Adaptive divergence in shoot gravitropism creates hybrid sterility in an Australian wildflower

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Natural selection is responsible for much of the diversity we see in nature. Just as it drives the evolution of new traits, it can also lead to new species. However, it is unclear whether natural selection conferring adaptation to local environments can drive speciation through the evolution of hybrid sterility between populations. Here, we show that adaptive divergence in shoot gravitropism, the ability of a plant's shoot to bend upwards in response to the downward pull of gravity, contributes to the evolution of hybrid sterility in an Australian wildflower, _Senecio lautus_. We find that shoot gravitropism has evolved multiple times in association with plant height between adjacent populations inhabiting contrasting environments, suggesting that these traits have evolved by natural selection. We directly tested this prediction using a hybrid population subjected to eight rounds of recombination and three rounds of selection in the field. Our experiments revealed that shoot gravitropism responds to natural selection in the expected direction of the locally adapted population. Using the advanced hybrid population, we discovered that individuals with extreme differences in gravitropism had more sterile crosses than individuals with similar gravitropic responses, which were largely fertile, indicating that this adaptive trait is genetically correlated with hybrid sterility. Our results suggest that natural selection can drive the evolution of locally adaptive traits that also create hybrid sterility, thus revealing an evolutionary connection between local adaptation and the origin of new species.

local adaptation | intrinsic reproductive isolation | hybrid sterility | speciation | natural selection

Ever since Darwin's work on the origin of species by natural selection (1), researchers have sought to understand how natural selection creates reproductive barriers between populations (2). On one hand, many studies have established that adaptation to contrasting environments often reduces migrant and hybrid fitness in the wild, a process commonly known as extrinsic reproductive isolation (3). These extrinsic barriers to reproduction can dramatically reduce gene flow between populations (4, 5); however, they only act in the local environment of populations. On the other hand, intrinsic reproductive barriers such as hybrid sterility can accumulate regardless of the environment (6) and are therefore expected to be more stable over time and contribute to the completion of speciation (2). One way in which natural selection can simultaneously create both extrinsic and intrinsic reproductive isolation is when the beneficial mutations that drive local adaptation in each population also fail to interact properly between populations through negative epistasis (7) (i.e., Dobzhansky–Muller genetic incompatibilities). However, given the paucity of examples that directly link loci contributing to both local adaptation and intrinsic reproductive isolation, we remain largely ignorant as to whether natural selection drives speciation through the concomitant evolution of extrinsic and intrinsic barriers between populations (8).

Two notable empirical examples provide a genetic link between local adaptation and intrinsic reproductive isolation, one via pleiotropy (9) and the other via tight genetic linkage (10). Selection for pathogen resistance genes in _Arabidopsis thaliana_ results in a pleiotropic effect of hybrid necrosis, which dramatically lowers reproductive success (9). In contrast, tight genetic linkage between alleles selected for copper tolerance and alleles that cause hybrid lethality led to divergence between populations of _Mimulus guttatus_ growing next to copper mines and those occupying typical habitats (10). Although these mechanisms might often drive species divergence (11), we require more genetic and ecological studies to further understand when natural selection drives the correlated evolution of local adaptation and intrinsic reproductive isolation (12). To date, most research on the genetics of intrinsic reproductive isolation suggest that several mechanisms are driving the evolution of hybrid sterility across taxa including genetic conflict (e.g., the evolution of distorter genes and their suppressors) (13) and parental conflict (e.g., endosperm failure in plants) (14) [but refer to Presgraves (15) for a complete review]. However, it remains unclear whether the local environment of a population plays a key role in creating intrinsic reproductive

Significance

New species originate as populations become reproductively isolated from one another. Despite recent progress in uncovering the genetic basis of reproductive isolation, it remains unclear whether intrinsic reproductive barriers, such as hybrid sterility, can evolve as a by-product of local adaptation to contrasting environments. Here, we show that differences in a plant's response to the pull of gravity have repeatedly evolved amongst coastal populations of an Australian wildflower, thus implicating a role of natural selection in their evolution. We found a strong genetic association between variation in this adaptive trait and hybrid sterility, suggesting that intrinsic reproductive barriers contribute to the origin of new species as populations adapt to heterogeneous environments.

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barriers [refer to Fishman and Sweigart (16) for a review of the mechanisms], thus limiting our understanding of the mechanisms creating genetic correlations between local adaptation and intrinsic reproductive isolation.

To study the contribution of local adaptation to the evolution of intrinsic reproductive barriers, we take advantage of the repeated evolution of divergent growth habits in an Australian wildflower, *Senecio lautus* (G. Forst. ex Willd) (17, 18). Coastal and alpine populations exhibit erect or prostrate growth habits depending on whether they inhabit sheltered environments (sand dunes or alpine gullies) or exposed environments (rocky headlands or alpine meadows), respectively (Fig. 1). Previous population genetic studies in *S. lautus* found that different sets of genes related by similar biological functions were repeatedly differentiated between populations with these two contrasting growth habits (19, 20). Many of these sets were enriched with genes that had functions related to the transport and regulation of the hormone, auxin. Auxin is known for its role in plant growth and development, including contributions to variation in height (21, 22) and branching patterns (23, 24), as well as in multiple aspects of plant reproduction such as ovule development, fertilization, and seed development (25). Given the observed divergence in height, branching, and barriers to reproduction between erect and prostrate populations of *S. lautus* (20, 26), the evolution of auxin-related genes could be the key to explaining both adaptation and reproductive isolation in *S. lautus*. To explore this hypothesis, we examined traits that are directly controlled by the movement of auxin in the aerial section of the plant. Mutant surveys in *Arabidopsis* revealed that genes influencing the biosynthesis, transport, or signaling of the auxin hormone are required for shoot gravitropism or the reorientation of the stem after a 90° rotation (23, 27–29). We reasoned that if divergence in auxin-related genes contributed to local adaptation, then natural selection would create divergence in shoot gravitropism between adjacent erect and prostrate populations of *S. lautus*. Finally, because of the pleiotropic effects of auxin-related genes on reproduction (23, 25), adaptation via hormonal pathways could also lead to correlated responses that incidentally drive the evolution of intrinsic reproductive isolation between populations.

We tested these predictions on 16 *S. lautus* populations (Fig. 1 and *SI Appendix*, Fig. S1 and Table S1) inhabiting sand dunes (Dune populations), rocky headlands (Headland populations), alpine gullies (Alpine-sheltered), and alpine meadows (Alpine-exposed). Dune populations are continually exposed to high temperatures, high solar radiation, low salinity, and low-nutrient sand substrates, whereas Headland populations are exposed to high salinity, high nutrients, and powerful winds (30). These Dune and Headland ecotypes are genetically grouped into two major monophyletic clades based on geography (eastern and southeastern Australia) (31, 32). Adjacent Dune and Headland populations at each locality (population pair hereafter) are often sister taxa and experience very little gene flow (32), indicating that they have evolved their contrasting growth habits independently multiple times. Hybrid sterility in F1 Dune–Headland hybrids is generally weak (30, 33) but strong in the F2 generation (30), and hybrid sterility has also been observed in F2 hybrids created from the equal contribution of four ecotypes (Dune, Headland, Tableland, and Woodland) (34). The presence of reproductive isolation between multiple locally adapted erect and prostrate populations of *S. lautus* provides an excellent opportunity to understand how natural selection drove the evolution of morphological and physiological traits that incidentally created intrinsic reproductive barriers between them.

