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# The genetic basis of adaptive evolution and divergence in an Australian wildflower

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# Abstract

Independent populations that evolve similar traits when inhabiting similar environments provide a powerful way to study adaptation. This process is known as convergent evolution and has been found across species, from the evolution of armour plating in sticklebacks in freshwater environments, to the evolution of wings in bats, birds and insects. Although adaptation is an old concept and it has been studied intensely over the recent past, we still remain largely ignorant about the genetics underlying this process, and therefore some of the most fundamental aspects of adaptation remain untested. In particular, we know very little about how natural selection drives trait evolution in independent populations, and whether such populations utilise the same genetic variation to evolve similar traits. We also lack an understanding of how trait evolution contributes to population divergence and whether it can directly lead to formation of new species.

Here, I contribute to our understanding of the genetics of adaptation and divergence by studying an Australian wildflower, *Senecio lautus*, where two growth habits have evolved repeatedly and independently in adjacent and contrasting environments along the Australian coast. A prostrate growth habit is present in the rocky and windy headlands, whereas an erect growth habit is present in the adjacent sand dunes. A previous study found that sets of genes connected by similar functions and pathways were repeatedly differentiated in many of these adjacent Dune and Headland populations. One of these sets of genes was the auxin pathway. Auxin is a hormone that is transported from cell to cell to alter the direction of growth of the plant, from a stimulus such as light or gravity. I hypothesise that differences in the auxin pathway are responsible for the divergent growth habits between adjacent Dune and Headland populations. *Arabidopsis* mutants have shown that mutations in genes within the auxin pathway often result in a decreased ability to respond to an auxin controlled stimulus, such as gravity. Thus, to investigate divergence in the auxin pathway, I examined differences in the response to gravity (gravitropism). This was achieved through a gravitropism assay which assesses the ability of a seedling to alter its direction of growth after a 90° rotation.

My results suggest that the auxin pathway was repeatedly utilised in the evolution of growth habit in many *S. lautus* populations adapting to their environment. I found that families with parents with a prostrate growth habit survived for longer when planted into

the windy headland environment. This is coupled with a strong correlation between growth habit and gravitropism across *S. lautus* populations. For example, Dune populations are often erect and gravitropic (respond strongly to gravity stimuli) and Headland populations are often prostrate and agravitropic (respond poorly to gravity stimuli). To investigate whether gravitropism was driven by natural selection, I exposed an advanced recombinant population, derived from a cross between a pair of Dune and Headland populations, to three generations of natural selection in the dune and headland environment. I found evidence that natural selection was targeting gravitropic plants in the dune environment, whereby the fittest families in the dune environment produce offspring that are more gravitropic. Additionally, I found gravitropism variation is coupled with intrinsic reproductive isolation, suggesting gravitropism (or a tightly correlated trait) is not only important for adaptation but also for divergence between Dune and Headland populations. To further explore the genes involved in the evolution of gravitropism, an F11 recombinant population derived from Dune and Headland individuals was phenotyped and grouped into gravitropic and agravitropic individuals. These groups were sequenced and the genetic differences between them appear to lie in genes with functions related to the auxin pathway, the abscisic acid pathway, transport, localisation and salt tolerance. Some of the alleles with the greatest genetic differences between gravitropic and agravitropic individuals can predict fertilisation success. These results support my hypothesis that the auxin pathway is under selection and contributing to the divergence of adjacent Dune and Headland populations.

Overall, my results indicate that independent populations adapting to similar environments can utilise the variation contained in a genetic pathway to evolve similar traits. I provide evidence that the auxin pathway is repeatedly utilised by *S. lautus* populations in the evolution of growth habit and gravitropism when adapting to dune and headland environments. Additionally, the evolution of gravitropism appears to contribute to population divergence by reductions in fertilisation success. Thus, my research has furthered our understanding of the genetics underlying the process of how natural selection drives adaptation and how this can directly lead to the formation of new species. Widening our knowledge on the genetic basis of adaptation and divergence in new systems will increase our ability to make generalisations across taxa for how evolution and speciation occur at the genetic level.

## Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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## Publications included in this thesis

No publications included.

## Submitted manuscripts included in this thesis

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## Other publications during candidature

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## Contributions by others to the thesis

| Contributor | Specific contribution to: |
|---|---|
| Federico Roda | Gravitropism assay design in Chapter 3. Auxin treatments and inhibitor experiment design and execution in Chapter 3. Creation of initial BC2F1 generation used in Chapter 2. |
| Greg Walter | Design and execution of glasshouse height measurements for all 16 natural populations in Chapter 3. Field experiment design, co-execution and analysis in Chapter 4. |
| Maddie James | Assisted with field measures of height in 12 natural populations in Chapter 3. Large contribution to field experiment execution in Chapter 4. |
| Scott Allen | Creation of *Senecio lautus* genome used in Chapter 5. |
| Rick Nipper and Jessica Walsh | Creation and sequencing of RAD libraries and production of genotypes used in Chapter 5. |
| Daniel Ortiz-Barrientos | Supervised research, contributed to design, data analysis and writing of thesis. |

## Statement of parts of the thesis submitted to qualify for the award of another degree

None

## Research Involving Human or Animal Subjects

No animal or human subjects were involved in this research.

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Natural selection, adaptive evolution, convergent evolution, auxin pathway, gravitropism, local adaptation, reciprocal transplant experiment, speciation, intrinsic reproductive isolation

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# Table of Contents

# List of Tables and Figures

## Chapter 2: Growth habit contributes to local adaptation in an Australian wildflower

## Chapter 3: Convergent evolution of gravitropism and growth habit in an Australian wildflower

**Chapter 4: Adaptive evolution of gravitropism causes intrinsic reproductive isolation between coastal ecotypes of an Australian wildflower**

**Chapter 5: A genetic link between adaptation and speciation in an Australian wildflower**

## List of Abbreviations

DNA: Deoxyribonucleic acid

RADs: Restriction-site associated DNA

RI: Reproductive isolation

SNP: Single nucleotide polymorphism

# Chapter 1: General introduction

From the diversity of beak morphologies in Darwin's finches (Lamichhaney et al., 2015; Kleindorfer et al., 2006) to the assortment of colour patterns for camouflage in mice and lizards (Linnen et al., 2013; Steiner et al., 2007; Rosenblum et al., 2010), the diversity we see in nature is largely shaped by the evolution of adaptive phenotypes. Natural selection drives the evolution of phenotypes that improve an individual's survival in the environment in which it lives. As a consequence of this adaptive evolution, reproductive isolation (RI) might arise between populations and prevent interbreeding (Coyne and Orr, 2004; Hendry, 2004). Yet understanding how natural selection can drive both adaptive evolution and divergence remains a fundamental question in evolutionary biology (Schluter and Nagel, 1995; Chan et al., 2010; Baxter et al., 2008). More specifically, we remain largely ignorant about the genetics underlying this process (Barrett and Hoekstra, 2011); whether independent populations adapting to similar environments utilise the same genetic variation (Nadeau and Jiggins, 2010; Stinchcombe and Hoekstra, 2007); and lastly, how this adaptive evolution can lead to the formation of new species.

Traits might evolve in parallel because the same mutation, gene, or gene network increases fitness in each population. In deer mice, the same gene, but not the same mutation causes changes in coat colouration across multiple populations of the species. These mutations allow mice to camouflage in specific dark or light sand environments (Linnen et al., 2013). Similarly, in sticklebacks inhabiting freshwater lakes where predators are rare, different mutations within the PITX1 gene have resulted in loss of pelvic armour plating (Chan et al., 2010). In contrast, identical mutations in the EDA gene have contributed to reductions in armour plating in these populations (Colosimo et al., 2005). These and other examples (Gallant et al., 2014; Pfenninger et al., 2014; Dobler et al., 2012; Zhen et al., 2012) suggest that convergent evolution can occur through the recruitment of the same mutation or gene. However, can different genes within the same or different genetic pathways lead to the same phenotypic outcome?

Here, I study the genetic basis of convergent evolution of growth habit in an Australian wildflower, *Senecio lautus*. Previous studies in *S. lautus* indicate that many genes belonging to the same functional network might be responsible for the repeated evolution of adaptive phenotypes. Two growth habits have evolved repeatedly and independently in adjacent and contrasting environments along the Australian coast. A prostrate growth habit is present in the rocky and windy headlands, whereas a tall and erect growth habit is present in the adjacent sand dunes (Radford et al., 2004; Ali, 1968; Ali, 1964). These traits have a genetic basis as they maintain their morphology when grown in the glasshouse. Distinct genes and genetic changes seem to have caused these traits in each population, but these genes can often be grouped into common biological functions. For example, genes that are involved in the transport of the plant hormone auxin, a major controller of plant growth, consistently contain genetic differences between erect and prostrate populations (Roda et al., 2013b). Interestingly, these populations often utilise different genes within the auxin pathway, indicating that there might be some genetic constraint but at the level of the genetic pathway. Overall, this suggests a possible role for auxin related genes in the convergent evolution of growth habit in *Senecio*.

### Convergent evolution as a tool for exploring the genetic basis of adaptation

Phenotypic convergence can occur across distant lineages, such as the evolution of wings in birds and bats (Feduccia, 1999) and the evolution of the camera eye in cephalopods, vertebrates and cnidaria (Land and Fernald, 1992). Convergence can also occur among closely related lineages, for instance, evolution of human resistance to malaria (Tishkoff et al., 2001), beak morphology in Darwin's finches in relation to seed size and shape (De León et al., 2014) and the evolution of body size and shape of the walking-stick insect with different host-plants (Nosil et al., 2002). Despite these examples, and more (Landry and Bernatchez, 2010; Landry et al., 2007; Baxter et al., 2008; Dobler et al., 2012; Westram et al., 2014; Steiner et al., 2009), we remain largely ignorant of whether phenotypic convergence also implies genetic convergence, and whether the former could arise from old or new segregating mutations (Conte et al., 2012; Arendt and Reznick, 2008; Rosenblum et al., 2014; Storz, 2016).

The MC1R gene illustrates a case where genotypic variation in a single gene explains phenotypic variation across disparate taxa. MC1R is responsible for changes in pigment colour in beach mice (Hoekstra et al., 2006; Hoekstra, 2010), lizards (Rosenblum et al., 2004), birds (Mundy et al., 2004; Theron et al., 2001) and woolly mammoths (Römpler et al., 2006). It is also involved in the loss of skin colouration in Mexican cave fish, suggesting that a mutation in MC1R can also lead to non-adaptive evolution (Gross et al., 2009). Although these examples show that evolution can be repeated at the gene level, it is unclear whether this is common in nature. More problematic is that we do not know whether reuse of genes stems from developmental constraints, weak pleiotropic effects on other traits, increases in mutation rates, or whether they simply tend to produce mutations of large effect that researchers are inclined to study over those of weak effect (Orr, 2005; Weinreich et al., 2006; Gompel and Prud'homme, 2009; Stern and Orgogozo, 2009; Streisfeld and Rausher, 2011). Uncovering the causes of repeated gene use can help us understand trait evolution, and how it proceeds at different levels of biological organisation.

At biological levels of organisation above genes, studies have found that populations sometimes utilised different genes during the evolution of the same trait (Westram et al., 2014; Steiner et al., 2009). However, whether these genes are functionally independent or participate in the same genetic or developmental pathway, remains largely untested. A major exception is found in studies of the anthocyanin pathway, which controls flower colour evolution in angiosperms. For example, different genes within this pathway have led to changes in anthocyanin production and subsequent evolution of red petals in *Penstemon barbatus*, generally associated with selection to hummingbird pollination (Wessinger and Rausher, 2013). A major result from these studies is that genes repeatedly recruited in flower colour evolution tend to be expressed only in specific flower tissues, suggesting that they are not functionally pleiotropic (Wessinger and Rausher, 2013). However, with few studies looking for biological replication at the genetic pathway level, we cannot make assumptions about the repeatability of evolution across taxa.

Here, I used the *Senecio lautus* complex, a system undergoing convergent evolution of multiple traits (Bernal, 2015; Roda et al., 2013b), to help to fill this gap. I made physiological and phenotypic predictions based on genetic differences, between dune and headland populations, at the pathway level, and explored these in 16 natural populations of *S. lautus*. This enabled me to investigate the repeatability of evolution in a genetic pathway in many biological replicates. Next, I made predictions on the consequences of adaptive evolution in *Senecio* and how this might contribute to speciation. I will discuss the adaptation and speciation connection below as I explore how speciation can be driven by natural selection.

### Adaptive evolution as a mechanism for speciation

The evolution of new species via natural selection is not a new concept. Darwin (1859) reasoned that the origin of new species can be a by-product of adaptation to different environments. From there, speciation research has developed into the search for speciation genes, which are defined as genes that make a significant contribution to the evolution of RI between populations (Nosil and Schluter, 2011; Rieseberg and Blackman, 2010). Reproductive isolation barriers can be prezygotic and occur before reproduction, or can be postzygotic and occur after reproduction (Rieseberg and Blackman, 2010). When these RI barriers accumulate, they reduce gene flow between populations and drive speciation (Weissing et al., 2011). Reproductive isolation barriers can also be extrinsic or intrinsic. Extrinsic RI is driven by the environment and can be the result of maladaptive hybrids or physical barriers that separate populations from interbreeding (Hendry, 2004; Nosil et al., 2005). Intrinsic RI, on the other hand, occurs in any environment, as it is driven by genic conflicts, such as genetic incompatibilities (Dobzhansky, 1937; Muller, 1942) and sometimes chromosomal inversions (Lowry and Willis, 2010). Intrinsic RI can also be driven by genetic differences that cause alterations to behaviour, such as those that change preferences in mates i.e. sexual selection (Lande, 1981; Panhuis et al., 2001) and in the case of flower colour evolution, attraction of different pollinators (Bradshaw and Schemske, 2003; Sheehan et al., 2016; Hoballah et al., 2007). Overall, there are few examples where adaptation and speciation genes have been linked, and therefore we remain ignorant about the role of natural selection in the formation of RI barriers, and thus its contribution to speciation (Christie and Strauss, 2018; Schluter, 2015; Presgraves, 2010).

When genes responsible for local adaptation and intrinsic RI are genetically associated, through linkage or pleiotropy, they can drive the rapid formation of new species. Association of adaptive and intrinsic RI genes through physical genetic linkage on the chromosome can lead to natural selection for adaptive genes and incidentally allow the accumulation of genetic incompatibilities (Coyne and Orr, 2004; Rundle and Nosil, 2005). *Mimulus* adapting to copper mines provides a clear example of this, where incompatible loci hitchhiked to high frequencies with selection for copper adaptation loci (Wright et al., 2013). This resulted in hybrid lethality between the copper mine population and other populations. If natural selection is strong, this can lead to the rapid formation of new species. Alternatively, genes that control adaptive traits might also pleiotropically cause RI, for example, evolution of genes involved in pathogen response not only provides a fitness advantage against pathogen attack, but can cause genetic incompatibilities (Bomblies and Weigel, 2007). This is one of the few examples where intrinsic RI is under direct selection (Willis and Donohue, 2017). These examples show how a genetic association of local adaptation and intrinsic RI genes is important for speciation driven by natural selection, particularly when there is homogenising gene flow in the system.

Here, I explore whether natural selection has driven the evolution of intrinsic RI between adjacent *S. lautus* populations. Previous studies have shown that adjacent coastal populations of *S. lautus* have strong extrinsic RI but weak intrinsic RI (Richards and Ortiz-Barrientos, 2016; Melo et al., 2014). Firstly, reciprocal transplant experiments in dune and headland environments displayed strong local adaptation and selection against non-local genotypes, illustrating strong extrinsic RI (Richards and Ortiz-Barrientos, 2016; Melo et al., 2014; Walter et al., 2016; Walter et al., 2018; Richards et al., 2016). In fact, there is a positive correlation between percentage of headland genomic background and fitness in the headland environment in multiple *S. lautus* populations (Richards et al., 2016), showing that the percentage of local genes underlies the effect of local adaptation on extrinsic RI between populations. The relationship between dune genomic background and fitness is skewed by heterosis in the F1 hybrids, where F1 hybrids have higher fitness than parentals. Heterosis has been observed in many Dune and Headland populations (Melo et al., 2014; Walter et al., 2016; Richards and Ortiz-Barrientos, 2016; Richards et al., 2016), which is most likely masking intrinsic RI in this generation (Lowry et al., 2008; Rundle and Whitlock, 2001). Walter et al. (2016) tested intrinsic RI in F2 hybrids, derived from a Dune and Headland population, and found reduced fitness compared to F1 hybrids and parentals. Fitness was regained in the F3 generation, suggesting few but strong negative epistatic interactions are responsible for intrinsic RI in the *S. lautus* system. Considering, the close proximity of Dune and Headland populations, and thus the potential for gene flow between them, natural selection could be driving the observed intrinsic RI. This could occur through genetic linkage or pleiotropy with the evolution of adaptive traits, such as growth habit. Finding the genes underlying adaptation and intrinsic RI in the *Senecio lautus* species complex will aid in our understanding of the genetic architecture responsible for the rapid evolution of new species.

## Discovery of adaptation and speciation genes

To isolate the loci underlying adaptation and speciation, mapping populations must be created to disassociate linked traits. When populations adapt to different environments, they might develop many genetic differences, some of which might not be under selection but be associated by genetic linkage to a trait under selection (Barrett and Hoekstra, 2011). Thus, when comparing divergent populations, we need to control the genomic background in which the traits of interest segregate. This can be achieved by constructing genetic lines where candidate genes segregate with either a randomised genomic background, such as a recombinant line, or a homogenous genomic background, such as an introgression line. In recombinant lines, genetic homogenisation between the divergent populations splits genetic associations that might have formed due to linkage. The more rounds of random crossing between these divergent populations the more the genes under selection will be disassociated from surrounding genes (Broman, 2005). This same concept applies to introgression lines, with the difference being that divergent phenotypes are repeatedly backcrossed to one of the original parents. This produces genetic lines with a single genomic background of one of the two populations, but with a few regions containing alleles from the alternative population, presumably responsible for the trait difference between them. These populations facilitate the first steps to identifying the genes responsible for the convergent evolution of divergent phenotypes (Abzhanov et al., 2008) and their association with genes that cause intrinsic RI.

To facilitate the discovery of genes involved in growth habit phenotypes in *S. lautus*, I created Dune and Headland recombinant and introgression lines. The desired genetic lines are easily created in this system, due to the ease with which *S. lautus* can be grown and crossed in controlled conditions and seeds stored for later use. I have grown an 11th generation Dune and Headland recombinant population (F11), which has substantial reductions in linkage. More importantly, siblings from these F10s have undergone three rounds of selection in the dune and headland environments at Lennox Head, thus allowing me to investigate fitness variation in growth habit-related phenotypes (See Chapter 4 - Figure 1 for a visual representation of this experimental design). I also tested whether adaptive traits in *S. lautus* were contributing to the formation of intrinsic RI barriers and therefore contributing to the divergence between Dune and Headland populations (Chapter 4). Next, I created an introgression line, where height was utilised as a proxy for growth habit and tall alleles were introgressed onto the short Headland genomic background (See Chapter 2 - Figure 1 for a visual representation of this genetic line). On the last round of crossing, these groups were crossed amongst one another to reduce heterozygosity because the original parents and those used for repeated backcrossing were not homozygous lines. Therefore, it is likely that some of these families will segregate for unrelated loci depending on the complexity of the height genetic architecture. To identify whether these growth habit related genes conferred a fitness advantage, I planted these seeds into the dune and headland environments at Lennox Head and compared fitness differences with height differences across a common genomic background (Chapter 2).

### Connecting growth habit and the auxin pathway

Recombinant and introgression lines are valuable for identifying the genomic targets of selection. They can further aid in identifying the genetic basis of adaptation and divergence when combined with genetic information derived from natural populations. Previous work in *S. lautus* has provided preliminary evidence about the candidate genes that might be involved in the evolution of contrasting growth habits in the *S. lautus* complex (Roda et al., 2013b). Analyses of genetic differentiation between Dune and Headland populations revealed enrichment of differentiated genes involved in development, particularly in those with functions related to auxin transport. More specifically, 16 genes involved in the regulation of auxin levels and transport were consistently divergent between eight Dune and Headland adjacent pairs along the Australian coastline (Roda et al., 2013b). Many of these genes code for PIN-FORMED (PIN) auxin efflux carriers that are involved in transporting auxin out of the cell. In a second experiment, PIN genes were further implicated to be involved in growth habit differences in *S. lautus*; four erect and prostrate pairs were crossed, and the upper and lower tails (10%) of three growth habit traits (vegetative height, angle of the main stem and the number of branches) were compared within each pair. The loci divergent between the upper and lower tails indicate those loci possibly involved in the evolution of growth habit. PIN genes were repeatedly divergent between the upper and lower tails (Roda, 2014). Overall, these two analyses indicate that the auxin pathway might have contributed to the evolution of adaptive phenotypes in *S. lautus* populations adapting to adjacent contrasting dune and headland environments. Thus, in this thesis I have tested the hypothesis that auxin genes, like these PIN genes, have contributed to the repeated evolution of growth habit in *S. lautus*. Below, I explain the extensive knowledge that exists on the auxin pathway and its control on growth habit.

### The auxin pathway

Auxins are a class of plant hormones heavily involved in plant growth. Auxin research dates back to tropism experiments in the 1880s by Darwin. Darwin and Darwin (1881) showed that plant growth was directed by light (phototropism) and gravity (gravitropism) stimuli; seedlings curved towards the light, possibly enhancing their ability to photosynthesise, and responded to changes in the gravity vector by re-orientating their growth so the stem grew upwards towards the light and against gravity, and the root grew into the soil in alignment with the gravity vector. Darwin and Darwin (1881) found that some kind of ‘fluid’ dispensed within the tip of the plant was controlling curvature in the lower part of the plant. This led to auxin being discovered as the first plant hormone, with Went (1935) showing that the amount of auxin determined the angle of curvature. Since then, research has accumulated in many areas of the auxin response, including signal transduction, auxin synthesis, transport of auxin and annotation of the genes involved in the auxin pathway (Lau et al., 2008; Brumos et al., 2014; Teale et al., 2006; Leyser, 2011; Mano and Nemoto, 2012).

Although we still remain fairly ignorant about how plants detect changes in a stimulus (Blancaflor, 2013), the cascade of signals and auxin movement during tropism and plant growth has been well studied (Mano and Nemoto, 2012; Leyser, 2018; Paque and Weijers, 2016). After a stimulus, such as gravity (e.g. from a change in orientation of a plant), auxin accumulates on the lower side of the shoot and root causing it to bend. In the stem, auxin accumulation causes cell elongation and subsequent lengthening of the lower side and curvature upwards. In roots, inhibition of cell elongation causes shortening of the lower side and curvature downwards (Woodward and Bartel, 2005). Regulation of these signals in most plants occurs through a simple two step IPA (indole-3-pyruvic acid) pathway, where Tryptophan is converted to IPA which is converted to IAA (Indole-3-acetic acid), the most common and active auxin (Brumos et al., 2014). Auxin is then transported through the phloem and via auxin transport proteins, generally moving from shoot tip to root tip. There are many different families of auxin transport proteins that have been identified, including the AUX/LAX family which moves auxin into the cell, and the PIN protein family, that move auxin out of the cell (Friml and Palme, 2002). These transport proteins are major determinants of auxin movement and gradient formation, thus helping to control the intensity and direction of a variety of developmental responses (Friml et al., 2004). Considering their major control in development, these transport genes, along with others involved in auxin signalling and synthesis, have been well annotated in model organisms, such as *Arabidopsis*, and studies are continuing to find new genes and functions (Sang et al., 2014; Yamaguchi et al., 2013; Wu et al., 2013; Liu et al., 2014), further adding to our pool of knowledge of the auxin pathway and its influence on plant growth.

### Testing the auxin response with a gravitropism experiment

A simple way to test whether auxin controls the direction of growth in a seedling, is a gravitropism bioassay, where the seedlings' ability to alter their growth in relation to a change in orientation is dependent on their ability to regulate auxin. It involves turning a seedling by 90° and measuring the angle at which the seedling re-orientates itself towards the new vector of gravity within a 24 hour time period (Sang et al., 2014; Lopez et al., 2014; Huang et al., 2014; Cho et al., 2014; Rigo et al., 2013; Ottenschläger et al., 2003; Blancaflor and Masson, 2003; Rashotte et al., 2000; Müller et al., 1998). A highly gravitropic plant will re-orientate its direction of stem and root growth with the new gravity vector. Agravitropic individuals on the other hand, have loss of function mutations that result in no response to gravity stimuli. These plants continue growing in the initial direction or a random direction (Friml and Palme, 2002). Alternatively, individuals with mutations in auxin related genes often lead to reduced gravitropic function (not complete loss of function) and respond poorly to a reorientation (Leyser, 2018; Baldwin et al., 2013). Interestingly, studies have shown that the gravitropic response in the root and stem are not necessarily linked, with some mutations causing agravitropism in the stem and not the root and vice versa (Tasaka et al., 1999). Considering I am interested in how auxin shapes the aerial parts of the plant, I have focussed the experiments in my thesis on stem gravitropism. I have utilised previous evidence (Roda, 2014; Roda et al., 2013b) implicating auxin pathway genes in the divergence of the Dune and Headland ecotypes and tested whether genes within the auxin pathway are contributing to the repeated evolution of divergent growth habits between Dune and Headland populations. I then tested whether the evolution of these adaptive phenotypes could be contributing to the evolution of intrinsic RI between Dune and Headland ecotypes.

### ***Senecio lautus*** **as a system for understanding the genetic basis of adaptation and divergence**

Here, I use the *Senecio lautus* species complex, a diverse group of plants that have repeatedly adapted to contrasting environments, which have in turn shaped its morphology (Roda et al., 2013a; Roda et al., 2013b). Each time they colonised an environment, *S. lautus* populations evolved a unique set of traits. These forms are known as ecotypes and in *S. lautus* they persist on the coast and inland of Australia, and include Dune, Headland, Alpine, Tableland and Woodland types (Radford et al., 2004). The Dune and Headland coastal ecotypes have evolved in adjacent and contrasting environments repeatedly, thus providing a unique opportunity for studying deterministic processes in nature, adaptive convergence in growth habit and the early stages of the speciation process (Roda et al., 2013a; Thompson, 2005). The sand dunes are sheltered, and have nutrient-poor soils, which are prone to retaining heat and little water. The rocky headlands are exposed to high wind speeds, and have nutrient-rich soils, which contain large amounts of salts (Ali, 1964; Ali, 1968; Radford et al., 2004; Roda et al., 2013b). Adaptation to the conditions in these environments has led to Dune ecotypes that are erect with few branches, and Headland ecotypes that are prostrate with many branches. Interestingly, there are also some Alpine populations in this system that are exposed to major contrasting environmental differences and again produce these contrasting growth habits. For example, one of the Alpine populations is exposed to high winds and has a prostrate growth habit and another in a more sheltered alpine environment has an erect growth habit. Tableland and Woodland ecotypes also have an erect growth habit (Ali, 1964; Ali, 1968; Radford et al., 2004; Walter et al., 2016; Roda et al., 2013a), indicating that the erect growth habit might be the ancestral phenotype and the prostrate growth habit might be the derived phenotype. Altogether, the evolution of the prostrate growth habit in windy environments across ecotypes, implicates wind as a strong selective pressure in its evolution.

*Senecio lautus* is also an excellent system for performing genetic crosses and manipulative experiments. The life history of *S. lautus* ranges from annual to short lived perennial and consists of a moderately large effective population sizes (Melo et al., 2014). It has a relatively short life cycle (3-4 months seed to seed in controlled conditions), can be grown easily in controlled temperature rooms or glasshouses, and seeds can be stored at

4°C for later use. As a result, genetic material can easily be generated for manipulation in glasshouse or field conditions. The one genetic limitation that is not common to all plant systems is that most *Senecio* populations are obligate outcrossers, displaying strong self-incompatibility, even in controlled conditions (Ornduff, 1964), limiting our ability to generate homozygous genetic lines. Instead, I have used recombinant and introgression genetic lines, where variation has been induced to make associations between phenotype and fitness. Overall, *Senecio* is an ideal system to uncover the genetic and ecological basis of adaptation and divergence.

## Thesis outline

To investigate whether the auxin pathway is responsible for the evolution of growth habit in the *S. lautus* system, I have completed a number of genotypic, phenotypic and physiological experiments. In Chapter 2, I observed whether the prostrate growth habit might provide a fitness advantage in the headland environment, when compared on a common genomic background (introgression line). In Chapter 3, to indicate whether the auxin pathway might be repeatedly utilised across many natural populations of *S. lautus* adapting to their environment, I completed gravitropism bioassays in 16 *S. lautus* natural populations. Next, in Chapter 4, I tested whether gravitropism has been targeted by selection in an F11 recombinant population that has undergone three rounds of selection in the dune and headland environments at Lennox Head. In Chapter 4, I also tested whether gravitropism was coupled with the formation of intrinsic RI barriers, to understand whether this trait might be contributing to divergence between adjacent Dune and Headland populations. Finally, in Chapter 5, I aimed to isolate the genes underlying gravitropism and intrinsic RI in *S. lautus*, to understand the genetic architecture that leads to rapid adaptation and the formation of new species.

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# Chapter 2: Growth habit contributes to local adaptation in an Australian wildflower

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Keywords: natural selection; local adaptation; field selection experiment; growth habit

## Abstract

Natural selection can drive the evolution of traits that increase survival in certain environments. Investigating the process of adaptation and the specific drivers of this evolution is vital for our understanding of how natural selection drives evolutionary change. Here I use the *Senecio lautus* species complex, an Australian wildflower that is locally adapted to two contrasting adjacent dune and headland environments, to investigate the possible traits that might be shaped by natural selection. I isolated a major phenotypic difference between these populations, growth habit, on a common genomic background and tested its contribution to fitness variation in a field selection experiment. I found that short parents produce offspring with a higher field fitness in the headland environment, which is in support of natural selection driving the evolution of a prostrate growth habit in the windy headland environment. Overall, my results provide insights into the influence of parental trait value on fitness of their offspring in their native environment, providing a platform to investigate how natural selection might be shaping trait evolution.

## Introduction

From salt tolerance in coastal plants (Lowry et al., 2009) to cold tolerance in freshwater sticklebacks (Barrett et al., 2011), natural selection drives the evolution of adaptive traits in different environments (Kawecki and Ebert, 2004). The evolution of adaptive traits often leads to local adaptation, whereby populations have a higher fitness in their local environment compared to foreign populations (Lowry, 2012). Local adaptation is widespread in plants (Baack et al., 2015; Joe Hereford, 2009). Due to their sessile nature, plants often cannot escape abiotic differences in the environment and tend to evolve traits to survive (Wu et al., 2007). Although it is well accepted that populations adapt to their local environment, the targets of natural selection and the factors driving this evolution often remain obscure (Kawecki and Ebert, 2004). Thus, the role of natural selection in the evolution of divergence within species requires further investigation (Lowry, 2012; Kawecki and Ebert, 2004).

Isolating adaptive traits that are under selection can sometimes be difficult due to correlations between traits. Trait correlations are often caused by genetic linkage on a chromosome, where selection for one trait leads to the coupled inheritance of other traits (Barrett and Hoekstra, 2011). As a consequence, differentiating the trait targeted by natural selection from co-segregating traits is difficult in natural populations. Backcross populations, such as nearly isogenic lines and introgression lines, can be used to overcome trait correlations. These breeding designs allow us to study the relationship between fitness and specific phenotypic differences in isolation from the genetic transmission of other traits. Backcross breeding with phenotypic selection has been used to identify abiotic stress tolerance traits in rice (He et al., 2010; Lafitte et al., 2006; Ali et al., 2006). Lafitte et al. (2006) created a BC2F2 genetic line in rice, where drought tolerant plants were backcrossed to the parental generation and drought tolerant traits were identified through a field selection experiment.

Field selection experiments are the most common approach for testing for local adaptation (Lowry, 2012). Clausen et al. (1940) was the first to use a field selection experiment to illustrate local adaptation. Clausen et al. (1940) planted a number of plant populations at or near their original collection sites and found that native populations have higher fitness than foreign populations. In other words, they were locally adapted. Local adaptation has been found using field selection experiments on a range of species, including three-spine stickleback (Kaufmann et al., 2017), lizards (Iraeta et al., 2006; Niewiarowski and Roosenburg, 1993) and many flowering plants (e.g. Ågren and Schemske, 2012; Lowry and Willis, 2010; Walter et al., 2016; Busoms et al., 2015). When field experiments are combined with a backcross population, they can be used to identify which traits are contributing to local adaptation. For example Lowry and Willis (2010) introgressed an chromosomal inversion causing flowering time and morphological trait differences between ecotypes of the yellow monkeyflower, *Mimulus guttatus,* into a common genomic background, and performed a field selection experiment to find that the genes contained in the inversion contributed to local adaptation and reproductive isolation (RI). This illustrates the power of combining a field selection experiment with a mapping population to investigate the targets of natural selection in populations that are adapting to their local environment.

Here, I combined the use of introgression lines with a field selection experiment in an Australian wildflower, *Senecio lautus*, to understand the contribution of parental traits on offspring fitness in the species. This species complex occurs in a variety of environments that range from dune and headland coastal environments, to inland tableland, woodland and alpine environments (Ali, 1964; Radford et al., 2004; Roda et al., 2013; Walter et al., 2016). The adjacent Dune and Headland coastal populations are particularly interesting as they have evolved contrasting traits repeatedly and independently, and most likely in the face of gene flow (Roda et al., 2013), indicating natural selection is driving trait evolution in this system. Dune and Headland ecotypes are annual to short lived perennial and are insect pollinated. They have a moderately large population size and are obligate outcrossers. Dune populations are exposed to high temperatures, low salinity, and high dew point, whereas Headland populations are exposed to high salinity, powerful winds and have lower water availability due to osmotic stress (Walter et al., 2016). These environmental differences have most likely driven the evolution of major plant architectural (Walter et al., 2016) and leaf (Bernal, 2015) differences that exist between adjacent Dune and Headland populations. Populations inhabiting hot dune environments display an erect growth habit with few branches, whereas populations inhabiting windy headland environments display a prostrate growth habit with many branches. Growth habit has a genetic basis as it retains its architecture when grown in the glasshouse (Roda et al., 2017) and the prostrate growth habit appears to be the derived trait as most ecotypes have an erect growth habit, including Tableland, Woodland and Alpine (excluding one population in a windy exposed alpine environment) (Ali, 1964; Ali, 1968; Radford et al., 2004; Walter et al., 2016; Roda et al., 2013).

In this initial work, I investigated variation in parental growth habit with offspring fitness values in the windy headland environment in *S. lautus*. In the following chapters, I delve into the genetic mechanisms underlying growth habit in *S. lautus*. Growth habit consists of both qualitative (e.g., prostate versus erect growth habit) and quantitative descriptions of the plant (e.g., plant height and angle of main stem). In my thesis, I have utilised the easily repeatable quantitative measure of plant height. To isolate height alleles on a common genomic background, I introgressed tall alleles onto the headland genomic background, by crossing the tallest (20%) individuals to a Headland parental for two generations. Then, to understand whether height alleles provide a fitness advantage, I subjected these genetic lines to natural selection in the native environments of their parents. To investigate which abiotic factors might be driving local adaptation, during the experiment I tracked major environmental differences between the dune and headland environments (wind and temperature). These results provide insights into the traits that might contribute to local adaptation in *S. lautus* and provide a platform for, in the following chapters, investigating the genetic and molecular mechanisms underlying adaptation.

## Methods

### Seed production

All seeds used in this experiment were derived from individuals collected at Lennox Head, NSW (dune environment: S 28° 47’ 1.23’’, E 153° 35’ 38.56’’ and headland environment: S 28° 48’ 47.22’’, E 153° 36’ 19.15’’). I collected individuals at a 10m distance to reduce the risk of sampling related individuals. To reduce maternal effects, one round of crossing between individuals collected from the same location was completed under glasshouse conditions. Seeds were then stored at four degrees in Turdor seed envelopes until required.

### Creation of genetic lines

To investigate the hypothesis that being short on a windy headland increases chances of survival, I created genetic lines that aimed to isolate height differences on a common genomic background (Figure 1). Firstly, Lennox Head Dune and Headland parentals were crossed to create an F1 generation. Secondly, to isolate the height alleles on a common genomic background, I introgressed the tall trait onto a headland genomic background by backcrossing the tallest F1 to a Headland parental. This created a BC1F1 generation, where the tallest individuals (N=11, tallest 20% of the population) were again backcrossed to the Headland parental, creating a BC2F1 generation. Surprisingly, this genetic line showed no average increase in height compared to Headland parentals grown at the same time in the same conditions: the average height of BC2F1s was 7.9±0.2cm (N=96) and the Headland parentals were 8.1±0.7cm (N=14). The same was noticed when this was continued to the 5th generation: the average height of BC5F1s was 9.7±0.4cm (N=10) and the Headland parentals grown in the same conditions were 11.6±1.4cm (N=5). However, the BC2F1 generation segregated variation in height, so I crossed the tallest individuals (N=18, tallest 10% of the population) among one another and the shortest individuals (N=19, shortest 10% of the population) among one another. This increased homozygosity and created BC2F2-short and BC2F2-tall genetic lines for comparing height alleles on a common genomic background with approximately the same proportion of Headland genome in each genetic line.

### Field selection experiment

I conducted a field selection experiment to test for 1) local adaptation in the contrasting dune and the headland environments (using parental seeds) and 2) the fitness effects of the prostrate growth habit in the headland environment (using BC2F2 seeds). Parental and BC2F2 seeds were planted into each environment on the 2nd October 2016 (Australian spring) in the same location where the parental seeds were collected. I planted five replicate plots in each environment, where each plot (1.08x0.33m) consisted of the same 68 families with four (occasionally three) individuals per family in each plot, totalling 2,712 parental and BC2F2 seeds. The entire field experiment had a total of 4,708 seeds (108 families) but the other genotypes were not relevant to this study. To track each individual, every seed was glued to a toothpick and placed 1-2mm under the ground within a grid cell that was randomly assigned (Figure S1). All 10 plots were identical with the same designated grid cells. To minimise edge effects, 3-4 replicate individuals from each family were planted in each plot and it was ensured that every seed received the same treatment e.g. shade cloth covered edges and surrounding edge of each plot was watered.

*Senecio lautus* often germinate with the protection of surrounding vegetation, particularly in the dune environment where the native grass spinifex is plentiful; however, this surrounding vegetation must be cleared for the seeds planted in the experiment to be tracked. Therefore, 50% shade cloth was suspended over all 10 plots to replicate the shade given by surrounding vegetation. Seeds were watered twice a day to keep the soil moist and replicate ideal germination conditions to maximise the number of seeds in the experiment. Once germination plateaued for all genotypes in the headland environment, watering was ceased (day 11) and when germination plateaued in the dune environment, watering was reduced to once a day (day 14). Watering in the dune environment was eased off more slowly as sand retains much less water than the soil in the headland, and thus a dramatic shift in the water given might shock and kill all plants. The first month of the experiment was particularly dry: rainfall at Ballina airport (6-7km from field sites) recorded a total rainfall of 11% of the average rainfall in October (October 2016=11.4mm and October average for years 1992-2019=102.9mm). Due to the abnormally dry summer, I continued watering in the dune environment during extreme dryness and heat. Shade cloth was replaced with bird netting in the headland environment on day 44 once plants were established, but shade cloth was kept in the dune environment for the remainder of the experiment to replicate shade cover from spinifex grass. Germination and mortality were recorded every day for 49 days, then every 3-4 days until day 79 and then weekly for the remainder of the experiment. Table 1 shows the number of seeds and families planted into the dune and headland environments.

