# The Genetic Basis of Plant Functional Traits and the Evolution of Plant-Environment Interactions

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The genetic basis of plant functional traits and the evolution of plant-environment interactions

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Abstract
Leaves are the most conspicuous organs of plants and their form and function are key determinants of plant ecology. Moreover, energy captured by leaves through photosynthetic carbon reduction forms the base of nearly every terrestrial ecosystem. As such, the morphology and physiology of leaves have been a central focus of research on plant ecophysiology, development, and evolution. We review recent research on the genetic basis of leaf structure and nutrient profile, as well as stomatal patterning as exemplar traits for understanding the evolution of plant functional traits. We discuss available and emerging methods for determining the genetic basis of plant traits and then present a synthetic assessment of the molecular basis of each trait and the extent to which patterns of natural diversity are relevant to eco-evolutionary analysis. Overall, we find that research on the three traits has emerged from different sub-disciplines in biology. We have a deep understanding of the developmental genetics of leaf size and stomatal patterning and, to a lesser degree, leaf shape, though research on these has been limited to a small number of plant species. By contrast, there is a deep literature describing natural genetic diversity of leaf nitrogen content due, in part, to the ease of measuring this trait in large genetic mapping populations. The molecular control of leaf P concentration, on the other hand, has been severely understudied. For all three traits, there are few examples of studies that have empirically linked molecular genetic variation in specific genes with phenotypic diversity observed in natural populations of plants. We conclude by discussing present challenges with synthesizing different traditions in genetics, physiology, development and evolution and prospects for progress in the coming years.

Introduction
Evolution by natural selection requires that individuals in a population express heritable differences in a trait that affects reproductive success. This central principle of organic evolution places an understanding of the genetic basis of adaptive traits as a central goal for modern biology (Orr 2005). More broadly, determining the causal link between genetic variants -- specific differences among individuals in their genetic make-up -- and phenotypic variation -- comprising any trait from the level of gene expression to whole organisms -- is a common goal of many fields of biology (Rockman 2008). Medical genetics aims to identify variants in the human population that affect health outcomes, including both variants that directly cause heritable disease or those that determine the efficacy of medical intervention. Agricultural genetics is concerned with identifying the genetic basis of yield-enhancing traits that might be used in marker-assisted selection breeding strategies or, increasingly, through biotechnology. Evolutionary biologists both contribute to and benefit from conceptual, empirical, and analytical advancements in these related fields to understand the so-called genotype to phenotype map.
“Adaptation” can refer either to the traits that are adaptive or the process of evolving traits that are adaptive. In either usage, herein we restrict adaptation to refer to heritable traits that increase the relative fitness of an individual relative to other individuals in a population. Using this distinction, studying the genetics of adaptation often entails identifying the specific genes or genetic features responsible for the expression of an adaptive trait, and variants which describe phenotypic differences in a population. It is generally unknown what fraction of genes involved in the expression of an adaptive trait were specifically targeted by natural selection during the evolution of that trait. It remains a considerable challenge to formally demonstrate that gene A was shaped by natural selection in the evolution of trait B, though substantial progress has been made to this end over the past 20 years as tools from genomics and molecular genetics have become accessible in an increasing number of plant species. Presently, the “gold standard” for causally linking genetic and phenotypic variation is the direct integration of genetic material from a genotype possessing a trait or trait value with one lacking it using either genetic crosses and/or stable genetic transformation (e.g. Prasad et al. 2012, Des Marais et al. 2014). The gold-standard for demonstrating the adaptive value of a trait is direct measurement of fitness measured in the natural environment, ideally over multiple seasons (e.g. Anderson et al. 2014), although phylogenetic tests for adaptation are also useful for many evolutionary questions (Ackerly 2004). An additional approach for detecting the role of natural selection in shaping a trait is to compare phenotypic divergence among populations as compared to neutral genetic divergence among populations, the so-called QST/FST approach (Leinonen et al. 2013).