Results

Divergence in Shoot Gravitropism Is Auxin Dependent in *S. lautus*. We found that synthetic auxin and auxin transport inhibitors reduce the gravitropic response in *S. lautus*. We grew Dune ($n = 90$) and Headland ($n = 98$) seeds collected from Lennox Head treated with synthetic auxin 2,4-D (2,4-dichlorophenoxyacetic acid), and polar auxin transport inhibitor NPA (naphthylphthalamic acid) (35, 36). Because a gravitropic response requires an auxin concentration gradient, we reasoned that removing the gradient would reduce the gravitropic angle (*SI Appendix*, Fig. S2). As expected, the addition of synthetic auxin 2,4-D reduced the gravitropic angle in both Dune and Headland individuals ($F_{1,39} = 63.58$, $P < 0.0001$, and $R^2 = 0.70$). Similarly, the addition of auxin transport inhibitor NPA also reduced the gravitropic angle in Dune and Headland individuals ($F_{1,38} = 10.83$, $P = 0.0022$, and $R^2 = 0.31$). Consistent with observations in other plant species, these results imply that shoot gravitropism is auxin dependent in *S. lautus*.
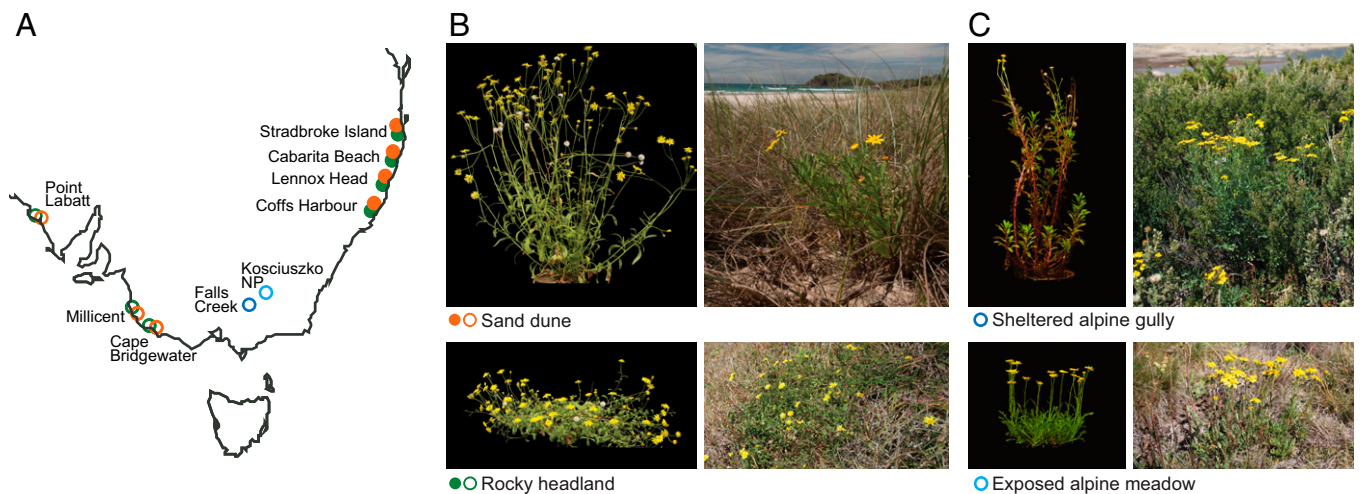

Fig. 1. Sample locations and growth habit differences between adjacent *S. lautus* populations. (*A*) Map of Australia showing locations of the 16 populations used in this study. The seven coastal localities have a Dune (orange) and Headland (green) population occurring adjacent to each other. The populations are split into two monophyletic clades (31), the eastern clade (closed circles) and the southeastern clade (open circles). (*B*) *S. lautus* native to the sand dunes have an erect growth habit, and *S. lautus* native to the rocky headlands have a prostrate growth habit. (*C*) Alpine populations of *S. lautus* include a sheltered alpine gully and an exposed alpine meadow, containing individuals with an erect and prostrate growth habit, respectively.

Repeated Height and Gravitropism Divergence across *S. lautus* Populations. We found that plant height (a growth habit trait) predicts shoot gravitropism in *S. lautus* natural populations. First, we show that plant height in natural environments (field) strongly correlates with estimates of plant height in common garden conditions (glasshouse) ($F_{1,9} = 12.41$, $P = 0.0065$, and $R^2 = 0.66$), indicating that differences in plant height are genetic and do not arise from plastic responses to the environment. Secondly, we measured the change in angle of a seedling's shoot 24 h after a 90° rotation (28, 37), in which 0° reflects a lack of a gravitropic response and 90° describes a complete reorientation of the shoot and a large gravitropic response. Under the assumption that plant height and gravitropism are genetically correlated, we expected that short populations would have a smaller gravitropic angle than their adjacent tall population. Across all 16 natural populations, the average height of a population predicted its average gravitropic angle, with the average magnitude of gravitropism differing across the two monophyletic clades of this system (Fig. 2*A*; Height: $F_{1,13} = 17.65$, and $P = 0.0010$; Clade: $F_{1,13} = 32.58$, $P < 0.0001$; and $R^2 = 0.74$). Therefore, changes in gravitropism appear to be biologically correlated with divergent growth habit traits such as plant height, and this relationship has evolved independently in each clade.

We then looked for replicated patterns of evolution of gravitropism across the seven adjacent Dune and Headland (and Alpine) population pairs. Four population pairs showed the expected correlation, in which plants from the Headland population exhibited a smaller gravitropic angle and were shorter than plants from their adjacent Dune population (Fig. 2 *B* and *C*). The expected pattern was also observed in divergent populations from the alpine region of Australia, where the exposed Alpine population in the meadow was shorter and had a smaller gravitropic angle than the population in the sheltered alpine gully (Fig. 2*C*). In the population pair at Millicent, plant height and gravitropism did not differ between the Dune and Headland (Fig. 2*C*), possibly due to their similarity in environmental variables (31). At Stradbroke Island, we observed a difference in height in the expected direction but not gravitropism (Fig. 2*B*), and at Point Labatt, we observed a difference in gravitropism in the expected direction but not height (Fig. 2*C*). Our results show that height and gravitropism have evolved in parallel across coastal and alpine ecotypes in this system and that height and gravitropism are genetically correlated in most but not all populations, suggesting that alternate genes or pathways might be utilized (e.g., gibberellin controls dwarfism in some plants) (38, 39).

Local Adaptation Drives the Evolution of Height and Gravitropism. Through two independent sets of field adaptation experiments, we provide strong evidence that natural selection drove the evolution of height and gravitropism. In each field experiment, Dune and Headland parental and hybrid seeds were transplanted into replicated blocks at the sand dune and rocky headland at Lennox Head (*SI Appendix*, Table S1). In all field experiments, natural selection consistently favored the local Dune or Headland population over the foreign population (Fig. 3), indicating that our experiments consistently captured the divergent natural selection that drove local adaptation.

First, we predicted that short plants would have greater fitness in the rocky headland. If natural selection was driving the evolution of height, offspring produced by short hybrid parents would live longer than offspring produced by tall hybrid parents in the rocky headland. We focused on prostrate growth because it is likely the derived trait, given that the majority of *S. lautus* populations have an erect growth habit (40). Our goal was to introgress Dune alleles associated with height onto a Headland genomic background to examine their effect on fitness in the
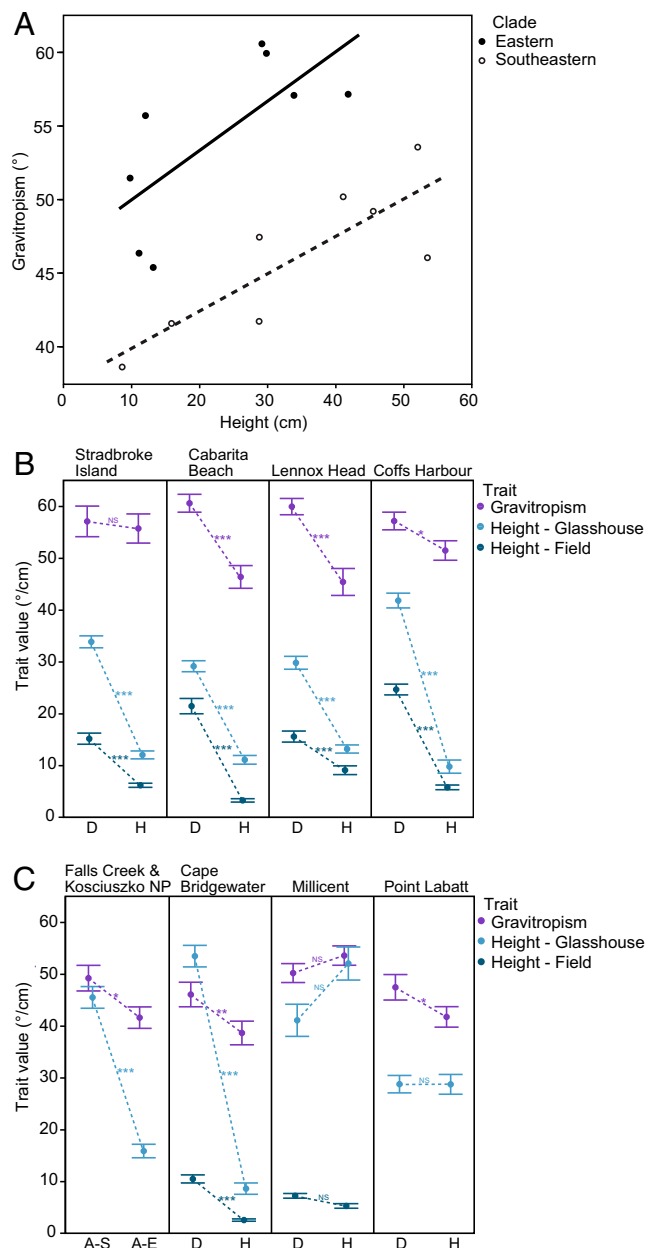