### Environmental variables

Digitech weather stations were placed at field sites during the experiment to measure differences in temperature (°C) and wind (km/hr) between the dune and headland environments. The maximum temperature and maximum wind per day were plotted in Figure 2 with a smoothed curve (wind: lambda=0.4 and temperature: lambda=0.05) to illustrate the major trends.

### Survival analysis

To investigate patterns of local adaptation for the parental and hybrid genotypes, I performed a survival analysis using the nonparametric Kaplan-Meier method. The proportion of surviving individuals from each genotype was compared across the length of the experiment. I performed a generalised Wilcoxon chi-square to test homogeneity of the estimated survival function across genotypes. All statistical results reported here were produced in JMP v13 (SAS 2015).

### Growth habit fitness correlations

The percentage of genome belonging to Dune or Headland is a major determinant of fitness in dune and headland environments. For example the higher the percentage of Headland genome, the higher the survival in the headland environment (Richards et al., 2016). This means that to understand the effects of variation in height on fitness, I must compare genetic lines that have similar genetic content. Therefore, I used a single type of backcross (BC2F2) in my field experiments. I then asked if height variation in BC2F1 parents predicted the fitness value of their offspring (BC2F2-short and BC2F2-tall) in the headland environment. I implemented a mixed linear model to test the hypothesis that individuals with short parents will have higher fitness in the headland environment:

$$y_{ijkl} = H_i + F_j + B_k + e_{l(ijk)} \qquad (1)$$

where parental height ($H_i$) was the average height of the parents measured in the glasshouse, family ($F_j$) was individuals with the same parents, and block ($B_k$) is the five replicate plots across the headland environment. Parental height is a fixed effect, and family and block are random effects, and $e_{l(ijk)}$ was the residual error. I applied the model to two offspring fitness measures independently, the number of days alive and ability to produce 10 leaves. All statistical results reported here were produced in JMP v13 (SAS 2015).

## Results

### Temperature and wind measurements at field sites

To understand the major differences in environmental variables between dune and headland environments that might be shaping growth habit, I placed weather stations next to field sites during the experiment. As expected, maximum wind speeds were highest in the headland environment and maximum temperatures were highest in the dune environment (Figure 2). The maximum wind speed recorded at the headland was 14.7km/h higher than the maximum wind speed recorded in the dune (headland=29.5km/h on day 96 and dune=14.8km/h on day 64) and on average, the maximum wind speed was 2.9km/h higher at the headland than the dune over the length of the experiment (headland=10.1±0.8km/h and dune=7.2±0.3km/h). Additionally, the headland had a maximum wind speed 5km/hr greater than the dune environment on 38% of the days where wind was recorded (N=97) and 10km/hr greater on 24% of days. The maximum temperature in the dunes was on average 2.7°C higher than at the headland (dune=32.0±0.3°C and headland=29.3±0.4°C). There was a heatwave at Lennox Head during the experiment, which resulted in the death of 277 individuals (20% of total) in the dune environment on day 33 (Figure 3). The peak of the heatwave was on day 34 where temperatures reached 40.8°C in the dune environment (Figure 2).

### Local adaptation of Dune and Headland parentals

If natural selection targeted individuals with local trait values in this experiment, then I would expect to observe higher survival of the local parent in comparison to the non-local parent and hybrids. I found strong differences in survival between the genotypes both in the dune environment (Wilcoxon chi-square, $X^2$=174.37, df=3, p<0.0001) and the headland environment (Wilcoxon chi-square, $X^2$=38.18, df=3, p<0.0001), where the local parent had the highest proportion of individuals surviving (Figure 3; Table S1). In the headland environment, the local Headland population had the highest percentage of individuals that reached 10 leaves, produced a bud and produced a flower (Table 1). In the dune environment, only a total of 16 individuals produced 10 leaves across all genotypes, 14 of which were dune parentals. No plants in the dune environment produced a bud or a flower (Table 1), most likely due to the harsh dry environment. Germination was induced by artificial watering and therefore a similar number of individuals germinated across the genotypes in both environments. These results are consistent with expectations for strong local adaptation to adjacent dune and headland environments.

### Variation in parental growth habit predicts variation in offspring fitness in the headland environment

If natural selection had driven the evolution of prostrate growth habit in Headland populations, then I would expect to find tall parents produce maladapted offspring (BC2F2-tall), and short parents produce fit offspring (BC2F2-short). I used height as a proxy for growth habit and measured the height of the parentals (BC2F1) in the glasshouse. A field selection experiment with the offspring (BC2F2) enabled parental height variation to be compared to offspring fitness on a common genomic background. The results were consistent with my expectations and showed that parental height contributes to survival in the headland environment (Figure 3; $F_{1,26.19}$=4.65, p=0.0404), where shorter parents produced offspring that lived longer than the offspring produced from tall parents. Parental height also contributes to whether an individual reaches 10 leaves in the headland environment ($F_{1,26.13}$=5.45, p=0.0276). Comparisons were not made between other fitness measures such as producing a bud or a flower, as there were only a small number of hybrids that reached this developmental stage (Table 1).

## Discussion

Natural selection can drive the evolution of traits that increase survival in certain environments. This is often driven by environmental variables that impose harsh selection pressures; for instance, in plants, saline soils can drive water loss and therefore many species have evolved salt tolerance mechanisms to survive in these saline environments (Lexer et al., 2003; Parida and Das, 2005). Furthermore, in mice, different coloured terrain can reduce camouflage from predators and so some mice populations have evolved coat colourations to survive in these different environments (Steiner et al., 2007; Steiner et al., 2009; Nachman et al., 2003; Hoekstra et al., 2006). These examples show that populations evolve beneficial traits in response to their surrounding environment, however, isolating the traits targeted by selection and the abiotic factors responsible remain obscure across taxa (Kawecki and Ebert, 2004).

In the present study, the results are consistent with natural selection for short plants in the windy headland environment. I found that on a common genomic background, individuals with shorter parents survived for more days and had more individuals reaching 10 leaves in the headland environment than individuals with taller parents. Although, I do not provide direct evidence for the environmental variable/s driving the evolution of the prostrate growth habit in the headland environment, I suggest wind as a possible selective agent to be investigated in the future. Hotter temperatures might be contributing to the evolution of an erect growth habit in the dune environment as local adaptation to environmental temperature has occurred in a number of species (Gorter et al., 2016; Wang et al., 2016; Ohlberger et al., 2008; Partridge et al., 1994), but this remains untested in this experiment and the prostrate growth habit, that was most likely derived from the erect growth habit (Ali, 1964; Ali, 1968; Radford et al., 2004; Walter et al., 2016; Roda et al., 2013; Walter et al., 2018), was investigated.

In this study, Dune and Headland parentals had high fitness in their native, but not in the alternative environment. They also outperformed all hybrids across the two environments. Reduced fitness in hybrids could arise from a mixture of environmental differences (extrinsic RI) (Hendry, 2004; Nosil et al., 2005) or from genic conflicts (intrinsic RI) that can result in hybrids having a poor performance in any environment (Dobzhansky, 1937; Muller, 1942). The fitness differences found between the parental populations is consistent with local adaptation and natural selection creating strong extrinsic RI barriers (Schuler et al., 2016) between them. These findings are also consistent with field experiments in other systems (e.g. Ågren and Schemske, 2012; Lowry and Willis, 2010; Busoms et al., 2015; Kaufmann et al., 2017; Iraeta et al., 2006; Niewiarowski and Roosenburg, 1993) and previous Dune and Headland field experiments in the *S. lautus* system (Richards and Ortiz-Barrientos, 2016; Walter et al., 2016; Richards et al., 2016; Melo et al., 2014). Together, these results suggest that natural selection has driven the evolution of divergent traits in adjacent contrasting dune and headland environments. However, few reciprocal transplant experiments and previous Dune and Headland field experiments have directly tested which traits drive local adaptation.

The association shown between parental height and offspring fitness in the headland environment using introgression lines, suggests that natural selection possibly drove the evolution of the prostate growth habit in headland environments. In my experiments, I controlled for genomic background by creating genetic lines with variation in height through repeated backcrossing to Headland individuals. By backcrossing the tall alleles onto the headland genomic background, I induced differences in the headland genome around loci controlling height. This variation in height allowed me to experimentally associate height variation with fitness variation in a selection experiment in the headland environment, on a common genomic background. It was particularly important to control for genomic background, as previous studies in *S. lautus* have shown that the higher the percentage of headland genome, the higher the survival rate in the headland environment (Richards et al., 2016). BC2F2-short and BC2F2-tall genetic lines have the same percentage of headland genome, and therefore height differences can be observed in isolation from the effect of percentage genome.

There are two caveats to my experimental results. First, I measured height of the parental generation in glasshouse conditions and the height of the offspring in the field. The reason is simple: field height would be skewed by fitness of the individuals, with unfit individuals not surviving to be measured and fit individuals growing tall. As height was not measured on the generation that underwent selection, this experimental design supports the hypothesis that natural selection has (directly or indirectly via pleiotropy) driven the evolution of a prostrate growth habit in the headland environment. Second, I did not include flowering data as a fitness measure due to the low number of individuals reaching this developmental stage during the experiment. The harsh conditions in the first month of the experiment likely contributed to the low survivorship to maturity, whereby a nearby site recorded only 11% of the average rainfall in October (October 2016 = 11.4mm and October average for years 1992-2019 = 102.9mm). The correlation between low rainfall and low survivorship to maturity has also been found in other *S. lautus* field experiments (Richards et al., 2016).

There is a strong association between prostrate growth habit and windy environments across the *Senecio lautus* species complex. In addition to windy headland environments, there is also a windy and exposed alpine environment that contains plants that have a prostrate growth habit (Roda, 2014). This is in contrast to most *S. lautus* alpine environments that are more sheltered and have individuals with erect growth habits (Roda, 2014). The repeated selection in independent populations for prostrate plants in the headland and alpine environments (Roda et al., 2013), suggests that strong environmental factors common to these environments are driving the evolution of prostrate growth habit. In this study I found a large environmental difference in maximum wind speed between the dune and headland environments (14.7km/h), where on 38% of the days when wind was recorded, the headland had a maximum wind speed 5km/hr greater than the dune environment. I suggest that prostrate growth habit might be beneficial in the headland environment, as it protects the plants from mechanical stress such as broken branches, caused by these high wind speeds (Read and Stokes, 2006; Auld and Morrison, 1992).

Transitions from erect to prostrate growth are common in plants that colonise coastal headlands (Beeftink et al., 1985; Auld and Morrison, 1992; Morrison and Rupp, 1995a; Crutsinger et al., 2010). For example, Morrison and Rupp (1995b) found that a short-phyllode headland form of *Acacia suaveolens* that occurs only on windswept coastal headlands, is twice as wide as it is tall (Auld and Morrison, 1992). This is consistent with *S. lautus* and indicates that a prostrate architecture occurs across plant taxa in these rocky, wind-exposed, saline and serpentine environments. Additionally, Morrison and Rupp (1995b) show that this prostrate growth has a genetic basis as it retains its architecture when grown in the glasshouse, indicating that a prostrate growth habit has likely evolved in many plant species to improve survival in these headland conditions. In fact in *S. lautus* the prostrate growth habit appears to be derived from the erect growth habit (Ali, 1964; Ali, 1968; Radford et al., 2004; Walter et al., 2016; Roda et al., 2013; Walter et al., 2018), further suggesting that repeated evolution of prostrate growth is occurring when *S. lautus* populations inhabit windy environments. These results indicate that differences in the environment can drive the rapid evolution of advantageous traits, contributing to our understanding of the role of natural selection in the evolution of divergence within species (Lowry, 2012; Kawecki and Ebert, 2004). Future studies should complete direct tests of the effect of wind on growth habit.

## Conclusions

Here, I provided evidence consistent with natural selection for a prostrate growth habit in the headland environment, as I correlated parental height variation with fitness variation on a common genomic background. I suggest that selection for a prostrate growth habit in the headland environment might be driven by differences in wind speed. These results aid in our understanding of the possible targets of natural selection in populations adapting to their environment. Future research should investigate the genes underlying this adaptive evolution to have a more complete picture of how natural selection contributes to evolutionary change.

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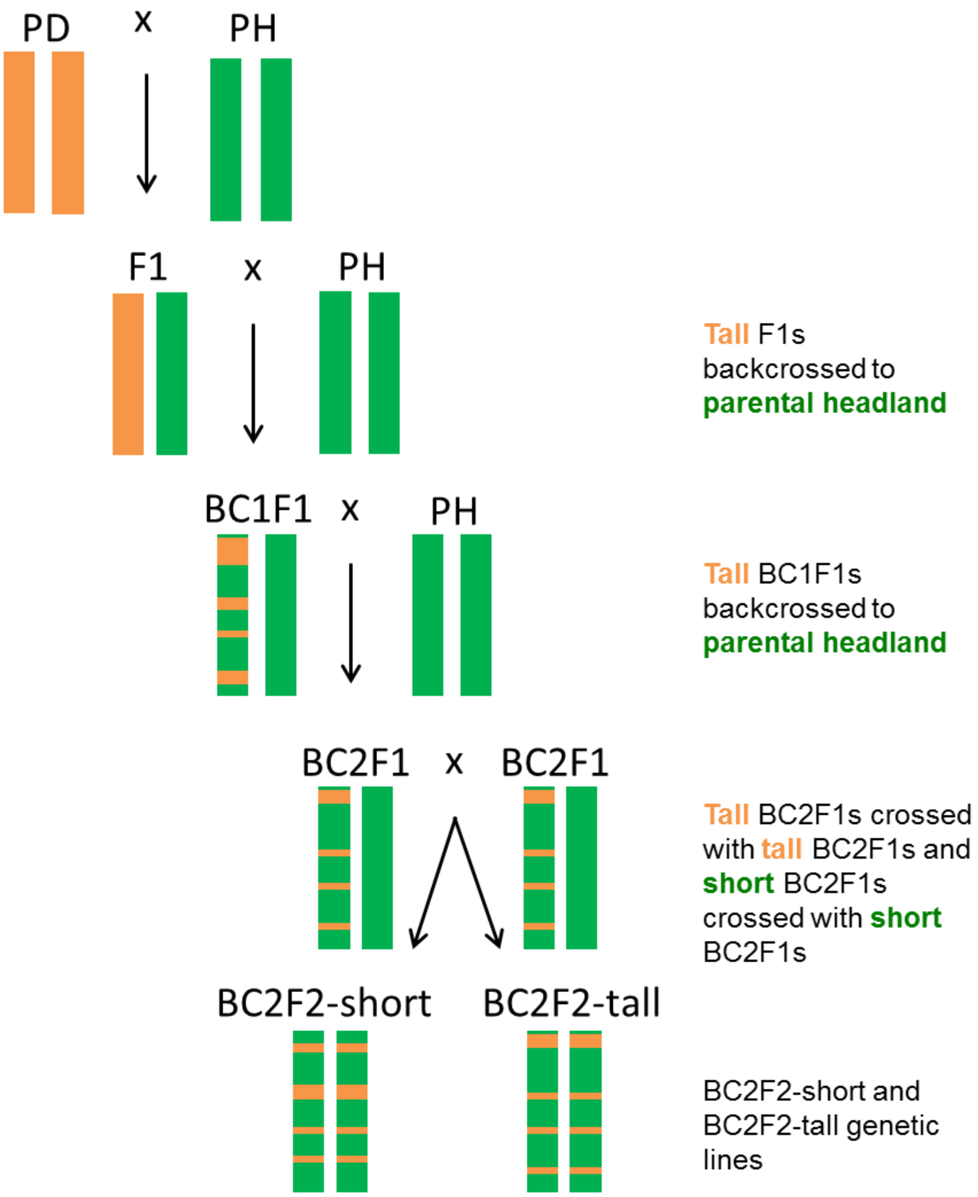
## Tables

**Table 1**. **Field selection experiment sample information.**

The number of seeds and families planted into the dune and headland environments at Lennox Head, NSW. The mean days alive of each genotype in each environment and the standard error (SE) are shown. For each genotype the percentages of individuals that germinated, reached 10 leaves, produced a bud and a flower are also shown.

| Env | Genotype | Seeds planted | Families | Mean days alive | SE days alive | % Germinated | % 10 leaves | % Bud | % Flower |
|---|---|---|---|---|---|---|---|---|---|
| Dune | BC2F2-short | 300 | 15 | 28.02 | 1.43 | 85 | 0 | 0 | 0 |
| Dune | BC2F2-tall | 258 | 13 | 24.07 | 1.59 | 73 | 0 | 0 | 0 |
| Dune | D01 | 399 | 20 | 60.05 | 2.46 | 89 | 4 | 0 | 0 |
| Dune | H01 | 399 | 20 | 38.52 | 1.68 | 91 | 0 | 0 | 0 |
| Headland | BC2F2-short | 300 | 15 | 57.12 | 3.19 | 82 | 19 | 4 | 2 |
| Headland | BC2F2-tall | 258 | 13 | 50.38 | 3.19 | 78 | 15 | 2 | 2 |
| Headland | D01 | 398 | 20 | 47.39 | 2.39 | 79 | 13 | 0 | 0 |
| Headland | H01 | 400 | 20 | 71.53 | 3.02 | 87 | 34 | 10 | 6 |

# Figures



Tall F1s backcrossed to **parental headland**

Tall BC1F1s backcrossed to **parental headland**

Tall BC2F1s crossed with tall BC2F1s and **short** BC2F1s crossed with **short** BC2F1s

BC2F2-short and BC2F2-tall genetic lines

**Figure 1. The creation of the genetic lines for isolation of growth habit loci.** These genetic lines were created by crossing the parental headland (PH) and parental dune (PD) to create an F1 generation containing variation in the erect and prostrate phenotype. Here, I selected the tallest individuals (top 20%) and crossed them to PH. This created a BC1F1 generation (first generation backcross), where the tallest individuals of this generation were again backcrossed to PH, to create a BC2F1 generation. The tallest BC2F1 individuals were then crossed amongst each other (BC2F2-tall) and the shortest BC2F1 individuals were crossed amongst each other (BC2F2-short).

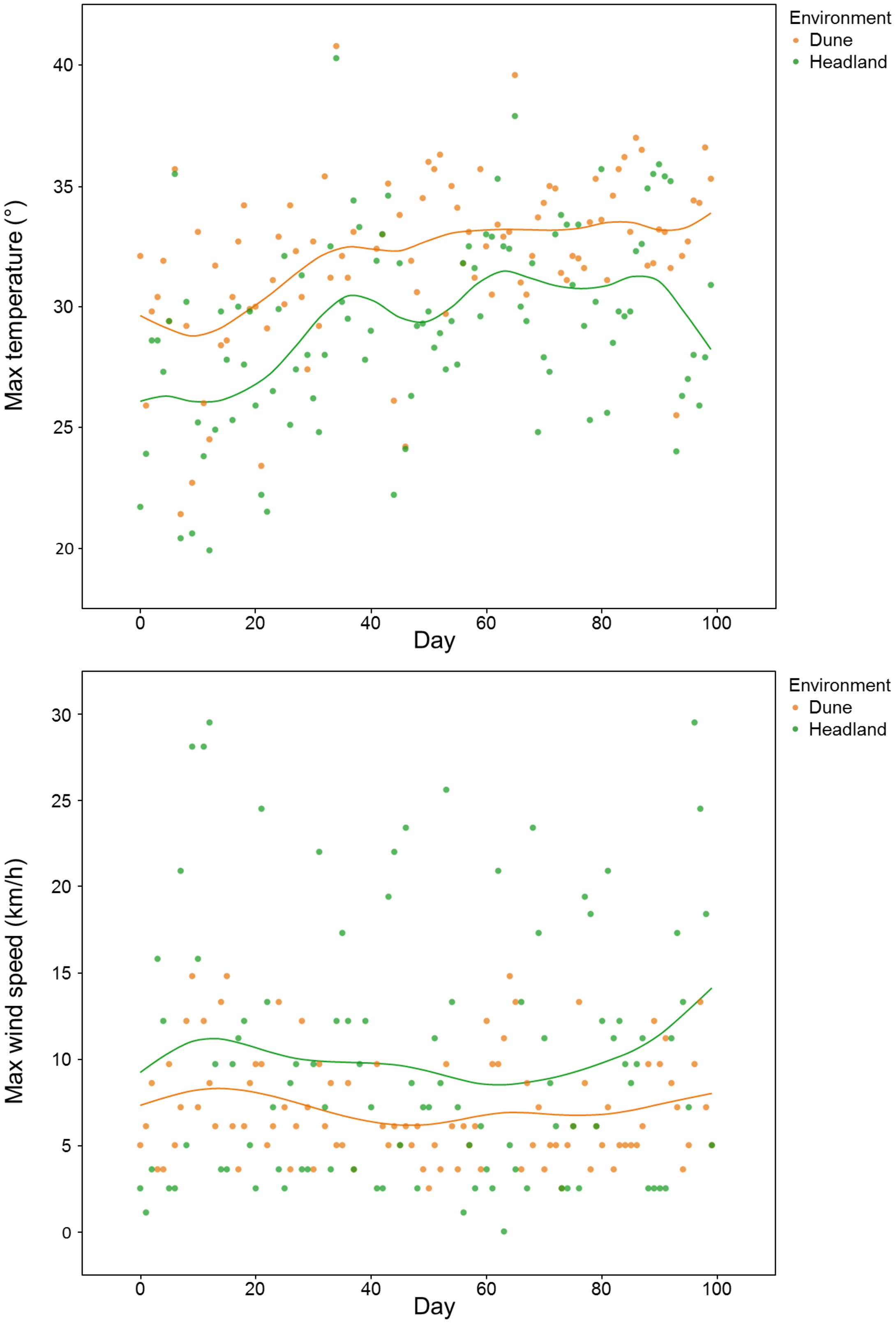


**Figure 2. Maximum temperature (°) and maximum wind speed (km/h) recorded from weather stations placed at field sites.**

The maximum temperature and maximum wind speed were recorded per day in the dune and headland environments at Lennox Head, NSW.

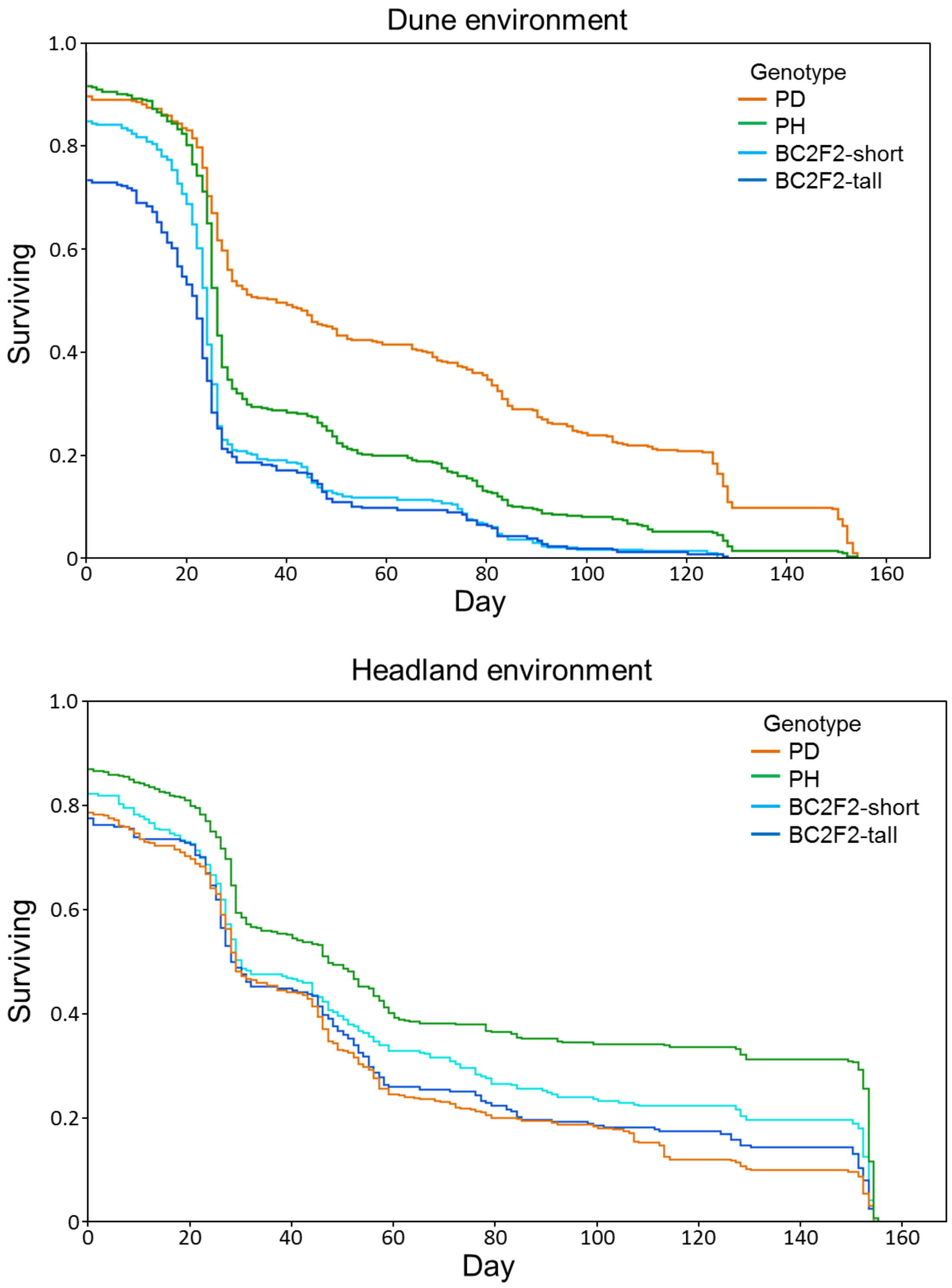


**Figure 3. Survival curves for parentals and hybrids.**

The proportion of individuals surviving for each genotype during the field selection experiment at the dune and headland environments at Lennox Head, NSW. The local parent (PD=parental dune and PH=parental headland) has a higher proportion of individuals surviving in both environments.

# Supplementary Tables and Figures

## Table S1. Survival proportions for parentals and hybrids.

The median time (days) alive for each genotype (Group) in each environment (Env) and the 95% confidence intervals are shown.

| Env | Group | Median Time | Lower 95% | Upper 95% | 25% Failures | 75% Failures |
|---|---|---|---|---|---|---|
| Dune | BC2F2-short | 24 | 23 | 24 | 18 | 27 |
| Dune | BC2F2-tall | 22 | 19 | 23 | 0 | 27 |
| Dune | PD | 38 | 29 | 47 | 24 | 97 |
| Dune | PH | 26 | 25 | 26 | 22 | 48 |
| Dune | Combined | 25 | 25 | 26 | 19 | 49 |
| Headland | BC2F2-short | 30 | 28 | 44 | 16 | 90.5 |
| Headland | BC2F2-tall | 28.5 | 27 | 43 | 9 | 76 |
| Headland | PD | 29 | 28 | 37 | 9 | 59 |
| Headland | PH | 47 | 40 | 55 | 24.5 | 153 |
| Headland | Combined | 32 | 29 | 42 | 19 | 92 |

**Figure S1**. **Photos of the plots and seedlings in the field selection experiment.** The field selection experiment was performed in the dune (top) and headland (bottom) environments at Lennox Head, NSW. Seeds were glued to toothpicks and planted into designated grid cells to track germination and survival. Toothpicks were marked with blue when the seed germinated and black when the plant died.

# Chapter 3: Convergent evolution of gravitropism and growth habit in an Australian wildflower

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Keywords: genetic basis of adaptation; convergent evolution; gravitropism; the auxin pathway

## Abstract

The diversity we see in nature is often driven by populations adapting to different environments. Yet understanding how natural selection shapes traits remains a fundamental question in evolutionary biology. The difficulty often lies in unravelling the genetic targets of selection. Harnessing cases of convergent trait evolution, whereby independent populations evolve similar traits when inhabiting a similar environment, provides us with a unique opportunity to study the role of natural selection on the origins of diversity. Here, I used related taxa from the *Senecio lautus* species complex, an Australian wildflower that has evolved similar traits in many independent populations in similar environments. *Senecio lautus* have adapted to contrasting adjacent dune and headland environments along the Australian coastline and two contrasting alpine environments. In the windy alpine and headland environments, the plants have evolved a prostrate growth habit, whereas in the sheltered alpine and dune environments, the plants have evolved an erect growth habit. Previous studies comparing adjacent Dune and Headland populations have shown that genetic differentiation is enriched in genes with functions related to the auxin pathway, which controls plant growth and development. Here, I used phenotypic and physiological experiments to evaluate the hypothesis that differences in auxin concentrations have contributed to the evolution of growth habit differences in *S. lautus*. I explored whether plant bending in response to gravity effects (gravitropism), a trait directly controlled by auxin concentration, is differentiated between 16 *S. lautus* erect and prostrate populations. My results are consistent with my hypothesis and show that populations with an erect growth habit are often better at altering their direction of growth

after a 90° change in orientation, in comparison to their adjacent prostrate population. Next, I found that modifying the auxin content and the transport of auxin with synthetic auxins and auxin transport inhibitors, had a concentration dependent effect on gravitropism, particularly when the movement of auxin out of the cell was impaired, suggesting that differences in auxin efflux is vital for gravitropism in *S. lautus*. These results provide further support for allelic differences in auxin genes driving the repeated evolution of erect and prostrate growth habits in many independent *S. lautus* populations. This research provides a platform to study the genes underlying traits that are contributing to population divergence and leading to the diversity we see in nature.

## Introduction

The repeated but independent origin of the same trait in populations inhabiting similar environments provides strong evidence for the role of natural selection in their evolution (Schluter and Nagel, 1995; Chan et al., 2010; Baxter et al., 2008; Endler, 1986). In cases of convergent evolution, a question arises: is selection acting on the same mutations, genes or pathways to evolve the adaptive traits? There are already some examples of genetic convergence; for instance, the same gene, but not the same mutation, appears to be responsible for the repeated evolution of light coat colour in beach mice that have colonised different light sand environments (Hoekstra et al., 2006; Steiner et al., 2009; Steiner et al., 2007; Linnen et al., 2013; Vignieri et al., 2010; Linnen et al., 2009). In sticklebacks, the same mutation in the EDA gene causes parallel evolution of body armour loss in freshwater environments, suggesting that evolutionary recycling can be highly constrained (Colosimo et al., 2005). However, with only few examples, it still remains unclear whether genetic convergence is common and which organisational level it is most common at; thus it remains unclear how adaptive evolution occurs at the genetic level (Conte et al., 2012).

One way in which seemingly different genetic changes might be responsible for the repeated evolution of traits is through evolution of different genes connected by the same genetic pathway (Weiss, 2000; Wessinger and Rausher, 2013). Genetic pathways are often characterised by genetic interactions amongst many genes and their products. For instance, the repeated evolution of flower colour in plants relies heavily on changes accrued in the anthocyanin pathway (Wessinger and Rausher, 2013; Hopkins et al., 2012), which controls the accumulation of pigment in the petals of a flower (Weiss, 2000). Although many populations (and species) have evolved the same flower colour, the genes involved in flower colour evolution are often different amongst them, yet always belong to the anthocyanin pathway (Rausher, 2013; Rausher, 2006). Whether there are other ways to change flower colour in plants remains unknown, but current data seems to suggest that there are genetic constraints localising flower colour evolution to this pathway. Examples from more taxa will allow us to analyse the nature of genetic constraint and whether it is common in adaptive evolution.

Many traits in the *Senecio lautus* species complex appear to have evolved under similar genetic constraints (Roda et al., 2013b). More specifically, the same genetic pathways are independently evolving in populations adapting to similar environments. Allelic differences between adjacent Dune and Headland populations were consistently found in genes that often belonged to the same functional category or genetic pathway, even if not always the exact same genes were involved. For example, genetic pathways involved in stress responses, morphogenesis, and movement of hormones were enriched with allelic differences between populations with different growth habits (Roda et al., 2013b). One of the most enriched genetic pathways was the auxin pathway, whereby 16 auxin genes contained allelic differences between multiple Dune and Headland population pairs (Roda et al., 2013b). Auxin is a plant hormone that is involved in plant growth and development and is known to play a role in phenotypes that define Dune and Headland populations, such as height (Wallace et al., 2016; Xu et al., 2005) and branching (Gallavotti, 2013; Domagalska and Leyser, 2011). Dune populations are usually tall and erect with few branches and Headland populations are usually short and prostrate with many branches (Ali, 1964; Radford et al., 2004; Roda et al., 2013a). This genotypic and phenotypic evidence advocates an investigation into the role of the auxin pathway in the evolution and divergence of *S. lautus* coastal populations.

Auxins are essential to mediate morphogenic responses based on environmental cues (Viaene et al., 2014b; Bennett et al., 2014; Langdale, 2014; Roychoudhry and Kepinski, 2015; Rakusová et al., 2015; Bennett, 2015). Auxins have played a central role at multiple scales of plant evolution: on one hand, auxins are associated with the appearance of complex architectures during the origin of land plants (Langdale, 2014; Pires and Dolan, 2012; Pires et al., 2013; Viaene et al., 2014a; Rensing et al., 2008; Kenrick and Crane, 1997; Fujita et al., 2008). On the other hand, mutations in auxin genes also underlie recent phenotypic transitions associated with plant domestication (Tan et al., 2008; Li et al., 2010; Sang et al., 2014; McSteen, 2010; Zhao et al., 2015). The role of auxins in plant evolution largely results from its role in mediating tropisms, directional movement or growth responses that occur in response to directional stimuli (Langin et al., 2015). These include responses to light (phototropism) (Whippo and Hangarter, 2006), and to gravity (gravitropism) (Paque and Weijers, 2016; Tasaka et al., 1999; Darwin and Darwin, 1881). In most plant systems, the shoot tip grows towards light and against gravity while the root tip grows away from light and down with gravity (Gilroy, 2008). A change in orientation of a plant usually causes the shoot and root tip to correct its direction of growth in relation to gravity. This occurs through auxin signalling inducing the plant to transport auxin to the lower side of the shoot and root. In the shoot, accumulation of auxin on the lower side stimulates cell elongation and causes bending of the shoot upwards (Figure 1; Gallavotti, 2013; Tasaka et al., 1999) and in the root, accumulation of auxin on the lower side induces inhibition of cell elongation and causes bending of the root downwards (Woodward and Bartel, 2005; Blancaflor and Masson, 2003; Vanneste and Friml, 2009).

Auxin genes are diverse and are involved in auxin signalling, transport and biosynthesis and any part of this pathway might affect a variety of phenotypes. The aim of this study was to investigate whether the auxin pathway is involved in the evolution of the major phenotypic differences between adjacent Dune and Headland populations of *S. lautus*. Exposing Dune and Headland seedlings to synthetic auxins that increase auxin content and to auxin transport inhibitors that block the activity of auxin transporters can 1) show the involvement of the auxin pathway in gravitropism in *S. lautus* and 2) indicate which part of the auxin pathway is vital for gravitropism in *S. lautus* (Figure 2). For example, plants with mutations in their auxin influx carriers have a diminished ability to respond to gravity, but gravitropism can be rescued when synthetic auxin 1-Naphthaleneacetic acid (1-NAA) is applied (Marchant et al., 1999; Yamamoto and Yamamoto, 1998). Unlike the naturally occurring auxin, Indole-3-acetic acid (IAA), and the synthetic auxin, 2,4-Dichlorophenoxyacetic acid (2,4-D), 1-NAA can enter the cell through diffusion and not rely on this influx carrier to perform its function (Yamamoto and Yamamoto, 1998). Another way of testing the influence of the influx carrier on gravitropism is to block the influx carrier with 1-Naphthoxyacetic acid (1-NOA) (Laňková et al., 2010; Parry et al., 2001; Yang et al., 2006). 1-NOA reduces auxin uptake through the influx carrier which has been shown to reduce root gravitropism in *Arabidopsis* (Ottenschläger et al., 2003; Parry et al., 2001). Efflux carriers have a major role in root gravitropism with studies in *Arabidopsis* finding that disrupting the efflux carriers reduces the formation of the auxin concentration gradient and subsequent reduction in gravitropism (Benková et al., 2003; Yamamoto and Yamamoto, 1999; Ottenschläger et al., 2003). The importance of the efflux carrier can be tested with 1) 1-N-naphthylphthalamic acid (NPA), which blocks the activity of the carrier (Friml et al., 2003); and 2) synthetic auxin 2,4-D that has poor affinity with the efflux carrier compared to IAA and NAA. NPA and 2,4-D both remove the control of the auxin carrier in natural auxin and synthetic auxin movement. Thus reductions in gravitropism with addition of NPA and 2,4-D suggest that auxin efflux carriers are vital for creation of the auxin concentration gradient required for gravitropic bending to occur (Benková et al., 2003; Ottenschläger et al., 2003; Yamamoto and Yamamoto, 1998; Delbarre et al., 1998; Enders and Strader, 2015).

Here, I used phenotypic and physiological experiments to test the hypothesis that allelic variation in auxin genes drove the repeated evolution of divergent growth habits in *S. lautus.* Despite an erect growth habit being common to dune environments and a prostrate growth habit being common to headland environments, adjacent dune and headland *S. lautus* populations are more genetically similar (Roda et al., 2013a; Melo, 2014), providing a platform to test for repeated evolution of growth habit. Furthermore, these populations consist of two monophyletic clades (Roda et al., 2013a; Melo, 2014), grouped by two genetically distinct regions (eastern and southern), allowing for tests of repeated evolution with unique genetic backgrounds. In previous analyses that aimed to understand the genes responsible for major phenotypic differences between Dune and Headland populations, genes within the auxin pathway were repeatedly involved (Roda et al., 2013b). In this study, I predicted that if there were differences in the auxin pathway between Dune and Headland populations, then there would also be differences in auxin controlled phenotypes such as gravitropism. In other words, I used the gravitropism phenotype as an avenue to explore divergence in the auxin pathway. Furthermore, I predicted that the direction of the gravitropism difference would be consistent with plant architectural differences between Dune and Headland populations, more gravitropic in the Dune to achieve an erect growth habit and less gravitropic in the Headland to achieve a prostrate growth habit. To test these hypotheses, I measured gravitropism in 1,278 plants across 14 Dune and Headland populations, by recording the angle of the stem (within a 24 hour period) after a 90° change in orientation. Then to understand whether differences in the auxin pathway were correlated to differences in growth habit, I looked at the relationship between gravitropism and a quantitative measure of erect and prostrate growth habits, plant height. Next, I physiologically investigated the involvement of the auxin pathway for gravitropism in *S. lautus*. This was achieved through modifying the auxin content and the transport of auxin with synthetic auxins and auxin transport inhibitors, respectively, and observing how this influenced gravitropism in Dune and Headland seedlings.

## Methods

### Study System

The *Senecio lautus* species complex consists of multiple taxonomic species (Roda et al., 2013a) that inhabit a variety of environments (Radford et al., 2004; Thompson, 2005). There is a strong correlation between the morphology of populations and the environment in which they live (Figure 3; Figure S1), with the *S. lautus* system containing many ecotypes from the coast and inland of Australia, including - Headland, Dune, Alpine, Tableland and Woodland ecotypes (Radford et al., 2004). One of the most striking patterns occurs along the coast, where adjacent Dune and Headland population pairs show contrasting growth habits despite high potential for gene flow (Roda et al., 2013a; Melo, 2014). Populations inhabiting hot sand dunes often have an erect growth habit and populations found in the adjacent windy rocky headlands often have a prostrate growth habit (Figure 3; Figure S1). Dune and Headland population pairs are often sister taxa but local adaptation has created strong extrinsic reproductive isolation between the populations (Melo et al., 2014; Richards and Ortiz-Barrientos, 2016; Richards et al., 2016; Walter et al., 2016).