Our reliance on a formal definition of an adaptation is motivated by the sustained interest in plant functional traits as key determinants of organismal ecology and by the relevance of functional traits for applied plant science (Ackerly et al. 2000). Writing nearly 20 years ago, Ackerly and Monson (2003) highlighted an empirical and conceptual gap between plant ecology and physiology on the one hand and plant evolutionary biology on the other. Ackerly and Monson specifically noted the paucity of studies documenting the “fitness consequences, heritability, genetic architecture, and phylogenetic distribution” of physiological traits in plants. Understanding the fitness and heritability of functional traits is greatly facilitated by first studying the genetic architecture of the trait in question: which and how many genetic loci drive natural variation in a trait? Are traits genetically correlated with one another, are these correlations positive or negative, and do the correlations arise due to genetic linkage or pleiotropic effects of individual loci? By studying the genetic basis of traits directly related to plant-environment interactions, including physiological traits, a researcher might isolate the effects of individual functional traits and natural variation in those traits so as to determine their ecological and evolutionary significance. Genetic analysis can also determine whether evolving combinations of traits and trait values which might appear ecologically beneficial are, in fact, difficult to realize due to genetic linkage or pleiotropy.
In the current review, we use leaves as a means to explore our understanding of the genetic basis of plant functional traits and discuss new and emerging tools related to these efforts. Throughout, we highlight three principle themes that have facilitated the study of the genetic basis of plant functional trait evolution over the past 20 years. The first is the continual expansion of detailed molecular studies in a few genetic model systems which provides a fundamental understanding of how plant cells and tissues develop and how they function under variable environmental conditions. The second major development is the expansion of genomic and other -omic technologies and their application to an ever-expanding number of plant species of ecological and evolutionary interest. While considerable limitations to -omic approaches persist for many plant species, we anticipate that such barriers to functional inference will continue to erode in the coming years. The third major theme is the growing synergy and cross-talk between the disciplines of molecular genetics, systems biology, quantitative genetics, evolutionary genetics, and ecological genetics. In particular, the expanded study of natural variants by molecular geneticists – including association mapping analyses based on large panels of natural variants – creates a natural first step to uncover the role played by evolutionary and ecological processes in shaping plant functional traits.

**Tools and methods for reconstructing the genetic basis of adaptive change**

An essential first step in reconstructing the genotype to phenotype map is perhaps obvious but often overlooked: determining whether observed phenotypic variation in a trait is heritable or, conversely, what proportion of phenotypic variation is expressly due to the effects of environmental variation. Heritability measures the proportion of total phenotypic variance observed in a population (V_P) that can be attributed to genetic effects, or V_G, and ranges from zero -- when there is no variance component attributable to genetics -- and one -- when there the effect of genotype on population variance is complete. Formally speaking, a trait is heritable if heritability is statistically greater than zero. The importance of heritability to evolutionary studies is readily appreciated by considering the classic breeder’s equation which states that the change in trait value after one bout of selection – one generation – is the product of heritability and the strength of selection imposed. If heritability is zero then selection has no power to affect trait values; if heritability is one then selection may be very efficient. V_G may itself be partitioned into additive effects, the dominance of alleles, epistatic effects, and epigenetic effects, among others, but we will not further consider those effects here. A second component of phenotypic variance is the effect of the environment, V_E, or phenotypic plasticity. Phenotypic plasticity is commonly observed in studies of plant-environment interaction (Bradshaw 1965) and can often be a confounding effect in ecological studies. Formally speaking, phenotypic plasticity is the capacity of a single genotype to manifest a range of trait values and can, itself, be genetically determined. That is, the ability to respond plastically to the
environment is determined by the genetic make-up of a plant and this ability varies among individuals. This variation in plasticity among individuals in a population is the third critical component of total phenotypic variation, genotype by environment interaction (Des Marais et al. 2013); the proportion of phenotypic variance attributable to GxE is denoted $V_{GxE}$. Partitioning these variance components can be readily accomplished using classical quantitative genetic approaches (Cheverud 2006). Note that $V_G$, or any other component of $V_P$, can be observed in any kind of trait regardless of the role of past or ongoing adaptation.

Our extended discussion of the components of population-level phenotypic variation highlight the complicated role played by the environment in ecological genetic studies. Within a generation, the environment may shape the distribution of phenotypes in a population if there is environmental heterogeneity and if individuals in a population express phenotypic plasticity in some traits. Note that this type of variation could be observed even among genotypically identical individuals if environmental gradients are particularly steep, or if individuals germinate at different times of year when conditions differ. If GxE is prevalent in populations, within-generation phenotypic variation will also be driven by the effects of environmental variation interacting with genetic variation. Both of these properties of individuals are relevant for ecological studies but the effects of phenotypic plasticity are often underappreciated in ecological studies (Miner et al. 2005). As we turn to studying plant functional traits on evolutionary time scales, the environment will shape the frequencies of genetic variants affecting these traits between generations. Here, the phenotypic variation observed in populations will change through time owing to natural selection acting on heritable trait differences, $V_G$, on heritable differences in response to the environment, $V_{GxE}$, and due to the effects of neutral genetic drift on either. With the exception of the still poorly-understood trait effects manifested through transgenerational epigenetics (Richards et al. 2010), phenotypic diversity manifested strictly as $V_E$ will not participate in evolutionary change because, by definition, this phenotypic component is not heritable.