Fig. 2. Gravitropism and height variation across 16 *S. lautus* populations. (*A*) The correlation between gravitropism and height across *S. lautus* populations split into their monophyletic clades; refer to Fig. 1 for details. Each point in the graph represents a population mean where height was measured in the glasshouse and gravitropism was measured 24 h after a 90° rotation. (*B* and *C*) Divergence in gravitropism (°), height (centimeters) in the glasshouse, and height in the field between adjacent *S. lautus* populations (D = Dune, H = Headland, A-S = Alpine Sheltered, and A-E = Alpine Exposed). (*B*) Eastern clade and (*C*) Southeastern clade. Height in the field for Falls Creek, Kosciuszko National Park, and Point Labatt were not measured. Data are mean ± SEM; one-tailed Student's *t* test (*SI Appendix*, Table S6), *$P \le 0.05$, **$P \le 0.01$, ***$P \le 0.001$, and NS not significant.

headland environment. Briefly, we created a F1 hybrid by crossing a Dune and Headland individual from Lennox Head, which we backcrossed to the Headland population for two generations followed by one round of crossing between the tallest 10% and the shortest 10% (*SI Appendix*, Table S2 and Fig. S3). We transplanted 558 of these seeds (from 28 families) into the rocky headland. As predicted, shorter hybrid parents produced offspring that lived longer in the rocky headland relative to
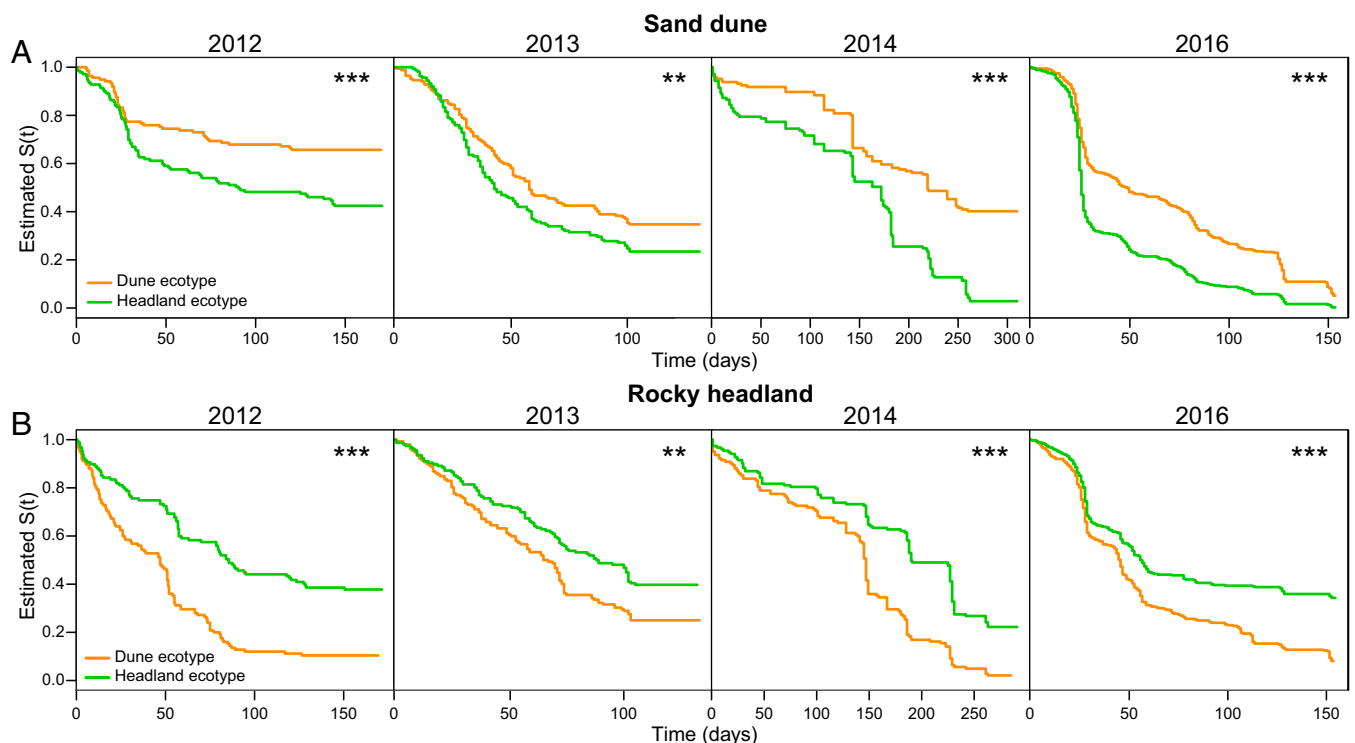
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Fig. 3. Parental survival curves in the height (2016) and gravitropism (2012 through 2014) adaptation experiments at the sand dune and rocky headland at Lennox Head. (*A* and *B*) Survival (Estimated S(t)) over the length of the field experiments is shown for the Lennox Head Dune population (orange) and the Lennox Head Headland population (green) for four independent field selection experiments in the (*A*) sand dune and (*B*) rocky headland. Asterisks indicate a significant difference in mortality risk between the Dune and Headland ecotypes (**$P \leq 0.01$ and ***$P \leq 0.001$).

offspring from taller hybrid parents ($F_{1,26.23} = 4.87$, $P = 0.0362$, and $R^2 = 0.23$). These results suggest that plant height (or traits genetically correlated) contributed to variation in early developmental fitness in the rocky headland and directly or indirectly to the evolution of divergent growth habit.

Next, we hypothesized that adaptation to contrasting dune and headland environments would lead to divergence in shoot gravitropism. If natural selection drove the evolution of gravitropism, then exposing an advanced recombinant population to multiple rounds of strong viability selection in the field would uncover a genetic correlation between fitness and gravitropism. We created a recombinant hybrid population by mating 23 Dune and 22 Headland individuals from Lennox Head and maintaining three independent genetic lines for eight generations (*SI Appendix*, Table S3), which disassembled allelic combinations from each ecotype (26) (Fig. 4*A*). We planted 2,403 F8 seeds (from 89 families) into both the sand dune and rocky headland (Fig. 4*B* and *SI Appendix*, Table S4) and conducted family based truncation selection (41). The fittest families in each environment were selected based on the highest emergence and survival (top 50%; see *Materials and Methods*). Siblings from these families were crossed in the glasshouse (within a genetic line) to produce the next generation. After three rounds of selection, we tested whether F10 families with the largest number of survivors produced offspring (F11) with the local gravitropic response under controlled conditions. Consistent with our prediction, F10 families with the largest number of survivors in the sand dune produced F11 offspring with a higher gravitropic angle (Table 1). The relationship between fitness and gravitropism was strongly associated with the F10 dam and not the F10 sire (Table 1), suggesting that maternal genetic effects might contribute to the evolution of gravitropism in the sand dunes. In contrast, we did not detect an association between the fitness of the F10 families and gravitropism of their

offspring when transplanted in the rocky headland. Instead, Headland survivors showed a positive association between the number of days until death in a controlled environment (intrinsic viability) and gravitropism (Table 1), in which individuals that died early in development had a smaller gravitropic angle (agravitropic). We therefore believe that there could be intrinsic fitness costs for agravitropic alleles on a hybrid genomic background.