To explore the genetic basis of parallel growth habit in plants, I studied 16 coastal populations of the Australian wildflower, *Senecio lautus*. Of these populations, 14 occur along the eastern and southern coastline of Australia and occupy adjacent dune and headland environments. The remaining two populations are inland Alpine populations at Kosciusko National Park (A07) and Falls Creek (A03). The divergence in growth habit also occurs in these Alpine populations, where the sheltered alpine environment, A03, contains erect plants and the exposed windy ridge alpine environment, A07, contains prostrate plants. Phylogenetic analysis has shown that the *S. lautus* system is genetically split into two monophyletic clades: populations from the east coast of Australia are in the eastern clade, whereas populations from the south coast of Australia and the two inland Alpine populations are in the southern clade (Roda et al., 2013a; Melo, 2014).

### Height variation in *S. lautus*

To quantitatively capture growth habit differences between prostrate and erect growth habits, I measured vegetative height in both controlled glasshouse conditions, and in the native field environment. Similar height measurements taken in both the glasshouse and the field indicate height has a strong genetic basis and that this is unlikely to be due to phenotypic plasticity. Population locations are shown in Table S1 and the number of individuals per population where height was measured is shown in Table S2.

I measured vegetative height in 12 Dune and Headland populations in their native environment. At least 32 individuals per population were measured. This excluded four populations that were only measured in the glasshouse as they could not be visited for field measures (D23 and H21 at Point Labatt, A03 at Falls Creek and A07 at Kosciusko NP). To reduce variation in height caused by different developmental stages, I phenotyped each population in the field during the flowering season specified for Coffs Harbour in Richards and Ortiz-Barrientos (2016). The Dune population at Cape Bridgewater (D32) was the only population where less than half of the individuals were flowering (Table S2). Nonetheless, the vegetative height remained consistent with glasshouse measures (Figure 4). To get an even distribution of individuals across a population, I measured the total length of the range of the population and took 32 individuals from evenly spaced points across the range. In populations with a small range, I ensured that the individuals measured were growing at least a metre apart to reduce the chances of measuring related individuals.

I measured vegetative height in all 16 Dune and Headland populations in controlled glasshouse conditions. An average of 14 individuals per population was measured (Table S2). Briefly, seeds collected from field sites (Table S1) were germinated by cutting 1mm off the micropyle side of the seed and placed in petri dishes with dampened filter paper. The seeds were placed in darkness for two days for roots to grow and then transferred to a 12 hour light/day cycle in a constant temperature room at 25°C for seven days for shoots to grow. Seedlings were then transferred into pots with standard potting mix and grown in the glasshouse. Plants were watered every day. In order to remove maternal effects, one round of crossing in the glasshouse occurred amongst plants from the same locality. Crossing involved rubbing flower heads of individuals together and collecting the seeds produced. These parental seeds were then germinated and grown under the same conditions as those mentioned above and only one individual per family was used to avoid family effects. Vegetative height (cm) was measured once plants were mature and flowering, and was measured as the vertical distance from the soil to the highest point of the plant that had vegetative leaves (flowers and stems are not included).

For both the glasshouse and field measurements, I used a linear model to determine whether Dune populations were taller than their adjacent Headland pair:

$$y_{ijk} = P_i + E_{j(i)} + e_{k(ij)} \tag{1}$$

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. Population height ($y_{ijk}$) for each pair was compared using a one-tailed t-test. The Alpine populations were also included with the prediction that the sheltered Alpine population (A03) would be taller than the wind exposed Alpine population (A07). All statistical results reported here were produced in JMP v13 (SAS 2015).

### Gravitropism variation in *S. lautus*

As the auxin hormone is known to control gravitropism, I hypothesised that allelic differences in the auxin pathway between *S. lautus* populations might lead to differences in gravitropism. Specifically, I expected that tall Dune populations would be more gravitropic than their short Headland pair. To test this hypothesis, I measured the angle of the stem after a 90° rotation of a seedling (Sang et al., 2014; Lopez et al., 2014; Huang et al., 2014; Cho et al., 2014; Rigo et al., 2013; Ottenschläger et al., 2003; Blancaflor and Masson, 2003; Rashotte et al., 2000; Müller et al., 1998). I performed this gravitropism experiment on seedlings from the same populations as that whose height had been measured (Table S1). To reduce maternal effects, 10 populations had one round of random crossing in the glasshouse amongst individuals of the same population. The remaining six populations' utilised seeds collected straight from the field (Table S3). I grew 2-4 seeds per family for ~40 maternal families (1,278 seeds in total; Table S3).

The gravitropism assay was performed *in vitro* on agar plates in a dark growth cabinet. The seeds from a population were combined in a falcon tube and were first sterilised with a quick rinse in 70% EtOH, followed by four 10 minute inversions in a sterilising 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% MES, 0.15% sucrose and 1% agar. I placed eight seeds on each plate and incubated the plates at 21°C in the dark to avoid any light effects. After seven days, or once the majority of seeds had reached a length greater than 4mm, all plates were rotated clockwise by 90°. I took a photograph of each plate 24 hours after rotation. The photographs were imported into ImageJ (Schneider et al., 2012) to measure gravitropism, defined as the angle to which the stem reorientated itself in relation to the new gravity vector (Figure S2).

Overall there was a 63.8% germination success but some seedlings were removed from analyses, as the angle could not be measured as they did not meet the following criteria: minimum stem length of 5mm, germinated after rotation, or growth was not initially upright so angle was not reflective of the response to the change in the gravity vector. This left a total of 736 seedlings across 16 populations where gravitropism was measured (57.6% of the total number of seeds planted). For the exact number of seeds for each population that germinated and that were used in analyses, refer to Table S3. To test the hypothesis that Dune populations would have a stronger gravitropic response in their stem than their adjacent Headland pair, I used a mixed linear model:

$$y_{ijkl} = P_i + E_{j(i)} + A_k + e_{l(ijk)} \qquad (2)$$

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 measures were averaged for each population and compared between each population pair using a one-tailed t-test. This linear model was also performed on root gravitropism to observe whether there were similar differences in shoot and root gravitropism between Dune and Headland pairs. As there was no expectation for the direction of root gravitropism, a two-tailed t-test was used. All statistical results reported here were produced in JMP v13 (SAS 2015).

If a plant's gravitropic ability determines its growth habit and height, then I would expect to observe a positive correlation between gravitropism and height at the population level. I hypothesised that populations with a strong gravitropic response would grow upright and be taller. To test this, I performed a linear regression with mean vegetative height against mean gravitropism for all 16 populations, where populations were grouped into their respective clades (eastern and southern) (Table S1).

### Auxin treatments and inhibitor experiments in *S. lautus*

To investigate the involvement of the auxin pathway for gravitropism in *S. lautus* and indicate which points in the auxin pathway are vital for gravitropism in *S. lautus*, I applied synthetic auxins and auxin transport inhibitors to Headland and Dune seedlings. Firstly, to increase the auxin content, I used two synthetic auxins, 1-Naphthaleneacetic acid (1-NAA) that travels into the cell by diffusion (Marchant et al., 1999; Yamamoto and Yamamoto, 1998) and 2,4-Dichlorophenoxyacetic acid (2,4-D) that relies on influx carriers to enter the cell (Yang et al., 2006; Yamamoto and Yamamoto, 1998) but is poorly transported out of the cell by auxin efflux carriers (Delbarre et al., 1998; Rinne and van der Schoot, 1998). I predicted that if Headlands had a mutation in the influx carrier or were deficient in auxin, then 1-NAA that diffuses into the cell would rescue gravitropism. I also used two transport inhibitors, 1-naphthoxyacetic acid (1-NOA) an influx inhibitor (Parry et al., 2001) and 1-N-naphthylphthalamic acid (NPA) an efflux inhibitor (Ottenschläger et al., 2003). These inhibitors reduce the function of the carriers in transporting auxin into the cell and transporting auxin out of the cell, respectively (Figure 2). Studies in *Arabidopsis* have found that these influx and efflux carriers are used for transport of auxin to create an auxin concentration gradient which is necessary for gravitropic bending (Benková et al., 2003; Yamamoto and Yamamoto, 1999; Ottenschläger et al., 2003; Laňková et al., 2010; Parry et al., 2001; Yang et al., 2006). I predicted that *S. lautus* is similar to the *Arabidopsis* model and thus both transport inhibitors would decrease gravitropism. I show the expected movement of auxin with the addition of each chemical in Figure 2.

The auxin treatments and the inhibitor experiment were completed using sterilised seeds (as detailed above) and grown on MS agar plates containing the different chemicals at concentrations of 0µM, 0.5µM, 5µM and 50µM. For this I used the following stock solutions: NPA: 10mM in DMSO; 1-NAA: 10mM in DMSO; 1-NOA: 500mM in DMSO; 2,4-D: 1mM in Ethanol. I created 1ml dilutions of stock solutions (in ethanol of DMSO), which were dissolved in 500ml of media. Plates were incubated, rotated, photographed and gravitropism measured as described above. Only one population pair, Lennox Head (H01 and D01), was used in this auxin assay in order to maximise the sample size for each chemical and concentration. Lennox Head was chosen due to strong differences in height and gravitropism between its Dune and Headland populations. Approximately 40 individuals with one individual per family were used for each chemical concentration, resulting in a total of 1,273 seeds. Refer to Table S4 for the number of individuals grown for each chemical, concentration and ecotype.

To determine the effect of the chemicals on gravitropism, I performed a non-parametric Wilcoxon rank sum test using a total of 421 individuals. Addition of chemicals has negatively skewed the distribution due to an increase of individuals unable to respond to a change in orientation. There are no gravitropism measures for synthetic auxins 1-NAA and 2,4-D at 50µM, as the chemicals were lethal at this very high concentration. In addition, gravitropism could not be measured on more than 90% of individuals exposed to 50µM of the efflux inhibitor, NPA, and so it was removed from the analysis. For both ecotypes and each of the four chemicals, I determined whether there was a significant shift in gravitropism at different chemical concentrations. Table S5 contains the mean and standard error values for each chemical, concentration and ecotype. All statistical results reported here were produced in JMP v13 (SAS 2015).

## Results

### Phenotypic variation in the *Senecio lautus* species complex

Height was measured in populations of the *S. lautus* species complex across Australia to quantitatively assess variation in growth habit. Considering the Dune ecotype is defined as having an erect growth habit and the Headland ecotype is defined as having a prostrate growth habit (Radford et al., 2004; Ali, 1964), I hypothesised, that for each pair, the Dune population would be taller than its Headland pair. I also expected that height would have a genetic basis (not be driven by plasticity) and therefore measurements in the field and glasshouse conditions would follow the same pattern. Population pairs from the eastern clade supported this hypothesis, both in field and glasshouse conditions, where Dune populations produced taller individuals in comparison to their Headland pair (Figure 4). In the southern clade on the other hand, the Dune population was taller than its Headland pair in two out of four pairs, that is, at Falls Creek/Kosciusko NP and Cape Bridgewater. At Millicent and Point Labatt I found no difference in height between the Dune and Headland populations (Figure 4). This might be due to greater similarity between the dune and headland environments at these localities than in the eastern clade, which I will detail in the discussion. Cape Bridgewater and Millicent both have field and glasshouse height measurements that follow the same pattern, while Falls Creek, Kosciusko NP and Point Labatt could not be measured in the field, and so only glasshouse measures are shown.

The statistical results for the one tailed t-tests between Dune and Headland pairs measured in the glasshouse (Table S6) and field (Table S7) are visually represented in Figure 4.

To investigate whether the auxin pathway is responsible for the major phenotypic differences between Dune and Headland populations, I measured their ability to respond to gravity, a trait that is controlled by auxin (Dong et al., 2013; Li et al., 2007; Friml et al., 2002; Blancaflor and Masson, 2003). Population gravitropism differences between adjacent Dune and Headland populations were compared to the differences I observed in height at each locality. I predicted that if the differences in height between Dune and Headland population pairs are controlled by allelic variation in genes in the auxin pathway, then I would find differences in auxin controlled phenotypes, such as gravitropism. More specifically, I predicted that Dune populations that are taller than their Headland pair would also have stronger gravitropic responses in their shoots. I measured gravitropism as the angle to which the plant corrected its direction of growth after a 90° rotation, thus larger angles would indicate stronger gravitropic responses. I found that in four out of five coastal population pairs with a significantly taller Dune population, the Dune population also had stronger shoot gravitropic responses than its Headland pair (Table S8; Figure 4). The Alpine populations showed the same pattern, where the sheltered Alpine meadow population was taller and had stronger gravitropic responses than the rocky wind-exposed Alpine population, suggesting that the correlation between the two phenotypes has also evolved across ecotypes. The two population pairs that were not differentiated in plant height were Millicent which, as expected, had no differentiation in gravitropism, and Point Labatt, which unexpectedly had slight differences in gravitropism between its Dune and Headland populations (Figure 4). Lastly, Stradbroke Island showed strong differences in plant height but no differences in shoot gravitropism between the Dune and the Headland populations, suggesting that not all *S. lautus* populations show the same pattern. The results for root gravitropism showed most localities did not differ in root gravitropic responses between Dune and Headland populations (Table S9). Lennox Head and Point Labatt were the exceptions, as these Dune populations had stronger root gravitropic responses than their adjacent Headland population. This indicates that the differences in the gravitropism phenotype are focussed on the aerial architecture in many of these populations.

I then investigated the overall pattern between height and gravitropism across all *S. lautus* populations in this study. The analysis was performed with height measures in the glasshouse and populations were split into their respective clade, the eastern clade, which consists of populations from the east coast of Australia and the southern clade, which consists of populations from the south coast of Australia. I found a strong positive correlation between height and gravitropism across *S. lautus* populations and the direction of the correlation is consistent with my predictions, with the taller the *S. lautus* population, the more gravitropic the population (Figure 5; eastern clade $F_{1,6}$=6.26, p=0.0464, r=0.71; southern clade $F_{1,6}$=13.26, p=0.0108, r=0.83). Overall, the eastern clade, in comparison to the southern clade, has a higher magnitude in their gravitropic ability but a weaker correlation between height and gravitropism (Figure 5).

### Auxin efflux carrier vital for gravitropism in *S. lautus*

To further explore the role of auxin in the evolution of growth habit, I exposed Dune and Headland seedlings to synthetic auxin and auxin transport inhibitors. I hypothesised that if there was a mutation in the Headland influx carrier, synthetic auxin 1-NAA (that enters the cell through diffusion) would bypass the influx carrier and rescue gravitropism. However, 1-NAA did not rescue gravitropism in Headland seedlings. Instead, it did not influence Headland gravitropism but reduced Dune gravitropism (Figure 6). The influx inhibitor, 1-NOA, slightly reduced Dune gravitropism but not Headland gravitropism (Figure 6), showing 1-NOA has an effect on *S. lautus* but the Headland is not hypersensitive to it. Synthetic auxin 2,4-D, (that enters the cell through the influx transporters but has little affinity for exiting the cell through the efflux carrier), strongly reduced gravitropism in the Dune and the Headland (Figure 6). This supports the hypothesis that the auxin efflux carrier is important for the redistribution of auxin to create an auxin differential to initiate bending. This is further supported by the efflux inhibitor, NPA, dramatically reducing gravitropism in both the Dune and the Headland (Figure 6). At high concentrations (50 µM), the synthetic auxins, 1-NAA and 2,4-D, were lethal. Table S10 contains the chi-square results used for significance values shown in Figure 6.

## Discussion

To understand the genetic basis of growth habit in *S. lautus*, I studied 16 populations evolving erect and prostrate growth habits. I found that Dune populations are often taller and more gravitropic than their adjacent Headland pair. The strong correlation observed between gravitropism and height supports the hypothesis that genes within the auxin pathway contributed to the evolution of growth habit. This relationship between gravitropism and height occurs in many but not all *S. lautus* populations, suggesting that genetic convergence at the pathway level is likely to be partially responsible for the evolution of growth habit phenotypes. Here I discuss the differences observed in gravitropism and height between the eastern and southern clades and how this might be related to environmental differences between the clades. I also discuss possible genes within the auxin pathway that might be underlying these differences in gravitropism for future studies to investigate. Overall, my results provide a major leap towards understanding the genetic basis of adaptation in this system, contributing to our understanding of the fundamental questions about the process of adaptation.

### Convergent evolution of growth habit phenotypes

The genetic mechanisms used by populations evolving similar traits under similar selective pressures still remains a vital question in evolutionary biology (Stern, 2013; Stern and Orgogozo, 2009; Losos, 2011; Arendt and Reznick, 2008). Assessing whether populations utilise genes connected by a genetic pathway is the first step in understanding the genetic constraints that populations might face. This will ultimately enable us to understand the fundamental processes of how populations adapt to their environment. Studies in periwinkle (Ravinet et al., 2016), whitefish (Landry et al., 2007; Renaut et al., 2013), stickleback (Erickson et al., 2016) and beach mice (Hoekstra et al., 2006) have shown that populations can use both the same and different genetic mechanisms to evolve similar phenotypes. Beach mice populations, for instance, have evolved a lighter coat colour to camouflage themselves against their new, lighter sand environment. Evolution has occurred through the MC1R gene in some beach mice populations (Hoekstra et al., 2006) and the AGOUTI gene has been used in other beach mice populations (Steiner et al., 2007). The results in this study also suggest that the same and different genetic mechanisms were used in different *S. lautus* populations evolving similar growth habits in response to the environment. Five of the six population pairs that had differences in height also had differences in the auxin controlled shoot gravitropism phenotype in the expected direction, which is consistent with these populations utilising the same genetic pathway to evolve the same trait. One exception is the Dune and Headland population pair at Stradbroke Island that did not differ in gravitropism despite differences in height. It is possible that genes in the auxin pathway not involved in gravitropism were utilised in the evolution of the erect and prostrate phenotype in this population pair, but gravitropism evolution could just as likely have occurred through an alternative genetic route. Non-auxin candidate genes with functions in environmental adaptation, phenotypic divergence and reproductive isolation have previously been identified as containing allelic differences between Dune and Headland pairs (Roda, 2014). This supports the idea that another genetic route could have been used to solve the same phenotypic problem and suggests that evolution of growth habit might not be completely constrained to one genetic pathway in the *Senecio lautus* complex.

Environmental differences might be responsible for the observed differences in height and shoot gravitropism between the eastern and southern *S. lautus* phylogenetic clades. Six out of eight wind exposed populations (Headlands and the exposed Alpine) have strikingly short growth compared to their sheltered population pair (Dunes and sheltered Alpine). This is not surprising as many plant species growing in very windy environments are known to develop strong and woody root systems, a prostrate growth and branched aerial parts to adhere to the substrate (Mirabet et al., 2011; Monshausen and Haswell, 2013; Garrido et al., 2015; Hamant, 2013; Wilson and Archer, 1979). Interestingly, the two Dune and Headland populations in the southern clade which do not have striking differences in height (Millicent and Point Labatt) also do not have striking differences in environmental variables (Roda et al., 2013a). For example, these Dune populations are at the top of high dunes (approx. 7m from sea level), leaving them exposed to higher winds relative to the eastern clade Dune populations that are at sea level. The southern clade as a whole is also less gravitropic by a magnitude of ~10° compared to the eastern clade, supporting the idea that there might be different selective pressures acting on the two clades. Unfortunately, there are no quantitative measures of wind in the southern clade environments to make direct comparisons between wind speed and growth habit. Future research should directly investigate the role of wind in the evolution of growth habit in *S. lautus* and the environmental differences between the clades that might explain the observed phenotypic differences.

### Adaptation to coastal environments through the auxin pathway

Overall my results indicate that selection on auxin genes could have contributed to the evolution of divergent growth habits in *S. lautus*. I found a strong correlation between a growth habit phenotype, height, and an auxin controlled phenotype, shoot gravitropism in many *S. lautus* populations. I did not find the same correlation with height and root gravitropism, indicating that the differences in the auxin pathway are specific to the aerial parts of the plant that might influence their growth habit. Auxins are known to modulate growth and development via their role in regulating mechanosensing, phototropism, gravitropism, apical dominance and branching (Hamant, 2013; Wilson and Archer, 1979). In *S. lautus,* functions related to morphogenesis and development are enriched in differentiation outliers from adjacent Dune and Headland populations (Roda et al., 2013b), suggesting natural selection is operating in a large number of *S. lautus* morphogenic genes. Several of these morphogenic genes are involved in the auxin pathway, including auxin influx and efflux carriers, as well as the auxin response factors (ARF), which control expression in response to auxin (Roda et al., 2013b).

Here, I provided evidence that differences in the auxin efflux carrier might be contributing to the evolution of divergent growth habits in *S. lautus*. Firstly, the genes controlling the efflux carriers contain alleles that are highly differentiated in more than three pairs of Dune and Headland natural populations (Roda et al., 2013b), indicating that a simple genetic change might have been replicated across some population pairs and thus contributed to the evolution of divergent growth habits. Secondly, studies in maize (Wallace et al., 2016) and rice (Chen et al., 2012) have also found strong associations between plant height and auxin efflux carriers. For example, overexpression of an auxin efflux carrier (PIN2) in rice results in a larger tiller angle and reduced plant height (Chen et al., 2012). Thirdly, the synthetic auxins and auxin transport inhibitors applied to Lennox Head Dune and Headland seedlings in the current study, further implicates the importance of efflux carriers in the control of gravitropism, whereby the synthetic auxin, 2,4-D, and auxin efflux inhibitor, NPA, considerably reduced shoot gravitropism in Dune and Headland seedlings. 2,4-D has low affinity for efflux carriers and NPA blocks the efflux carriers, both lowering the control of the efflux carrier in creating an auxin differential for bending to occur. Studies in other plant systems have found that 2,4-D and NPA reduced root gravitropism as the auxin gradient was lost (Yamamoto and Yamamoto, 1998; Ottenschläger et al., 2003; Robert et al., 2015). Overall the results are in support of defects in polar transport of auxin through mutations in efflux genes being responsible for the reduction in gravitropism in some Headland populations.

Interestingly, blocking the influx carrier did not reduce gravitropism in *S. lautus* Headland seedlings as I hypothesised, and only reduced Dune gravitropism at high concentrations (50µM). It is possible that the Dune seedlings are slightly more sensitive to this inhibitor and the Headland seedlings were not exposed to the required dose to see an effect. Understanding a biological reason behind this might be difficult as the molecular mechanism of how 1-NOA reduces auxin influx remains unknown (Parry et al., 2001). Thus, more research on the molecular mechanisms of 1-NOA must occur before conclusions about the importance of auxin influx in gravitropism in *S. lautus* can be made. Future research should apply these synthetic auxins and transport inhibitors to different population pairs. Considering allelic differences between Dune and Headland pairs are often in different auxin genes (Roda et al., 2013b), I suspect that not all population pairs would respond to the chemicals in the same manner. For example, a different Headland population might have a mutation in the influx carrier and therefore shoot gravitropism would be rescued with addition of synthetic auxin, 1-NAA that bypasses the influx carrier.

## Conclusions

Overall the results of this study, and previous studies, are consistent with natural selection for growth habit related phenotypes in *S. lautus*, occurring through allelic differences in the auxin pathway in many, but possibly not all, populations of *S. lautus.* These results have important implications for our understanding of how populations adapt to their environment and how common gene reuse is, in independent populations, faced with the same selective pressures.

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## Figures

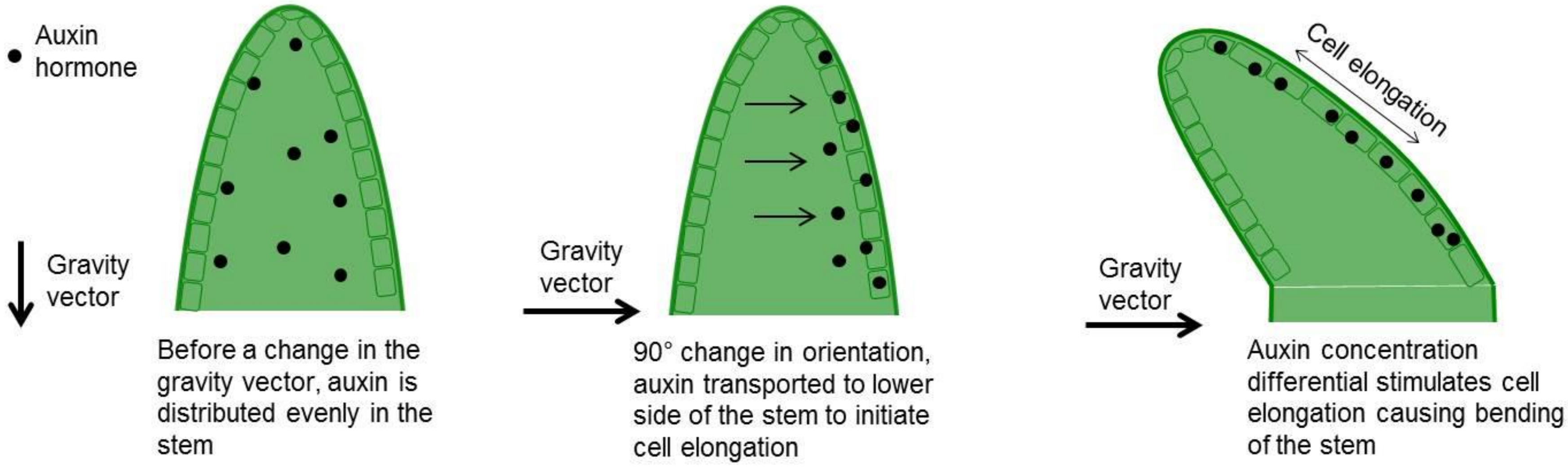

**Figure 1. The role of auxin in gravitropism in the stem.**

Before a change in the gravity vector, the auxin hormone is distributed evenly in the stem and the stem grows against gravity. When the stem is re-orientated by 90°, auxin is transported to the lower side of the stem to initiate cell elongation. The auxin concentration gradient stimulates the cells to elongate, causing bending of the stem in response to the change in orientation. The stem continues to bend until it is growing against gravity.

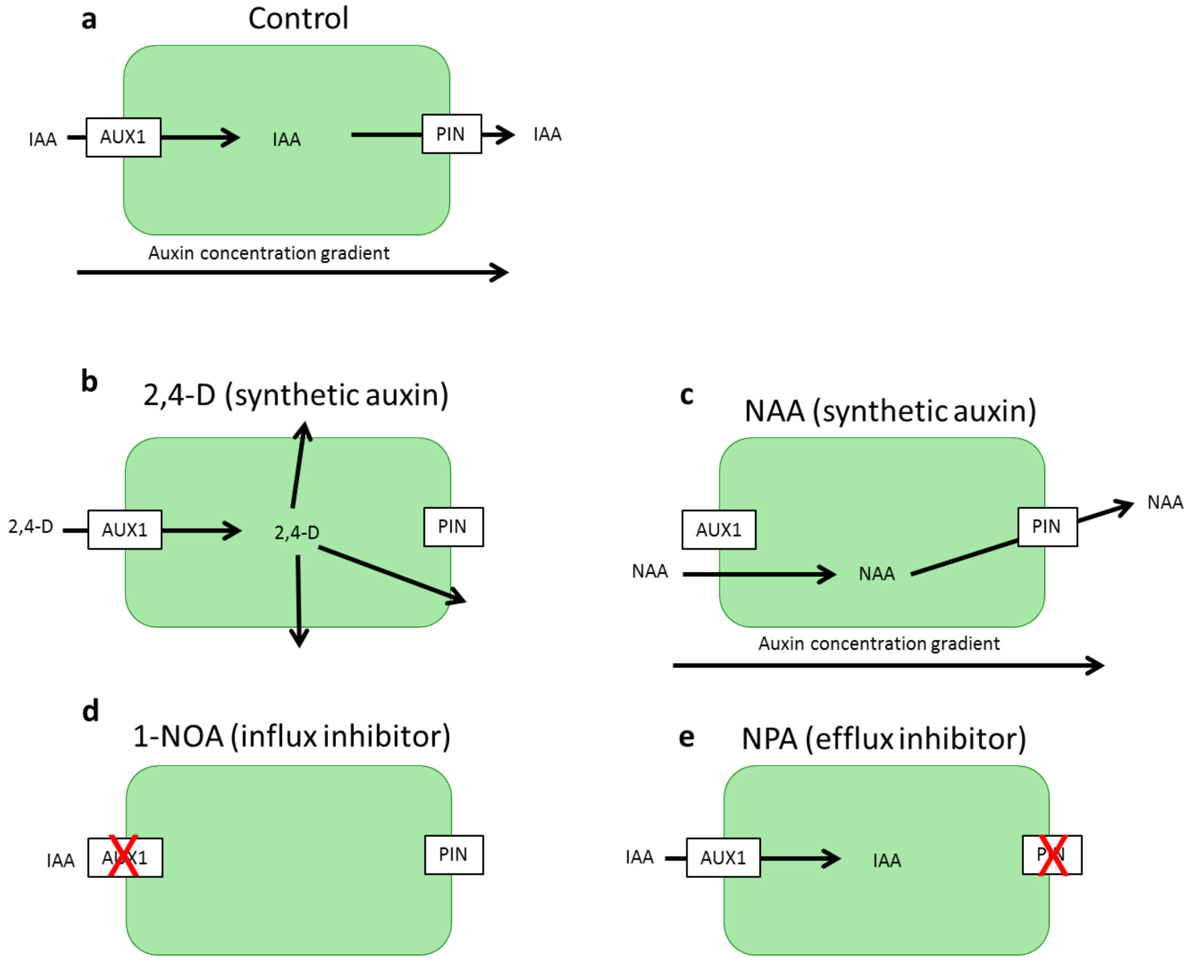

**Figure 2. The effect of synthetic auxins and auxin transport inhibitors on gravitropism in plant cells.**

**a**. When wild type cells (control) respond to a change in the gravity vector, naturally occurring auxin, IAA, enters the cell through AUX1 influx carrier and exits the cell through a PIN efflux carrier. The movement of auxin creates an auxin concentration gradient to initiate bending of the stem. **b**. When synthetic auxin 2,4-D is added, it enters the cell through AUX1 but has low affinity with PIN so diffuses out of the cell, diminishing the ability to create an auxin concentration gradient. **c**. Synthetic auxin NAA has low affinity with AUX1, thus diffuses into the cell and an auxin concentration gradient is created by PIN controlling the movement out of the cell. **d**. 1-NOA influx inhibitor blocks AUX1 activity and stops IAA from entering the cell. **e**. NPA efflux inhibitor blocks PIN activity and stops IAA from exiting the cell.

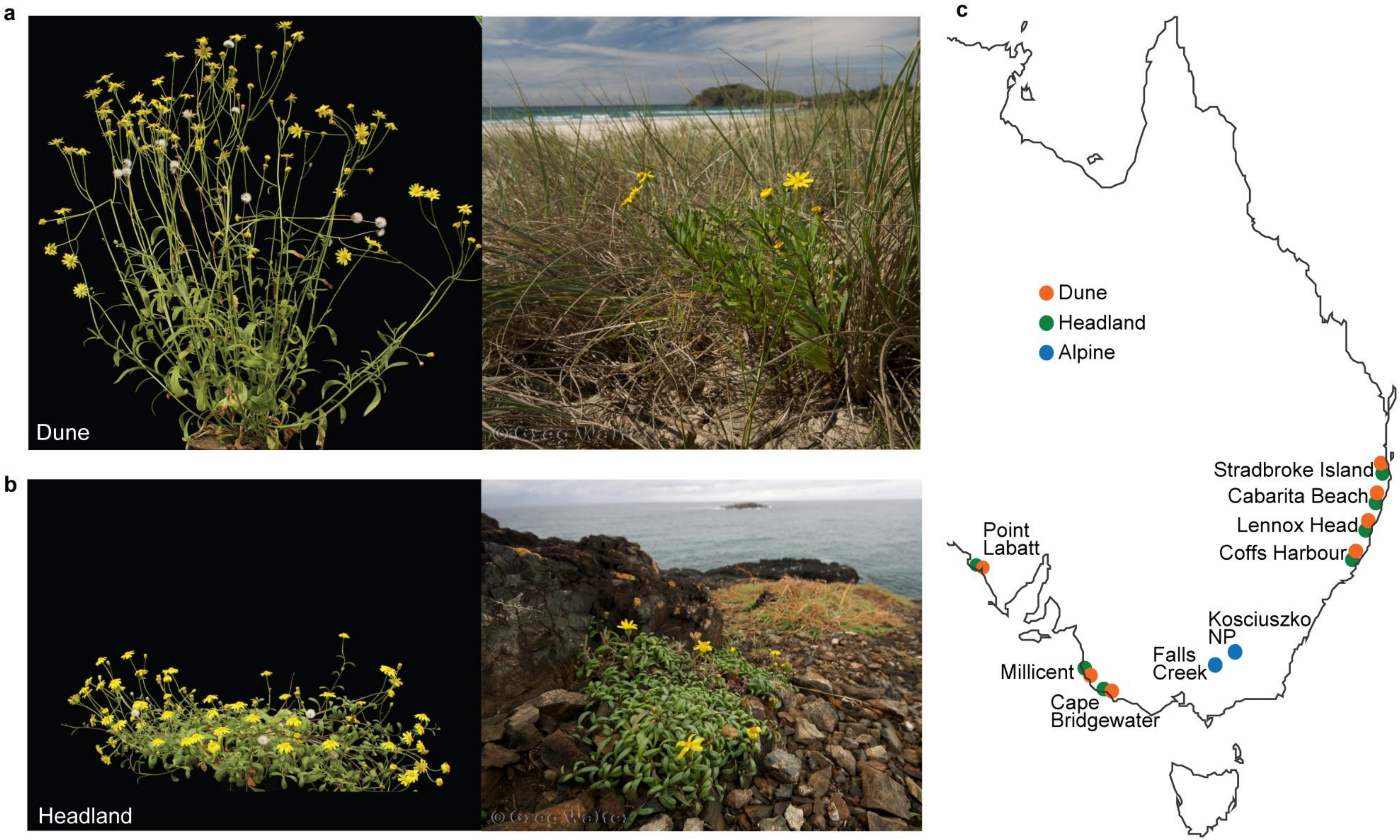
a
Dune
©Greg Walter
b
Headland
©Greg Walter
c
● Dune
● Headland
● Alpine
Stradbroke Island
Cabarita Beach
Lennox Head
Coffs Harbour
Point Labatt
Kosciuszko NP
Falls Creek
Millicent
Cape Bridgewater

**Figure 3. Phenotype, environment and location of *S. lautus* populations.**

**a**. *Senecio lautus* in the hot, sandy, sheltered dune environment have an erect growth. **b**. *Senecio lautus* growing on the windy rocky headlands have a prostrate growth. **c.** Each locality has a Dune (orange) and Headland (green) population occurring adjacent to each other. Two Alpine populations have also been included, one in a sheltered alpine environment with an erect growth, the other in a wind exposed alpine environment with prostrate growth. The populations are split into two monophyletic clades (Roda et al. 2013a; Melo, 2014). The four population pairs on the eastern coastline of Australia are in the eastern clade, while the populations on the southern coastline and the Alpine populations are in the southern clade.

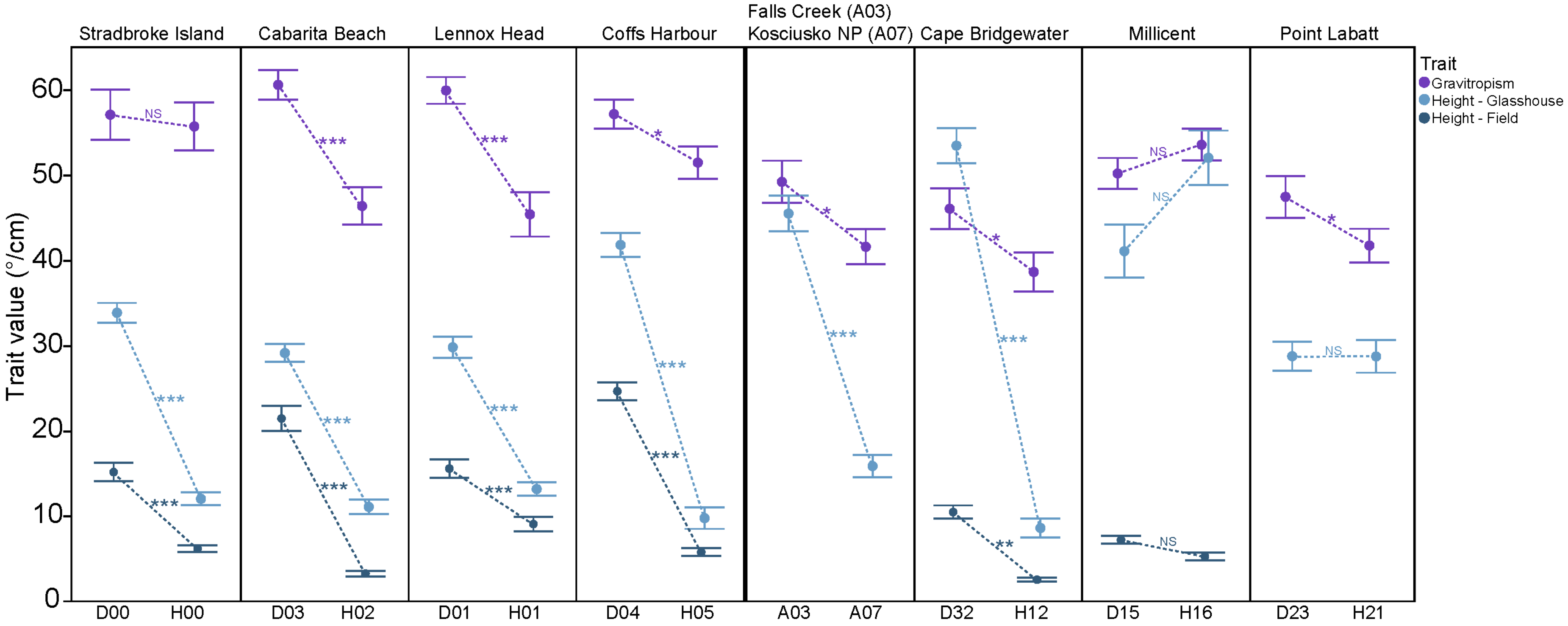

**Figure 4. Gravitropism and height variation across *S. lautus* populations.**

Gravitropism (purple), height in the glasshouse (light blue) and height in the field (dark blue) have been measured for each population (height in the field couldn't be measured for Falls Creek, Kosciusko NP and Point Labatt). Gravitropism (°) is the angle that the plant bends in response to a 90° rotation, where an angle of 0° reflects no gravitropic response. Height (cm) was measured from the base of the plant to the top of the vegetative leaves (excludes flower stems) in controlled conditions in the glasshouse and in a given populations native field environment.  Population pairs are split into clades with the four pairs from the eastern clade on the left and the four pairs from the southern clade on the right. Mean and standard error of each trait in each population is given. A one tailed t-test was used to determine significant differences between populations at a locality (NS $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

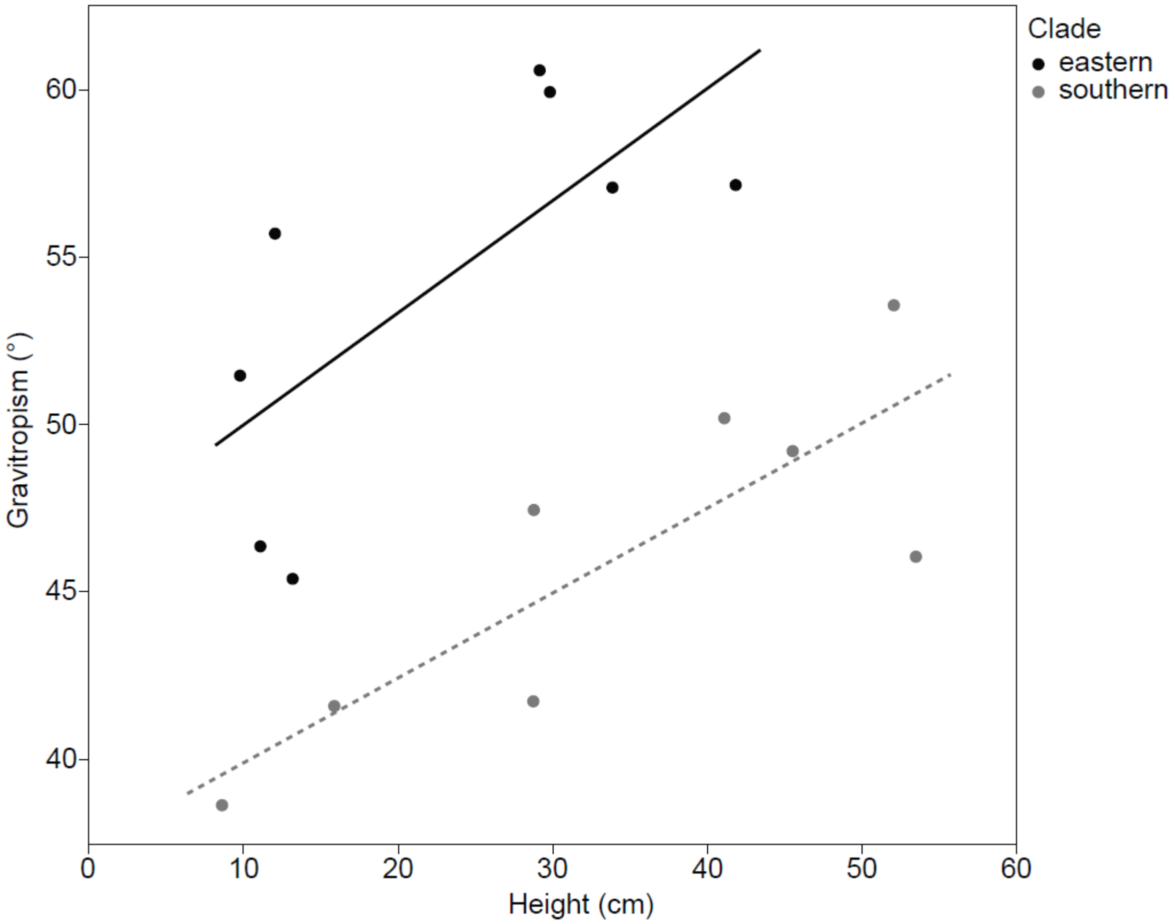

**Figure 5. Gravitropism and height are strongly correlated across *S. lautus* populations.**

The solid line is the regression line for populations from the eastern clade ($F_{1,6}$=6.26, p=0.0464, r=0.71) whereas the dotted line is the regression line for populations from the southern clade ($F_{1,6}$=13.26, p=0.0108, r=0.83). Each point in the graph represents a population mean where height was measured in the glasshouse and gravitropism was measured 24 hours after a 90° rotation.

