wedde, A.E., Kim, J.Y., Schwieterman, M.L., Levin, L.A., Van Moerkercke, A., Schuurink, R.C., and Clark, D.G. (2012). A peroxisomally localized acyl-activating enzyme is required for volatile benzenoid formation in a Petuniaxhybrida cv. 'Mitchell Diploid' flower. J. Exp. Bot. *63*, 4821–4833.
32. Klempien, A., Kaminaga, Y., Qualley, A., Nagegowda, D.A., Widhalm, J.R., Orlova, I., Shasany, A.K., Taguchi, G., Kish, C.M., Cooper, B.R., et al. (2012). Contribution of CoA ligases to benzenoid biosynthesis in petunia flowers. Plant Cell *24*, 2015–2030.
33. Josephs, E.B., Lee, Y.W., Stinchcombe, J.R., and Wright, S.I. (2015). Association mapping reveals the role of purifying selection in the maintenance of genomic variation in gene expression. Proc. Natl. Acad. Sci. USA *112*, 15390–15395.
34. Theis, N. (2006). Fragrance of Canada thistle (Cirsium arvense) attracts both floral herbivores and pollinators. J. Chem. Ecol. *32*, 917–927.
35. MacKintosh, R.W., and Fewson, C.A. (1988). Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II from Acinetobacter calcoaceticus. Purification and preliminary characterization. Biochem. J. *250*, 743–751.
36. Shaw, J.P., and Harayama, S. (1990). Purification and characterisation of TOL plasmid-encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase of Pseudomonas putida. Eur. J. Biochem. *191*, 705–714.
37. He, A., Li, T., Daniels, L., Fotheringham, I., and Rosazza, J.P. (2004). Nocardia sp. carboxylic acid reductase: cloning, expression, and characterization of a new aldehyde oxidoreductase family. Appl. Environ. Microbiol. *70*, 1874–1881.