We then hypothesized that adaptation to contrasting environments can recreate trait correlations observed in nature. As we described before, there is a strong correlation between gravitropism and height across many (but not all) natural populations of *S. lautus*. We expect to break this trait correlation in creating the hybrid population if different genes control these traits. If natural selection for these traits were strong, we could reconstruct the correlation after several rounds of selection in the coastal environments of the Dune and Headland populations. We found no phenotypic correlation between height and gravitropism in the F11 control population grown only in the glasshouse ($F_{1,114.3} = 0.08$, $P = 0.7801$, and $R^2 = 0.04$), indicating different genes that are not tightly linked contribute to gravitropism and height. As predicted, the phenotypic correlation between height and gravitropism was strong after three rounds of selection in the rocky headland ($F_{1,169.5} = 7.09$, $P = 0.0085$, and $R^2 = 0.27$) and weak after selection in the sand dune ($F_{1,151.3} = 3.20$, $P = 0.0756$, and $R^2 = 0.09$). Together, these results indicate that natural selection can act on standing genetic variation and recreate genetic architectures favored in these coastal environments.

The Genetics Underlying Gravitropism in *S. lautus*. We identified candidate genes underlying shoot gravitropism in *S. lautus* through selective genotyping on the tails of the gravitropic distribution of the F11 hybrid populations (Fig. 4*C*; Dune
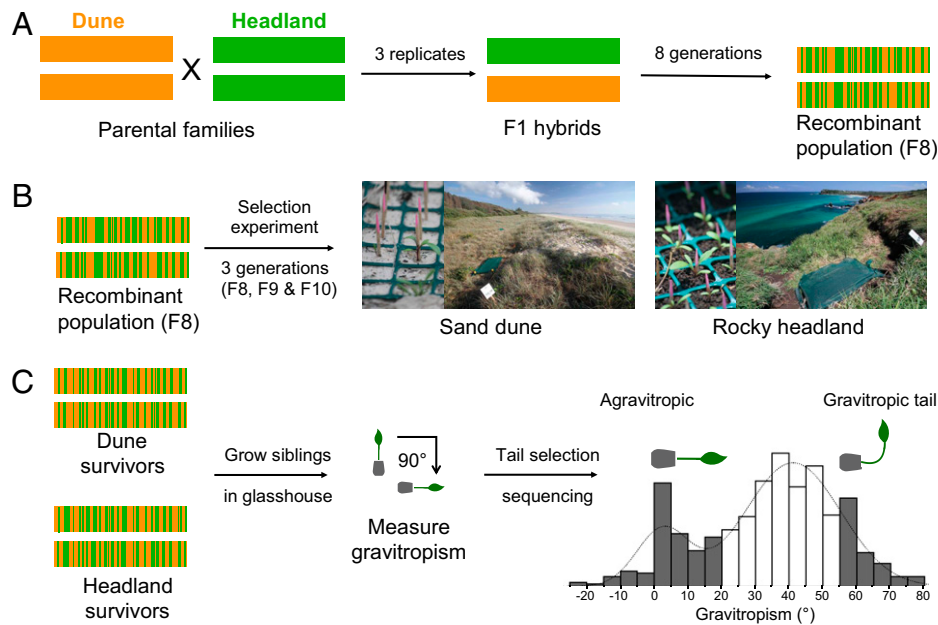
Fig. 4. The creation of the recombinant hybrid generation, the design of the gravitropism adaptation experiments, and sequencing of the tails of the gravitropic distribution. (*A*) 23 parental Dune and 22 parental Headland individuals from Lennox Head were crossed randomly and with equal contribution for eight generations. (*B*) Seeds from this F8 recombinant population were glued to toothpicks and transplanted into the sand dune and rocky headland at Lennox Head. Among family–based selection occurred for three generations (F8, F9, and F10), in which full siblings from the fittest families were grown in the glasshouse and crossed among their respective genetic lines (A, B, and C) and their environment (Dune survivors or Headland survivors). An inbred control was kept in the glasshouse and underwent the same crossing scheme but free from viability selection. (*C*) Gravitropism was measured in the F11 recombinant population by reorientating the plant by 90°. Here, agravitropic plants are defined as individuals with gravitropic angles <20°, while gravitropic plants have gravitropic angles >56° as they reorient their growth and subsequently grow upright. Individuals in the tails of the gravitropism distribution were sequenced on four lanes of an Illumina sequencing platform (HiSeq 4000).

survivors, Headland survivors, and Control). We genotyped individuals with the smallest gravitropic angle (agravitropic tail, <20°; mean of tail = 6.46 ± 1.10°, and $n = 68$) and the largest gravitropic angle (gravitropic tail, >56°; mean of tail = 62.03 ± 0.45°, and $n = 77$). We found 55 loci (0.2% of all single nucleotide polymorphisms [SNPs]) across 49 genomic contigs (Dataset S1) with an allelic difference in the 99.8% quantile ($0.15 < p < 0.22$), indicating a polygenic basis for gravitropism. We discovered that these candidate gene regions disproportionally contained homologous genes with predicted gene ontology categories of transport and localization of molecules within and between cells (*SI Appendix*, Table S5). This is consistent with expectations, as redistribution of auxin is required for a gravitropic response (27).

Five of the 55 loci (11%) are in gene homologs with functions related to the auxin pathway, including the second (*ENODL1*; early nodulin-like protein 1) and fourth (*ABA3*; molybdenum cofactor sulfurase) most-differentiated SNPs between the agravitropic and gravitropic F11 tails. *Arabidopsis wat1* mutants, an ortholog of *ENODL1*, are deficient in auxin production, display reduced auxin basipetal transport, and have down-regulated expression of many auxin-related genes, including those involved in response to auxin, auxin biosynthesis, and transport (42, 43). *ABA3*, also named *LOS5* and *SIR3*, encodes a molybdenum cofactor sulfurase essential for the activity of several enzymes controlling the biosynthesis of the plant hormone ABA and of sirtinol, which activates auxin-signaling pathways (44, 45). Furthermore, genetic loss of function of *ABA3* causes impaired auxin signaling as well as reduced ABA levels (46). In the F11 tails, *ENODL1* and *ABA3* SNPs (or tightly linked SNPs) are associated with a substantial reduction in gravitropism. Individuals homozygous for the cytosine allele (C/C) in *ENODL1* and homozygous for the guanine allele (G/G) in *ABA3* were associated with a 25° reduction in the

Table 1. General linear model for the effect of dam and sire on gravitropism (°) after a field selection experiment on a recombinant hybrid Dune and Headland population

Source	Dune				Headland			
	DF	SS	F-Ratio	*P* value	DF	SS	F-Ratio	*P* value
Dam family fitness	6	8,515.77	4.779	**<0.001**	6	1,884.31	0.701	0.650
Sire family fitness	6	1,806.62	1.014	0.424	5	2,315.36	1.033	0.405
Intrinsic viability	1	260.85	0.878	0.352	1	5,209.38	11.624	**0.001**
Genetic lines	3	1,135.49	1.275	0.290	3	3,357.19	2.497	0.067
Temporal block	2	193.14	0.325	0.724	2	2,234.96	2.494	0.090

Field selection experiments were performed on F8, F9, and F10 hybrid generations to achieve three rounds of selection in the sand dune and rocky headland at Lennox Head (refer to Fig. 4 for the experimental design). Dam and sire fitness are the F10 family fitness values for the individuals that were crossed to create the F11 offspring in which gravitropism was measured. Intrinsic viability is the number of days until death of the F11 generation in the controlled temperature room. This experiment was conducted three times (temporal block) with three independent genetic lines. DF, degrees of freedom and SS, sum of squares.
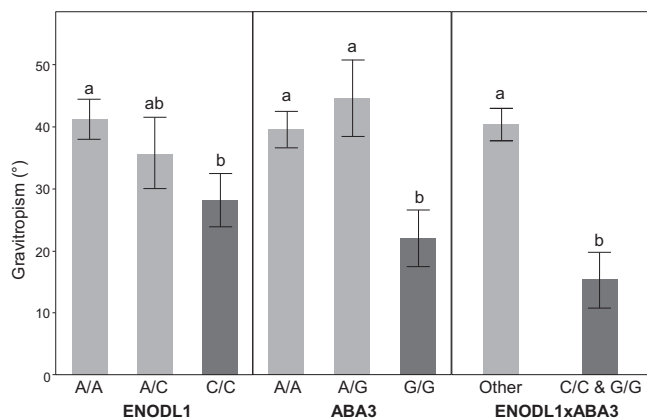
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Fig. 5. Association between *ENODL1* and *ABA3* alleles and gravitropism in *S. lautus*. The average gravitropism angle is shown for each allelic combination, independently and when the agravitropic alleles (dark gray) are combined. Different letters denote significant differences between genotypes at a significance level of α = 0.05.

gravitropic angle relative to all other genotype combinations (Fig. 5; $t_{34.30}$ = 4.86, P < 0.0001). This indicates that these genotypes covary with the recessive trait of not sensing or reacting to the pull of gravity. These results are consistent with auxin-related genes contributing to the evolution of gravitropism in *S. lautus*, with *ENODL1* and *ABA3* being strong candidates.