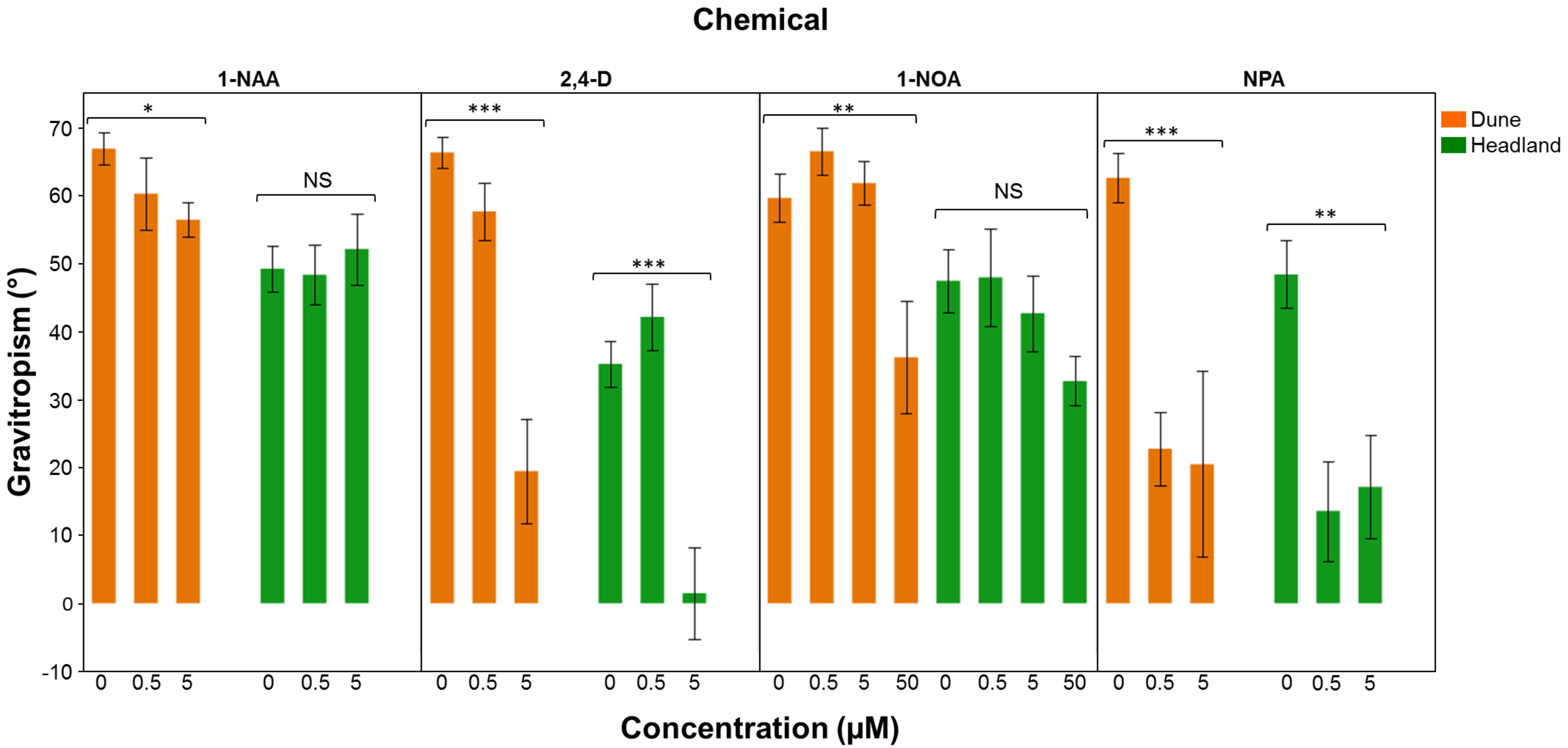
Chemical
1-NAA
2,4-D
1-NOA
NPA
Dune
Headland
Gravitropism (°)
70
60
50
40
30
20
10
0
-10
*
NS
***
***
**
NS
***
**
0
0.5
5
50
Concentration (μM)

**Figure 6. 2,4-D and NPA strongly reduce shoot gravitropism in Dune and Headland *S. lautus* seedlings.**

Dune and Headland seeds were grown on agar plates containing one of the four chemicals: synthetic auxins (2,4-D and 1-NAA) and two transport inhibitors, an influx (1-NOA) and efflux (NPA) inhibitor. The effect of each of the four chemicals on gravitropism in plant cells is visually represented in Figure 2. Four concentrations of each chemical were used (0µM, 0.5µM, 5µM and 50µM). Gravitropism (°) is the angle that the plant bends in response to a 90° rotation, where an angle of 0° reflects no gravitropic response. Mean and standard error of each chemical concentration in both populations are shown. A non-parametric chi-squared test was used to determine significant differences between concentrations of each chemical (NS $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

## Supplementary Tables and Figures

**Table S1. *Senecio lautus* population pairs and geographic locations.**

The geographic locations of each population used in this study are shown. Populations (Pop) located adjacently to each other have been grouped into population pairs (Pop pair). ‘E’ refers to the eastern clade and ‘S’ refers to the southern clade.

| Clade | Ecotype | Pop | Pop pair | Location | Latitude, longitude |
|---|---|---|---|---|---|
| E | Dune | D00 | D00-H00 | Stradbroke Island, Flinders Beach* | -27.405604, 153.469734 |
| E | Headland | H00 | D00-H00 | Stradbroke Island, Point Lookout | -27.436051, 153.545201 |
| E | Dune | D03 | D03-H02 | Cabarita Beach | -28.33179, 153.571379 |
| E | Headland | H02 | D03-H02 | Cabarita, Hasting-Point | -28.362477, 153.579662 |
| E | Dune | D01 | D01-H01 | Lennox Head, Surf club | -28.787454, 153.593867 |
| E | Headland | H01 | D01-H01 | Lennox Head | -28.80607, 153.60275 |
| E | Dune | D04 | D04-H05 | Coffs Harbour | -30.312681, 153.140016 |
| E | Headland | H05 | D04-H05 | Coffs Harbour, Corambirra Point | -30.311598, 153.143771 |
| S | Alpine | A03 | A03-A07 | Falls Creek | -36.87236, 147.288594 |
| S | Alpine | A07 | A03-A07 | Kosciuszko NP | -36.40744, 148.30431 |
| S | Dune | D32 | D32-H12 | Cape Bridgewater | -38.324472, 141.395222 |
| S | Headland | H12 | D32-H12 | Cape Bridgewater | -38.376115, 141.366577 |
| S | Dune | D15 | D15-H16 | Millicent, Canunda National Park | -37.658021, 140.224814 |
| S | Headland | H16 | D15-H16 | Millicent, Southend, Cape Buffon | -37.565807, 140.108063 |
| S | Dune | D23 | D23-H21 | Point Labatt, Salmon Beach | -33.125215, 134.265836 |
| S | Headland | H21 | D23-H21 | Point Labatt | -33.152508, 134.261963 |

*Different locations were used for D00 seeds in the glasshouse and measurements in the field. The location and GPS coordinates above are for the glasshouse seeds and the field plants were measured at Stradbroke Island, Main beach (-27.51944672, 153.5031086).

**Table S2. *Senecio lautus* population sample information.**

The number of individuals used to calculate the mean height of the population (Pop) in the glasshouse (GH) and in the field are shown. N is the number of individuals that were measured in the glasshouse and the field for calculations of height for each population. The date the population was phenotyped and the percentage of individuals flowering (of those that were phenotyped) have also been shown.

| | | **GH** | **Field** | | |
|---|---|---|---|---|---|
| **Pop** | **Location** | **N** | **N** | **Date phenotyped** | **% Flowering** |
| D00 | Stradbroke Island, Flinders Beach* | 16 | 32 | Apr-15 | 65 |
| H00 | Stradbroke Island | 17 | 32 | Apr-15 | 100 |
| D03 | Cabarita Beach | 17 | 32 | Mar-15 | 84 |
| H02 | Cabarita Beach | 17 | 32 | Mar-15 | 100 |
| D01 | Lennox Head | 17 | 32 | Mar-15 | 50 |
| H01 | Lennox Head | 16 | 32 | Mar-15 | 100 |
| D04 | Coffs Harbour | 13 | 54 | Feb-15 | 100 |
| H05 | Coffs Harbour | 16 | 52 | Feb-15 | 100 |
| A03 | Falls Creek | 18 | - | | |
| A07 | Kosciuszko NP | 13 | - | | |
| D32 | Cape Bridgewater | 12 | 32 | May-15 | 22 |
| H12 | Cape Bridgewater | 9 | 32 | May-15 | 68 |
| D15 | Millicent, Canunda National Park | 12 | 32 | May-15 | 56 |
| H16 | Millicent, Southend, Cape Buffon | 12 | 32 | May-15 | 56 |
| D23 | Point Labatt, Salmon Beach | 10 | - | | |
| H21 | Point Labatt | 11 | - | | |

*Different locations were used for D00 seeds in the glasshouse and measurements in the field. The locations above are for the glasshouse seeds and the field plants were measured at Stradbroke Island, Main beach.

**Table S3. The seed information for the natural population gravitropism measures in *S. lautus*.**

The number of families and the number of individuals per family are shown for each population (Pop). ‘Parental’ seeds refers to seeds that were collected from the field and then underwent one round of random crossing in the glasshouse to remove maternal effects; while ‘ancestral’ seeds do not have this round of crossing and are seeds directly from the field. The number of seeds that germinated and the number that were used for analysis are also shown.

| Pop | Location | Families | Individuals per family | Ancestral or Parental seeds | Seeds that germinated | Seeds used for analysis |
|---|---|---|---|---|---|---|
| D00 | Stradbroke Island | 40 | 2 | Ancestral | 39 | 31 |
| H00 | Stradbroke Island | 27 | 3 | Ancestral | 39 | 37 |
| D03 | Cabarita Beach | 40 | 2 | Parental | 58 | 54 |
| H02 | Cabarita Beach | 40 | 2 | Parental | 56 | 49 |
| D01 | Lennox Head | 40 | 2 | Parental | 50 | 47 |
| H01 | Lennox Head | 40 | 2 | Parental | 43 | 38 |
| D04 | Coffs Harbour | 28 | 3 | Parental | 71 | 65 |
| H05 | Coffs Harbour | 40 | 2 | Parental | 39 | 32 |
| A03 | Falls Creek | 29 | 3 | Parental | 36 | 31 |
| A07 | Kosciuszko NP | 21 | 4 | Parental | 32 | 24 |
| D32 | Cape Bridgewater | 40 | 2 | Ancestral | 61 | 59 |
| H12 | Cape Bridgewater | 40 | 2 | Ancestral | 63 | 58 |
| D15 | Millicent, Canunda National Park | 40 | 2 | Ancestral | 64 | 62 |
| H16 | Millicent, Southend, Cape Buffon | 40 | 2 | Ancestral | 46 | 43 |
| D23 | Point Labatt, Salmon Beach | 40 | 2 | Parental | 52 | 48 |
| H21 | Point Labatt | 40 | 2 | Parental | 68 | 58 |

**Table S4. Sample information for auxin treatments and inhibitor experiments in *S. lautus.***

Dune and Headland seeds were grown on agar plates containing one of the four chemicals: synthetic auxins (2,4-D and 1-NAA) and two transport inhibitors, an influx (1-NOA) and efflux (NPA) inhibitor. The effect of each of the four chemicals on gravitropism in plant cells is visually represented in Figure 2. Four concentrations (Conc) of each chemical were used (0µM, 0.5µM, 5µM and 50µM). N refers to the number of seedlings grown under each condition.

| Chemical | Function | Ecotype | Conc (µM) | N |
|---|---|---|---|---|
| 1-NAA | Synthetic auxin | Dune | 0 | 40 |
| 1-NAA | Synthetic auxin | Dune | 0.5 | 42 |
| 1-NAA | Synthetic auxin | Dune | 5 | 42 |
| 1-NAA | Synthetic auxin | Dune | 50 | 35 |
| 1-NAA | Synthetic auxin | Headland | 0 | 45 |
| 1-NAA | Synthetic auxin | Headland | 0.5 | 42 |
| 1-NAA | Synthetic auxin | Headland | 5 | 44 |
| 1-NAA | Synthetic auxin | Headland | 50 | 35 |
| 2,4-D | Synthetic auxin | Dune | 0 | 40 |
| 2,4-D | Synthetic auxin | Dune | 0.5 | 42 |
| 2,4-D | Synthetic auxin | Dune | 5 | 42 |
| 2,4-D | Synthetic auxin | Dune | 50 | 44 |
| 2,4-D | Synthetic auxin | Headland | 0 | 46 |
| 2,4-D | Synthetic auxin | Headland | 0.5 | 44 |
| 2,4-D | Synthetic auxin | Headland | 5 | 44 |
| 2,4-D | Synthetic auxin | Headland | 50 | 44 |
| 1-NOA | Influx inhibitor | Dune | 0 | 34 |
| 1-NOA | Influx inhibitor | Dune | 0.5 | 40 |
| 1-NOA | Influx inhibitor | Dune | 5 | 44 |
| 1-NOA | Influx inhibitor | Dune | 50 | 35 |
| 1-NOA | Influx inhibitor | Headland | 0 | 37 |
| 1-NOA | Influx inhibitor | Headland | 0.5 | 35 |
| 1-NOA | Influx inhibitor | Headland | 5 | 36 |
| 1-NOA | Influx inhibitor | Headland | 50 | 30 |
| NPA | Efflux inhibitor | Dune | 0 | 38 |
| NPA | Efflux inhibitor | Dune | 0.5 | 35 |
| NPA | Efflux inhibitor | Dune | 5 | 36 |
| NPA | Efflux inhibitor | Dune | 50 | 39 |
| NPA | Efflux inhibitor | Headland | 0 | 39 |
| NPA | Efflux inhibitor | Headland | 0.5 | 42 |
| NPA | Efflux inhibitor | Headland | 5 | 43 |
| NPA | Efflux inhibitor | Headland | 50 | 39 |

**Table S5. Mean gravitropism and standard error (SE) for each ecotype and chemical concentration with four auxin chemicals.**

Dune and Headland seeds were grown on agar plates containing one of the four chemicals: synthetic auxins (2,4-D and 1-NAA) and two transport inhibitors, an influx (1-NOA) and efflux (NPA) inhibitor. The effect of each of the four chemicals on gravitropism in plant cells is visually represented in Figure 2. Four concentrations (Conc) of each chemical were used (0µM, 0.5µM, 5µM and 50µM). Synthetic auxins 1-NAA and 2,4-D are lethal at high concentrations (50µM). Additionally, gravitropism could not be measured on more than 90% of individuals exposed to 50µM of NPA and so gravitropism measurements were not included.

| Chemical | Function | Ecotype | Conc (µM) | N | Mean gravitropism (°) | SE gravitropism (°) |
|---|---|---|---|---|---|---|
| 1-NAA | Synthetic auxin | Dune | 0 | 20 | 66.90 | 2.34 |
| 1-NAA | Synthetic auxin | Dune | 0.5 | 24 | 60.25 | 5.27 |
| 1-NAA | Synthetic auxin | Dune | 5 | 13 | 56.41 | 2.49 |
| 1-NAA | Synthetic auxin | Headland | 0 | 22 | 49.21 | 3.33 |
| 1-NAA | Synthetic auxin | Headland | 0.5 | 18 | 48.34 | 4.43 |
| 1-NAA | Synthetic auxin | Headland | 5 | 14 | 52.11 | 5.23 |
| 2,4-D | Synthetic auxin | Dune | 0 | 27 | 66.34 | 2.27 |
| 2,4-D | Synthetic auxin | Dune | 0.5 | 19 | 57.64 | 4.20 |
| 2,4-D | Synthetic auxin | Dune | 5 | 11 | 19.44 | 7.64 |
| 2,4-D | Synthetic auxin | Headland | 0 | 29 | 35.24 | 3.39 |
| 2,4-D | Synthetic auxin | Headland | 0.5 | 19 | 42.14 | 4.87 |
| 2,4-D | Synthetic auxin | Headland | 5 | 13 | 1.49 | 6.78 |
| 1-NOA | Influx inhibitor | Dune | 0 | 18 | 59.62 | 3.50 |
| 1-NOA | Influx inhibitor | Dune | 0.5 | 20 | 66.49 | 3.51 |
| 1-NOA | Influx inhibitor | Dune | 5 | 22 | 61.84 | 3.15 |
| 1-NOA | Influx inhibitor | Dune | 50 | 7 | 36.17 | 8.30 |
| 1-NOA | Influx inhibitor | Headland | 0 | 21 | 47.43 | 4.56 |
| 1-NOA | Influx inhibitor | Headland | 0.5 | 17 | 47.96 | 7.18 |
| 1-NOA | Influx inhibitor | Headland | 5 | 14 | 42.67 | 5.54 |
| 1-NOA | Influx inhibitor | Headland | 50 | 9 | 32.69 | 3.61 |
| NPA | Efflux inhibitor | Dune | 0 | 15 | 62.57 | 3.62 |
| NPA | Efflux inhibitor | Dune | 0.5 | 7 | 22.75 | 5.41 |
| NPA | Efflux inhibitor | Dune | 5 | 8 | 20.47 | 13.67 |
| NPA | Efflux inhibitor | Headland | 0 | 19 | 48.39 | 4.97 |
| NPA | Efflux inhibitor | Headland | 0.5 | 9 | 13.58 | 7.35 |
| NPA | Efflux inhibitor | Headland | 5 | 6 | 17.15 | 7.52 |

**Table S6. Glasshouse height differences between *S. lautus* population pairs.** Population height (cm) in the glasshouse for each population pair (Pop pair) was compared using a one-tailed t-test. Dune populations are taller than their adjacent Headland population except for Millicent and Point Labatt. The sheltered Alpine population (A03) is also significantly taller than the exposed Alpine population (A07). The number of individuals per population used for each comparison is shown in Table S2 (GH N). Standard error (SE) of the difference in means and 95% confidence intervals are shown. The degrees of freedom for each comparison is 210.

| Pop pair | Location | Difference | SE Difference | Lower CL | Upper CL | P>t |
|---|---|---|---|---|---|---|
| **D00-H00** | Stradbroke Island | 21.8210 | 2.1192 | 17.6434 | 25.9985 | ≤ 0.001 |
| **D03-H02** | Cabarita Beach | 18.0588 | 2.0868 | 13.9451 | 22.1726 | ≤ 0.001 |
| **D01-H01** | Lennox Head | 16.6368 | 2.1192 | 12.4592 | 20.8143 | ≤ 0.001 |
| **D04-H05** | Coffs Harbour | 32.0481 | 2.2717 | 27.5697 | 36.5264 | ≤ 0.001 |
| **A03-A07** | Falls Creek & Kosciuszko NP | 29.6419 | 2.2144 | 25.2765 | 34.0073 | ≤ 0.001 |
| **D32-H12** | Cape Bridgewater | 44.8639 | 2.6828 | 39.5752 | 50.1526 | ≤ 0.001 |
| **D15-H16** | Millicent | -10.9583 | 2.4838 | -15.8547 | -6.0620 | 1 |
| **D23-H21** | Point Labatt | 0.0336 | 2.6583 | -5.2067 | 5.2740 | 0.4950 |

**Table S7. Field height differences between *S. lautus* population pairs.** Population height (cm) in the field for each population pair (Pop pair) was compared using a one-tailed t-test. Dune populations are taller than their adjacent Headland population except for Millicent. The number of individuals per population used for each comparison is shown in Table S2 (Field N). Standard error (SE) of the difference in means and 95% confidence intervals are shown. The degrees of freedom for each comparison is 414.

| Pop pair | Location | Difference | SE Difference | Lower CL | Upper CL | P>t |
|---|---|---|---|---|---|---|
| **D00-H00** | Stradbroke Island | 8.9938 | 1.2342 | 6.5676 | 11.4199 | ≤ 0.001 |
| **D03-H02** | Cabarita Beach | 18.2625 | 1.2342 | 15.8364 | 20.6886 | ≤ 0.001 |
| **D01-H01** | Lennox Head | 6.5000 | 1.2342 | 4.0739 | 8.9261 | ≤ 0.001 |
| **D04-H05** | Coffs Harbour | 18.8787 | 0.9592 | 16.9932 | 20.7642 | ≤ 0.001 |
| **D32-H12** | Cape Bridgewater | 7.9375 | 1.2342 | 5.5114 | 10.3636 | ≤ 0.001 |
| **D15-H16** | Millicent | 1.9531 | 1.2342 | -0.4730 | 4.3792 | 0.0572 |

**Table S8. Shoot gravitropism differences between *S. lautus* population pairs.** Shoot gravitropism for each population pair (Pop pair) was compared using a one-tailed t-test. Gravitropism (°) is the angle that the plant bends in response to a 90° rotation, where an angle of 0° reflects no gravitropic response. Dune populations are more gravitropic than their adjacent Headland population except for Stradbroke Island, Millicent and Point Labatt. The sheltered Alpine population (A03) is also significantly more gravitropic than the exposed Alpine population (A07). The number of individuals per population used for each comparison is shown in Table S5. Standard error (SE) of the difference in means and 95% confidence intervals are shown.

| Pop pair | Location | Difference | SE Difference | Lower CL | Upper CL | P>t |
|---|---|---|---|---|---|---|
| **D00-H00** | Stradbroke Island | 1.3581 | 3.6623 | -5.8609 | 8.5771 | 0.3556 |
| **D03-H02** | Cabarita Beach | 14.1985 | 2.9901 | 8.2679 | 20.1291 | ≤ 0.001 |
| **D01-H01** | Lennox Head | 14.5072 | 3.2950 | 7.9914 | 21.0230 | ≤ 0.001 |
| **D04-H05** | Coffs Harbour | 5.7064 | 3.2551 | -0.7224 | 12.1351 | 0.0408 |
| **A03-A07** | Falls Creek & Kosciuszko NP | 7.6431 | 4.0897 | -0.4135 | 15.6997 | 0.0314 |
| **D32-H12** | Cape Bridgewater | 7.3986 | 2.8067 | 1.8201 | 12.9771 | 0.0050 |
| **D15-H16** | Millicent | -3.4232 | 3.0087 | -9.3925 | 2.5462 | 1 |
| **D23-H21** | Point Labatt | 5.6892 | 2.9551 | -0.1696 | 11.5480 | 0.0284 |

**Table S9. Root gravitropism differences between *S. lautus* population pairs.** Root gravitropism for each population pair (Pop pair) was compared using a two-tailed t-test. Root gravitropism (°) is the angle the root bends in response to a 90° rotation, where an angle of 0° reflects no gravitropic response. The number of individuals per population used for each comparison is shown in Table S5. Standard error (SE) of the difference in means and 95% confidence intervals are shown.

| Pop pair | Location | Difference | SE Difference | Lower CL | Upper CL | P>ltl |
|---|---|---|---|---|---|---|
| **D00-H00** | Stradbroke Island | -2.8258 | 4.0154 | -10.7365 | 5.0850 | 0.4823 |
| **D03-H02** | Cabarita Beach | 0.0180 | 3.2268 | -6.3778 | 6.4139 | 0.9955 |
| **D01-H01** | Lennox Head | 11.4075 | 3.5804 | 4.3325 | 18.4825 | 0.0018 |
| **D04-H05** | Coffs Harbour | -5.9720 | 3.5491 | -12.9762 | 1.0322 | 0.0942 |
| **A03-A07** | Falls Creek & Kosciuszko NP | 5.4470 | 4.4924 | -3.3994 | 14.2934 | 0.2264 |
| **D32-H12** | Cape Bridgewater | 3.9399 | 3.0164 | -2.0512 | 9.9310 | 0.1948 |
| **D15-H16** | Millicent | -2.1876 | 3.2450 | -8.6217 | 4.2464 | 0.5017 |
| **D23-H21** | Point Labatt | 6.7223 | 3.1922 | 0.3982 | 13.0464 | 0.0374 |

**Table S10. The effect of synthetic auxin and auxin transport inhibitors on gravitropism in *S. lautus* seeds.**

Dune and Headland seeds were grown on agar plates containing one of the four chemicals: synthetic auxins (2,4-D and 1-NAA) and two transport inhibitors, an influx (1-NOA) and efflux (NPA) inhibitor. The effect of each of the four chemicals on gravitropism in plant cells is visually represented in Figure 2. Four concentrations of each chemical were used (0µM, 0.5µM, 5µM and 50µM). Non parametric Chi-sq test was used to assess whether gravitropism was significantly different at different chemical concentrations. Each ecotype was assessed independently to capture the effect of the chemicals and not the naturally occurring ecotype differences.

| Chemical | Function | Ecotype | ChiSq | DF | Prob>ChiSq |
|---|---|---|---|---|---|
| 1-NAA | Synthetic auxin | Dune | 6.5518 | 2 | ≤ 0.05 |
| 1-NAA | Synthetic auxin | Headland | 0.5393 | 2 | 0.764 |
| 2,4-D | Synthetic auxin | Dune | 23.6600 | 2 | ≤ 0.001 |
| 2,4-D | Synthetic auxin | Headland | 18.3580 | 2 | ≤ 0.001 |
| 1-NOA | Influx inhibitor | Dune | 11.6630 | 3 | ≤ 0.01 |
| 1-NOA | Influx inhibitor | Headland | 6.2697 | 3 | 0.099 |
| NPA | Efflux inhibitor | Dune | 14.2265 | 2 | ≤ 0.001 |
| NPA | Efflux inhibitor | Headland | 12.1642 | 2 | ≤ 0.01 |


a
Stradbroke Island
Lennox Head
Cabarita Beach
Coffs Harbour
DUNE
HEADLAND

**Figure S1.** ***Senecio lautus*** **populations in their native environment.**

**a**. The eastern clade where the dune environment is sandy and the plants grow erect, and where the headland environment is rocky and the plants grow prostrate. **b**. The southern clade where the environments are less discrete but in general the Dune plants are erect and the Headland plants are prostrate.

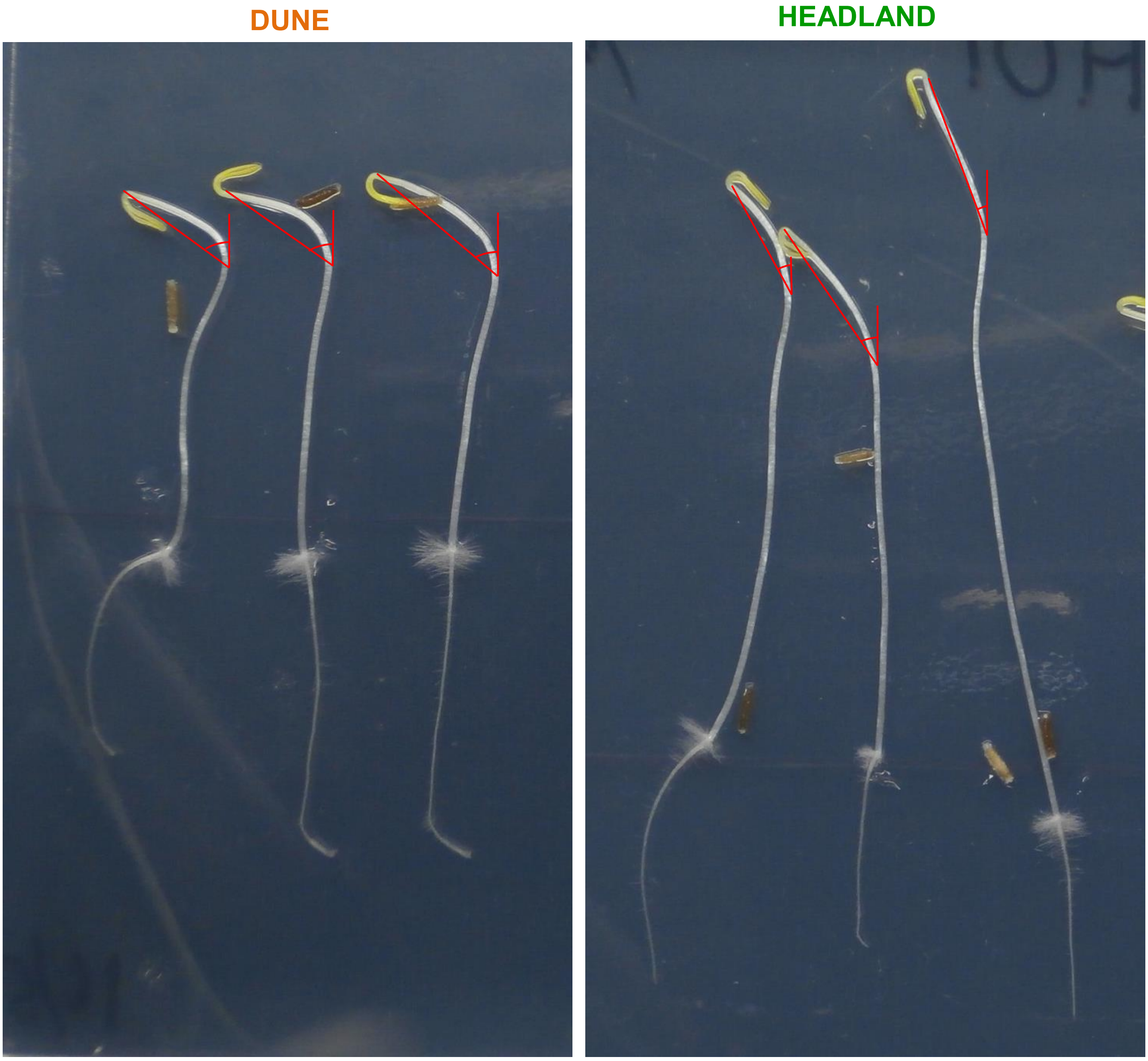

**Figure S2. Gravitropism assay in *S. lautus* Headland and Dune seedlings.**
The seedlings were reorientated clockwise by 90° for 24 hours. The Dune seedlings are more gravitropic as they altered their angle of growth more than the Headland seedlings did, in response to the change in gravity.

# Chapter 4: Adaptive evolution of gravitropism causes intrinsic reproductive isolation between coastal ecotypes of an Australian wildflower

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## Abstract

Adaptation to contrasting environments can drive population divergence and lead to the formation of new species. Investigating the process of adaptation and the specific drivers of divergence is therefore vital for understanding how diversity originates in nature. Here, I discover that adaptive trait evolution to adjacent contrasting environments is driving the evolution of intrinsic reproductive isolation (RI) between closely related populations of the native Australian wildflower, *Senecio lautus*. Populations of *S. lautus* inhabiting hot sand dunes often have an erect growth habit and populations of *S. lautus* inhabiting windy headlands often have a prostrate growth habit. In Chapter 3, I found growth habit to be strongly correlated to growth responses in relation to gravity (gravitropism). For example, erect Dune populations that are rotated by 90° recover their upwards growth in the stem more than their adjacent prostrate Headland pair, within a 24 hour period. To investigate whether gravitropism was driven by natural selection, I exposed an advanced recombinant population, derived from a cross between a pair of Dune and Headland populations, to three generations of natural selection in the dune and headland environment. I found evidence that natural selection was targeting gravitropic plants in the dune environment, whereby the fittest families in the dune environment produce offspring that are more gravitropic. Next, I found that in this advanced recombinant population, whereby gravitropism is segregated from other traits, gravitropic plants crossed with agravitropic plants (poor at responding to gravity stimuli) are more likely to fail to reproduce. This indicates that the gravitropism trait (or a tightly correlated trait) can create intrinsic RI barriers in *S. lautus*. Overall, my results provide insights into the co-segregation of

adaptive traits and traits mediating intrinsic RI during divergence, thus suggesting that natural selection plays a direct role in the origin of new plant species.

## Introduction

From the evolution of coat colour for camouflaging in different terrains (Linnen et al., 2013; Steiner et al., 2007; Rosenblum et al., 2010), to beak size evolution for different seed sizes in Darwin's finches (Lamichhaney et al., 2015; Kleindorfer et al., 2006), populations have evolved adaptations to improve their chances of survival in a variety of environments. As a consequence of adaptation, reproductive isolation (RI) might arise between populations (Coyne and Orr, 2004; Hendry, 2004). Reproductive isolation evolves when breeding between populations is difficult or comes to a halt. Reproductive isolation can arise in many ways and range from physical barriers that prevent populations from interbreeding to natural selection purging hybrids from the population (Coyne and Orr, 2004). Populations inhabiting and adapting to divergent environments can quickly become separated by formation of RI barriers (Lowry, 2012; Fitzpatrick, 2008). Studies that investigate how adaptation creates RI improve our understanding of the evolutionary processes driving the formation of new species (Coyne and Orr, 2004), yet they remain rare, particularly those uncovering the genetic associations between the evolution of adaptive traits and RI.

Reproductive isolation barriers can form in two ways: one that is dependent on the environment (extrinsic RI), and another one that is independent of the environment (intrinsic RI) (Schluter, 2000; Schluter and Conte, 2009) and occur before reproduction (prezygotic) or after reproduction is complete (postzygotic). Extrinsic RI includes natural selection against both immigrants and hybrids that are unfit in the local environment (postzygotic) and also physical separation of the two populations (prezygotic) (Hendry, 2004; Nosil et al., 2005). On the other hand, intrinsic postzygotic RI includes hybrid sterility and inviability that arise from the evolution of genetically incompatible differences between populations (Dobzhansky, 1937; Muller, 1942; Turelli and Orr, 2000). In plants, pollen-pistil interactions can also create intrinsic prezygotic RI; one example is rejection of incompatible pollen by the female pistil, which has been observed in *Arabidopsis* (Hou et al., 2016) and *Brassicaceae* (Chapman and Goring, 2010). Although intrinsic RI will inevitably accumulate between geographically separated populations (Coyne and Orr, 1989), it still remains unclear how natural selection or other forces, such as sexual selection, accelerate the speciation process (Christie and Strauss, 2018; Schluter, 2015; Presgraves, 2010).

Intrinsic RI might be caused by mutations throughout the speciation process (Bomblies, 2010; Wright et al., 2013; Gavrilets, 2004; Bolnick and Fitzpatrick, 2007; Nosil, 2008). However, it is often difficult to piece together when the intrinsic RI barriers arose and whether it was a consequence of adaptation to local conditions. One way to address the role of local adaptation to the origins of intrinsic RI is to directly associate adaptive traits with hybrid fitness. For instance, one can segregate traits by shuffling the genomes of two divergent populations, and then expose such recombinant population to the original environments for multiple generations. The more rounds of crossing between the populations, the more genotype and phenotype associations are broken down, enabling smaller chunks of the genome to co-vary with selection (Clark, 1987; Barrett and Hoekstra, 2011; Mackay, 2001). For instance, in a selection experiment in the flowering plant, *Gilia capitate*, F2 hybrids were transplanted into the parental environments and after crossing survivors in the field, key traits in the F3 hybrids shifted towards the trait value typically found in the environment in which they were planted (Nagy, 1997). Although field experiments with hybrids are common, they have rarely examined the co-segregation of adaptive traits with RI. One notable exception exists in *Mimulus* populations adapted to mines, in which linked loci of adaptation and hybrid mortality were identified (Wright et al., 2013). Here, I perform a field experiment over three rounds of selection using an advanced (F11 generation) recombinant population to 1) reconstruct evolution of an adaptive trait and 2) assess the trait’s contribution to intrinsic RI barriers and thus population divergence.