We discovered that these candidate SNPs in *ENODL1* and *ABA3* evolved in the expected direction of natural selection both in nature and under experimental conditions. In the F11 tails, the allelic frequency of the candidate SNP in *ENODL1* and in *ABA3* shifted toward the naturally occurring allelic frequency of the Dune and Headland population at Lennox Head, with the same gravitropism phenotype (Dataset S1). For instance, the alleles favored in the agravitropic F11 tail occur at high frequencies in the Headland natural population (*ENODL1* C = 0.87 and *ABA3* G = 0.89), with the alternate alleles favored in the gravitropic F11 tail and abundant in the Dune natural population (*ENODL1* adenine allele [A] = 0.69 and *ABA3* A = 0.97). Our results also show that an interaction between alleles of the two genes was reconstituted during the multiyear transplant experiment at Lennox Head. Specifically, we discovered that *ENODL1* and *ABA3* were in strong linkage disequilibrium in the survivors of the gravitropism adaptation experiment in the rocky headland (Fisher's exact test, n = 57, and P = 0.0008) and sand dune (Fisher's exact test, n = 48, and P = 0.0107) but not in the control population reared in the glasshouse (Fisher's exact test, n = 37, and P = 0.4093). These results suggest that local adaptation is driving the evolution of these loci. Overall, our results are consistent with polygenic adaptation of *S. lautus* to coastal environments via the evolution of auxin-related genes.

The Consequences of Gravitropism Divergence for the Evolution of Hybrid Sterility. By testing whether variation in an adaptive trait in *S. lautus* leads to reproductive incompatibilities, we discovered that gravitropism divergence in F11 hybrids leads to hybrid sterility. We performed 28 crosses in families within the agravitropic tail, 37 crosses in families within the gravitropic tail, and 67 crosses between these agravitropic and gravitropic tails. Consistent with a strong genetic association between gravitropism and intrinsic reproductive isolation in *S. lautus*, we found that hybrid sterility was more common (Odd's ratio = 4.8×, P = 0.0188) in crosses between F11 agravitropic and gravitropic plants (21%) than within each of these tails (5%; LR χ^2 = 6.86, df = 1, and P = 0.0088). In other words, we observed

hybrid sterility between F11 agravitropic and gravitropic individuals and reproductive compatibility between individuals with similar gravitropism values. Additionally, these crosses displayed largely symmetric effects on reducing hybrid fitness: 12.3% of crosses were sterile in reciprocal crosses (n = 17), while 4.3% had differences in sterility in reciprocal crosses (n = 6; these were removed from analyses). This pattern of crossing failures indicates that gravitropism alleles have linked or pleiotropic effects on hybrid sterility in *S. lautus*.

We found that genetic line, F11 population, or specific individuals did not drive the association found between gravitropism and hybrid sterility. Crosses within (n = 77) and between (n = 55) the three genetic lines, regardless of their gravitropic response, did not affect hybrid sterility (LR χ^2 = 1.10, df = 1, and P = 0.2937). Therefore, the hybrid sterility observed in the F11s is unlikely a result of genetic drift creating incompatible differences between the genetic lines. Crosses within (n = 80) and between (n = 52) the three F11 populations (Dune survivors, Headland survivors, and Control) did not affect hybrid sterility in agravitropic and gravitropic individuals (LR χ^2 = 0.15, df = 1, and P = 0.6995), increasing the likelihood that gravitropism and not another trait responding to selection in one of these environments causes intrinsic reproductive isolation in these populations. Next, we determined whether specific F11 individuals (n = 129) drove this association between gravitropism and hybrid sterility. Of the 32 individuals that participated as one of the parents in a sterile cross, 26 of them successfully produced seed when crossed against a separate individual. Thus, sterility is specific to each parental combination, consistent with the idea that hybrid sterility is polymorphic in the system, a result consistent with the segregation of postzygotic reproductive isolation across populations in other systems (47, 48).

Lastly, we found that the interaction between *ENODL1* and *ABA3* SNPs did not have a significant effect on sterility (Table 2). However, the *ABA3* A allele was positively associated with hybrid sterility, in which individuals with the gravitropic favored A allele, which is dominant to the G allele (Fig. 5), had a higher percentage of failed crosses (Table 2). This suggests that *ABA3* or a tightly linked gene might play a role in creating the observed genetic correlation between adaptive evolution of shoot gravitropism and hybrid sterility in *S. lautus*.

Discussion

Whether local adaptation commonly drives the formation of hybrid sterility and inviability is still a topic of debate (5, 16). These intrinsic reproductive barriers are believed to evolve late in the speciation process, often after extrinsic and prezygotic barriers have formed (e.g., immigrant inviability or assortative mating) (4), and genetic drift or natural selection has led to the subsequent accumulation of genetic incompatibilities (2). There are few studies (9, 10) on the genetics of local adaptation and intrinsic reproductive isolation within a species, with most studies having

Table 2. General linear model for the effect of *ENODL1* and *ABA3* alleles on hybrid sterility

Source	DF	SS	F-Ratio	*P* value
ENODL1	1	6.51	0.078	0.791
ABA3	1	925.52	11.076	**0.021**
ENODL1 x *ABA3*	1	9.77	0.117	0.746

The genotypes of the F12s were predicted based on F11 parental genotypes with ambiguous genotypes removed. *ENODL1* is the allele counts for the *ENODL1* C allele in the F12's, while *ABA3* is the allele counts for the *ABA3* G allele. *ENODL1* x *ABA3* is the effect of all observed allelic combinations between these two gravitropism candidate loci and the percentage of failed crosses. DF, degrees of freedom and SS, sum of squares.

focused on the effect of nonecological evolution (e.g., genetic conflict on the evolution of Dobzhansky–Muller incompatibilities) (15). Our study is a step forward for understanding the role of polygenic adaptation in creating hybrid sterility during the early stages of speciation. Here, we provide empirical evidence to suggest that natural selection is a major driving force behind the hybrid sterility found between recently derived erect and prostrate populations of *S. lautus*. Further, we identified variation in a trait in natural populations that is dependent on divergence in specific hormonal pathways, thus suggesting that the joint function of many genes could underlie the evolution of divergent traits correlated with reproductive barriers. The results from our experiments suggest a broadly applicable explanation to the elusive link between the genetics of local adaptation and the evolution of intrinsic reproductive isolation.

Our results suggest that natural selection has contributed to the colonization of harsh environments in *S. lautus*, like those found on rocky headlands along the coast. Previous population genetic results in this system (19, 26, 31) along with those presented here suggest that the auxin pathway could facilitate plant adaptation to new habitats through concomitant changes in multiple developmental and architectural traits. Transitions from erect to prostrate growth and the associated traits of short stature and many branches are common in plants that colonize coastal headlands (49–52), indicating that there are strong selective agents common to headland environments. For example, powerful winds or salty substrates could impose early selective pressures on traits controlled by auxins such as responses to mechanical cues (53) and halotropism (54–56). As the structure of hormonal pathways is generally conserved across plant species, the evolution of similar architectures in similar environments might prove to be a general mechanism to link adaptation with the incidental evolution of traits affecting species interactions such as ecological competition and reproductive success. For instance, in *Mimulus* (38, 57) and *Arabidopsis* (39), gibberellin, another essential plant hormone, has been implicated in the evolution of dwarfism in coastal environments. In *Mimulus*, a gene from the *GA20* oxidase family is located within a chromosomal inversion previously identified to contribute to reproductive isolation between coastal and inland populations along the coast of California (38, 57, 58). These results, together with the findings we report in *Senecio*, suggest that hormonal pathways are likely involved in creating complex phenotypic differences and possibly in reproductive isolation between locally adapted populations (38, 59, 60).