To explore the link between adaptation and intrinsic RI, I focus on studying traits involved in adaptation and divergence of *Senecio lautus* Dune and Headland coastal populations. *Senecio lautus* is an Australian wildflower that has produced multiple ecotypes through adaptation to divergent environments across the Australian coastline. For example, the Dune ecotype that inhabits sand dune environments often has an erect growth habit with few branches and the Headland ecotype that inhabits rocky windy headland environments often has a prostrate growth habit with many branches (Ali, 1964; Ali, 1968; Radford et al., 2004; Walter et al., 2016; Roda et al., 2013a). Phylogenetic analyses show that geographically adjacent Dune and Headland populations are often each other’s closest relatives (Roda et al., 2013a; Melo et al., 2014; Roda et al., 2013b), indicating that evolution of these phenotypes has occurred independently, multiple times. Previous results in *S. lautus* revealed that multiple genes from the auxin pathway were divergent across coastal pairs (Roda et al., 2013b). Divergence in the auxin pathway predicts multiple phenotypes, including shoot gravitropism, which is defined as the bending of the shoot in response to gravity stimuli (Gilroy, 2008). In Chapter 3, I found that most tall Dune populations respond well to gravity stimuli (gravitropic), whereas most short Headland populations respond poorly to gravity stimuli (agravitropic). This suggests gravitropism and growth habit have repeatedly evolved in combination in response to the environment. In this Chapter I test whether natural selection has targeted gravitropism (or a tightly correlated trait) and whether variation in the trait also contributes to intrinsic RI in *S. lautus*. I measured gravitropism in an F11 recombinant population, which had been subjected to three rounds of selection in dune and headland environments at Lennox Head, a locality where strong local adaptation has been found in Chapter 2 and previous *S. lautus* field experiments (Walter et al., 2016; Walter et al., 2018; Richards et al., 2016). Considering Dune populations are often more gravitropic than their adjacent Headland population (Chapter 3), I expected that after selection in the dune environment, the F11 individuals would be more gravitropic, and that after selection in the headland environment, the F11 individuals would be less gravitropic. To then assess whether this trait is correlated with intrinsic RI in *S. lautus*, I measured fertilisation success after crossing F11 gravitropic individuals with F11 agravitropic individuals. I expected that if gravitropism was contributing to intrinsic RI in *S. lautus*, this would produce more failed crosses relative to crosses within each of these groups. These results provide insights into adaptation and divergence of populations and how this can lead to the creation of ecotypes and new species.

## Methods

### Field selection experiments

To determine the traits under selection in *S. lautus* I created an advanced recombinant population and exposed it to three rounds of selection in the native environments of the parental families at Lennox Head, NSW (Figure 1). Firstly, an F8 population was created by crossing randomly, and with equal contribution, 30 different individuals derived from the Dune and Headland at Lennox Head (Rockman & Kruglyak, 2008). This was carried out for three independent replicate genetic lines (A, B and C) for eight generations, but from the same 30 families. Seeds from this F8 recombinant population were glued to toothpicks and then planted under native soil at Lennox Head dune and headland environments in a randomised block design. 50% of the top surviving families within an environment and genetic line were chosen based on the between-family selection estimates detailed below. Seeds from the full siblings of the surviving families were grown and crossed in the glasshouse to produce the next generation (F9). A second selection experiment was performed with the F9 generation following the same process of choosing the top 50% of the surviving families to germinate and grow in the glasshouse. This happened to another generation (F10), so selection in the field was applied for a total of three generations (in the F8, F9 and F10s). A schematic of the crossing scheme for the selection and production of seeds for each field selection experiment is shown in Figure S1. For further experimental details see Walter et al. (2018) and Walter et al. (2016).

## Between-family based selection

I used a between-family based selection scheme which selects the top 50% of families with the highest field performance (Walsh and Lynch, 2013). The full sibs of these families were used to produce the next generation in the glasshouse. This is known as full-sib selection (Walsh and Lynch, 2013) and avoids crossing individuals in the field where there is open pollination and therefore avoids the risk of unknown parentage. I completed full sib selection on the top 50% of families with the highest field performance in the F8, F9 and F10 generation that underwent selection experiments in the dune and headland environments at Lennox Head (see details above). In the first field experiment with the F8 generation, families were chosen based on survival to 85 days with the formula below:

$$1-((1 - \text{Proportion emerged})*(1 - \text{Proportion present day 85}))$$

To select the top 50% of surviving families in the F9 and F10 field selection experiment, I used Aster modelling (Geyer et al., 2007; Shaw et al., 2008), which I implemented in the Aster package in R (R Core Team, 2014). Aster modelling allows multiple components of fitness to be combined into one analysis, while controlling for different sampling distributions (Shaw et al., 2008). The fitness components included in the model were family germination and survival success, where germination was the total number of individuals that germinated in each family and survival success was the total number of individuals per family that survived to day 138 in the F9s and day 232 in the F10s. See Supplementary Material for the R codes for the Aster modelling.

### F11 seed production

To produce the F11 recombinant population for the gravitropism experiment, one individual from each of the chosen F10 families was crossed using a nested full-sib, half-sib crossing design. To assess whether there was a shift in gravitropism after selection in the dune and headland environments, a Control population was grown in the glasshouse that underwent the same crossing scheme but with random mating rather than viability selection. *Senecio lautus* are hermaphrodites and so each plant was randomly assigned as a dam or a sire. Every dam (F10) was randomly mated with two sires (F10) within a given replicate genetic line (A, B and C) and population (Dune, Headland and Control). Each dam and sire had a field fitness value based on the F10 family fitness values given to their full-siblings in the field (Table S1). This produced an F11 recombinant population with 39 Dune survivor families, 37 Headland survivor families and 25 Control families.

### F11 gravitropism measurements

I hypothesised that if there was strong selection on gravitropism loci then I would expect to see divergence in gravitropism in a recombinant population after selection. Gravitropism was quantified by measuring the angle of the stem 12 hours after a 90° rotation of a seedling. I performed this experiment on the 101 F11 families mentioned above, with 12 individuals per family (1,212 seeds in total were germinated). The families were germinated in three separate time blocks ~seven days apart as the gravitropism experiment could not be accurately performed on all 1,212 individuals at once.

The gravitropism experiment was performed on two-week old F11 seedlings in small square pots. I germinated the F11 seeds by cutting 1mm from the micropyle side of the seeds and then placing them in petri dishes with dampened filter paper. The seeds were placed in darkness for two days for roots to grow and then transferred to light for four days 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 hour light/day cycle. After seven days of growing in the pot, the plants were rotated by 90° at the end of their light cycle. I took a photograph of each individual 12 hours after rotation and before the start of the light cycle to avoid any light effects. The photographs were imported into ImageJ (Schneider et al., 2012) to measure gravitropism, defined as the angle to which the stem had reorientated itself in relation to the new gravity vector.

Overall there was an 89% germination success but gravitropism was not measured on seedlings where the stem was not clearly visible above the soil. This left gravitropism measurements for a total of 788 seedlings across 100 families (73% of the total number of seedlings planted). Refer to Table S1 for the total number of individuals per replicate and time block, and mean gravitropism.

### Gravitropism tests of selection

I implemented a linear model to test the hypothesis that high fitness dune families will produce gravitropic plants and high fitness headland families will produce agravitropic plants. Independent models were used for the dune and headland environments to test the effect of gravitropism on fitness in each environment:

$$y_{ijklmn} = B_i + V_j + R_{k(i)} + D_{l(ik)} + S_{m(ik)} + e_{n(ijklm)} \qquad (1)$$

where time-block ($B_i$) is the three time points in which the F11 seeds were grown (~seven days apart); viability ($V_j$) is the number of days until the death of F11 plants in controlled conditions; and replicate, which consists of the three independent genetic replicates, is nested within time-block ($R_{k(i)}$). Dam fitness was nested in replicate and time-block ($D_{l(ik)}$) and sire fitness was also nested in replicate and time-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. Viability of the offspring was included in the model to control for the influence of survival in controlled conditions on gravitropism. Replicate C was removed from analyses as it has little variation in fitness values which means it did not converge. Shapiro-Wilk W test shows the residuals from the model are normally distributed for both the dune (W=0.98, p=0.3879) and the headland environment (W=0.98, p=0.2776). The linear model was performed in JMP v13 (SAS 2015).

### Gravitropism heritability

I calculated narrow sense heritability ($h^2$) of gravitropism to understand the proportion of the variance in gravitropism that is due to genetic variation in the population. The sire variance was multiplied by four as an estimate of the genetic variance. $h^2$ was calculated using a linear regression with 147 F11 Control individuals in replicates A and B:

$$y_{ijkl} = B_i + S_{j(i)} + D_{k(i)} + e_{l(ijk)} \qquad (2)$$

where time-block ($B_i$) is a fixed effect and is the three time points at which the F11 seeds were grown (~seven days apart). Sire was nested in time-block ($S_{k(i)}$) and Dam was nested in time-block ($D_{j(i)}$) as random effects, and $e_{l(ijk)}$ was the residual error. The linear regression was performed in JMP v13 (SAS 2015).

### Estimating intrinsic RI

I tested whether intrinsic RI was associated with the gravitropism phenotype by measuring fertilisation success of crosses conducted within and between gravitropic and agravitropic groups. Individuals with a gravitropism angle of less than 20° were classified as agravitropic and greater than 56° were classified as gravitropic. If gravitropism was contributing to ecotype divergence in *S. lautus* then I expected to observe a decrease in successful crosses between gravitropic and agravitropic individuals. To test this hypothesis, I randomly crossed individuals in the F11 recombinant population used above in a controlled temperature room. To maximise sample size, all three replicate genetic lines (A, B and C) were used across all three populations (Dune survivors, Headland survivors and Control). A total of 132 crosses were completed (65 crosses within the tails and 67 between the tails) by rubbing multiple flower heads of two individuals together and collecting the seeds produced from both parents. To remove genetic incompatibilities that might be caused by relatedness, crosses within the same family were not performed. A failed cross was considered when, in the presence of pollen, less than three seeds were produced per seed head with at least three mating attempts. Failed crosses in this context might be caused by pollen-pistil interactions (prezygotic) or genetic incompatibilities (postzygotic). I performed a chi-square test of independence in JMP v13 (SAS. 2015) to determine whether there was a significant association between cross type (within vs between) and failed crosses.

## Results

### Natural selection drives the evolution of genetically based gravitropism

Local adaptation is strong in the transplant sites at Lennox Head, with local parents outperforming the foreign parent in each generation of the field experiments (Roda et al., 2017; Greg M. Walter personal communication). With this in mind, I asked if natural selection drove the divergence of gravitropism between the Dune and Headland natural populations. Firstly, I estimated a narrow sense heritability for gravitropism in control families of 0.35 (95% CI = 0.33-0.66) under controlled conditions (Table S2), suggesting gravitropism in the recombinant population has the potential to respond to selection. Next, I tested a simple prediction: Natural selection should recreate phenotypic divergence in gravitropism in an advanced recombinant population subject to selection in the native environments of the parents. I expected that families that have high fitness in the dune environment would produce offspring with strong gravitropic values relative to families that have low fitness in the dune environment. The reverse expectation would be true for families in the headland environment. Consistent with a strong response to selection, F10 family fitness in the dune environment strongly predicted levels of gravitropism in the F11 families grown in controlled conditions. However, this was true when considering the dam but not the sire of the cross (Table 1). In the headland environment, I did not find a correlation between F10 family fitness and gravitropism of F11 families grown in controlled conditions (Table 1).

Because lack of gravitropism could result from a stressed plant that lacks the ability to respond normally to environmental cues, I explored whether plants that died early during development were responsible for the direction and magnitude of agravitropic measurements in the F11 generation. I found that the number of days until death (viability) of the F11 plants grown in controlled conditions did not have an effect on gravitropism in the offspring of the Dune survivors (Table 1), but I found a viability effect on gravitropism in the offspring of the Headland survivors (Table 1), where individuals that died early in development were agravitropic. I found that neither genetic replicate nor time-block had an effect on gravitropism of the F11 offspring of Dune and Headland survivors (Table 1).

The strong response to selection of gravitropism in the dune environment could be explained by the dominant nature of the Dune-favoured gravitropic phenotype. The gravitropic phenotype was three times more prevalent than the agravitropic phenotype in the F11 generation, indicating the gravitropic phenotype is dominant over the agravitropic phenotype. The bimodal distribution of the gravitropism trait has two peaks: the agravitropic peak at 2.56° (95% CIs=1.17-3.95) and the gravitropic peak at 41.13° (95% CIs=39.85-42.40), shown in Figure S2.

### Gravitropism correlates with intrinsic RI

I then asked if a trait that contributes to adaptation to novel conditions, also could be causing the evolution of intrinsic RI in the system. Different from studies where the relationships between intrinsic RI and trait evolution are difficult to study, I was able to investigate the effects of gravitropism, segregated from other traits in an advanced recombinant population (F11 generation) on reproductive success. Crosses were grouped into cross type (within or between the tails of the gravitropic distribution), gravitropic x agravitropic (between), gravitropic x gravitropic (within) and agravitropic x agravitropic (within). I found a significant association between crosses (within vs between) and failed crosses (Table 2; LR chi-square, $X^2$=8.40, df=1, p=0.004), where there were more failed crosses between gravitropic and agravitropic plants than crosses within each of these groups. More specifically, 21% of the crosses between gravitropic and agravitropic F11 plants failed, whereas 5% of crosses within the tails failed. When the within tail crosses were analysed independently, 8% of the crosses within gravitropic plants failed and 0% of the crosses within agravitropic plants failed (Table S3).

## Discussion

Although it is well accepted that adaptation to different environments can drive population divergence, the process of how adaptive evolution leads to the formation of intrinsic RI remains largely unknown (Coyne and Orr, 2004; Hendry, 2004). Here, I used an F11 recombinant population in a field experiment to study the role of natural selection on the evolution of gravitropism and the consequences of variation in gravitropism on the origins of intrinsic RI. I found that after an advanced recombinant population was exposed to selection in the dune environment for three generations, the fitness of families in the field (F10 generation) could predict the gravitropism strength of the F11 generation. However, the ability to predict gravitropism in the F11 generation was only possible when I tracked the fitness of the dam’s family and not the fitness of the sire’s family in the wild. This suggests that evolution of the dominant gravitropic phenotype in the dune environment is occurring through the maternal genotype. In contrast, fitness of the F10 families in the headland environment did not predict gravitropism of the F11 families. I also found intrinsic RI when gravitropic individuals were crossed (in controlled conditions) with agravitropic individuals, suggesting that this trait might not only have a major role in adaptation to the environment but might also be contributing to the reproductive divergence of these ecotypes in *S. lautus.* The association between gravitropism and intrinsic RI might be occurring through genetic linkage or pleiotropism, which I discuss below, and explore in detail in Chapter 5. Overall, the results indicate that natural selection drove the evolution of gravitropism (or a tightly correlated trait) in *S. lautus* and this could be contributing to the divergence of some Dune and Headland populations, providing an insight into species formation.

### Adaptive evolution of a dominant gravitropic phenotype

The narrow sense heritability measure of 0.35 for gravitropism found in this experiment suggests the recombinant population has the potential to respond to selection (Lande and Arnold, 1983), which I tested using experimental evolution by combining field and glasshouse experiments. I found that gravitropic *S. lautus* plants with an erect growth habit are advantageous in the dune environment. Chapter 3 showed that gravitropism and growth habit are repeatedly divergent between independent natural Dune and Headland populations across Australia, whereby Dune populations are often erect and gravitropic and Headland populations are often prostrate and agravitropic. The strong connection between gravitropism and growth habit is not surprising as evidence is accumulating to suggest that plant architecture (tiller angle in many grasses) is controlled by the same genetic pathway as gravitropism (Li et al., 2007; Yoshihara and Iino, 2007). Furthermore, the independent and repeated evolution of these traits alone suggests that natural selection is most likely targeting these traits in these environments (Stern, 2013), but does not rule out the possibility that genetically linked loci might be the targets of selection.

I provided evidence that the dominant gravitropic phenotype is under selection in the dune environment as I partially reconstructed its evolution in a population with reduced genotypic and phenotypic associations. In previous work, Roda et al. (2017) showed that phenotypes related to gravitropism, like height and angle of main stem, are dominant in the dune population and recessive in the Headland population. Consistent with these observations, I found a ~3:1 relationship between the prevalence of the gravitropic phenotype over the agravitropic phenotype, which could be indicative of the gravitropic phenotype being dominant. I suggest that gravitropic plants, controlled by dominant alleles, have an erect growth habit that might be advantageous in the dune environment as it allows the plants to grow upright away from the hot sand and prevent sand covering. On the other hand, I suggest that agravitropic plants, controlled by recessive alleles, have a prostrate growth habit that might be beneficial in the headland environment as it protects the plants from mechanical stress caused by wind (Read and Stokes, 2006; Auld and Morrison, 1992). Evolution of prostrate plants on windswept coastal cliffs is common across the plant kingdom (Beeftink et al., 1985; Auld and Morrison, 1992; Morrison and Rupp, 1995; Crutsinger et al., 2010), and results from Chapter 2 were consistent with natural selection for a prostrate growth habit in the headland environment at Lennox Head, which makes it puzzling why I did not detect selection for agravitropic plants in the headland environment in this study. I provide possible reasons for the inability to detect evolution for agravitropism below.

### Inability to detect evolution of recessive agravitropic phenotype

To explore why I was able to detect a gravitropic response to selection in the dune environment but not the headland environment, I must first consider the opportunity a new dominant allele might have to rise in frequency over a new recessive allele (Whitlock, 2003; Andrews, 2010; Haldane, 1924). Considering rare alleles are found mostly in heterozygotes, where only the dominant alleles are “visible” to selection, it is more likely for the dominant alleles to have the chance to rise in frequency. Recessive alleles are “invisible” to selection until recessive homozygotes are more common in the population (Nuismer et al., 2012). The ~3:1 genetic architecture might explain why gravitropism was able to respond to selection in just three generations of selection in the dune but not in the headland environment (see Chapter 5 for a novel explanation of this result using genotypic data). That is, the recessive agravitropic phenotype, hypothesised to be favoured in the headland environment, might have been at low frequencies there, and therefore more likely to be subjected to strong random behaviour than to the deterministic increase in frequency by natural selection (Haldane, 1924). However, it must be noted that this argument is only valid for new mutations or rare standing genetic variants (possibly under mutation-selection balance) (Olson-Manning et al., 2012). Cases of adaptive evolution through recessive alleles are not uncommon and include: colouration of lizards to white sands (Rosenblum et al., 2010), body armour in stickleback fish (Chan et al., 2010), wing colour in butterflies (Chamberlain et al., 2009) and flower colour in monkeyflowers (Bradshaw and Schemske, 2003). The parental headland population used to derive this F11 recombinant population contains the genetic variation to produce agravitropic plants, suggesting that these agravitropic alleles had the potential to rise in frequency after selection in the headland environment. Therefore I provide other reasons for not detecting agravitropic evolution below.

The response to selection of the agravitropic phenotype might have been masked by maladaptive plants causing agravitropism, whereby stressed plants were unable to respond to a stimulus. This is supported by reductions in gravitropism in cold stressed plants (Shibasaki et al., 2009; Du et al., 2013) and salt stressed plants (Sun et al., 2008). In the present study, I found that low viability in controlled conditions reduced gravitropism in offspring of Headland survivors but not in those of Dune survivors. This indicates that alleles beneficial in the headland environment might be maladaptive on the dune and headland intermixed genome. Studies in tomatoes have found that introgressing specific sections of the genome containing QTLs onto an alternative background can cause genetic incompatibilities (Moyle and Graham, 2004). Additionally, experiments in yeast show that just one mutation in a common genomic background can cause genetic incompatibilities (Ono et al., 2017; Anderson et al., 2010), indicating that incompatibilities can arise very rapidly at specific loci. In the present chapter, on a Dune and Headland intermixed genome, I observed reproductive failure between gravitropic and agravitropic individuals. I also found that at low frequencies there was reproductive failure between gravitropic individuals, but not between agravitropic individuals. This could indicate that the homozygous agravitropic individuals have the specific genomic background to avoid genetic incompatibilities, while the heterozygous gravitropic individuals might be segregating the genetic incompatibility at low frequencies. Overall, it might be difficult for agravitropic allelic combinations to rise in frequency in populations with certain genomic backgrounds. It might take many generations of selection for individuals inhabiting the headland environment to purge these incompatibilities and evolve agravitropic alleles.

### Evolution of a maternally controlled phenotype

In the present study, the dam and not the sire affected the gravitropic response of recombinant individuals after the field experiment in the dune environment. Other experiments in the *S. lautus* system have also shown that the maternal genotype has an important role in fitness. For example, in reciprocal transplant experiments with F1 generation plants (derived from Dune and Headland populations), individuals with the maternally inherited local cytoplasm were more fit at Cabarita beach (Melo et al., 2014) and Coffs Harbour (Richards and Ortiz-Barrientos, 2016). In these examples and the present study, non-genetic environmental maternal effects are removed as the parental generation was grown in the glasshouse (Roach and Wulff, 1987; Wolf and Wade, 2009).

This means that the maternal genotype is controlling trait value, which could occur through cytoplasmic inheritance, including the mitochondria and chloroplast, genetic imprinting or maternal provisioning in the seed via the endosperm (Wolf and Hager, 2006; Lawson et al., 2013; Jarvis et al., 2005; Kearsey and Pooni, 1998). To comment further on the process of the maternal evolution found in this study, the genetics of gravitropism needs to be investigated in greater detail.

### Adaptive evolution correlates with intrinsic RI

The current study contributes to the few empirical examples we have that indicate that adaptive trait evolution might cause intrinsic RI, which continues to be debated (Nosil, 2012). One example of adaptive evolution causing intrinsic RI is copper tolerance in *Mimulus* monkeyflowers (Macnair and Christie, 1983; Christie and Macnair, 1984). Wright et al. (2013) found that a gene tightly linked to a copper tolerance gene interacts with a small number of genes from the other population to cause hybrid lethality (inviable progeny average is 45–62%). Although the gene that causes the intrinsic RI is not the gene under direct selection, adaptive evolution and tight genetic linkage caused the incompatible gene to rise in frequency in the population. The barrier to gene flow arose quickly in the population and independently of extrinsic RI, which is often considered to be the cause of intrinsic RI (Nosil, 2012). In the current study, I found evidence to suggest that gravitropism is associated with intrinsic RI in *S. lautus* populations. As the sample size of the cross failures is not large enough to investigate the F11 Control population in isolation from the F11 Dune and Headland survivor populations, I am unable to determine whether natural selection directly caused this intrinsic RI in this experiment. However, it is clear from these results that 1) gravitropism (or a strongly correlated trait) is targeted by natural selection and 2) gravitropism variation correlates with intrinsic RI, suggesting that adaptive trait evolution can lead to intrinsic RI.

Gravitropic and agravitropic plants fail to reproduce in 21% of crosses, indicating that genetic incompatibilities might be polymorphic in populations and might have an allelic basis (Cutter, 2012; Charron et al., 2014; Corbett-Detig et al., 2013). Examples of polymorphic incompatibilities, where alleles contribute a partial barrier to reproduction, have been found in fruit flies (Davis and Wu, 1996; Tao et al., 2003), monkeyflower (Wright et al., 2013), house mice (Larson et al., 2018) and sunfish (López-Fernández and Bolnick, 2007). Additionally, in the present study, 8% of crosses within gravitropic plants failed and 0% of crosses within agravitropic plants failed, suggesting that the genetic incompatibilities are not associated with agravitropic recessive alleles. Genetic linkage, as observed in copper tolerance, or pleiotropism might be responsible for this association between gravitropic alleles and intrinsic RI. Below I discuss probable ways this barrier to gene flow evolved.

The intrinsic RI barriers observed in this study were most likely caused by genetic incompatibilities (postzygotic) or pollen-pistil interactions (prezygotic). Firstly, genetic incompatibilities might have reduced fertilisation success by negative genetic interactions between differentiated alleles of gravitropic and agravitropic plants causing the seed to abort. This could occur through either genetic linkage e.g. gravitropism is genetically linked to incompatible loci, or pleiotropy e.g. gravitropism loci is incompatible. I will continue this discussion in Chapter 5, where I explore the genetic basis of gravitropism. Secondly, pollen-pistil interactions might prevent the egg from being fertilised. Pollen-pistil interactions range from pollen tube reception, where the females might reject unrecognised pollen (Müller, 2014; Hou et al., 2016; Escobar-Restrepo et al., 2007) to pollen tube growth, where elongation of the pollen tube towards the ovary for fertilisation might be arrested (Chen and Zhao, 2008; Wu et al., 2008; Zerzour et al., 2009). Further investigation is required to narrow down the specific intrinsic RI barrier that occurs between gravitropic and agravitropic plants.

Failure to create offspring from crossing between gravitropic and agravitropic plants might imply that Dune and Headland populations with extreme and contrasting values of gravitropism would show signs of being reproductively incompatible. However, intrinsic RI is not observed when gravitropism is not segregated from other traits. Melo et al. (2014) found that there was no difference in fertilisation success between parental crosses and F1 crosses of Dune and Headland adjacent pairs at Cabarita and Lennox Head. These populations both display Dune and Headland divergence in gravitropism (Chapter 3) and the F11 population used in this study was derived from the Lennox Head natural population. However, heterosis and little to no intrinsic RI has repeatedly been found in the F1 generation between Dune and Headland populations at Cabarita Beach (Melo et al., 2014), Lennox Head (Walter et al., 2016) and Coffs Harbour (Richards and Ortiz-Barrientos, 2016; Richards et al., 2016). This is not surprising as heterosis is known to mask intrinsic and extrinsic RI in F1s (Lowry et al., 2008; Rundle and Whitlock, 2001). In the F2 generation, where hybrid breakdown begins to occur (Oka et al., 2004; Johansen-Morris and Latta, 2006; Stelkens et al., 2015), intrinsic RI was found to arise in the Lennox Head population, but fitness was quickly regained in the F3 generation (Walter et al., 2016). Based on these results and the results of the present study, I suggest that strong but few genetic incompatibilities (or pollen-pistil interactions) have accumulated in *S. lautus* and they are pleiotropic or genetically linked to gravitropism loci. Future research should investigate the connection between gravitropism and intrinsic RI at the genetic level in order to understand whether gravitropism loci are directly creating intrinsic RI in the *S. lautus* system.

## Conclusions

Here, I sought to determine the targets of natural selection and understand its contribution to ecotype divergence. Differences in growth habit define these ecotypes and after finding a strong connection between growth habit and gravitropism in Chapter 3, the direct evidence here that natural selection is targeting gravitropism, suggests it is important for ecotype evolution in *S. lautus*. Furthermore, identifying the consequences of this adaptation in the form of intrinsic RI barriers implicates a major role for gravitropism in ecotype divergence and furthers our understanding of the early stages of speciation. Overall, my results suggest that natural selection can create intrinsic RI barriers and thus natural selection has a direct role in the origin of new species. I continue this research in Chapter 5 by investigating the genes controlling gravitropism to understand the genetic basis of adaptation and divergence.

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# Tables

**Table 1. General linear model for the effect of dam and sire on gravitropism after an advanced recombinant population was subject to selection in the parental dune and headland environments.**

Three reciprocal transplant experiments were performed on an advanced recombinant population (F8, F9 and F10 generation) to achieve three rounds of selection in the dune and headland environments at Lennox Head, NSW. 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. After selection in the dune environment, the field family fitness of the dam (F10) predicts the gravitropic response of its offspring (F11), with the fitter Dune dams producing more gravitropic offspring.

| | Dune | | | | Headland | | | |
|---|---|---|---|---|---|---|---|---|
| **Source** | Df | Sum of Squares | F Ratio | Prob > F | Df | Sum of Squares | F Ratio | Prob > F |
| **Dam fitness** | 6 | 8515.77 | 4.7793 | 0.0004 | 6 | 1884.31 | 0.7008 | 0.6498 |
| **Sire fitness** | 6 | 1806.62 | 1.0139 | 0.4237 | 5 | 2315.36 | 1.0333 | 0.4047 |
| **Viability** | 1 | 260.85 | 0.8784 | 0.352 | 1 | 5209.38 | 11.6244 | 0.0011 |
| **Replicate[Time Block]** | 3 | 1135.49 | 1.2745 | 0.2901 | 3 | 3357.19 | 2.4971 | 0.0665 |
| **Time Block** | 2 | 193.14 | 0.3252 | 0.7235 | 2 | 2234.96 | 2.4936 | 0.0897 |

**Table 2 . Crosses between gravitropic and agravitropic individuals fail to produce seeds more than crosses within each of these groups.**

The F11 recombinant population, described in Figure 1, were randomly crossed and fertilisation success measured based on successful production of seeds (successful crosses) or repeated failure to produce more than three seeds (failed crosses). Crosses were grouped into whether the crosses occurred between or within the tails of the gravitropic distribution – gravitropic x agravitropic (between), gravitropic x gravitropic (within) and agravitropic x agravitropic (within). A chi-square test shows crosses between gravitropic and agravitropic plants, produced more than expected failed crosses relative to crosses within each of these groups (LR chi-square, $X^2$=8.40, df=1, p=0.004).

| **Gravitropism distribution tail** | **Successful crosses** | **Failed crosses** | **Total crosses** |
|---|---|---|---|
| Within | 62 (95%) | 3 (5%) | 65 |
| Between | 53 (79%) | 14 (21%) | 67 |

## Figures

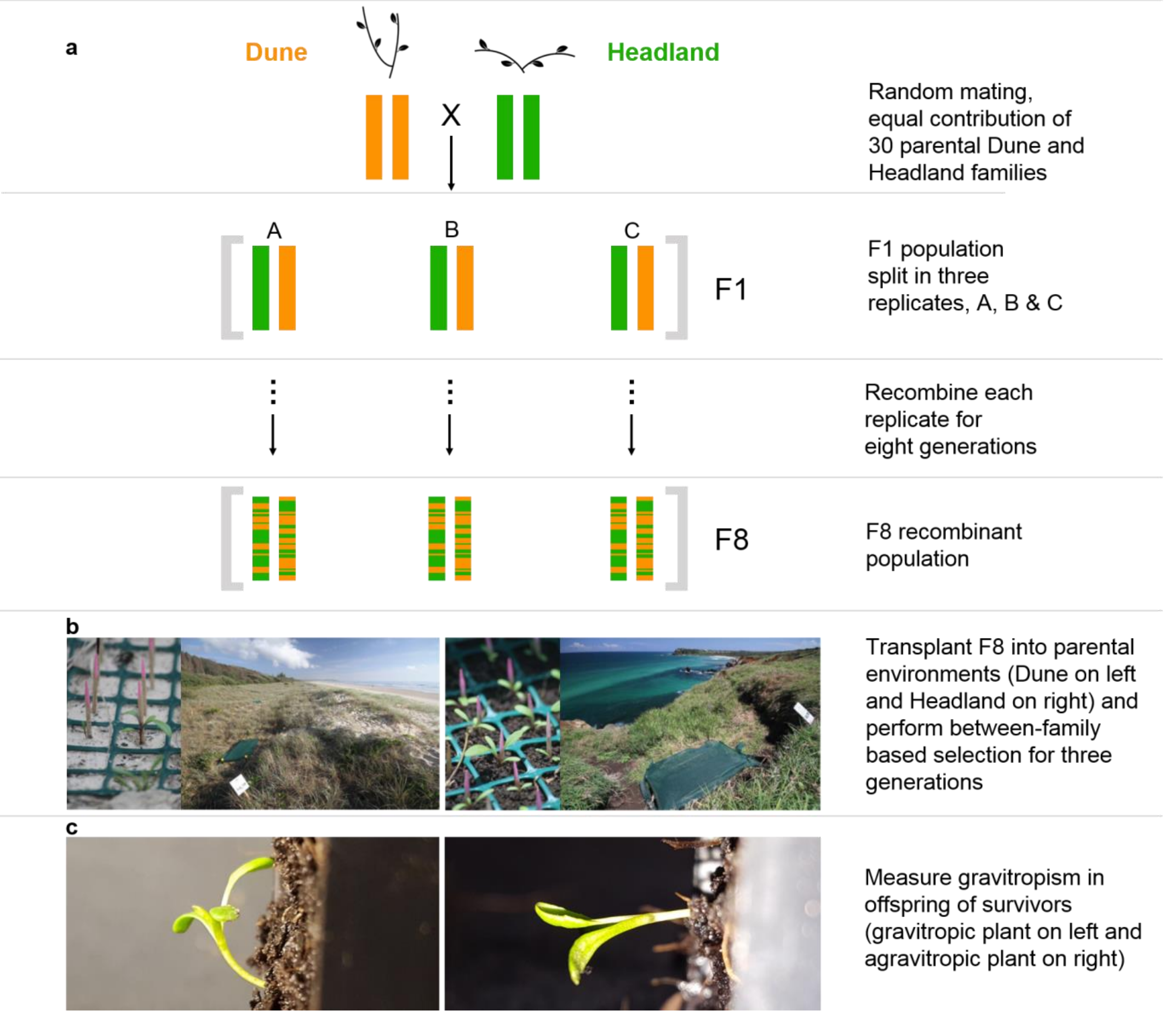

**Figure 1. The creation of the F11 recombinant population for the selection experiment and measurement of gravitropism.**

**a**. A total of 30 parental Dune (erect) and Headland (prostrate) individuals from Lennox Head (NSW) 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 parental dune and headland environments at Lennox Head. Between-family based selection occurred for three generations (F8, F9 and F10), where full-siblings from the fittest families were grown in the glasshouse and crossed amongst 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°. Agravitropic plants are not able to respond to this change in the gravity vector, while gravitropic plants are able to reorient their growth and grow upright.

## Supplementary Tables and Figures

**Table S1. Gravitropism and fitness information for the between-family based selection analysis.**

The total number of individuals per replicate (A, B or C) and time block (1, 2 or 3) are shown for the F11 recombinant individuals used to calculate gravitropism in each environment (Env). Gravitropism (°) was measured in the F11 recombinant population by rotating plants by 90°. An angle of 0° reflects no gravitropic response. Viability is the number of days until death of the F11 generation in the controlled temperature room. Dam and sire fitness are the F10 family fitness values for the full-siblings that were crossed to create the F11 offspring where gravitropism was measured. There are no fitness values for the Control population because they were not exposed to field selection.

| Env | Replicate | Time Block | N | Mean gravitropism (°) | Mean viability | Mean dam fitness | Mean sire fitness |
|---|---|---|---|---|---|---|---|
| Dune | A | 1 | 48 | 36.09 | 42.28 | 0.53 | 0.51 |
| Dune | A | 2 | 37 | 32.56 | 38.71 | 0.56 | 0.49 |
| Dune | A | 3 | 24 | 38.80 | 44.05 | 0.60 | 0.49 |
| Dune | B | 1 | 44 | 27.77 | 48.00 | 0.60 | 0.62 |
| Dune | B | 2 | 34 | 34.34 | 47.57 | 0.52 | 0.63 |
| Dune | B | 3 | 34 | 33.21 | 41.38 | 0.54 | 0.61 |
| Dune | C | 1 | 18 | 42.93 | 41.75 | 0.46 | 0.61 |
| Dune | C | 2 | 25 | 31.24 | 56.28 | 0.50 | 0.54 |
| Dune | C | 3 | 26 | 43.52 | 36.44 | 0.47 | 0.66 |
| Headland | A | 1 | 50 | 30.55 | 50.67 | 0.51 | 0.46 |
| Headland | A | 2 | 51 | 36.04 | 48.43 | 0.51 | 0.47 |
| Headland | A | 3 | 46 | 30.16 | 49.53 | 0.53 | 0.46 |
| Headland | B | 1 | 26 | 37.01 | 40.00 | 0.47 | 0.54 |
| Headland | B | 2 | 22 | 40.52 | 47.00 | 0.53 | 0.55 |
| Headland | B | 3 | 24 | 30.20 | 43.73 | 0.45 | 0.46 |
| Headland | C | 1 | 26 | 41.29 | 47.75 | 0.46 | 0.58 |
| Headland | C | 2 | 24 | 44.94 | 39.63 | 0.48 | 0.42 |
| Headland | C | 3 | 21 | 49.32 | 47.88 | 0.46 | 0.50 |
| Control | A | 1 | 26 | 31.83 | 52.82 | | |
| Control | A | 2 | 29 | 30.13 | 41.33 | | |
| Control | A | 3 | 29 | 37.44 | 42.12 | | |
| Control | B | 1 | 22 | 26.05 | 48.14 | | |
| Control | B | 2 | 18 | 36.06 | 43.11 | | |
| Control | B | 3 | 23 | 36.92 | 57.63 | | |
| Control | C | 1 | 14 | 39.12 | 40.00 | | |
| Control | C | 2 | 19 | 31.72 | 40.11 | | |
| Control | C | 3 | 26 | 39.48 | 53.78 | | |

**Table S2. Gravitropism heritability.**

Restricted maximum likelihood variance (Var) component estimates for the dam and sire at the three time-blocks. To calculate the narrow sense heritability ($h^2$), the percentage (Pct) of the total variance explained by the sire was multiplied by four.

| Random Effect | Var Ratio | Var Component | Pct of Total |
|---|---|---|---|
| Dam[time-block] | 0.0463 | 18.6243 | 4.04 |
| Sire[time-block] | 0.1000 | 40.1823 | 8.72 |
| Residual | | 401.8450 | 87.23 |
| Total | | 460.6516 | 100 |

**Table S3. Crosses between gravitropic and agravitropic individuals fail to produce seeds more than crosses within each of these groups.**

The F11 recombinant population, described in Figure 1, were randomly crossed and fertilisation success measured, based on successful production of seeds (success) or failure to produce more than three seeds on three mating attempts (fail). Crosses were grouped into whether the crosses occurred between or within the tails of the gravitropism distribution – gravitropic x agravitropic (between), gravitropic x gravitropic (within) and agravitropic x agravitropic (within). The percentage of successful and failed crosses is shown based on the total number of crosses completed within each group.

| Gravitropism tail | Cross | Successful cross | Failed cross | Total crosses |
|---|---|---|---|---|
| Within | gravitropic x gravitropic | 34 (92%) | 3 (8%) | 37 |
| Within | agravitropic x agravitropic | 28 (100%) | 0 (0%) | 28 |
| Between | gravitropic x agravitropic | 53 (79%) | 14 (21%) | 67 |

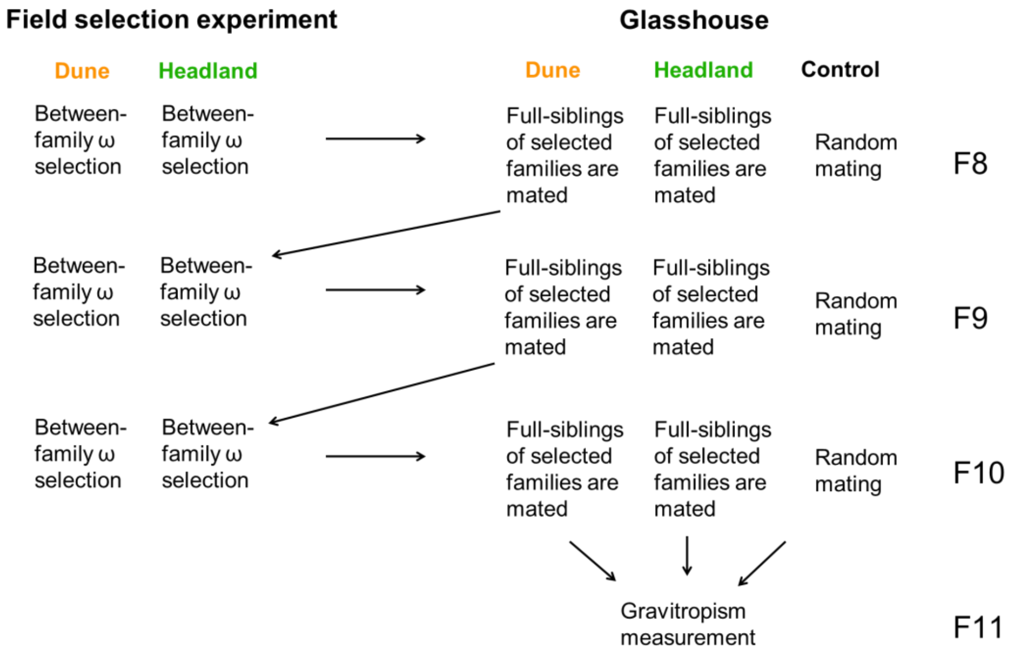

**Figure S1. The crossing scheme for the fitness (ω) selection and production of seeds for each field selection experiment.**

The fittest families were selected in the field selection experiment in the dune and headland environments and their full-siblings were grown and crossed in the glasshouse to produce the next generation. This occurred for three generations (F8, F9 and F10). The selection of the top 50% of families was based on family germination and survival. All crossing was completed within a genetic line and within the three environmental groups (Dune survivors, Headland survivors and Control). Gravitropism was measured in the F11 generation across all three environmental groups.

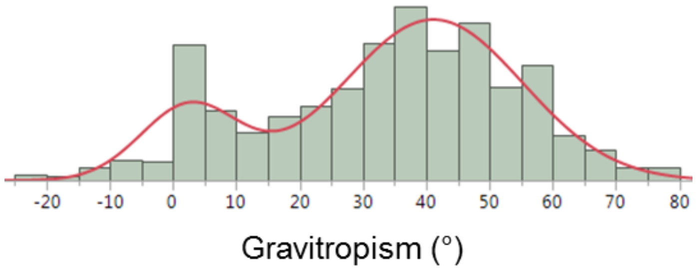

**Figure S2. The distribution of the gravitropism trait is bimodal.**

The gravitropic phenotype is three times more prevalent than the agravitropic phenotype. The agravitropic peak is at 2.56° (95% CIs=1.17-3.95) and the gravitropic peak is at 41.13° (95% CIs=39.85-42.40).

# Supplementary Material

## R code for Aster modelling.

```
library(aster)

#####################################
# DUNE
#####################################

data <- read.csv("F10.csv",header=T)
data <- droplevels(subset(data, ENV == "DUNE")) # Get only Dune Environment
data <- droplevels(subset(data, Type == "F10_D")) # Get only Dune F10 genotype
names(data)
sapply(data,class)
data$ENVblock<-factor(data$block)

# 1. ASTER DUNE Surv

vars <- c("GE", "Surv")
redata <- reshape(data, varying = list(vars), direction = "long",timevar = "varb", times = as.factor(vars), v.names = "resp")
names(redata)

class(redata$varb)
levels(redata$varb)
redata<-data.frame(redata,root=1)
names(redata)

pred <- c(0, 1)
fam <- c(1, 1)
sapply(fam.default(), as.character)[fam]

Surv <- grep("Surv", as.character(redata$varb))
Surv <- is.element(seq(along = redata$varb), Surv)
redata <- data.frame(redata, Surv = as.integer(Surv))
names(redata)

aout4 <- aster(resp ~ varb + Surv:Family, pred, fam, varb, id, root, data = redata)
summary(aout4)

#Using the aster model summary

newdata <- data.frame(Family = levels(data$Family))
for (v in vars) newdata[[v]] <- 1
newdata$root <- 1

renewdata<-reshape(newdata,varying=list(vars),direction="long",timevar="varb",times=as.factor(vars),v.names="resp")

##ESTIMATING CANONICAL PARAMETERS
Surv <- grep("Surv", as.character(renewdata$varb))
Surv <- is.element(seq(along = renewdata$varb), Surv)
renewdata <- data.frame(renewdata, Surv = as.integer(Surv))
names(redata)
names(renewdata)

nind <- nrow(newdata)
nnode <- length(vars)
amat <- array(0, c(nind, nnode, nind))
for (i in 1:nind) amat[i, grep("Surv", vars), i] <- 1

foo <- predict(aout4, varvar = varb, idvar = id, root = root, newdata = renewdata, se.fit = TRUE, amat = amat, parm.type = "canon")
bar <- cbind(foo$fit, foo$se.fit)
dimnames(bar) <- list(as.character(newdata$pop), c("Estimate","Std. Error"))
print(bar)


##ESTIMATING MEAN VALUE PARAMETERS (probabilities and expectation are the oL10y meaningful parts of the model)

pout4 <- predict(aout4, varvar = varb, idvar = id, root = root,newdata = renewdata, se.fit = TRUE, amat = amat)

#Code for FIG 1
popnames <- as.character(newdata$Family)
```

```
fit4 <- pout4$fit
i <- seq(along = popnames)
foo <- 0.1
y4top <- fit4 + pout4$se.fit
y4bot <- fit4 - pout4$se.fit

plot(c(i - 1.5 * foo, i - 1.5 * foo), c(y4top, y4bot), type = "n", axes = FALSE, xlab = "", ylab = "")
segments(i - 1.5 * foo, y4bot, i - 1.5 * foo, y4top)
segments(i - 2.5 * foo, y4bot, i - 0.5 * foo, y4bot)
segments(i - 2.5 * foo, y4top, i - 0.5 * foo, y4top)
segments(i - 2.5 * foo, fit4, i - 0.5 * foo, fit4)

axis(side = 2)
title(ylab = "unconditional mean value parameter")
axis(side = 1, at = i, labels = popnames)
title(xlab = "Surv DUNE FAMILY")

tbl<-data.frame(popnames,pout4$fit,pout4$se.fit)
write.csv(tbl, "F10_DuneSurvivors.csv")


with(data,
    table(Surv = Surv > 0, Family, useNA = "ifany"))


rm(list = ls(all = TRUE))


####################################
# HEADLAND
####################################

data <- read.csv("F10.csv",header=T)
data <- droplevels(subset(data, ENV == "HEAD")) # Get only Headland Environment
data <- droplevels(subset(data, Type == "F10_H")) # Get only Headland F10 genotype
names(data)
sapply(data,class)
data$ENVblock<-factor(data$block)

# 1. ASTER HEAD Surv

vars <- c("GE", "Surv")
redata <- reshape(data, varying = list(vars), direction = "long",timevar = "varb", times = as.factor(vars), v.names = "resp")
names(redata)

class(redata$varb)
levels(redata$varb)
redata<-data.frame(redata,root=1)
names(redata)

pred <- c(0, 1)
fam <- c(1, 1)
sapply(fam.default(), as.character)[fam]

Surv <- grep("Surv", as.character(redata$varb))
Surv <- is.element(seq(along = redata$varb), Surv)
redata <- data.frame(redata, Surv = as.integer(Surv))
names(redata)

aout4 <- aster(resp ~ varb + Surv:Family, pred, fam, varb, id, root, data = redata)
summary(aout4)

#Using the aster model summary

newdata <- data.frame(Family = levels(data$Family))
for (v in vars) newdata[[v]] <- 1
newdata$root <- 1

renewdata<-reshape(newdata,varying=list(vars),direction="long",timevar="varb",times=as.factor(vars),v.names="resp")

##ESTIMATING CANONICAL PARAMETERS
Surv <- grep("Surv", as.character(renewdata$varb))
Surv <- is.element(seq(along = renewdata$varb), Surv)
renewdata <- data.frame(renewdata, Surv = as.integer(Surv))
names(redata)
names(renewdata)
```

```
nind <- nrow(newdata)
nnode <- length(vars)
amat <- array(0, c(nind, nnode, nind))
for (i in 1:nind) amat[i, grep("Surv", vars), i] <- 1

foo <- predict(aout4, varvar = varb, idvar = id, root = root, newdata = renewdata, se.fit = TRUE, amat = amat, parm.type = "canon")
bar <- cbind(foo$fit, foo$se.fit)
dimnames(bar) <- list(as.character(newdata$pop), c("Estimate","Std. Error"))
print(bar)


##ESTIMATING MEAN VALUE PARAMETERS (probabilities and expectation are the oL10y meaningful parts of the model)

pout4 <- predict(aout4, varvar = varb, idvar = id, root = root,newdata = renewdata, se.fit = TRUE, amat = amat)

#Code for FIG 1
popnames <- as.character(newdata$Family)

fit4 <- pout4$fit
i <- seq(along = popnames)
foo <- 0.1
y4top <- fit4 + pout4$se.fit
y4bot <- fit4 - pout4$se.fit

plot(c(i - 1.5 * foo, i - 1.5 * foo), c(y4top, y4bot), type = "n", axes = FALSE, xlab = "", ylab = "")
segments(i - 1.5 * foo, y4bot, i - 1.5 * foo, y4top)
segments(i - 2.5 * foo, y4bot, i - 0.5 * foo, y4bot)
segments(i - 2.5 * foo, y4top, i - 0.5 * foo, y4top)
segments(i - 2.5 * foo, fit4, i - 0.5 * foo, fit4)

axis(side = 2)
title(ylab = "unconditional mean value parameter")
axis(side = 1, at = i, labels = popnames)
title(xlab = "Surv HEAD FAMILY")

tbl<-data.frame(popnames,pout4$fit,pout4$se.fit)
write.csv(tbl, "F10_HeadSurvivors.csv")


with(data,
   table(Surv = Surv > 0, Family, useNA = "ifany"))
```

# Chapter 5: A genetic link between adaptation and speciation in an Australian wildflower

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Keywords: genetic basis of adaptation; speciation; auxin pathway; gravitropism; ENODL; ABA

## Abstract

The diversity we see in nature is largely driven by populations evolving new traits as they adapt to different environments. Yet understanding how natural selection drives the evolution of adaptive traits and its consequences for the formation of new species remains a fundamental question in evolutionary biology. More specifically, we know very little about the genetics linking these two processes. I have previously provided evidence that is consistent with natural selection driving the evolution of growth habit in an Australian wildflower (*Senecio lautus)* that is adapting to adjacent contrasting coastal (dune and headland) environments. Plant growth habit is coupled with ability to respond to gravity stimuli (gravitropism), whereby *S. lautus* inhabiting rocky and windy headlands have a prostrate growth habit and respond poorly to gravity stimuli, while *S. lautus* inhabiting the hot sand dunes have an erect growth habit and respond well to gravity stimuli. Furthermore, gravitropism appears to be genetically coupled with intrinsic reproductive isolation (RI), thus linking adaptation and divergence in this system. Here I explore in greater detail the genetic links between an adaptive trait, gravitropism, and a form of intrinsic RI, hybrid seed production. I performed selective genotyping, where I genotyped only the tails of the gravitropism distribution in an F11 recombinant population. I found that gravitropism candidate genes in *S. lautus* have functions related to the auxin and abscisic acid pathways and the gravitropism candidate gene set is overrepresented in the gene ontology categories of transport and localisation. I also found that variation in some of the alleles contained in the top gravitropism candidate genes can predict fertilisation success. Overall, my results provide insights into the genetic and molecular basis of adaptive trait evolution and its contribution to the formation of new species.

## Introduction

Adaptive evolution of new traits in new environments can drive divergence between populations and can explain a large amount of the diversity we see in nature (Coyne and Orr, 2004; Hendry, 2004). A key goal in evolutionary biology is to understand the genes responsible for this adaptive evolution and reproductive isolation (RI) (Pardo-Diaz et al., 2015; Hoekstra and Coyne, 2007; Stern and Orgogozo, 2009; Nadeau and Jiggins, 2010; Butlin and Smadja, 2018). However, there are currently few systems where the genetic coupling of adaptation and speciation genes has been tested (Wright et al., 2013; Ono et al., 2017; Ferris et al., 2017), particularly in those involved in intrinsic RI such as hybrid sterility and inviability (but see Wright et al., 2013 for an example). Therefore, we remain ignorant about the genetic architecture that links adaptation and important features of population divergence, and thus the mechanisms driving the rapid formation of new species (Coyne and Orr, 2004).

Reproductive isolation barriers can occur through adaptive evolution of traits that directly prevent reproduction (prezygotic) or occur after reproduction and reduce hybrid fitness (postzygotic). These barriers can also be extrinsic, and be dependent on the local environment, or intrinsic, and manifest in any environment. For example, flowering time, an intrinsic prezygotic isolation barrier, can directly prevent the opportunity for reproduction by up to 90% between closely related populations of *Mimulus* (Ferris et al., 2017; Fishman et al., 2013; Lowry et al., 2008). Similarly, evolution of flower colour can directly change visiting pollinators and lead to reductions in gene flow via intrinsic prezygotic isolation between populations, as seen in *Petunia* (Hoballah et al., 2007), *Mimulus* (Byers et al., 2014) and *Solanum* (Li and Chetelat, 2015). Perhaps the clearest link between adaptation and speciation genes is via extrinsic postzygotic isolation, when populations adapt to local conditions and as a consequence migrants fail to establish, and hybrid offspring with intermediate values have reduced fitness in the parental environment (Hendry, 2004; Nosil et al., 2005; Lowry et al., 2008). However, there are few examples that investigate concomitantly the genetics of adaptation and intrinsic RI and therefore a major gap remains in our understanding of how natural selection creates hybrid dysfunction in nature (Presgraves, 2010), a problem that has daunted the field of evolutionary biology ever since Darwin suggested natural selection as the major mechanism for the origin of new species (Darwin, 1859).

The evolution of intrinsic RI barriers by natural selection can occur indirectly by the coupling of adaptation and speciation genes through genetic linkage (Nosil and Schluter, 2011; Nosil, 2012; Blackman, 2016; Brideau et al., 2006; Orr et al., 2004; Rieseberg and Willis, 2007). The closer these genes are on a chromosome, the less likely recombination will genetically disassociate them (Schwander et al., 2014; Thompson and Jiggins, 2014; Yeaman, 2013), therefore increasing the chance that natural selection will drive intrinsic RI between populations as a by-product of adaptive evolution. For example, tight genetic linkage between alleles selected for copper tolerance and alleles that cause hybrid mortality led to divergence between populations growing next to copper mines and those occupying typical *Mimulus* habitats (Wright et al., 2013). Additionally, in very rare examples, intrinsic RI loci are directly under selection (Ono et al., 2017; Bomblies and Weigel, 2007). For example, Ono et al. (2017) found that a single mutation arising in each yeast population, exposed to a common environment, created genetic incompatibilities, despite the mutations occurring in the same genetic pathway in response to the same selective pressure. These examples show that natural selection for adaptive loci genetically linked with speciation loci, can quickly drive population divergence and potentially lead to the formation of new species. However, empirical studies remain extremely rare, and so our understanding of the genetic mechanisms driving the formation of new species remains incomplete.

Here, I sought to understand the genetic architecture that links adaptive evolution and intrinsic RI using *Senecio lautus* populations inhabiting and adapting to adjacent dune and headland environments. I investigated the genetic basis of gravitropism, a trait that is not only targeted by natural selection but is coupled with intrinsic RI barriers in *S. lautus* (Chapter 4). Gravitropism, defined as the bending of a plant in response to gravity, is often correlated with growth habit in natural populations of *S. lautus* (Chapter 3), whereby *S. lautus* in dune environments have an erect growth habit and respond well to gravity stimuli (gravitropic) and *S. lautus* in headland environments have a prostrate growth habit and respond poorly to gravity stimuli (agravitropic). Adjacent Dune and Headland populations are often sister taxa (Melo et al., 2014; Richards and Ortiz-Barrientos, 2016; Richards et al., 2016; Walter et al., 2016), therefore suggesting that natural selection has driven the evolution of these phenotypes independently multiple times. To understand the genetic architecture involved in adaptation and divergence in *S. lautus*, I investigated the genes underlying gravitropism.

Tropic responses, such as gravitropism, are controlled by the auxin pathway in most plant systems. In fact, auxin was first suggested to control gravitropism in 1937 by Went and Thimann, and since then a number of studies have supported this, particularly in relation to the importance of auxin transport in creating a concentration gradient to induce gravitropic bending (Li et al., 2007). For example, mutants of *lazy plant1* (and orthologs) disrupt auxin transport and lead to reduced shoot gravitropism in rice (Li et al., 2007; Wu et al., 2013; Yoshihara and Iino, 2007), maize (Dong et al., 2013; Zhang et al., 2014) and tomatoes (Roberts Jeremy, 1984). These mutants also have reduced plant tiller angle in rice (Li et al., 2007; Wu et al., 2013; Yoshihara and Iino, 2007) and have a prostrate growth in maize (Dong et al., 2013). Mutations in other genes that reduce auxin transport have also been identified to lead to cell elongation defects that result in shorter plants, such as the maize *brachytic2* gene and the sorghum *dwarf3* gene (Multani et al., 2003). Shoot gravitropism can also be disrupted at other points in the auxin pathway, including, but not limited to biosynthesis of auxin (Sang et al., 2014; Song and Xu, 2013) and the auxin pathway is known to interact with a number of other genetic pathways to modify growth and development (Sang et al., 2014; Trupkin et al., 2017; Ohta et al., 2003; Li and Zhang, 2008; Sun et al., 2008; Zhao et al., 2014). These examples show that there are many genes within the auxin pathway that can modify major developmental phenotypes, such as gravitropism and growth habit.

Therefore, genes within the auxin pathway could be responsible for the major traits that contributed to the origin of Dune and Headland populations. It is not surprising that previous studies investigating the allelic differences between many Dune and Headland populations found that the auxin pathway was enriched with allelic differences (Roda et al., 2013). Here, I hypothesise that genes within the auxin pathway are responsible for the adaptive evolution of gravitropism and this is linked to the formation of intrinsic RI barriers in *S. lautus*. To test this, I genotyped the high and low tails of the F11 gravitropism distribution, a process called selective genotyping. The tails consisted of 68 agravitropic individuals and 77 gravitropic individuals from an F11 recombinant population derived from a cross between a pair of coastal Dune and Headland populations. These individuals were sequenced using Restriction-site Associated DNA (RAD) sequencing on one lane of the Illumina HiSeq platform. To isolate gravitropism candidate genes, I extracted the most differentiated SNPs between the gravitropic and agravitropic groups (99.9% quantile). In this gravitropism candidate gene set, I tested for overrepresentation in gene ontology categories and investigated linkage disequilibrium (LD) between gravitropism candidate genes. I compared allelic differences in this F11 recombinant population to the parental populations, to understand whether natural selection might have targeted these alleles in natural populations. Lastly, to narrow the source of intrinsic RI in *S. lautus*, I tested whether variation in gravitropism candidate genes predicted fertilisation success. These results provide insights into the genetic and molecular basis of adaptive traits and their link with speciation.

## Methods

### F11 recombinant population

The F11 recombinant population created in Chapter 4 was utilised to uncover the genetic basis of gravitropism. Briefly, the F8 recombinant population was created from random mating with equal contribution between 30 Dune and Headland parentals from Lennox Head, NSW. A field selection experiment at the dune and headland environments at Lennox Head was completed for three generations starting with this F8 recombinant population (F8, F9 and F10 generation), whereby the fittest families contributed to the next generation in each round of selection. The F11 recombinant population was created from the fittest F10 families and gravitropism was measured in a controlled temperature room by measuring the angle of the stem 12 hours after a 90° rotation. For more detail on the three field selection experiments, the F11 seed production and the gravitropism measurements, refer to Chapter 4 Methods.

### Genetic association between height and gravitropism

To understand whether height and gravitropism loci are genetically linked, I measured these traits in an F11 recombinant population. I have previously shown that height and gravitropism are strongly correlated in natural populations (Chapter 3), but here I tested their association after they had been segregated in an advanced recombinant population with reduced phenotypic and genotypic associations. I implemented a mixed linear model for the three F11 populations (Dune survivors, Headland survivors and a Control population) that accounts for family variation:

$$y_{ijk} = H_i + F_j + e_{k(ij)} \qquad (1)$$

where gravitropism ($y_{ij}$) is the angle of the growth response 12 hours after a 90° rotation, height ($H_i$) is the vertical distance from the soil to the top of the vegetative leaves (measured after maturity) 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 v13 (SAS 2015).

### Reference genome

Two Headland individuals from Lennox Head were used to create the reference genome. Firstly, seeds were collected from two wild individuals and these individuals were grown and crossed in the glasshouse. Siblings from the seeds produced were grown and crossed to produce a family of individuals capable of selfing (rare in *S. lautus*). Lastly, one generation of selfing was completed to increase homozygosity. A genome was de novo assembled using second-generation short reads and AllPaths-LG (version 6.1.2) by utilising a series of eight HiSeq read libraries (Table S1). All sequencing was performed by Beijing Genomics Institute (BGI). The reads were trimmed to remove residual adapter sequence and low quality bases (minimum quality 15). The final assembly was ~843MB long and consisted of 96,372 scaffolds with an N50 of 21K. Although 843MB is much shorter than the expected haploid size of 1.38GB (Liu, 2015) of the full genome, the 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.

### Genotyping of gravitropism distribution tails

To find genes responsible for gravitropism, I genotyped the high and low tails of the F11 gravitropism distribution, a process called selective genotyping (Darvasi and Soller, 1992). The gravitropism distribution in the Dune, Headland and Control populations is similar (Figure S1), thus I used individuals across all of these populations to increase sample size. F11 individuals were grouped into the gravitropic tail if they had a gravitropism angle of greater than 56° and the agravitropic tail if they had a gravitropism angle of less than 20°. This gave 81 individuals in each tail for genotyping but three of the individuals were removed as there was not enough leaf tissue for DNA extraction. Thus, leaf tissue from a total of 159 individuals across the two tails was collected and put into 96 well extraction plates for DNA extraction of the fresh tissue.

I extracted the DNA following the CTAB protocol (Clarke, 2009) with column cleaning and overnight incubation. I quantified the DNA using the Picogreen reagent (Invitrogen, Carlsbad, CA) and checked quality using the nanodrop 260/230 and 260/280 scores. This left 77 gravitropic individuals and 68 agravitropic individuals to identify gravitropism candidate genes. Each of these individuals was duplicated in independent wells for sequencing to ensure genotypes were accurate. To determine which parent the alleles were derived from, I also included 39 Dune parentals (D01) and 41 Headland parentals (H01). Leaves from these populations were collected directly from natives growing at Lennox Head and the same DNA extraction protocol was followed. Libraries of Restriction-site Associated DNA (RAD) tags (Baird et al., 2008) were created and sequenced at Floragenex (University of Oregon High Throughput Sequencing Facility) following the Baird et al. (2008) protocol but using the PstI restriction enzyme. Genomic libraries were sequenced with Illumina HiSeq, producing single reads (91 bp).

To genotype the 225 individuals, a total of 1.26 billion reads was produced with a mean of 3.41 million reads per sample. These reads were aligned to the reference genome using Bowtie 1.1.1 (Langmead et al., 2009) and a FASTQ quality score of above 20. The genotypes produced had an average individual sequencing coverage of 73x with an average individual genotype quality of 142.5 (Phred-scaled). After filtering, 27,743 SNPs were identified across 3,861 genome scaffolds and a total of 6.2 million genotypes were produced.

To acquire the top candidate SNPs for gravitropism, I extracted the SNPs in the 99.9% quantile of the distribution of differentiated SNPs between the gravitropism tails. This meant an allelic difference of greater than 0.15 was considered a gravitropism candidate. 67 SNPs were identified but 12 SNPs were then removed due to the individual duplicates having an allelic difference of more than 0.1. The region of the scaffold containing the SNP was annotated using the blastx NCBI database (Altschul et al., 1990). This produced an *S. lautus* gravitropism candidate gene set of 55 SNPs across 49 genomic scaffolds (Table S2).

### ***Senecio lautus*** **gravitropism candidate genes**

#### *Overrepresentation of functional categories*

To identify whether functional categories of genes were overrepresented in my *S. lautus* gravitropism candidates, I conducted a statistical overrepresentation test in PANTHER (http://pantherdb.org/). I extracted TAIR identification for 32 unique genes in my *S. lautus* gravitropism candidate gene set and matched these to a reference list of 27,502 *Arabidopsis thaliana* genes.

#### *Linkage disequilibrium*

To understand whether gravitropism candidate genes were more likely to be co-inherited, I performed a chi-square test to observe whether genotypic combinations between candidate genes were more common than expected by chance. Chi-square tests were performed with all individuals and with each F11 group independently (Control population, Dune survivors and Headland survivors), to observe whether natural selection could be driving the association.

#### *Intrinsic RI*

To identify the loci that might be responsible for the intrinsic RI found in correlation with gravitropism (Chapter 4), I investigated whether the genetic differences in gravitropism candidate genes correlated with failed crosses. I performed random crossing within and between gravitropic and agravitropic groups (F11) and recorded successful and failed crosses (more detail in Chapter 4). A failed cross was considered when, in the presence of pollen, less than three seeds were produced per flower head with three mating attempts. From these F11 individuals that were crossed, I extracted their genotypes and I predicted the genotypes of their offspring (F12). I then investigated whether certain candidate gene genotypes were more likely to result in a failed cross and also whether genotypic combinations from two candidate genes were more likely to result in a failed cross. For example, does an F11 individual with two copies of gene1 A/A produce successful crosses with an F11 individual with two copies of gene2 A/A? I only used genotypes where the offspring genotype was not ambiguous i.e., only homozygous parents were used, which reduced the sample size considerably.

*Dominance relationships*

To understand possible dominance relationships between the alleles for the top gravitropism candidate genes, I explored the effect of genotype on gravitropism. The mean and standard error of gravitropism was found for the top candidate genes genotypes independently and in combination. A Tukey-Kramer test was used to determine significant differences in gravitropism values between genotypes at a locus. For example are A/A gravitropism values significantly different from A/a and a/a genotypes? All statistical results reported here were produced in JMP v13 (SAS 2015).

## Results

### Height and gravitropism correlation regained after selection

The genetic relationship between growth habit and gravitropism is important for our understanding of ecotype adaptation and divergence. It connects a major morphological defining phenotype between the ecotypes with a trait that is controlled by a genetic pathway in which the genes have been well studied (Gallavotti, 2013; Enders and Strader, 2015; Paque and Weijers, 2016; Woodward and Bartel, 2005; Vanneste and Friml, 2009; Friml, 2003; Lau et al., 2008), and its magnitude correlates with levels of intrinsic RI (Chapter 4). I previously found that height and gravitropism are strongly correlated in many natural *S. lautus* populations (see Chapter 3). Here I tested the strength of the genetic association after these two traits were segregated in an F11 recombinant population. I found that in the F11 recombinant Control population, height and gravitropism were genetically disassociated ($F_{1,114.3}$=0.08, p=0.7801), indicating that these traits are not controlled by the same loci (i.e., they are not pleiotropic). The association was quickly regained after three rounds of selection in the headland environment ($F_{1,169.5}$=7.09, p=0.0085), but only marginally in the dune environment ($F_{1,151.3}$=3.20, p=0.0756), suggesting that natural selection can drive the rapid formation of allelic combinations controlling both traits in the headland environment, and greater segregation of variance for growth habit traits.

### ***Senecio lautus*** gravitropism candidate genes

To understand the genes underlying adaptation and intrinsic RI in the *S. lautus* species complex, I performed selective genotyping of the gravitropism phenotype, in an F11 recombinant population, created from divergent Dune and Headland populations. I identified a total of 27,743 SNPs (3,861 genome scaffolds) and then defined outlier SNPs as the largest allelic differences (99.9% quantile) between the gravitropic and agravitropic F11 groups. This left a gravitropism candidate gene set with allelic differences ranging from 0.15 to 0.22. The genomic regions surrounding these SNPs were annotated to create a *S. lautus* gravitropism candidate gene set, which included 55 SNPs across 49 genomic scaffolds (Table S2). This gravitropism candidate gene set was used for all subsequent analyses.

### *Gravitropism candidate genes are overrepresented in the gene ontology categories of transport (GO:0006810) and localisation (GO:0051179)*

I conducted a statistical overrepresentation test in PANTHER to identify the functional categories overrepresented in the *S. lautus* gravitropism candidate gene set. I found 13 gene ontology (GO) terms were significantly overrepresented (Table S3), the parent terms overrepresented were transport (GO:0006810, $p<0.0001$) and localisation (GO:0051179, $p<0.0001$). Transport is defined as "the directed movement of substances (such as macromolecules, small molecules, ions) into, out of or within a cell, or between cells, or within a multicellular organism by means of some agent such as a transporter or pore", while localisation (GO:0051179) is defined as “any process in which a cell, a substance, or a cellular entity, such as a protein complex or organelle, is transported, tethered to or otherwise maintained in a specific location.” Refer to Table S3 for the overrepresented sub-classes under the transport and localisation parent terms. These results indicate that genes containing SNPs with a change in allele frequency between gravitropic and agravitropic groups of more than 0.15 were more likely to have functions in the movement of proteins and substances within and between the cell than any other functional category. Eight of the gravitropism candidate genes bind metals such as zinc, magnesium, copper, calcium and manganese, and therefore might be important for the transport of these metals from cell to cell.

### *Variation in transport and localisation genes in S. lautus*

The first and third most differentiated SNPs between the tails of the gravitropism distribution are located in genes with functions in transport and localisation and include: Ccrd_009761 (Gelsolin domain-containing protein) and TIM23-1 (Mitochondrial import inner membrane). Ccrd_009761 has functions in actin filament binding and bundle assembly (uniprot: A0A118K785) and TIM23-1 transports transit peptide-containing proteins across the mitochondrial inner membrane (uniprot: Q9LNQ1). There is a large change in allele frequency ($\Delta p$) between the gravitropic and agravitropic groups at the SNP located in the Ccrd_009761 gene (Δ*p*=0.22) and this SNP changes the amino acid from a negatively charged Asp to a hydrophilic non-charged Asn (Table 1). However, this shift in allele frequency is in the opposite direction to the parent with the same gravitropism phenotype. For example, the gravitropic tail allele frequency shifts towards the favoured allele in the Headland parental population and not the Dune that contains gravitropic plants. TIM23-1 also has a large change in allele frequency between gravitropic and agravitropic groups (Δ*p*=0.20) and changes the amino acid from a hydrophilic non-charged Tyr to a negatively charged Asp. For TIM23-1, the allele frequency moves towards the favoured alleles in the expected parent (Table 1; Δ*p*=0.44). These results suggest that transport and localisation might be important for gravitropism evolution in *S. lautus.*

### *Variation in auxin-related genes explain variation in gravitropism in S. lautus*

Orthologs of genes with functions related to the auxin pathway appear to largely control gravitropism in *S. lautus*. Of the 55 SNPs across 49 genomic scaffolds that I identified as gravitropism candidates, five of these SNPs were mapped to four unique gene orthologs with functions related to the auxin pathway, including: ENODL1 (early nodulin-like protein 1), ABA3 (molybdenum cofactor sulfurase), ABP19A (auxin binding protein ABP19A-like) and MYB44 (transcription factor MYB44-like). I mapped two of these SNPs, that are on different *Senecio* scaffolds (see details below), to the same ENODL1 gene. *Arabidopsis* WAT1 is an ortholog of ENODL1, and a loss-of-function *wat1* mutant has defects in cell elongation, displays a dwarfed-habit, is deficient in auxin production, displays reduced auxin basipetal transport and deregulates the expression of many auxin-related genes, including those involved in response to auxin, auxin biosynthesis and transport (Ranocha et al., 2010). ABA3 is a modulator of auxin signalling and a key regulator of abscisic acid (ABA) biosynthesis, where it is upregulated in response to abiotic stressors, including temperature, drought and presence of salt (tair: AT1G16540; uniprot:Q9C5X8). *aba3* mutants have reduced ability to synthesise ABA (Schwartz et al., 1997), reduced sensitivity to salt and osmotic stress during germination (Léon-Kloosterziel et al., 1996). ABA treatment reduces the expression levels of the PIN-FORMEDs (PIN) auxin efflux carriers (PIN1, PIN3, PIN4, and PIN7) (Promchuea et al., 2017). ABP19A is a probable receptor for the auxin hormone (uniprot: Q9ZRA4), evidenced from homology to a putative auxin binding site in ABP1 (Ohmiya et al., 1998). Lastly, MYB44 is a transcription factor (uniprot: Q9FDW1). Low auxin concentrations stimulate MYB44 to activate the transcription of the auxin-responsive gene IAA19 (Zhao et al., 2014) and overexpression of

MYB44 increases salt and drought tolerance (Persak and Pitzschke, 2014). These results suggest that the gravitropism candidate genes in this study have many auxin-related functions.

Two of the top four most differentiated SNPs in allelic frequency between the tails of the gravitropism distribution are ENODL1 ($\Delta p$ = 0.20) and ABA3 ($\Delta p$ = -0.20). In both ENODL1 and ABA3 genes, the allele frequency of the F11 tails moves towards the favoured alleles in the expected parent. For example, the gravitropic tail allele frequency moves towards the favoured allele in the Dune parental population and the agravitropic tail allele frequency moves towards the allele favoured in the Headland parental population, as you would expect if these alleles were respectively contributing to gravitropic plants in the dune and agravitropic plants in the headland (Table 1). The remaining two auxin genes, ABP194 and MYB44, had no change in allele frequency between the Dune and Headland parentals (Table 1). The ENODL1 SNP (A/C) is a non-synonymous substitution; the amino acid Tyr (A allele) is more common in the Dune population (A=0.69) and gravitropic plants (A=0.70), while the amino acid Asp (C allele) is more common in the Headland population (C=0.87) and agravitropic plants (C=0.55). This non-synonymous substitution changes the hydrophobicity and charge from a hydrophilic non-charged Tyr to a negatively charged Asp, suggesting possible structural changes to the protein and thus functional differences between gravitropic and agravitropic plants. The ABA3 SNP (A/G) is a synonymous substitution (Table 1) and therefore might only impact gene expression or protein folding; (Bailey et al., 2014) or it might be closely linked to a non-synonymous mutation that I have not sampled.

There is a second differentiated SNP (scaffold-13139, pos-1534), between F11 gravitropic and agravitropic individuals that also maps to ENODL1 using blastx (Altschul et al., 1990). There is a 91.5% protein pairwise identity (nucleotide identity 87%) between these two *Senecio* scaffolds in the overlapping region, which suggests that they are independent genes within the *Senecio* genome. The SNP (scaffold-13139, pos-1534) has a large change in allele frequency between parentals ($\Delta p$=-0.59) and is a non-synonymous substitution, so that Leu (A allele) is more common in Dune (A=0.9) and gravitropic plants (A=0.68) relative to Gln (T) which is more common in Headland (T=0.68) and agravitropic plants (T=0.5; Table 1). This amino acid change alters hydrophobicity, from hydrophobic Leu to hydrophilic Gln. Furthermore, this ENODL1 ortholog is in linkage disequilibrium (LD) with a transport protein, NIP1-1 (Aquaporin NIP1-1-like; LR chi-square, $X^2=20.51$, df=4, p=0.0004), which is involved in transporting arsenite and water across cell membranes (uniprot: Q8VZW1). Altogether, these results are consistent with the hypothesis that the auxin pathway has contributed to the evolution of gravitropism in *S. lautus* populations that are adapting to their environment.

### *Linkage disequilibrium between ENODL1 and ABA3 genotypes*

ENODL1 and ABA3 genotypes are in strong LD (LR chi-square, $X^2=26.40$, df=4, p<0.0001). The ENODL1 C allele is more likely to be inherited with the ABA3 G allele (Figure 1a). This co-inheritance is not observed in the Control recombinant population when it is tested independently from the Dune and Headland survivors (LR chi-square, $X^2=4.61$, df=4, p=0.3295). However, ENODL1 and ABA3 genotypes regain LD after exposure to natural selection in the dune environment (LR chi-square, $X^2=12.48$, df=4, p=0.0141) and the headland environment (LR chi-square, $X^2=18.16$, df=4, p=0.0011), indicating that natural selection likely drives the correlation between these two alleles.

### *ENODL1 and ABA3 genotypes predict levels of intrinsic RI*

ENODL1 and ABA3 genotypic combinations correlate with the intrinsic RI found in Chapter 4, where F11 gravitropic and agravitropic plants fail to reproduce in 21% of crosses, in comparison to 8% between gravitropic individuals and 0% between agravitropic individuals. Here, when parents with ENODL1 C/C and ABA3 G/G genotypes are crossed, they consistently produce successful crosses (0% failed crosses; Table S4). In contrast, when parents have the alternative ENODL1 A/A and ABA3 A/A genotypes, 19% of the crosses between these individuals fail, with the highest failure (31%) in offspring with ENODL1 A/C and ABA3 A/A (Table S4). Furthermore, parents crossed with ABA3 G/G genotype always produce successful crosses, irrespective of the ENODL1 genotype (Table S5), indicating that intrinsic incompatibilities might be associated with the ABA3 A allele. The offspring with ABA3 A/G genotype have 13% cross failure and offspring with ABA3 A/A genotype have 15% failure (Table S5). On the other hand, there is 7.7% cross failure in the ENODL1 C/C genotype and 18.4% and 16.0% for ENODL1 A/C and A/A genotypes, respectively (Table S5). These results suggest that intrinsic RI might increase the frequency of the ENODL1 C/C and ABA3 G/G agravitropic allele combination that appears to be advantageous in the headland environment, by creating positive LD in the population.

### *The effect of ENODL1 and ABA3 genotypes on gravitropism*

ENODL1 C/C and ABA3 G/G genotypes reduce gravitropism. ABA3 shows a dominant inheritance, whereby one copy of the A allele contributes to the gravitropic phenotype and two copies of the C allele contributes to the agravitropic phenotype (Figure 1b). More specifically, individuals with an ABA3 G/G genotype have significantly lower gravitropism values than both ABA3 A/A and A/G genotypes (Figure 1b), indicating complete dominance. On the other hand, individuals with an ENODL1 C/C genotype have significantly lower gravitropism values than the A/A ENODL1 genotype, but neither is significantly different from the heterozygous genotype (A/C) (Figure 1b). When ENODL1 C/C and ABA3 G/G genotypes are co-inherited, gravitropism significantly reduces (25.10°) relative to all other genotype combinations (Figure 1b; t=4.86, df=34.30, p<0.0001). Considering the agravitropic peak is at 2.56° (95% CIs=1.17-3.95) and the gravitropic peak is at 41.13° (95% CIs=39.85-42.40), shown in Chapter 4, the ENODL1 C/C and ABA3 G/G genotype combination explains 65% of the difference in gravitropism. Additionally, 86% of individuals with this genotype combination are in the agravitropic tail (<20°) with the remaining 14% in the gravitropic tail (>56°), illustrating the large combined effect these alleles have on gravitropism. The mean gravitropism values and standard errors used in Figure 1b are in Table S6 and Table S7, while the Tukey-Kramer test results associated with Figure 1b are shown in Table S8. Altogether, these results suggest natural selection is driving the co-inheritance of large effect loci, which is likely contributing to intrinsic RI barriers between Dune and Headland populations.

## Discussion

To understand the genetic basis of adaptation and intrinsic RI in *S. lautus*, I sequenced the phenotypic tails of a trait that contributes to adaptive evolution and is coupled with intrinsic RI in *S. lautus* (Chapter 4). I show that the strong correlation between growth habit and gravitropism that was found in natural populations in Chapter 3 is unlikely to be due to pleiotropism but suggest these traits could still be loosely genetically linked. I also found candidate genes underlying gravitropism in *S. lautus* have functions related to the auxin pathway, abscisic acid pathway, transport, localisation and salt tolerance. Below, I discuss how natural selection might be driving the co-inheritance of gravitropism candidate loci and reducing reproductive failure in the Headland favoured agravitropic alleles. Overall, the results indicate that genes with auxin-related functions were recruited for the evolution of gravitropism and this is coupled with intrinsic RI barriers, thus providing us with a deeper understanding of the underlying genetic and molecular processes of natural selection in adaptive and divergent evolution.