In *S. lautus*, previous results support a general link between local adaptation and intrinsic reproductive isolation. G.M.W. (34) found that additive variance responsible for local adaptation in four ecotypes was lost in the creation of an F2 hybrid generation that displayed elevated levels of hybrid sterility. A similar occurrence of hybrid sterility occurred in the F2's that created the F11 generation used here (30), suggesting that genetic incompatibilities are segregating in multiple populations and ecotypes of *S. lautus*. The other generations of the hybrid population (F3 through F11) are highly fertile, implying that a large proportion of the incompatible alleles between Dune and Headland populations were removed in the F2 generation. Remarkably, we can recreate intrinsic reproductive isolation in the F11 hybrid population when we sort F11 families according to the strength of their gravitropic response; crosses between individuals with divergent gravitropism values have a reduced ability to produce seed. A likely explanation is that incompatibility alleles segregate at low frequencies in the population after the F2 generation, but we artificially increased the frequency of the incompatible alleles by selecting individuals with extreme gravitropism. This observation implies that complex multilocus epistasis could underlie the sterility in crosses between gravitropic and agravitropic individuals.

Reproductive isolation in plants can be asymmetric when some of the incompatible genes are uniparentally inherited or expressed. Asymmetries can arise from the evolution of phenomena such as cytonuclear interactions, endosperm development, maternal effects, and pollen–pistil interactions (61). Asymmetric incompatibilities in plants often affect early developmental stages, because mothers provision resources to the endosperm and contribute organelles to offspring and other processes that control the growth of embryos and gametes. In our experiments, we found two types of asymmetric effects on fitness. We discovered that maternal genotypic effects explained the correlation between parental variation in fitness in the sand dune and offspring variation in gravitropism. Additionally, we found that the strength of hybrid sterility between agravitropic and gravitropic individuals was specific to the parental combination of the cross, with a few cases having asymmetric incompatibilities. Together, these results suggest that uniparental effects might be generally important for understanding the genetic link between local adaptation and intrinsic reproductive isolation. Future work will help discover whether the asymmetric effects on fitness presented here have a common genetic basis and therefore influence the tempo of evolution of hybrid inviability and hybrid sterility in plants.

The mechanisms by which gravitropism and hybrid sterility are genetically correlated could be pleiotropy (e.g., Bomblies and Weigel) (9) or genetic linkage (e.g., Wright, Lloyd, Lowry, Macnair, and Willis) (10). Functional annotations in *Arabidopsis* support pleiotropic roles of *ABA* and *ENODL* gene families in the auxin pathway (as outlined earlier) and hybrid sterility. The ABA hormone mediates pollen sterility (62), and members of the *ENODL* gene family have a female-specific role in pollen tube reception (63), which could lead to the evolution of asymmetric incompatibilities. Furthermore, we expect our advanced recombinant population to have extremely little linkage disequilibrium; *S. lautus* natural populations have a mean haploblock size of 359 bp (20), and 11 rounds of crossing in the recombinant population would reduce this even further. However, without whole-genome sequencing, we do not know the haplotype size and frequency around these candidate genes. Therefore, we cannot convincingly rule out the possibility that a gene linked to *ABA3* or *ENODL1* contributed to the correlation that we observed between gravitropism and hybrid sterility. Only genetic experiments, including gene knockouts, will be able to directly test this hypothesis.

Given that gravitropism has a polygenic basis and auxin-related genes have many pleiotropic effects on growth, development, and reproduction, our results suggest that many genes likely drove local adaptation and only a subset of them contribute to hybrid sterility as they become part of a Dobzhansky–Muller incompatibility. This systems' view of the evolutionary process, in which pleiotropic effects are pervasive, could provide fertile ground for the origin of adaptations and new species. Future genetic studies focusing on isolating sets of loci causing hybrid sterility in *S. lautus* will reveal the molecular mechanisms by which intrinsic reproductive barriers evolve together with local adaptation and will allow testing of these predictions on the origin of new species. Overall, our study showcases a powerful strategy to explore the genetic basis of local adaptation and speciation in natural systems. We postulate that the evolution of hormonal pathways in plants provides a simple mechanism for the rapid modification of coregulated traits that facilitate the colonization of new habitats and the correlated evolution of hybrid sterility.

Materials and Methods

Synthetic Auxin and Auxin Transport Inhibitor Experiments. We tested if auxins govern gravitropism in *S. lautus* by evaluating gravitropic responses in

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seedlings treated with chemicals affecting auxin signaling. Specifically, we used 2,4-D, a carrier-dependent synthetic auxin (35, 64), and NPA, an efflux inhibitor (36). Gravitropism was measured in vitro on agar plates on ~40 maternal families from Lennox Head sand dune and rocky headland populations. Seeds from an ecotype were combined in a falcon tube and were first sterilized with a quick rinse in 70% EtOH followed by four 10-min inversions in a sterilizing solution of 6% sodium hypochlorite and 1% Tween 20. Seeds were then rinsed three times with distilled water and vertically orientated on Murashiga and Skoog (MS) agar plates containing 0.15% MS, 0.05% 2-(N-morpholino)-ethanesulfonic acid, 0.15% sucrose, 1% agar, and 2,4-D and NPA at concentrations of either 0 μM, 0.5 μM, 5 μM, and 50 μM. For the chemical concentrations, we used the following stock solutions: 2,4-D: 1 mM in Ethanol and NPA: 10 mM in dimethyl sulfoxide (DMSO). We created 1 mL dilutions of stock solutions (in ethanol or DMSO), which were dissolved in 500 mL of media.

We placed eight seeds on each MS plate and incubated the plates at 21 °C in a dark growth cabinet to avoid any light effects. After 7 d, all plates were rotated clockwise by 90°, and a photograph of each plate was taken 24 h after rotation. All photographs were imported into ImageJ (65) to determine gravitropism by measuring the angle to which the stem reorientated compared to the horizontal plane (29, 66, 67). Seedlings were excluded from analyses if they were shorter than 5 mm, contacted the plate's edge, or germinated after rotation. We were left with a total of 188 seedlings to quantify the gravitropic response. The 50 μM concentration of 2,4-D treatment was excluded, as none of the seeds germinated under this high concentration. For each chemical, we used a linear model $y_{ijkl} = E_i + C_j + e_{l(ijk)}$, where ecotype ($E_i$) is Dune or Headland and concentration (C_j) are the three or four different chemical concentrations. All factors were fixed effects, and $e_{l(ijk)}$ is the residual error. Gravitropism (y_{ijkl}) was the average gravitropism value of seedlings on each agar plate (Dataset S2). All statistical results reported here were produced in JMP 15 (SAS 2015).

Phenotyping of Natural Populations.

Height measurements. We measured plant height in all 16 populations in the glasshouse and 12 of the populations in their native field environment (Datasets S3 and S4). Height (vegetative) was measured as the vertical distance from the soil to the plant's highest point that has vegetative leaves (flowers and stems are not included). In the controlled conditions of the glasshouse, we sampled an average of 14 individuals per population after plants reached maturity. In the field, we measured height in 32 individuals evenly across the range of each population.

For both the glasshouse and field measurements, we used a linear model to determine whether Dune populations were taller than their adjacent Headland pair: $y_{ijk} = P_i + E_{j(i)} + e_{k(ij)}$, where pair ($P_i$) is an adjacent Dune and Headland population at the same locality and ecotype ($E_{j(i)}$) is Dune or Headland and is nested within pair. All factors are fixed effects, and $e_{k(ij)}$ is the residual error. To examine replicated evolution in the system, we extracted fixed effects of ecotype on height (y_{ijk}) for each pair (SI Appendix, Table S6). The Alpine populations were also included with the prediction that the sheltered Alpine population (A03) would be taller than the exposed Alpine population (A07). All statistical results reported here were produced in JMP 13 (SAS 2015).

Gravitropism measurements. Gravitropism was measured in vitro on agar plates in a dark growth cabinet using seeds from all 16 natural populations. For each population, two to four seeds per family were grown for ~40 maternal families ($n = 1,278$ seeds). Plates were incubated, rotated, photographed, and gravitropism was measured in ImageJ (65), as outlined above. Overall, there was 63.8% germination success, but individuals were excluded based on the criteria mentioned in *Synthetic Auxin and Auxin Transport Inhibitor Experiments*. This left a total of 736 seedlings across all 16 populations (57.6% of the total number of seeds planted; Dataset S3).

To test the correlation between height and gravitropism, we performed a linear regression with mean height against mean gravitropism for all 16 populations: $y_{ijk} = H_i + C_j + e_{k(ij)}$, where height ($H_i$) is the average glasshouse plant height for each population and clade (C_j) is the eastern or southeastern clade within which the population is grouped (SI Appendix, Table S1). All factors are fixed effects, and $e_{k(ij)}$ is the residual error. We then tested the hypothesis that Dune populations would have a stronger gravitropic response in their stem than their adjacent Headland pair. We used a mixed linear model: $y_{ijkl} = P_i + E_{j(i)} + A_k + e_{l(ijk)}$, where pair ($P_i$) is an adjacent Dune and Headland population at the same locality, ecotype ($E_{j(i)}$) is Dune or Headland and is nested within pair, and agar plate (A_k) is the MS plate that the seeds were grown on. Agar plate was fitted as a random effect while the rest were fixed effects, and $e_{l(ijk)}$ is the residual error. Gravitropism (y_{ijkl}) measures were averaged for each population and compared between each population pair using a one-tailed t test

(SI Appendix, Table S6). All statistical results reported here were produced in JMP 13 (SAS 2015).

Field Experiments. All field experiments were conducted at the sand dune and rocky headland at Lennox Head, New South Wales, Australia in the exact location where native *S. lautus* grow (SI Appendix, Table S1). We tracked each individual by gluing each seed to a toothpick with nondrip superglue and placing them 1 to 2 mm under the ground within a grid cell (Fig. 4) that was randomly assigned [for details, refer to G.M.W. (30)]. A 50% shade cloth was suspended 15 cm above all plots to replicate the shade given by surrounding vegetation. This was later replaced with very-light bird netting. Seeds were watered twice a day to keep the soil moist and replicate ideal germination conditions to maximize the number of seeds in the experiment. Once germination plateaued for all genotypes, watering was gradually ceased.

Height adaptation experiments. We created genetic lines that aimed to isolate height differences on a common genomic background (SI Appendix, Fig. S3). Firstly, Lennox Head Dune and Headland seeds were grown and crossed to create an F1 generation. Secondly, we backcrossed to Headland parental plants for two generations to produce a BC2F1 generation. We then grew and crossed the tallest BC2F1 individuals among one another ($n = 16$, tallest 10% of the population) and the shortest individuals among one another ($n = 18$, shortest 10% of the population). Refer to SI Appendix, Table S2 for the number of individuals and families contributing to every generation to create these BC2F2 genetic lines. The BC2F2 seeds were planted into the rocky headland at Lennox Head in October 2016 (Australian spring). We planted five replicate plots in which each plot (1.08 × 0.33 m) consisted of the same 12 families with four (occasionally three) individuals per family in each plot, totaling 1,116 BC2F2 seeds. Germination and mortality were recorded every day for 49 d, then every 3 to 4 d until day 79, and then weekly for the remainder of the experiment, until day 159 (Dataset S5).

We implemented a mixed linear model to test the hypothesis that individuals with short parents would have higher fitness in the headland environment: $y_{ijkl} = H_i + F_j + B_k + e_{l(ijk)}$, where parental height (H_i) was the average height of the parents measured in the glasshouse, family (F_j) as individuals with the same parents, and block (B_k) as the five replicate plots across the rocky headland. Parental height is a fixed effect, family and block are random effects, and $e_{l(ijk)}$ was the residual error. Offspring fitness (y_{ijk}) was the total number of days alive in the rocky headland from planting. All statistical results reported here were produced in JMP 13 (SAS 2015).

Gravitropism adaptation experiments. We created an advanced recombinant population (F8) from 23 Dune and 22 Headland Lennox Head individuals, as described in F.R., G.M.W., R.N., and D.O.-B. (26). Briefly, we randomly mated each generation among families while ensuring all families contributed equally to the next generation. We replicated the construction of the F8 using three independent genetic lines (A, B, and C), all derived from the same base population. Refer to SI Appendix, Table S3 for the number of families per genetic line for every generation. F8 seeds were planted into the sand dune and rocky headland in 2012 as described in *Field Experiments*. The fittest families (top 50%) within an environment (and genetic line) were selected using Aster modeling (68, 69) implemented with the "Aster" package in R (70). Code is available at https://espace.library.uq.edu.au/view/UQ:2c603c6. The fitness components included emergence and survival success, in which emergence was the total number of individuals in each family that germinated and emerged above the soil line and survival success was the total number of individuals per family that survived to day 85 in the F8 generation. One full sibling from each of the selected F8 families (50 Dune survivor, 51 Headland survivor families, and 44 families randomly selected for the control) were then grown in the glasshouse. Each family was randomly mated with four other full-sibling families within genetic line (A, B, and C) and population (Dune survivors, Headland survivors, and Control) to maintain ~100 full-sibling families for each population after selection. The same field experiment and selection procedure was conducted on the F9 (in 2013) and F10 generations (in 2014) for a total of three rounds of selection. Survival success was measured at day 138 in the F9 generation and at day 232 in the F10 generation, which was when patterns of local adaptation were strong, and mortality had stabilized in each experiment. Refer to SI Appendix, Table S4 for the number of seeds and families planted per genetic line and environment for the three transplant experiments. To produce the F11 generation, one individual from each F10 full-sibling family was grown and randomly assigned as a pollen donor (sire) or pollen acceptor (dam). Each dam and sire were mated to two different families in a full-sibling, half-sibling crossing design with equal contribution, which produced 100 F11 families.

Reference Genome. Headland individuals from Lennox Head were used for the creation of an Illumina *S. lautus* reference genome. Firstly, we collected

seeds from the Headland at Lennox Head and germinated seeds from two individuals by cutting 1 mm off the micropyle side of the seed and placing in Petri dishes with dampened filter paper. The seeds were placed in darkness for 2 d to allow for roots to grow and then transferred to a 12 h light cycle in a constant temperature room at 25 °C for 7 d to allow for shoots to grow. Seedlings were then transferred into large individual pots with standard potting mix and grown in a glasshouse. Once mature, these two individuals were crossed by rubbing flower heads together and collecting the seeds produced. Siblings from the seeds produced were grown and crossed by rubbing flower heads together to produce a family of individuals capable of self-fertilization (rare in *S. lautus*). One generation of selfing was completed to increase homozygosity. We extracted DNA from the leaf tissue of one individual using a modified cetyltrimethylammonium bromide (CTAB) protocol (71).

A draft genome of *S. lautus* was de novo assembled using second-generation short reads and AllPaths-LG 6.1.2 using default settings. We utilized a series of eight read libraries (*SI Appendix*, Table S7). The reads were trimmed to remove residual adapter sequences and low-quality bases (minimum quality 15). The final assembly was ~843 MB long and consisted of 96,372 scaffolds with an N50 of 21 KB. Although 843 MB is much shorter than the expected haploid size of 1.38 GB (72) of the whole genome, the Benchmarking Universal Single-Copy Orthologs (BUSCO) gene content completeness of 84% (5% fragmented and 11% missing) suggests that this assembly is primarily missing intergenic repetitive DNA sequences, which are notoriously difficult to assemble. Genome is available at https://espace.library.uq.edu.au/view/UQ:2c603c6.

F11 Gravitropism Measurements. In the F11 generation described in *Gravitropism adaptation experiments*, we measured gravitropism as the angle of the stem after a 90° rotation of a seedling. This included 38 Dune survivor families, 37 Headland survivor families, and 25 inbred control families with 12 individuals per family (*n* = 1,212 seeds). These families were germinated in three separate batches approximately 7 d apart. Briefly, we germinated the F11 seeds by cutting 1 mm off the micropyle side of the seed and placing in Petri dishes with dampened filter paper. The seeds were placed in darkness for 2 d to enable roots to grow and then transferred to light for 4 d for shoots to grow. Seedlings were then transferred into small square pots with standard potting mix in a constant temperature room at 25 °C with a 12 h light cycle. After 1 wk of growing in the pot, the plants were rotated by 90°, and a photograph of each individual was taken 12 h after rotation. The photographs were imported into ImageJ (65) to measure gravitropism as described in *Synthetic Auxin and Auxin Transport Inhibitor Experiments*. Dataset S6 contains gravitropism measures and dam and sire fitness values.