### Natural selection drives co-inheritance of growth habit and gravitropism

Genetic linkage between adaptive traits reduces the likelihood of recombination separating the advantageous trait combination (Schwander et al., 2014; Thompson and Jiggins, 2014; Yeaman, 2013). Tight genetic linkage has driven the co-inheritance of adaptive traits across nature, including sticklebacks (Erickson et al., 2016), deer mice (Linnen et al., 2013), monkeyflower (Gilmartin and Li, 2010), snails (Murray and Clarke, 1976) and butterflies (Joron et al., 2006). Genetic linkage is particularly advantageous when populations are adapting to adjacent contrasting environments, but still experiencing gene flow (Lenormand and Otto, 2000; Bürger and Akerman, 2011). The tight genetic linkage aids in the tug of war between selection driving the evolution of adaptive alleles and recombination separating the adaptive alleles (Bürger and Akerman, 2011; Nosil et al., 2009). In *S. lautus*, the close proximity of the Dune and Headland populations suggests the potential for gene flow, yet distinct morphologies are maintained in the contrasting environments. For instance, populations at Coffs Harbour are morphologically different, almost genetically identical (mean $F_{ST}$~0), separated by a few meters, and they form a narrow hybrid zone where the two habitats abut (North, 2015). A powerful way to overcome this antagonism between natural selection and recombination during divergence is via pleiotropy or tight genetic linkage of beneficial alleles (Ortiz-Barrientos et al., 2002; Butlin, 2005). However, in this study I found that the strong correlation in natural populations between two possibly adaptive traits (Chapter 3) is unlikely to be due to pleiotropism and instead the two traits could be genetically linked as favoured by natural selection. I discuss these ideas and my results in more detail below.

Natural selection appears to have driven the association between growth habit and gravitropism in *S. lautus* populations that are adapting to contrasting environments. I have found direct evidence through field experiments that natural selection has targeted a gravitropic phenotype in the dune environment (Chapter 4) and evidence consistent with natural selection for a prostrate growth habit in the headland environment (Chapter 2). There is also a strong correlation between growth habit and gravitropism in natural populations (Chapter 3), which is particularly evident in the Lennox Head parental populations (Chapter 3) that the F11 recombinant population used in this study was derived from. Here, I found that the association between growth habit and gravitropism is broken through random mating in the F11 recombinant Control population, indicating that this trait association is not through pleiotropy and can be broken through 11 rounds of recombination (Bürger and Akerman, 2011; Nosil et al., 2009). If I use the commonly accepted genome average recombination rate of ~1cM/Mb (Collins and Morton, 1998; Ulgen and Li, 2005) and apply it to 11 generations of random mating, a recombination break would occur on average every 488bp. This means that only very tightly linked alleles would avoid being disassociated, illustrating that height and gravitropism alleles could be partially linked and co-inherited.

Surprisingly, plant studies have indicated that single point mutations can reduce both shoot gravitropism and lead to a prostrate phenotype, with the mutations most commonly occurring in genes within the auxin pathway (Xing et al., 2015; Li et al., 2007; Wu et al., 2013; Yoshihara and Iino, 2007; Dong et al., 2013). The regaining of the trait association after the selection experiment in the headland environment shows natural selection can quickly reconstruct this trait association, indicating that pleiotropy is not required for the co-inheritance of these adaptive traits. One caveat with this interpretation is that I did not evaluate LD and co-inheritance of these traits while creating the F11 recombinant population. Future work could evaluate the decay of LD between adaptive traits by observing how the covariance between height and gravitropism is reduced with increasing recombination events. I could then infer the role of linkage in creating associations between adaptation and RI. Future work should evaluate the genomic location of height and gravitropism quantitative trait loci that distinguish adjacent Dune and Headland populations. I posit that plant height, gravitropism, and candidate genes contributing to intrinsic RI will be genomic neighbours along a chromosome. Overall, these results suggest that selection can drive adaptive trait associations in unlinked or partially linked loci, giving us an insight into the genetic architecture that can lead to the evolution of multiple adaptive traits.

### Adaptive evolution of gravitropism in *S. lautus* might be occurring through variation in the auxin pathway

It is well accepted that the auxin pathway is a major controller of plant growth and development (Leyser, 2018; Paque and Weijers, 2016); for instance auxin controls a number of major developmental phenotypes, including plant height (Wallace et al., 2016;

Xu et al., 2005), branching (Gallavotti, 2013; Domagalska and Leyser, 2011), apical dominance (Phillips, 1975; Dun et al., 2006), phototropism (Whippo and Hangarter, 2006; Paque and Weijers, 2016) and gravitropism (Blancaflor and Masson, 2003; Hashiguchi et al., 2013). The coordinated action of auxin biosynthesis, auxin transport and auxin signal transduction dictates the phenotype produced. In particular, alterations to auxin transport through changes in auxin influx and efflux transporters have been shown in numerous cases to disrupt plant growth and development (Paque and Weijers, 2016; Gallavotti, 2013; Woodward and Bartel, 2005). Polar auxin transport is vital for creating auxin gradients, which is the basis for how auxin controls these major phenotypes. This has been well documented in most plant systems, particularly *Arabidopsis*, and in Chapter 3, I found evidence it also occurs in *Senecio*; I experimentally showed that disruptions to the efflux carrier caused major reductions in gravitropism in Dune and Headland seedlings. I then hypothesised that differences in gravitropism between Dune and Headland populations might be due to differences in the efflux carrier. Although no auxin transport genes were found to be differentiated between gravitropic and agravitropic individuals (these genes might not have been sequenced as RAD-seq does not cover the entire genome), I provide evidence, here, that other genes with functions that interact with auxin efflux transporters might be contributing to differences in gravitropism between Dune and Headland populations.

The current study provides another independent line of evidence implicating the auxin pathway in the adaptive evolution of gravitropism in *S. lautus*. I found that the genes that contain five of the 55 SNPs that are differentiated between gravitropic and agravitropic individuals have orthologs with auxin-related functions (ENODL1, ABA3, ABP19A and MYB44). The auxin-related functions range from activator of auxin repressors when auxin is present (MYB44, uniprot: Q9FDW1), a probable auxin receptor (ABP19A, uniprot: Q9ZRA4), modulator of auxin signalling (ABA, tair: AT1G16540) and a regulator of auxin gene expression and auxin production (ENODL, Ranocha et al., 2010). The non-synonymous substitution in ENODL that alters the hydrophobicity and charge of the amino acid implies that the differences between the gravitropic (Dune and F11 gravitropic group) and agravitropic (Headland and F11 agravitropic group) plants might cause structural changes to the protein that might ultimately lead to a change in function. Furthermore, mutants of an *Arabidopsis* homolog of ENODL1, walls are thin1 (WAT1), have defects in cell elongation causing a dwarf growth habit (Ranocha et al., 2010). These results link a non-synonymous allelic difference between a Dune and Headland population pair to a candidate gene with functions related to major phenotypic differences between the populations. This narrows down the targets of natural selection and provides a gateway to study the allelic differences responsible for population adaptation and divergence.

### The role of ENODL1 and ABA3 in salt tolerance

ENODL1 and ABA3 not only have functions related to gravitropism but also salt tolerance, another trait that differentiates Dune and Headland populations. Headland populations of *S. lautus* are exposed to high salt concentrations relative to their adjacent Dune population (Roda et al., 2013; Walter et al., 2016), and in controlled conditions, Headland plants have greater survival when exposed to high salt concentrations compared to their adjacent Dune population (unpublished results); indicating that Headland populations might have derived salt tolerance alleles. Furthermore, orthologs of ABA and ENODL both have major roles in salt tolerance. ENODL was found to improve osmotic tolerance in tobacco (Wu et al., 2011) and salt stress altered ENODL gene expression in rice (Ma et al., 2011) and wheat (Cao et al., 2015). ABA has been found to be crucial in salt accumulation signalling (Garcia de la Garma et al., 2015) and has been shown to interact with the salt overly sensitive (SOS) signalling pathway (Ohta et al., 2003). Furthermore, Sun et al. (2008) found that salt stress, which is modulated by the SOS signalling pathway, reduced gravitropism through reductions in an auxin efflux transporter (PIN2) in *Arabidopsis,* which has been shown to influence plant height (Chen et al., 2012). This indicates that auxin, ABA and SOS pathways all cross talk and modulate phenotypes such as gravitropism, height and salt tolerance (Ji et al., 2013). The agravitropic, prostrate and salt tolerant nature of Headland plants and the results of this study suggest that the adaptive evolution of these phenotypes might have occurred through interactions between the auxin, ABA and SOS pathways in *S. lautus* and ENODL1 and ABA3 alleles may be preferentially selected if they pleiotropically modulate multiple beneficial traits. I will discuss these ideas in more detail in Chapter 6.

### Towards the genetic and molecular basis of gravitropism

Understanding the genetic and molecular basis of adaptive evolution is a key goal in evolutionary biology. Investigating the effect size and dominance relationships between alleles contributing to adaptive trait evolution can provide insight into evolutionary processes (Rausher and Delph, 2015). My results suggest that gravitropism in *S. lautus* is controlled by few loci of large effect, some with dominance relationships. ENODL1 C/C and ABA3 G/G genotypes reduce gravitropism together by 25.10° relative to all other genotypic combinations. This explains 65% of the difference in gravitropism between gravitropic and agravitropic F11 individuals. Large effect loci is common across plant species, with major morphological differences between closely related species often being the genetic differences in one or two genes (Gottlieb, 1984). Additionally, ABA3 shows a dominant inheritance, whereby the agravitropic phenotype is only observed with 2 copies of the C allele, showing that the recessive alleles can contribute strongly to adaptive evolution. These results provide insights into the allelic interactions and dominance relationships that lead to adaptive trait evolution.

Natural selection appears to drive the association between ENODL1 and ABA3, which might contribute to the divergence between *S. lautus* populations. Individuals are more likely to co-inherit ENODL1 C and ABA3 G alleles together after selection in the dune and headland environments, but there is no association between these alleles in the F11 Control population. The 11 generations of random mating that were completed in the F11 Control population dissociated the allele combination, making strong genetic linkage (a distance of < 488bp, as discussed earlier) between these alleles unlikely. Instead, strong natural selection for these agravitropic alleles in the headland environment and for the alternate gravitropic alleles in the dune environment, most likely drove this association. The alleles might be in unlinked loci, as observed in *Drosophila*, where strong selection for unlinked chemoreceptor loci drove LD (Takano-Shimizu et al., 2004). Support for selection driving the agravitropic allele association is observed with the allelic frequencies of the natural populations being in the expected direction and in most cases close to fixation of the advantageous allele (ENODL1 dune allele is the furthest from fixation (A=0.79)). These results indicate that strong selection drove the co-inheritance of adaptive alleles (or closely linked alleles) in natural conditions in the absence of very tight linkage, providing insights into the genetic architecture that might drive the rapid formation of new species.

### The genetic architecture that links adaptation and speciation

There is currently a major gap in our understanding of how natural selection drives speciation (Presgraves, 2010; Nosil, 2012; Coyne and Orr, 2004), a problem arising ever since Darwin suggested natural selection as the major mechanism for the origin of new species (Darwin, 1859). Studies that link adaptation and RI provide insight into how natural selection might lead to the formation of new species, but few studies have linked these processes. In fact, only a handful of studies have linked adaptation and intrinsic RI (Ono et al., 2017; Bomblies and Weigel, 2007), therefore we lack an understanding of the direct role of natural selection in speciation. Here, I couple adaptive evolution with intrinsic isolation, through the gravitropism phenotype, where I found that 1) gravitropism is an adaptive trait (Chapter 4), 2) differences in gravitropism cause reduced fertilisation success (Chapter 4) and 3) in this chapter, genetic differences in gravitropism candidate genes can predict fertilisation success. For instance, I found that ENODL1 C and ABA3 G alleles strongly contribute to the agravitropic phenotype favoured in the headland environment, and these alleles are less likely to produce failed crosses in comparison to the other allelic combinations. This indicates that natural selection has targeted these alleles (or tightly linked) and this has major effects on both gravitropism and fertilisation success, suggesting that these alleles might be the link between adaptation and the early stages of speciation in *S. lautus*. Overall, these results support the hypothesis that natural selection can directly drive the formation of intrinsic RI barriers and lead to the formation of new species.

The mechanisms by which gravitropism couples adaptation and speciation genes in *S. lautus* are unknown, as research is currently in the early stages. The fertilisation success experiment (Chapter 4) was completed in controlled glasshouse conditions by rubbing multiple flower heads of two individuals together; therefore, failed crosses in this context might be caused by genetic incompatibilities or pollen-pistil interactions (e.g. pollen tube reception). Thus, gravitropism loci might be pleiotropic or genetically linked to incompatible loci or loci that control pollen-pistil interactions. One of the top gravitropism candidate genes in this study, ENODL1, has orthologs with functions in pollen tube reception (Hou et al., 2016), which controls the cross talk between males and females in fertilisation. For example, in *Arabidopsis* wild-type plants, male pollen tubes fail to arrest growth and rupture after entering the ovule of an *enodl* mutant, suggesting ENODL has a major role in pollen tube reception and therefore can reduce fertilisation success (Hou et al., 2016). Additionally, ENODL's role in pollen tube reception is female specific (Hou et al., 2016), which might help explain why the evolution of gravitropism, found in Chapter 4, occurred through the maternal genotype, but more research will need to investigate this and whether ENODL pleiotropically controls gravitropism and intrinsic RI in *S. lautus*. Thus, other mechanisms of intrinsic RI are still likely, including genetic incompatibilities from loci tightly linked to gravitropism loci, similar to those found in Wright et al. (2013) that rose in frequency from strong natural selection for tightly linked loci. Overall, my results indicate that natural selection plays a major role in driving the evolution of locally advantageous traits and that speciation might be a by-product of this evolution through tight linkage or pleiotropism. I will combine this discussion with results from the entire thesis in more detail in Chapter 6.

## Conclusions

Here, I sought to understand the genetic basis of adaptive phenotypes underlying the divergence between adjacent ecotypes in *S. lautus.* Through identifying candidate genes and exploring the genetic architecture responsible for the evolution of gravitropism, a trait I have found to be adaptive and coupled with barriers to reproduction, I have furthered our understanding of the genes underlying adaptation and divergence. This has major implications for our understanding of the mechanisms driving the formation of new species. Future research will continue to investigate the functional basis of these genes and the connection between salt tolerance, gravitropism, and pollen tube reception.

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# Tables

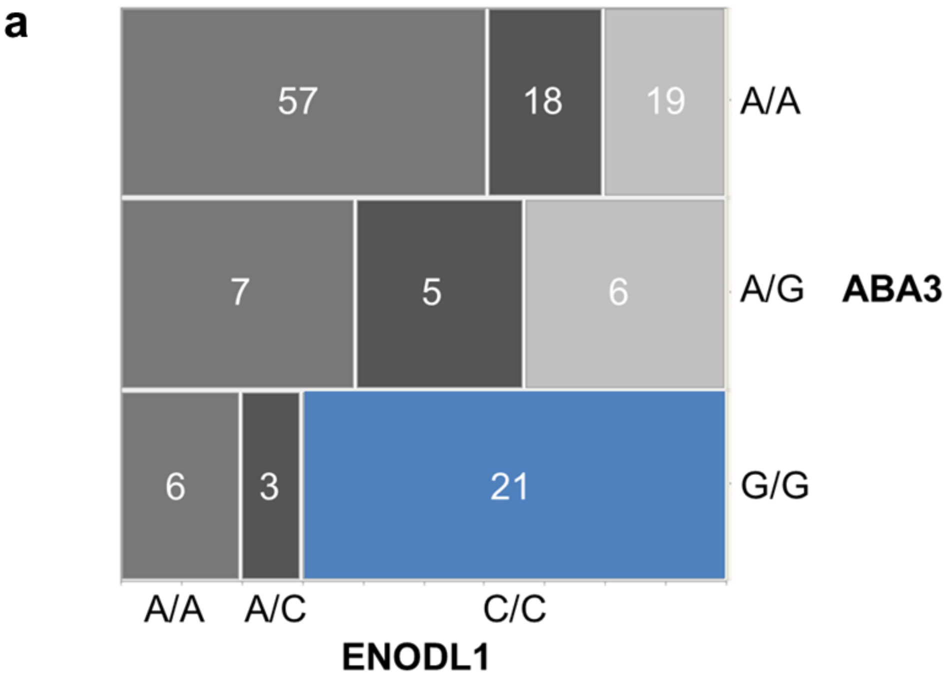
**Table 1. Gravitropism candidate genes with functions in auxin signalling, abscisic acid signalling, salt tolerance, transport and localisation in the *Senecio lautus* species complex.**

This data has been extracted from the *S. lautus* gravitropism candidate gene set (Table S2) produced from the most differentiated allelic frequency (Δp>0.15) between 77 gravitropic (g) and 68 agravitropic (ag) plants. *Senecio lautus* scaffolds and the position (Pos) of the SNP within the scaffold are shown. The SNP region was annotated using NCBI blastx and functions were extracted from uniprot and tair. The allele frequency of the reference allele is shown for the gravitropism tails and the wild type parentals: gravitropic (g), agravitropic (ag), Dune population (D01) and Headland population (H01). If the SNP is in the coding region, the amino acid (AA) produced from the reference allele is shown first and then the change in amino acid if the SNP is non-synonymous.

| ***Senecio* scaffold** | **Pos** | **Uniprot Gene** | **Protein** | **Function** | **Ref allele** | ***p* (g)** | ***p* (ag)** | ***p* (D01)** | ***p* (H01)** | **AA** |
|---|---|---|---|---|---|---|---|---|---|---|
| 29206 | 1935 | ENODL1 | Early nodulin-like protein 1 | Auxin signalling; Salt tolerance | A | 0.68 | 0.48 | 0.69 | 0.13 | Y/D |
| 10199 | 12546 | ABA3 | Molybdenum cofactor sulfurase | Auxin signalling; Abscisic acid signalling; Salt tolerance; Transport; Localisation | G | 0.18 | 0.38 | 0.03 | 0.89 | T |
| 34994 | 4007 | ENODL1 | Early nodulin-like protein 1 | Auxin signalling; Salt tolerance | T | 0.29 | 0.47 | 0.10 | 0.68 | Q/L |
| 19192 | 8093 | ABP19A | Auxin-binding protein ABP19a-like | Auxin signalling; Metal binding (Manganese) | T | 0.47 | 0.32 | 0.13 | 0.11 | V |
| 57053 | 1280 | MYB44 | Transcription factor MYB44-like | Auxin signalling; Abscisic acid signalling; Salt tolerance | G | 0.91 | 0.76 | 1.00 | 0.92 | A/P |

| Senecio scaffold | Pos | Uniprot Gene | Protein | Function | Ref allele | p (g) | p (ag) | p (D01) | p (H01) | AA |
|---|---|---|---|---|---|---|---|---|---|---|
| 9125 | 16392 | Ccrd_009761 | Gelsolin domain-containing protein | Transport; Localisation | C | 0.42 | 0.20 | 0.49 | 0.81 | D/N |
| 44863 | 1009 | TIM23-1 | Mitochondrial import inner membrane translocase subunit TIM23-1-like | Transport; Localisation | C | 0.91 | 0.72 | 0.90 | 0.46 | A/D |
| 327 | 50494 | AGD12 | Arf GTPase activating protein | Transport; Localisation; Metal binding (Zinc and Calcium) | C | 0.82 | 0.63 | 0.63 | 0.63 | P |
| 13139 | 2535 | NIP1-1 | Aquaporin NIP1-1-like | Transport; Localisation | T | 0.79 | 0.59 | 0.99 | 0.55 | |
| 13744 | 9948 | RABH1B | Ras-related protein RABH1b | Transport; Localisation | C | 0.77 | 0.60 | 0.66 | 0.54 | T/M |
| 20374 | 13262 | At3g03090 | D-xylose-proton symporter-like 1 isoform X1 | Transport; Localisation | A | 0.48 | 0.66 | 0.84 | 0.69 | A |
| 13139 | 2534 | NIP1-1 | Aquaporin NIP1-1-like | Transport; Localisation | T | 0.26 | 0.43 | 0.01 | 0.53 | |
| 4940 | 43836 | mcfB | Mitochondrial substrate carrier family protein B-like | Transport; Localisation; Metal binding (Calcium) | C | 0.41 | 0.58 | 0.59 | 0.35 | V/L |
| 504 | 14643 | VPS52 | Vacuolar protein sorting-associated protein 62 | Transport; Localisation | A | 0.41 | 0.57 | 0.33 | 0.58 | D |
| 11053 | 8590 | At1g31730 | AP-4 complex subunit epsilon | Transport; Localisation | G | 0.52 | 0.68 | 0.39 | 0.97 | S/F |
| 32038 | 3643 | HannXRQ_Chr17g0536851 | F-box protein/LRR-repeat protein | Transport; Localisation | A | 0.61 | 0.45 | 0.36 | 0.63 | S |
| 504 | 14660 | VPS52 | vacuolar protein sorting-associated protein 62 | Transport; Localisation | A | 0.42 | 0.57 | 0.34 | 0.58 | D |
| 11131 | 708 | ERD2 | ER lumen protein retaining receptor | Transport; Localisation | A | 0.75 | 0.90 | 0.91 | 0.77 | T/S |

## Figures


a
57
18
19
A/A
7
5
6
A/G
ABA3
6
3
21
G/G
A/A
A/C
C/C
ENODL1

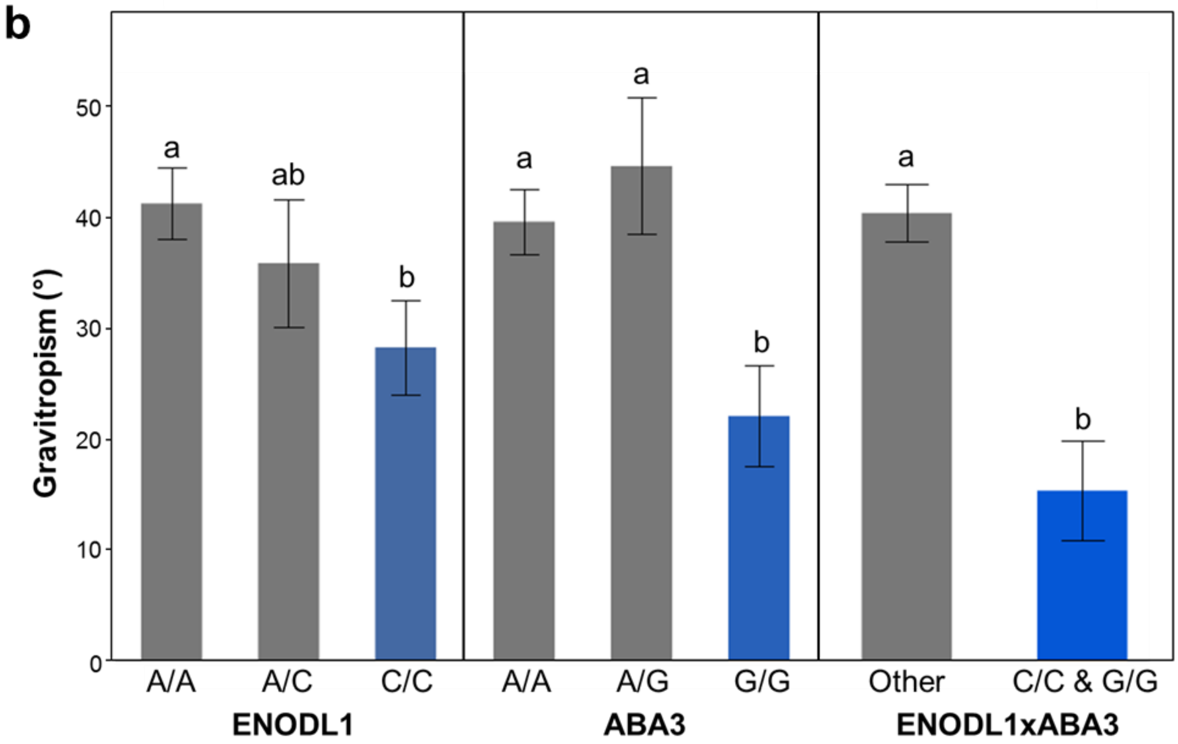

b
Gravitropism (°)
0
10
20
30
40
50
a
ab
b
a
a
b
a
b
A/A
A/C
C/C
ENODL1
A/A
A/G
G/G
ABA3
Other
C/C & G/G
ENODL1xABA3

**Figure 1. The co-inheritance and effect of ENODL1 and ABA3 alleles on gravitropism in *S. lautus*.**

To identify these gravitropism candidate genes, ENODL1 (early nodulin-like protein 1) and ABA3 (molybdenum cofactor sulfurase), I performed selective genotyping using the tails of the gravitropism distribution in an F11 recombinant population of *S. lautus*. **a)** The size of the box reflects the prevalence of ABA3 (y axis) and ENODL1 (x axis) alleles being co-inherited by F11 individuals. The number of F11 individuals with the allele combination is shown inside the box. ENODL1 and ABA3 are in linkage disequilibrium (LR chi-square, $X^2$=26.40, df=4, $p<0.0001$), where individuals are more likely to co-inherit ENODL1 C/C genotype with ABA3 G/G genotype (blue). **b)** The effect of ENODL1 and ABA3 alleles on gravitropism, independently and when combined (ENODL1xABA3). ENODL1 C/C and ABA3 G/G genotypes reduce gravitropism more than other genotypes and there is a greater reduction in gravitropism when these genotypes are co-inherited (C/C & G/G), compared to all other genotype combinations (Other). Different letters denote significant differences between genotypes ($t>1.98$, $p<0.05$).

## Supplementary Tables and Figures

**Table S1. The eight read libraries used in the de novo assembled *Senecio lautus* genome.**

The genome was assembled using second-generation short reads and AllPaths-LG (version 6.1.2).

| Platform | Read length (bp) | Insert size | Read count (M) | AllPaths-LG type |
|---|---|---|---|---|
| HiSeq2500 | 125 | 200bp | 47.5 | Fragment |
| HiSeq4000 | 150 | 270bp | 107.9 | Fragment |
| HiSeq2500 | 125 | 500bp | 94.3 | Jump |
| HiSeq2500 | 125 | 800bp | 94.4 | Jump |
| HiSeq4000 | 50 | 2 Kb | 72.4 | Jump |
| HiSeq4000 | 50 | 5 Kb | 89.7 | Jump |
| HiSeq4000 | 50 | 10 Kb | 80.3 | Jump |
| HiSeq4000 | 50 | 20 Kb | 78.9 | Long Jump |

**Table S2. The *S. lautus* gravitropism candidate gene set.**

The *Senecio* scaffolds and position (Pos) of the most differentiated SNPs (Δp>0.15) between gravitropic and agravitropic plants are shown. The SNP region was annotated using NCBI blastx, and functions were extracted from uniprot and tair. The allele frequency of the reference allele is shown for the gravitropism tails and the wild type parentals: gravitropic (g), agravitropic (ag), Dune population (D01) and Headland population (H01). If the SNP is in the coding region, the amino acid (AA) produced from the reference allele is shown first and then the change in amino acid if the SNP is non-synonymous. Gravitropism candidate genes are ordered from biggest to smallest change in allele frequency between gravitropic and agravitropic groups.

| ***Senecio* scaffold** | **Pos** | **Uniprot Gene** | **Protein** | **Function** | **Ref allele** | ***p* (g)** | ***p* (ag)** | ***p* (D01)** | ***p* (H01)** | **AA** |
|---|---|---|---|---|---|---|---|---|---|---|
| 9125 | 16392 | Ccrd_009761 | Gelsolin domain-containing protein | Transport; Localisation | C | 0.42 | 0.20 | 0.49 | 0.81 | D/N |
| 29206 | 1935 | ENODL1 | Early nodulin-like protein 1 | Auxin signalling pathway; Salt tolerance | A | 0.68 | 0.48 | 0.69 | 0.13 | Y/D |
| 44863 | 1009 | TIM23-1 | Mitochondrial import inner membrane translocase sub unit TIM23-1-like | Transport; Localisation | C | 0.91 | 0.72 | 0.90 | 0.46 | A/D |
| 10199 | 12546 | ABA3 | Molybdenum cofactor sulfurase | Auxin signalling pathway; Abscisic acid signalling pathway; Salt tolerance; Transport; Localisation | G | 0.18 | 0.38 | 0.03 | 0.89 | T |
| 327 | 50494 | AGD12 | Arf GTPase activating protein | Transport; Localisation; Metal binding (Zinc and Calcium) | C | 0.82 | 0.63 | 0.63 | 0.63 | P |
| 13139 | 2535 | NIP1-1 | Aquaporin NIP1-1-like | Transport; Localisation | T | 0.79 | 0.59 | 0.99 | 0.55 | |
| 273 | 57552 | At2g38810 | Histone H2A | | G | 0.61 | 0.80 | 0.24 | 0.60 | P |
| 68 | 61015 | Ccrd_018070 | NAD(P)-binding domain-containing protein | | A | 0.64 | 0.45 | 0.25 | 0.24 | S/T |
| 2864 | 37153 | MORF8 | multiple organellar RNA editing factor 8, chloroplastic/mitochondrial-like | Metal binding (Zinc) | C | 0.67 | 0.86 | 0.83 | 1.00 | S |

| ***Senecio* scaffold** | **Pos** | **Uniprot Gene** | **Protein** | **Function** | **Ref allele** | ***p* (g)** | ***p* (ag)** | ***p* (D01)** | ***p* (H01)** | **AA** |
|---|---|---|---|---|---|---|---|---|---|---|
| 22890 | 1043 | OBP2 | Zinc finger, Dof-type | Metal binding (Zinc) | T | 0.24 | 0.43 | 0.14 | 0.63 | I/L |
| 34 | 184942 | SAC3B | SAC3 family protein B | | G | 0.61 | 0.79 | 0.86 | 0.99 | L |
| 747 | 61945 | PNISR | Arginine/serine-rich coiled-coil protein 2 isoform X2 | | C | 0.85 | 0.67 | 0.69 | 0.94 | V |
| 13518 | 25785 | LCAT1 | Lecithin-cholesterol acyltransferase-like 1 | | A | 0.62 | 0.80 | 1.00 | 0.91 | |
| 20192 | 2779 | TSNAX | Translin-associated protein X | Metal binding (Magnesium) | T | 0.38 | 0.20 | 0.25 | 0.56 | L |
| 13744 | 9948 | RABH1B | Ras-related protein RABH1b | Transport; Localisation | C | 0.77 | 0.60 | 0.66 | 0.54 | T/M |
| 20374 | 13262 | At3g03090 | D-xylose-proton symporter-like 1 isoform X1 | Transport; Localisation | A | 0.48 | 0.66 | 0.84 | 0.69 | A |
| 35878 | 517 | At1g76210 | putative protein of unknown function DUF241 | | A | 0.74 | 0.57 | 0.46 | 0.83 | E/V |
| 3521 | 819 | SPL9 | SBP-like protein, partial | Metal binding (Zinc) | C | 0.81 | 0.63 | 0.69 | 0.93 | A/T |
| 34994 | 4007 | ENODL1 | Early nodulin-like protein 1 | Auxin signalling pathway; Salt tolerance | T | 0.29 | 0.47 | 0.10 | 0.68 | Q/L |
| 8038 | 9330 | DTX45 | protein detoxification 45, chloroplastic | | A | 0.58 | 0.76 | 0.89 | 0.99 | F/L |
| 13139 | 2534 | NIP1-1 | Aquaporin NIP1-1-like | Transport; Localisation | T | 0.26 | 0.43 | 0.01 | 0.53 | |
| 1889 | 29020 | At1g65750 | Putative ribonuclease H-like domain-containing protein | Metal binding (Magnesium) | G | 0.55 | 0.73 | 0.72 | 0.76 | |
| 9415 | 2717 | MKK5 | Mitogen-activated protein kinase 15-like isoform X2 | | A | 0.65 | 0.48 | 0.27 | 0.66 | D/G |
| 10652 | 9665 | At5g60850 | Zinc finger protein 4-like | Metal binding (Zinc) | A | 0.70 | 0.87 | 0.91 | 0.83 | T/S |
| 326 | 127921 | STX6 | Syntaxin 6, N-terminal | | A | 0.63 | 0.80 | 0.64 | 0.95 | S |
| 52 | 51858 | | Gag-pol polyprotein | | T | 0.72 | 0.56 | 0.63 | 0.84 | |
| 27133 | 815 | N/A | L-ascorbate oxidase homolog | Metal binding (Copper) | A | 0.65 | 0.48 | 0.97 | 0.81 | A |
| 1993 | 22802 | G3BP1 | Ras GTPase-activating protein-binding protein 1-like | | C | 0.30 | 0.46 | 0.10 | 0.18 | I/M |
| 4940 | 43836 | mcfB | Mitochondrial substrate carrier family protein B-like | Transport; Localisation; Metal binding (Calcium) | C | 0.41 | 0.58 | 0.59 | 0.35 | V/L |

| *Senecio* scaffold | Pos | Uniprot Gene | Protein | Function | Ref allele | *p* (g) | *p* (ag) | *p* (D01) | *p* (H01) | AA |
|---|---|---|---|---|---|---|---|---|---|---|
| 12858 | 5804 | At4g27190 | Disease resistance protein At4g27190-like |  | G | 0.51 | 0.35 | 0.26 | 0.55 | R |
| 13761 | 4232 | IPUT1 | Inositol phosphorylceramide glucuronosyltransferase 1 | Metal binding (Manganese) | T | 0.08 | 0.24 | 0.00 | 0.10 | F/I |
| 15472 | 1706 | OsI_15472 | Hypothetical protein OsI_15472 |  | A | 0.78 | 0.94 | 0.96 | 0.91 | T/A |
| 504 | 14643 | VPS52 | Vacuolar protein sorting-associated protein 62 | Transport; Localisation | A | 0.41 | 0.57 | 0.33 | 0.58 | D |
| 2138 | 57963 | SKIP32 | F-box protein 7 [Lactuca sativa] |  | C | 0.76 | 0.92 | 0.77 | 0.94 | P/H |
| 12570 | 7475 | PCMP-E36 | pentatricopeptide repeat-containing protein |  | T | 0.63 | 0.79 | 0.57 | 0.97 | I/V |
| 12570 | 7484 | PCMP-E36 | pentatricopeptide repeat-containing protein |  | C | 0.63 | 0.79 | 0.57 | 0.97 | D/N |
| 12570 | 7488 | PCMP-E36 | pentatricopeptide repeat-containing protein |  | G | 0.63 | 0.79 | 0.57 | 0.97 | N |
| 19859 | 4832 | HannXRQ_Chr15g0477161 | Nucleotide-binding, alpha-beta plait |  | G | 0.76 | 0.60 | 0.65 | 0.76 | V |
| 52 | 51890 |  | Gag-pol polyprotein |  | C | 0.75 | 0.59 | 0.68 | 0.84 |  |
| 105 | 63350 | RF298 | putative E3 ubiquitin-protein ligase RF298 | Metal binding (Zinc) | T | 0.13 | 0.29 | 0.00 | 0.51 | T/A |
| 8841 | 12010 | NEDP1 | NEDD8-specific protease 1 |  | G | 0.96 | 0.81 | 1.00 | 0.82 |  |
| 8162 | 11102 | LOC107840643 | Metal-independent phosphoserine phosphatase isoform X2 |  | G | 0.72 | 0.57 | 0.47 | 0.59 | A/V |
| 11053 | 8590 | At1g31730 | AP-4 complex subunit epsilon | Transport; Localisation | G | 0.52 | 0.68 | 0.39 | 0.97 | S/F |
| 185 | 41645 | LOC107923717 | Polyadenylate-binding protein-interacting protein 11-like isoform X1 |  | A | 0.76 | 0.60 | 0.36 | 0.73 | V |
| 2996 | 10237 | At4g19720 | Glycosyl hydrolase family protein with chitinase insertion domain-containing protein |  | G | 0.72 | 0.87 | 0.98 | 0.51 | T/I |

| ***Senecio* scaffold** | **Pos** | **Uniprot Gene** | **Protein** | **Function** | **Ref allele** | ***p* (g)** | ***p* (ag)** | ***p* (D01)** | ***p* (H01)** | **AA** |
|---|---|---|---|---|---|---|---|---|---|---|
| 19192 | 8093 | ABP19A | Auxin-binding protein ABP19a-like | Auxin signalling pathway; Metal binding (Manganese) | T | 0.47 | 0.32 | 0.13 | 0.11 | V |
| 57053 | 1280 | MYB44 | Transcription factor MYB44-like | Auxin signalling pathway; Abscisic acid signalling pathway; Salt tolerance | G | 0.91 | 0.76 | 1.00 | 0.92 | A/P |
| 19859 | 4901 | HannXRQ_Chr15g0477161 | Nucleotide-binding, alpha-beta plait | | A | 0.76 | 0.61 | 0.65 | 0.76 | A |
| 34118 | 11125 | RDR2 | RNA-dependent RNA polymerase 2 | | G | 0.76 | 0.91 | 0.84 | 1.00 | A |
| 2486 | 34228 | DDB_G0267686 | Probable serine/threonine-protein kinase DDB_G0267686 | | A | 0.89 | 0.74 | 0.76 | 0.97 | P |
| 19818 | 1271 | LOC103498143 | Elicitor-responsive protein 1-like | | C | 0.85 | 0.70 | 0.86 | 0.39 | Y |
| 32038 | 3643 | HannXRQ_Chr17g0536851 | F-box protein/LRR-repeat protein | Transport; Localisation | A | 0.61 | 0.45 | 0.36 | 0.63 | S |
| 504 | 14660 | VPS52 | vacuolar protein sorting-associated protein 62 | Transport; Localisation | A | 0.42 | 0.57 | 0.34 | 0.58 | D |
| 11131 | 708 | ERD2 | ER lumen protein retaining receptor | Transport; Localisation | A | 0.75 | 0.90 | 0.91 | 0.77 | T/S |
| 44907 | 1560 | | no hits in scaffold | | G | 0.71 | 0.86 | 0.77 | 0.65 | |

**Table S3. Gene ontology categories overrepresented in the *S. lautus* gravitropism candidate gene set.**

PANTHER overrepresentation test was used with *Arabidopsis thaliana* as a reference list (27502 mapped IDs) against 32 *Senecio* gravitropism candidate genes. The *S. lautus* gravitropism candidate gene set consists of loci with the most differentiated SNPs (Δp>0.15) between gravitropic and agravitropic plants. The number (N) of *Arabidopsis* and *Senecio* gravitropism candidate genes that matched to the overrepresented gene ontology (GO) categories and the expected number of *Senecio* individuals based on the *Arabidopsis* total in each GO category are shown. All GO categories are overrepresented (+). Transport (GO:0006810) and localisation (GO:0051179) are the parent terms.

| **GO biological process** | **N *Arabidopsis* genes** | **N *Senecio* gravitropism genes** | **Expected** | **Fold Enrichment** | **+/-** | ***P*** |
|---|---|---|---|---|---|---|
| transport (GO:0006810) | 2235 | 11 | 2.60 | + | 4.23 | 2.67E-05 |
| establishment of localisation (GO:0051234) | 2259 | 11 | 2.63 | + | 4.18 | 2.95E-05 |
| localisation (GO:0051179) | 2345 | 11 | 2.73 | + | 4.03 | 4.17E-05 |
| cellular localisation (GO:0051641) | 593 | 8 | 0.69 | + | 11.59 | 3.26E-07 |
| intracellular transport (GO:0046907) | 416 | 7 | 0.48 | + | 14.46 | 4.64E-07 |
| establishment of localisation in cell (GO:0051649) | 436 | 7 | 0.51 | + | 13.8 | 6.33E-07 |
| protein transport (GO:0015031) | 606 | 6 | 0.71 | + | 8.51 | 6.52E-05 |
| establishment of protein localisation (GO:0045184) | 612 | 6 | 0.71 | + | 8.43 | 6.88E-05 |
| protein localisation (GO:0008104) | 651 | 6 | 0.76 | + | 7.92 | 9.63E-05 |
| peptide transport (GO:0015833) | 665 | 6 | 0.77 | + | 7.75 | 1.08E-04 |
| amide transport (GO:0042886) | 674 | 6 | 0.78 | + | 7.65 | 1.16E-04 |
| cellular protein localisation (GO:0034613) | 408 | 5 | 0.47 | + | 10.53 | 1.07E-04 |
| cytosolic transport (GO:0016482) | 33 | 3 | 0.04 | + | 78.13 | 9.92E-06 |