Gravitropism tests of selection. We implemented a linear model to test the hypothesis that high-fitness Dune families will produce gravitropic offspring and high-fitness Headland families produce agravitropic offspring. Independent models were used for the sand dune and rocky headland to test the effect of gravitropism on fitness in each environment: $y_{ijklmn} = B_i + V_j + G_{k(i)} + D_{l(ik)} + S_{m(ik)} + e_{n(ijklm)}$, where temporal block ($B_i$) is the three time points in which the F11 seeds were grown (approximately 7 d apart), intrinsic viability (V_j) is the number of days until the death of F11 plants in controlled conditions, and genetic line, which consists of the three independent genetic lines (A, B, and C), nested within the temporal block ($G_{k(i)}$). Dam fitness was nested in genetic line and temporal block ($D_{l(ik)}$), and sire fitness was also nested in genetic line and temporal block ($S_{m(ik)}$). Dam and sire fitness are the F10 family fitness values for the individuals that were crossed to create the F11 offspring where gravitropism was measured. All factors were included as fixed effects, and $e_{n(ijklm)}$ was the residual error. Genetic line C was removed from analyses as it has little variation in fitness values, which means it did not converge. Shapiro–Wilk W test shows that the residuals from the model are normally distributed for both the sand dune ($W = 0.98$ and $P = 0.3879$) and rocky headland ($W = 0.98$ and $P = 0.2776$). The linear model was performed in JMP 13 (SAS 2015).

Genetic association between height and gravitropism. We tested the genetic association between height and gravitropism after segregation in an advanced recombinant population. We implemented a mixed linear model for the three F11 populations (Dune survivors, Headland survivors, and a Control population) that accounts for family variation: $y_{ijk} = H_i + F_j + e_{k(ij)}$, where gravitropism ($y_{ijk}$) is the angle of the growth response 12 h after a 90° rotation, height (H_i) is the vertical distance from the soil to the top of the vegetative leaves, measured after maturity in the glasshouse, and family (F_j) is a random effect that consists of individuals that have the same dam and sire. The mixed linear model was performed in JMP 13 (SAS 2015).

Genotyping of F11 gravitropism tails. To isolate gravitropism candidate genes, we genotyped 77 gravitropic (>56°) and 68 agravitropic (<20°) F11 individuals (Dataset S6). We extracted DNA from leaf tissue using a modified

CTAB protocol (71) and quantified the DNA using the PicoGreen reagent (Invitrogen, Carlsbad). To determine from which parent the alleles were derived, we included 39 Dune parentals (D01) and 41 Headland parentals (H01). Leaves from the Lennox Head Dune and Headland natural populations were collected directly from the field, and the same DNA extraction protocol was followed. Each F11 individual was duplicated in independent wells and libraries of Restriction-Site Associated DNA (RAD) tags were created at Floragenex following Baird et al. (73) but using the PstI restriction enzyme. We sequenced 380 samples on four lanes of an Illumina sequencing platform (HiSeq 4000) with 91-bp single-end reads at Floragenex. A total of 1.39 billion reads with a mean of 3.62 million reads per sample were produced. Reads were aligned to the Illumina reference genome using Bowtie 1.1.1 (74) and a quality score of above 20 (in FASTQ file format). We then used SAMtools 0.1.16 (75) to create an mpileup file of all samples with a minimum Phred quality score of 10, minimum sequencing depth per sample of 6×, and a minimum percent of population genotyped of 75%. This approach produced 26,800 variable positions (224,000 variants before filtering) with a minimum distance between variants of 50 bp. The variant call format (VCF) file is available at https://espace.library.uq.edu.au/view/UQ:2c603c6. The gravitropism candidate gene set consisted of SNPs in the 99.9% quantile of the distribution of differentiated SNPs between the gravitropism tails. Note that RAD sequencing is reduced representation sequencing, and therefore, we are not likely to identify all loci contributing to gravitropism, but we will capture a higher proportion of gene rich regions using the PstI restriction enzyme. The region of the scaffold containing the SNP was annotated using the local alignment search tool BLASTx using The National Center for Biotechnology Information (NCBI) database (76).

Senecio lautus gravitropism candidate genes. We tested for overrepresentation of gene function and linkage disequilibrium between gravitropism loci in the gravitropism candidate gene set. A statistical overrepresentation test was performed in the PANTHER Protein Classification Database (http://pantherdb.org/) using The Arabidopsis Information Resource (TAIR) identification for 32 unique gravitropism candidate genes matched to a reference list of 27,502 *A. thaliana* genes. To calculate linkage disequilibrium between loci, a likelihood-ratio χ^2 test was performed in JMP 13 (SAS 2015) with each F11 population independently (Dune survivors, Headland survivors, and Control; Dataset S7).

Intrinsic Reproductive Isolation.

Gravitropism and hybrid sterility correlation. We tested whether hybrid sterility was associated with gravitropism by randomly crossing within and between the F11 gravitropic and agravitropic groups in a controlled temperature room and recording successful and failed crosses. To maximize sample size, all three replicate genetic lines (A, B, and C) were used across all three environments (Dune survivors, Headland survivors, and Control). A total of 138 crosses were completed (Dataset S8), 71 crosses between the tails and 67 within the tails (agravitropic tail = 29 and gravitropic tail = 38). Crosses were completed by rubbing multiple flower heads of two individuals and collecting the seeds produced from both plants. To remove genetic incompatibilities that might be caused by relatedness, crosses within the same family were not performed. Hybrid sterility (a failed cross) was considered when, in the presence of pollen, less than three seeds were produced per flower head from both plants (reciprocal crosses) with three mating attempts (fertile plants produce at least 30 seeds per cross per flower). Here, hybrid sterility could be caused by failure to produce fertilized ovules (prezygotic) or by fertilized ovules failing to develop into viable seeds (postzygotic). Six crosses displayed differences in sterility in the reciprocal cross (asymmetry), with one successful and one failed cross when using the same parents and so were removed from the analysis (four crosses between the tails and two within the tails).

If gravitropism contributed to divergence in *S. lautus*, we expected to observe an increase in hybrid sterility in crosses between gravitropic and agravitropic individuals. We performed a linear model in JMP 14 (SAS 2018) to determine whether there was a significant association between cross-type (within versus between gravitropism tails) and hybrid sterility while accounting for genetic line and F11 population: $y_{ijkl} = T_i + G_j + P_k + e_{l(ijk)}$, where tails ($T_i$) are crosses within or between gravitropism tails, genetic line (G_j) is the three independent genetic lines (A, B, and C), and population (P_k) is the three F11 populations (Dune survivors, Headland survivors, and Control). All factors were included as fixed effects, and $e_{l(ijk)}$ was the residual error. Hybrid sterility (y_{ijkl}) was compared using a likelihood-ratio χ^2 test.

ENODL1 and ABA3 association with hybrid sterility. We implemented a linear model to test the hypothesis that *ENODL1* and *ABA3* alleles are correlated with hybrid sterility. From the F11 individuals that were crossed, we extracted their genotypes and predicted the genotypes of their offspring (F12). Genotypes that were ambiguous due to heterozygous parents were removed, which reduced the sample size to 61 crosses in which 11 were failed crosses (Dataset S9). We tested the effect of *ENODL1* and *ABA3* alleles and their

interaction on the percentage of failed crosses: $y_{ijk} = E_i + A_j + EA_{ij} + e_{k(ij)}$, where *ENODL1* ($E_i$) is the allele counts for the *ENODL1* C allele in the F12's, *ABA3* (A_j) is the allele counts for the *ABA3* G allele in the F12's, and *ENODL1* x *ABA3* (EA_{ij}) is their interaction. All factors were included as fixed effects, and $e_{k(ij)}$ was the residual error. Shapiro–Wilk W test shows the residuals from the model are normally distributed ($W = 0.94$ and $P = 0.6314$). The linear model was performed in JMP 15 (SAS 2015).

Data Availability. Sequence data have been deposited in The University of Queensland (https://espace.library.uq.edu.au/view/UQ:2c603c6).

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