**Table S4. The percentage of failed crosses for ENODL1 and ABA3 genotype combinations.**

The total number (N) of crosses that were performed between F11 recombinant individuals and the number of crosses of those that failed are shown.

| ENODL1 | ABA3 | N total crosses | N failed crosses | % failed crosses |
|---|---|---|---|---|
| A/A | A/A | 16 | 3 | 19 |
| A/C | A/A | 13 | 4 | 31 |
| C/C | A/A | 4 | 1 | 25 |
| A/A | A/G | 3 | 0 | 0 |
| A/C | A/G | 14 | 3 | 21 |
| C/C | A/G | 3 | 0 | 0 |
| A/A | G/G | 1 | 0 | 0 |
| A/C | G/G | 3 | 0 | 0 |
| C/C | G/G | 4 | 0 | 0 |

**Table S5. The percentage of failed crosses for each ENODL1 and ABA3 genotype.**

The total number (N) of crosses that were performed between F11 recombinant individuals and the number of crosses of those that failed are shown.

| Gene | Genotype | N total crosses | N failed crosses | % failed crosses |
|---|---|---|---|---|
| **ENODL1** | A/A | 25 | 4 | 16 |
| **ENODL1** | A/C | 38 | 7 | 18.4 |
| **ENODL1** | C/C | 13 | 1 | 7.7 |
| **ABA3** | A/A | 60 | 9 | 15 |
| **ABA3** | A/G | 31 | 4 | 12.9 |
| **ABA3** | G/G | 8 | 0 | 0 |

**Table S6. Mean gravitropism of ENODL1 and ABA3 genotypes.**

The number of individuals (N) used to calculate mean and standard error (SE) of gravitropism for the genotypes of two gravitropism candidate genes.

| Gene | Genotype | N | Mean gravitropism (°) | SE gravitropism (°) |
|---|---|---|---|---|
| **ENODL1** | A/A | 71 | 41.27 | 3.26 |
| **ENODL1** | A/C | 27 | 35.86 | 5.79 |
| **ENODL1** | C/C | 46 | 28.25 | 4.21 |
| **ABA3** | A/A | 95 | 39.62 | 2.93 |
| **ABA3** | A/G | 18 | 44.63 | 6.16 |
| **ABA3** | G/G | 30 | 22.06 | 4.52 |

**Table S7. Mean gravitropism of ENODL1 and ABA3 genotype combinations.** The number of individuals (N) used to calculate mean and standard error (SE) of gravitropism for the combined genotypes of two gravitropism candidate genes. The genotype combination in bold reduces gravitropism.

| ENODL1 | ABA3 | N | Mean gravitropism (°) | SE gravitropism (°) |
|---|---|---|---|---|
| A/A | A/A | 57 | 41.65 | 3.64 |
| A/C | A/A | 18 | 39.93 | 6.87 |
| C/C | A/A | 19 | 34.39 | 7.34 |
| A/A | G/A | 7 | 48.20 | 10.41 |
| A/C | G/A | 5 | 28.29 | 13.23 |
| C/C | G/A | 6 | 54.09 | 7.32 |
| A/A | G/G | 6 | 35.62 | 11.66 |
| A/C | G/G | 3 | 42.17 | 18.02 |
| **C/C** | **G/G** | **21** | **15.31** | **4.50** |

**Table S8. The effect of ENODL1 and ABA3 genotypes on gravitropism.** The mean gravitropic response angle was compared between ENODL1 and ABA3 genotypes using a Tukey-Kramer test. ENODL1 C/C genotype is significantly different from A/A genotype, while ABA3 G/G genotype is significantly different from both A/A and A/G genotypes. The number of individuals per population used for each comparison is shown in Table S7. Standard error (SE) of the difference in means and 95% confidence intervals are shown. The degrees of freedom for each comparison is 141.

| Gene | Genotype comparison | | Difference | SE Difference | Lower CL | Upper CL | P>ltl |
|---|---|---|---|---|---|---|---|
| **ENDOL1** | A/A | C/C | 13.0210 | 5.3607 | 0.3229 | 25.7192 | 0.0431 |
| **ENDOL1** | A/C | C/C | 7.6136 | 6.8665 | -8.6515 | 23.8786 | 0.5102 |
| **ENDOL1** | A/A | A/C | 5.4075 | 6.4038 | -9.7615 | 20.5765 | 0.6761 |
| **ABA3** | G/A | G/G | 22.5755 | 8.1984 | 3.1538 | 41.9971 | 0.0182 |
| **ABA3** | A/A | G/G | 17.5577 | 5.7589 | 3.9152 | 31.2002 | 0.0077 |
| **ABA3** | G/A | A/A | 5.0177 | 7.0689 | -11.7279 | 21.7634 | 0.7581 |

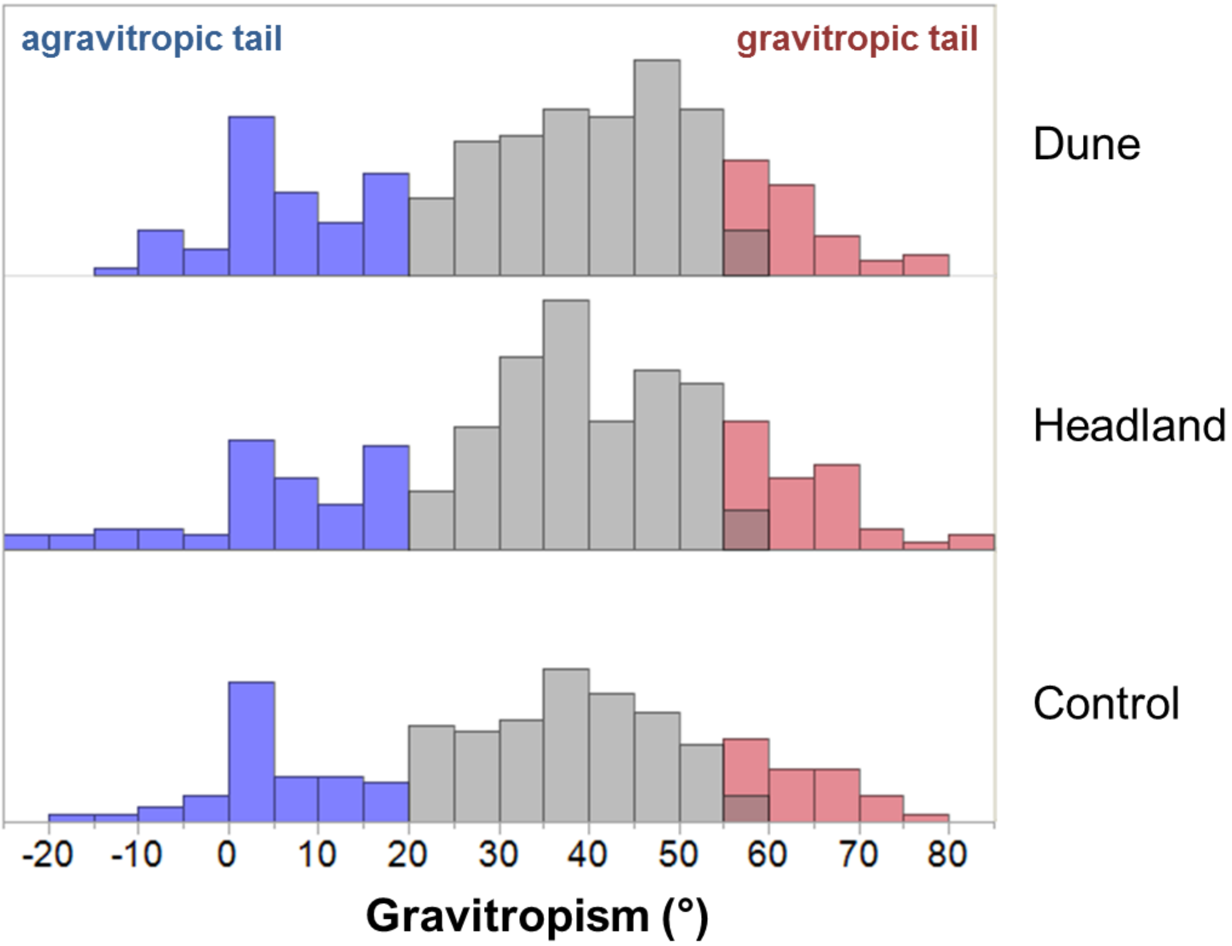


**Figure S1. Gravitropism distribution of the F11 recombinant individuals from the Dune survivors, Headland survivors and Control population.**

The tails of the gravitropism distribution were sequenced. The agravitropic tail (blue) contains individuals with gravitropism values less than 20° and the gravitropic tail (red) contains individuals with gravitropism values more than 56°. The Dune and Headland individuals underwent selection in the respective environments for three generations (F8, F9 and F10). The control population followed the same crossing scheme but was kept in controlled glasshouse conditions and was not exposed to viability selection.

# Chapter 6: General discussion

Natural selection is responsible for much of the diversity we see in nature, yet it is unclear how natural selection drives the evolution of traits and the consequences of this evolution (Schluter and Nagel, 1995; Chan et al., 2010; Baxter et al., 2008). Are the same genetic mechanisms involved in the evolution of adaptive traits across populations, and is the evolution of these traits directly causing intrinsic reproductive isolation (RI)? Here, I have found evidence that natural selection has repeatedly targeted genetic differences in the auxin pathway in the evolution of divergent traits in *S. lautus* populations inhabiting adjacent contrasting dune and headland environments. I have also found that genetic differences in auxin-controlled traits can reduce reproductive success, suggesting that the auxin pathway is not only important for adaptive evolution, but also population divergence and can potentially drive speciation. Overall, my results suggest that populations adapting to their environment can evolve similar traits utilising the genetic variation contained in a genetic pathway, and that adaptive evolution of a single trait might create barriers to reproduction and therefore contribute to the diversity we see in nature.

## Genetic convergence at the pathway level

It remains unclear why some populations but not others undergo genetic convergence to evolve similar traits (Stern, 2013; Arendt and Reznick, 2008). Factors such as gene flow and standing genetic variation dictate whether populations have the same loci for selection to target; and pleiotropy dictates whether evolution of these loci will be hindered by unwanted effects on other traits in certain populations (Wood et al., 2005; Lee and Coop, 2017; Losos, 2011). Although it is clear that the source of genetic variation and genetic architecture play a role in genetic convergence, we are yet to understand how these and other factors interact and lead to the evolution of similar phenotypes. The evolution of coat colour illustrates these points: despite closely related beach mice populations using different genetic routes to evolve light coat colour, different species, including lizards (Rosenblum et al., 2004), birds (Mundy et al., 2004; Theron et al., 2001) and pocket mice (Nachman et al., 2003) have repeatedly used the MC1R gene to adapt to colour changes in the environment. This indicates that although genetic convergence appears to be highly constrained in some populations and even across species, some populations within the same species are not genetically constrained.

In my thesis, I found evidence to suggest that many, but not necessarily all, populations of a plant species have repeatedly utilised a genetic pathway in adaptation to their environment. Specifically, *S. lautus*, have utilised the auxin pathway in the evolution of adaptive traits in contrasting dune and headland environments along the coast of Australia. This research stemmed from predictions based on a population genetic analysis of eight Dune and Headland population pairs (Roda et al., 2013). Roda et al. (2013) found that genetic divergence across pairs was often repeated in different genes connected by the same genetic pathways. One of these was the auxin pathway, which is known to control plant growth and development, and is therefore largely responsible for shaping plant architecture in many species (Gallavotti, 2013; Paque and Weijers, 2016; Vanneste and Friml, 2009). This provided a clear hypothesis: genes within the auxin pathway have contributed to the evolution of divergent traits between Dune and Headland populations. To explore this hypothesis, I asked what traits are common in auxin mutants (detailed in Chapter 1) and early studies revealed that *Arabidopsis* auxin mutants are often agravitropic and therefore unable to respond to gravity stimuli (Estelle, 1994). Thus, I tested for gravitropism differences between Dune and Headland populations (Chapter 3) to provide insight into whether the auxin pathway is repeatedly differentiated between adjacent Dune and Headland populations. I found that gravitropism is divergent in many, but not all population pairs of *S. lautus*, suggesting that the auxin pathway might not always be used to evolve adaptive growth habit in *S. lautus*. These results suggest that convergent evolution of adaptive traits might not be completely genetically constrained in the *Senecio lautus* complex, providing insights into how common convergence is within the same genetic pathway in closely related populations adapting to their environment with the potential for gene flow.

Convergence at the pathway level is also common in the evolution of flower colour in the anthocyanin pathway. Mutations at a single locus can lead to changes in flower colour (Rausher, 2006; Rausher, 2008; Mol et al., 1998), leading to attraction of different pollinators (Bradshaw and Schemske, 2003; Sheehan et al., 2016; Hoballah et al., 2007), which can cause RI between populations (Sheehan et al., 2016). Simple genetic changes appear to underlie both adaptive evolution and RI barriers in the anthocyanin pathway in *Petunia* (Hoballah et al., 2007), *Solanoideae* (Larter et al., 2018) and *Penstemon* (Wessinger and Rausher, 2013), and possibly in the auxin pathway in *Senecio,* detailed in this thesis. Despite the wide range of genetic mechanisms known to lead to flower colour evolution (Streisfeld and Rausher, 2011), sometimes adaptive evolution is predictable and utilises the same genetic mechanisms. For example, Larter et al. (2018) found that repeated losses of expression of late anthocyanin pathway genes led to the loss of the ancestral pigment that produces blue flowers. Although Larter et al. (2018) do not know the specific genetic differences that lead to changes in expression, the genes have been narrowed down to late in the anthocyanin pathway, where pleiotropic effects that might alter other plant tissues are likely to be avoided. Similar to the anthocyanin pathway, the auxin pathway controls many plant processes required for survival (Vanneste and Friml, 2009; Teale et al., 2006); thus similar restrictions might also apply for avoiding unwanted negative pleiotropic effects. In fact, considering root gravitropism does not have the same pattern of differentiation across *S. lautus* (Chapter 3), I suspect that shoot gravitropism evolution might be restricted to shoot specific genes.

Although convergence at the pathway level appears important for adaptive evolution in the *Senecio lautus* species complex, convergence at the allele level does not appear to be as common. I explored allelic convergence for the evolution of gravitropism, among populations with similar standing genetic variation, which provided insights into the genetic constraints that populations might face (Losos, 2011). More specifically, I looked at whether the same alleles, controlling gravitropism in an advanced recombinant population (F11 generation), could also have been utilised in the evolution of gravitropism in the parental Dune and Headland populations. I found that alleles in ABP19A (auxin binding protein ABP19A-like) and MYB44 (transcription factor MYB44-like) might have been utilised in the evolution of gravitropism in F11 individuals, but were not utilised in the parentals. These alleles were differentiated between gravitropic and agravitropic F11 individuals (Chapter 5), but were not differentiated between Lennox Head, Dune and Headland parental populations (D01 and H01). This indicates that the parental populations have recruited different alleles for the same phenotypic outcome. The remaining gravitropism candidate genes with functions related to the auxin pathway found in this study, ENODL1 (early nodulin-like protein 1) and ABA3 (molybdenum cofactor sulfurase), have allelic differences in the parental populations in the same direction as the F11 recombinant populations (Chapter 5), suggesting that allelic convergence could be occurring in these genes. Genetic convergence at the pathway level, but not always the allele/gene level, is supported by previous research from Roda et al. (2013) that found that different genes within the auxin pathway were often differentiated between many natural populations of *S. lautus*. These results, past and present, indicate that genetic convergence is not always present in the evolution of similar traits in populations with similar standing genetic variation.

Adaptive trait evolution is more likely to repeat itself through the same genetic mechanisms if the allele is widespread through the population (Innan and Kim, 2004; Barrett and Schluter, 2008). Factors such as allelic frequency of a beneficial allele dictate the chances the allele will be "visible" to selection (Haldane, 1924) and therefore whether the allele can become fixed in the population (Schluter et al., 2004). Thus genetic convergence from standing genetic variation is more likely than from new mutations (Barrett and Schluter, 2008), where beneficial new mutations must arise by chance and overcome the random effects of drift to spread through the population (Olson-Manning et al., 2012). Standing genetic variation of ENODL1 and ABA3 alleles in *S. lautus* might have been recruited for adaptive evolution in multiple populations. The recessive allele in MYB44, on the other hand, might be at frequencies too low in the population for it to be "visible" to selection. For instance, the parental Dune population is fixed (frequency of D01, $p$=1) and the parental Headland population is almost fixed (frequency of H01, $p$=0.92) for the G allele (Chapter 5). The MYB44 G allele is favoured in the F11 gravitropic plants (frequency of g, $p$=0.91) in comparison to the F11 agravitropic plants (frequency of ag, $p$=0.76). Considering the agravitropic phenotype appears to be controlled by recessive alleles (Chapter 5), the low frequency in the Headland population means that the trait is not likely to be expressed as it is likely to only occur and be "invisible" in heterozygotes (Haldane, 1924; Olson-Manning et al., 2012). Future studies will look at these gravitropism candidate genes in many natural populations of *S. lautus* to expand our understanding of replication of these genes in a phylogenetic context. This will ultimately enable us to understand whether populations adapting to similar environments use the same genetic mechanisms to evolve the same adaptive traits.

### Adaptive evolution of growth habit and gravitropism in *S. lautus*

Understanding the targets of natural selection is a key goal in evolutionary biology, as it provides insights into how diversity is created in nature (Lowry, 2012; Kawecki and Ebert, 2004). The repeated evolution of the same trait in a similar environment indicates natural selection most likely drove its evolution (Stern, 2013), but does not rule out that linked traits were the targets of selection. Here, I used an advanced recombinant population and an introgression line to isolate key traits that define Dune and Headland populations, to observe whether natural selection has repeatedly targeted these traits in these environments. I have found evidence through field experiments that natural selection has targeted a gravitropic phenotype in the dune environment (Chapter 4) and evidence consistent with natural selection for a prostrate growth habit in the headland environment (Chapter 2), thus suggesting that both growth habit and gravitropism are likely adaptive traits in *S. lautus,* as they correlate with survival in these environments.

A hypothesis arising from this thesis is that the prostrate growth habit might aid in avoiding branches breaking, due to the strong wind speeds in the headland environment (Chapter 2). This is supported by examples across the plant kingdom, where there is a strong correlation between prostrate growth habit and windy environments (Beeftink et al., 1985; Auld and Morrison, 1992; Crutsinger et al., 2010; Morrison and Rupp, 1995). In fact, Telewski and Pruyn (1998) replicated the mechanical effects caused by wind through flexing the stem of *Ulmus americana* and found that the more flexing, the shorter the plants grew, illustrating that the mechanical effects of wind causes direct changes in plant growth. Although this appears to be a plastic response in this example, phenotypic plasticity often leads to adaptive evolution through genetic assimilation (Lande, 2009). Additionally, mechanical stress has been found to cause changes in auxin signalling, gravitropism, branching, and stem elongation in other plants (Hamant et al., 2008; Sampathkumar et al., 2014; Nakayama et al., 2012; Braam, 2005; Toyota and Gilroy, 2013), further illustrating its strong effect on plant architecture. Further experiments are required to directly test the effect of wind on *S. lautus* traits, to understand the abiotic factors driving the evolution of adaptive traits in nature.

Another hypothesis arising from this thesis is that strong gravitropic ability is advantageous in the dune environment, as it allows the plants to grow upright away from the hot sand. This hypothesis suggests that gravitropic plants lead to erect plants and are therefore likely to be controlled by the same loci. This is supported by 1) a strong correlation between growth habit and gravitropism across *S. lautus* natural populations (Chapter 3), and 2) strong correlations between growth habit and gravitropism in other plant systems, often caused by single gene mutants (Xing et al., 2015; Li et al., 2007; Wu et al., 2013; Yoshihara and Iino, 2007; Dong et al., 2013). However, results from Chapter 5 suggest that growth habit and gravitropism are not tightly genetically linked, as the F11 Control population did not maintain a correlation between the traits after random mating. The physical distance in the genome between genes contributing to differences in these two traits is unclear, but from an F2 generation created from crossing Dune and Headland individuals from Coffs Harbour, the trait correlation was maintained ($F_{1,757}=85.24$, $p<0.0001$, $r=0.32$; unpublished results), indicating that they are likely to be located in the same region of a chromosome. This result does not rule out that the evolution of growth habit is occurring through differences in the auxin pathway, but suggests that the relationship between gravitropism and growth habit is complex and not completely pleiotropic. The reconstruction of the trait association after selection in the headland environment (Chapter 5), suggests that strong natural selection could be acting on these traits independently, thus indicating that differences in both gravitropism and growth habit are important for survival in dune and headland environments.

In general, it is accepted that adaptive evolution is more likely to occur when pleiotropy is minimised to reduce deleterious effects on unwanted traits (Stern et al., 2008; Stern and Orgogozo, 2009; Streisfeld and Rausher, 2011; Wray et al., 2003; Carroll, 2005). For example, regulatory genes in the anthocyanin pathway are repeatedly selected in flower colour evolution to avoid mutations influencing all flower tissues (Larter et al., 2018). This targeted approach minimises negative pleiotropic effects. In another example, the evolution of the light coat colour of deer mice for camouflage in sand hills involves multiple functionally related traits (e.g. dorsal (hue and brightness), ventral colour, tail stripe and d-v boundary) evolving in combination but controlled by independent SNPs (Linnen et al., 2013). These examples show how minimising pleiotropy can assist with rapid evolution, which might contribute to understanding the lack of pleiotropy found between adaptive growth habit and gravitropism alleles (Streisfeld and Rausher, 2011; Stern et al., 2008; Stern and Orgogozo, 2009).

Results from this thesis showed that growth habit and gravitropism are likely to be under direct or indirect selection in *S. lautus* populations adapting to contrasting adjacent environments and suggest that environmental selection pressures, such as wind, might be driving this adaptive evolution. These results provide insights into the relationship between natural selection and trait evolution and thus the origins of diversity.

### The genes underlying adaptive evolution in *S. lautus*

The genetic architecture that drives adaptive evolution remains unclear, as studies that isolate the genetic targets of natural selection remain few across taxa (Pardo-Diaz et al., 2015; Ehrenreich and Purugganan, 2006). Therefore, we remain ignorant about how evolution proceeds at the genetic level. Similar to the anthocyanin pathway in the evolution of red petals (Wessinger and Rausher, 2013), the auxin pathway appears to be repeatedly recruited in the evolution of gravitropism between adjacent Dune and Headland populations. Whether the genes repeatedly recruited tend to be expressed only in specific tissues, like the anthocyanin pathway (Wessinger and Rausher, 2013), is unclear; but by finding that root gravitropism does not follow the same pattern as shoot gravitropism across *S. lautus* populations (Chapter 3), the evolution of gravitropism through the auxin pathway appears to be restricted (at least partially) to shoot specific genes. These results contribute to our understanding of the genetic architecture that drives adaptive evolution.

The evolution of adaptive shoot gravitropism in *S. lautus* populations appears to be largely controlled by two major effect loci with functions related to the auxin pathway. This is consistent with examples in closely related plant species, where genetic differences in one or two genes appear to be responsible for major morphological differences (Gottlieb, 1984). The major effect loci in *S. lautus* were identified by comparing genetic differences between gravitropic and agravitropic F11 recombinant individuals (Chapter 5). Some of the most differentiated SNPs between these groups fell in genes with known functional importance in other plant species; for instance, ENODL1 and ABA3 have functions in gravitropism, growth habit and salt tolerance, and therefore could explain the adaptive evolution of the major phenotypic differences between Dune and Headland populations. In fact, the two alleles in these genes together explain 65% of the difference in gravitropism, between the gravitropic and agravitropic F11 groups (Chapter 5). Additionally, the allelic frequency change in ENODL1 and ABA3 between the gravitropic and agravitropic groups, matches the expected direction in the parents (Chapter 5), suggesting that natural selection has targeted these particular alleles (or tightly linked) in the evolution of adaptive traits in *S. lautus*. The ENODL1 amino acid change is from a hydrophilic non charged Tyr in the Dune to a negatively charged Asp in the Headland (Chapter 5), which might result in structural and functional changes of the protein and lead to alterations in phenotype. Similarly, Linnen et al. (2013) found that protein coding changes caused by single SNPs were associated with adaptive coat colour changes in sand mice, showing that small changes in the genome can lead to rapid adaptive evolution.

The remaining gravitropism candidate genes in this study appear to have functions related to gravitropism and some even interact to produce other traits differentiated between Dune and Headland populations. The most differentiated gene in this study, Ccrd_009761, is involved in bundling actin filaments and this is important for the movement of proteins and nutrients around the plant cell to enable auxin induced cell elongation (Tominaga et al., 2000), that is vital for bending of the stem in response to gravity stimuli (Baldwin et al., 2013). Therefore, Ccrd_009761 might have a vital role in the evolution of gravitropism in *S. lautus*. Additionally, MYB44 is a transcription factor (uniprot: Q9FDW1) that is activated with the presence of the signalling hormones ABA and auxin. For example, low auxin concentrations stimulate MYB44 to activate the transcription of the auxin-responsive gene IAA19 (Zhao et al., 2014), which functions as a repressor of auxin genes (Liscum and Reed, 2002). In fact, mutations in the IAA19 gene have led to shoot agravitropism but not root agravitropism (Tatematsu et al., 2004), indicating reduced function of MYB44 might lead to shoot specific reductions in gravitropism through alterations to the function of IAA19. ABA also stimulates MYB44 to repress the expression of protein phosphatases 2C and confer resistance to abiotic stresses such as dehydration, temperature, and salinity (Jung et al., 2008; Kirik et al., 1998). For example, the overexpression of MYB44, in an experiment by Persak and Pitzschke (2014), led to increased salt and drought tolerance. I speculate that differences in ABA3 and MYB44 might not only contribute to adaptive evolution of gravitropism, but also other traits that define Dune and Headland populations, such as salt tolerance. Below, I discuss how natural selection might drive the co-inheritance of growth habit and salt tolerance in *S. lautus* through positive pleiotropic effects.

### Is salt tolerance evolving in combination with growth habit in *S. lautus*?

Salt is a key environmental difference that is thought to drive evolution in the *Senecio lautus* complex (Roda et al., 2013; Wilkinson, 2012) and other coastal plants (Rus et al., 2006; Lowry et al., 2009; Lexer et al., 2003; Flowers and Colmer, 2015). In *Mimulus*, salt creates strong fitness differences between inland and coastal plants, where coastal plants have the greater salt tolerance and higher leaf sodium concentrations (Lowry et al., 2009). Through actively accumulating sodium ions, plants can achieve osmotic balance (Rus et al., 2006; Munns et al., 2006; Oh et al., 2010). Previous studies in *S. lautus* have found that Headland plants from Lennox Head have greater survival when exposed to high salt concentrations in controlled conditions compared to their sister Dune pair (unpublished results). The physiological mechanisms behind this are unknown, but in Chapter 5, I found that Dune and Headland parental populations from Lennox Head are differentiated in orthologs of ABA and ENODL, which both play a role in not just gravitropism, but also salt tolerance. For instance, ENODL was found to improve osmotic tolerance in tobacco (Wu et al., 2011) and changes in gene expression in ENODL after salt stress in rice (Ma et al., 2011) and wheat (Cao et al., 2015), further suggests a possible role for ENODL in salt tolerance. ABA3 is a key regulator in the abscisic acid (ABA) pathway, and is upregulated in response to abiotic stressors, including drought and salt (uniprot: Q9C5X8). ABA is likely to be contributing to the stress response by combatting oxidative stress through regulating reactive oxygen species (ROS) production in the mitochondria (Garcia de la Garma et al., 2015; Mittler and Blumwald, 2015).

In a previous study in *S. lautus*, salt candidate genes were found in a controlled selection experiment using an advanced recombinant population (F8 generation) derived from a cross between Lennox Head Dune and Headland populations (Wilkinson, 2012). F8 recombinant individuals were exposed to high salt concentrations and the allelic differences between survivors and a control population were identified. FER/AT3G51550 (Receptor-like protein kinase FERONIA) contained the SNP with the greatest allelic difference between these groups and has functions in salt tolerance (Feng et al., 2018), cell growth (Breuer et al., 2009) and pollen tube reception (Escobar-Restrepo et al., 2007). FER is vital for cell elongation during vegetative growth and is a positive regulator of auxin-promoted growth that represses ABA signalling via the activation of ABI2 phosphatase (uniprot: Q9SCZ4). Both auxin and ABA genes were found as gravitropism candidate genes in this thesis and therefore this result further promotes a functional connection between gravitropism and salt tolerance. Interestingly, unlike ENODL and ABA, where the recombinant population allele frequency after selection moves towards the allele frequency of the parent with the similar phenotype, FER does the opposite in the Lennox Head natural populations (Wilkinson, 2012). In fact, the same occurs for all four adjacent pairs from the eastern clade, with Lennox Head having the greatest difference in allele frequency ($\Delta p$=0.579) (Wilkinson, 2012). This suggests that salt tolerant FER alleles might be beneficial in controlled conditions with just variation in salt; but in nature, this might lead to negative pleiotropic effects of other adaptive traits, such as gravitropism.

Transport and localisation appear to be important for both gravitropism and salt tolerance. In Chapter 5, I found overrepresentation of transport and localisation in gravitropism candidate genes. Similar transport gene ontology categories were enriched in De Biasse et al. (2018) in salinity stress candidate genes, and De Biasse et al. (2018) suggest that intracellular transport of osmolytes allows osmotic regulation, e.g. accumulation of sodium in the vacuole to achieve osmotic balance (Rus et al., 2006; Munns et al., 2006; Oh et al., 2010). De Biasse et al. (2018) also found that osmotic stress can lead to elevated cellular respiration, which requires more transport of proteins across the mitochondria, using transport proteins such as TIM23-1 (Alberts et al., 2002), a gravitropism candidate gene found in Chapter 5. This suggests an overlap between the transport genes required for gravitropism and those required for salt tolerance. Additionally, direct evidence in tobacco cells showed that salt stress increases ABA (Garcia de la Garma et al., 2015) and ABA reduces the expression levels of the PIN-FORMED (PIN) auxin efflux carriers (Promchuea et al., 2017), that are vital for transporting auxin to create the hormone gradient that induces cell elongation and thus gravitropic bending (Benková et al., 2003; Ottenschläger et al., 2003; Yamamoto and Yamamoto, 1998; Delbarre et al., 1998; Enders and Strader, 2015). This indicates that exposure of *S. lautus* populations to high salt in the headland environment, might directly lead to the reduced gravitropism that is found in Headland individuals, through salt inducing reductions in the expression of the auxin efflux carrier. Altogether, these examples suggest that there might be a functional link between the transport proteins required in gravitropism and salt tolerance in *S. lautus*.

Gravitropism and salt tolerance genes might be co-inherited in *S. lautus* populations adapting to their environment. The connection between salt and gravitropism is further supported by salt stressed *Arabidopsis* plants which had a reduction in the gravitropic response in the roots and downregulation of PIN2 abundance and auxin distribution (Sun et al., 2008). Note that Sun et al. (2008) only looked at root gravitropism and the connection between root and shoot gravitropism in response to salt stress remains unclear (Li and Zhang, 2008). Thus, the effect of salt stress on shoot gravitropism needs to be directly tested. Overall, the research in this thesis did not seek to investigate the evolution of salt tolerance in *S. lautus*, but the evidence from the gravitropism candidate genes and previous studies continue to point to a connection between the two. I suggest that the co-inheritance of gravitropism and salt tolerance be further investigated to understand the genetic architecture of functionally related traits evolving in combination in response to the environment.

### The consequences of adaptive evolution in *S. lautus*

Whether natural selection can directly drive the formation of intrinsic RI is still a topic of debate. Intrinsic RI barriers are considered to occur late in the speciation process, after extrinsic RI barriers have been formed and genetic drift has led to the accumulation of genetic differences (Via, 2009; Baack et al., 2015). With few examples that directly link natural selection and intrinsic RI (see exceptions: Bomblies and Weigel, 2007; Ono et al., 2017; Wright et al., 2013), the role of natural selection in creating intrinsic RI barriers at the start of the speciation process is unclear (Baack et al., 2015). Here, I provide evidence to suggest that natural selection is a driving force behind the intrinsic RI found in *S. lautus*. I found that 1) gravitropism is under selection (Chapter 4); 2) gravitropism is linked with intrinsic RI, where gravitropic individuals crossed with agravitropic individuals have reduced fertilisation success (Chapter 4); and 3) genetic differences in gravitropism candidate genes can predict fertilisation success. This indicates that gravitropism, controlled by the auxin pathway, might be the link in *S. lautus* between adaptation and speciation, whereby natural selection for gravitropism loci drives the evolution of intrinsic RI, as a by-product. Although I do not provide direct evidence for the evolution of intrinsic RI through natural selection, I provide a strong correlation and tools for future studies to test for causation. These results suggest that intrinsic RI can be driven by selection at the start of the speciation process and without the need for extrinsic RI to separate the populations.

To further support the hypothesis that adaptive evolution in *S. lautus* is creating intrinsic RI and driving divergence, I found evidence that growth habit differences are also causing intrinsic RI in *S. lautus*. I found that attempts at introgressing tall alleles onto the Headland genomic background were not successful as doing so did not make the plants taller (Chapter 2), indicating that the alleles (or closely linked alleles) underlying the erect growth habit found in Dune populations cause pollen-pistil interactions or negative epistatic interactions with alleles in the Headland genome and this prevents successful reproduction. Payseur et al. (2004) has also found that certain areas of the genome have reduced introgression between two species of house mice, *Mus domesticus* and *M. musculus*, and suggest that this section of the genome might be important for intrinsic RI between house mice species. Similarly, in this thesis, alleles pleiotropic or genetically linked with alleles contributing to adaptive evolution of both gravitropism and growth habit, might be contributing to intrinsic RI in *S. lautus*.

Evolution of intrinsic RI through gravitropism and growth habit loci (or linked loci) is supported by a study in *S. lautus* by Walter et al. (2016) that compared fertilisation success in controlled conditions of hybrid generations, derived from Dune and Headland Lennox Head populations. Walter et al. (2016) found a fertilisation success of 42% in F2 hybrids compared to a fertilisation success of over 90% in the F1 and F3 hybrid generations, suggesting that F2 hybrids are experiencing intrinsic RI. Heterosis is most likely masking intrinsic RI in the F1 generation (Lowry et al., 2008; Rundle and Whitlock, 2001) and the loss of intrinsic RI in the F3 generation (Walter et al., 2016), suggests that there are only few negative epistatic interactions and these are removed in one generation. I suggest that genetic differences in growth habit and gravitropism alleles (or genetically linked) between Dune and Headland populations might be causing these negative epistatic interactions (or pollen-pistil interactions). Overall, it appears as though intrinsic RI barriers are formed before speciation is complete within this system and could possibly contribute to the speciation process in the future.

The mechanism by which adaptive evolution and intrinsic RI is linked in *S. lautus* could be through pleiotropy or genetic linkage (Coyne and Orr, 2004; Rundle and Nosil, 2005). The closer the speciation gene is to the target of selection in the genome, the more likely RI will evolve as a by-product. Thus, loci that pleiotropically control adaptation and speciation genes, are effective in driving divergence between populations. Examples include: divergence in flower colour (Sheehan et al., 2016), changes in flowering time (Ferris et al., 2017; Fishman et al., 2013; Lowry et al., 2008), differences in the plant pathogen response (Bomblies and Weigel, 2007) and pollen-pistil interactions (Müller, 2014; Hou et al., 2016; Escobar-Restrepo et al., 2007; Chen and Zhao, 2008; Wu et al., 2008; Zerzour et al., 2009). Pollen tube reception is a type of pollen-pistil interaction that is driven by the female’s interaction with the pollen tube when it enters the female gametophyte and is controlled by genes, such as ENODL (Hou et al., 2016) (*Senecio* gravitropism candidate) and FER (Escobar-Restrepo et al., 2007) (*Senecio* salt candidate). Therefore, these genes might have major roles in both adaptive evolution and intrinsic RI in *Senecio* and thus provide an exciting future avenue of research in this system, which I discuss in more detail below.

### Pollen tube reception, a mechanism for intrinsic RI in *S. lautus*?

Could the intrinsic RI found in *S. lautus* be explained by differences in pollen tube reception between Dune and Headland populations? I found that a gravitropism candidate gene in *S. lautus*, ENODL, has orthologs with functions related to pollen tube reception. For example, mutants of *enodl* failed to arrest growth and rupture the entering pollen tube (Hou et al., 2016), indicating a female specific role of ENODL in communicating with the male pollen tube and enabling reproduction. The evolution of gravitropism through the maternal genotype and not the paternal genotype (Chapter 4) supports selection occurring on the female specific role of ENODL. Pollen tube reception has the potential to explain the reproductive failures found in *S. lautus* (explained above), including 1) crosses between gravitropic and agravitropic individuals (Chapter 4); 2) introgressing tall alleles onto headland genomic background (Chapter 2); and 3) the F2 hybrid generation between Lennox Head, Dune and Headland populations (Walter et al., 2016). Furthermore, allelic differences in ENODL, between gravitropic and agravitropic individuals, alter hydrophobicity and charge of the amino acid. These structural changes might be altering the function of the ENODL protein in not just gravitropism, but also pollen tube reception. Thus, I propose the hypothesis that the female specific ENODL is driving the evolution of intrinsic RI through control over whether the pollen tube bursts to induce fertilisation. Future studies should investigate whether ENODL is pleiotropically controlling gravitropism and pollen tube reception and therefore contributing to both adaptation and divergence in *S. lautus*.

### Future directions

The goal of my research is to understand the genetic basis of adaptive evolution and divergence. This knowledge is vital for our understanding of the role of natural selection in driving the evolution of new species. The research discussed in this thesis has provided evidence that a common genetic pathway has been utilised in many independent populations of *S. lautus* adapting to their environment. Additionally, this adaptive evolution appears to be driving the formation of intrinsic RI barriers and therefore contributing to population divergence, and possibly speciation. The next step in this research is to sequence the candidate genes found in this thesis in many natural populations of *S. lautus* to understand the repeatability of evolution at the allelic and genic level. The development of a new *S. lautus* phylogeny (Maddie E. James personal communication) means that analyses can be done in a phylogenetic context to truly assess whether independent populations evolving the same traits are likely to be utilising the same genetic mechanisms with similar standing genetic variation. Future studies should also explore whether pollen tube reception is the mechanism by which intrinsic RI is evolving in *S. lautus*. Semi-in-vivo-fertilisation assays should be performed, that track the release of the sperm from the pollen tube (Hou et al., 2016; Liu et al., 2013). This would clarify whether a gravitropism candidate gene in this research, ENODL, might be pleiotropically controlling adaptive evolution and divergence in *S. lautus.*

To understand the environmental variables driving the adaptive evolution of growth habit and gravitropism found in this thesis, future research should strive to directly test the phenotypic effect of factors such as wind and salt. Firstly, selection pressures due to wind could be tested in a controlled temperature room with a recombinant population exposed to different wind speeds over multiple generations. I hypothesise that exposure to high wind speeds would lead to the evolution of shorter plants. Secondly, the co-inheritance of gravitropism and salt tolerance could be tested by investigating salt tolerance differences between gravitropic and agravitropic plants in the F11 recombinant population. If gravitropism and salt tolerance are pleiotropically controlled in *S. lautus*, I would expect that agravitropic plants favoured in the salty headland environment, would be more salt tolerant than the gravitropic plants.

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