**Molecular tools for studying the genotype-phenotype map**

With this quantitative genetic perspective on phenotypic variation as a background, what methods do we have available for identifying the genetic basis of plant functional traits? Two general strategies have been exploited to date. The first is to study natural genetic variation in genes previously identified and characterized in model systems. This body of work builds on decades of forward- and reverse-genetic studies of species such as *Arabidopsis thaliana* (Provart et al. 2015), maize (Strable and Scanlon 2009), poplar (Douglas 2017), *Solanum* (Kimura and Sinha 2008) and *Nicotiana* (Lewis 2011), among several other species. An advantage of these approaches is that considerable effort is usually made to understand the mechanisms through which a specific gene or pathway directly impacts cell function, and then
impacts higher-order tissue and whole-plant function. Empirical approaches include measuring the phenotypic effects of partial or complete loss-of-function of a gene, the effects of the over-expression of a gene, where, when, and under what conditions a gene is transcriptionally active and how the resulting protein’s activity may be regulated, and interactions between the target gene or protein and other genes or proteins. Through forward genetic screens – where researchers measure a trait of interest in large populations of synthetic mutants and then identify the mutants causing trait diversity – a study might reveal a considerable number of genes that play a role in the expression of some phenotype (at least those for which mutations are not lethal).

New tools from systems biology also provide a means to link genes with phenotypic traits in plants. One approach identifies correlations among transcriptomic, metabolomic, or phenomic data collected across a time series comprising, for example, a developmental transition or the application of an environmental challenge (e.g. Greenham et al. 2017). This approach is facilitated by recent developments in the inference of gene co-expression networks (Langfelder and Horvath 2008, Stone and Ayroles 2009), comparison of co-expression networks to identify topological differences between two classes of samples (e.g. control or treatment, proximal or distal leaf blade, prior to or following flowering; Langfelder et al. 2011, Ritchie et al. 2016), and correlations between groups of co-expressed genes and other traits (Langfelder and Horvath 2008). These and related systems biology approaches are particularly valuable for understanding what cellular processes are involved in metabolic, developmental, or physiological traits of interest and in developing lists of candidate genes for further study though considerable additional work is required to causally associate candidate genes with phenotypes. Such approaches are also very resource intensive and fully realizing the interpretation of systems biology data requires a well-annotated reference genome sequence.

These highly reductionist studies are of some relevance for the study of the evolution of plant functional traits. First, the functional information gleaned from such studies may provide context when orthologous genes are discovered to be associated with traits in other species (see below). Second, researchers working in non-model plants may use a “candidate gene” approach, specifically querying diverse species or populations for the role played by orthologous genes known to play a role in determining the phenotype of interest in model species.

Studies of gene function using natural variants
Molecular genetic approaches provide a “parts-list” of genes and genetics mechanisms that control the expression of traits. Directly applying such lists to evolutionary studies is challenging because, in the case of forward- and reverse-genetic methods, the studies typically rely on artificially induced genetic modifications which may not represent the types of mutations that segregate in natural populations. In the

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In the case of systems biology, well-annotated reference genomes are generally required to make meaningful functional inference. Accordingly, a suite of linkage mapping tools is typically preferred by evolutionary biologists for reconstructing the genotype-phenotype map. Here, we briefly outline these approaches and note exciting new developments since the earlier treatment of this subject by Remmington and Purugganan (2003).

The fundamental goal in linkage mapping is to identify a statistical association between a genetic marker and a trait of interest by exploiting the genetic linkage between a marker and the genetic variant which is actually responsible for causing trait differences among two or more focal genotypes. The value of this approach is that, given sufficient statistical power in the sampling design, genotype-phenotype associations can be made for traits with varied genetic architectures spanning from simple one locus-two allele models to the effects of multiple loci across the genome. Key determinants of power for such tests include the magnitude of the trait difference between genotypes, the relative contributions of individual loci to phenotypic traits, the size of the mapping population, the extent of linkage disequilibrium in the mapping population, and the heritability of the trait. Limitations of linkage mapping arise largely from these determinants of statistical power: inability to detect loci which affect traits but in very small or environmentally-dependent ways (Manolio et al. 2009), the confounding effects of demography on traits that are spatially structured across a landscape (Barton et al. 2019), or the effects of loci with multiple independent variants (Monroe et al. in press).

Linkage mapping experiments may provide insight into the number of genetic loci controlling natural variation in a trait and their relative effect sizes, the direction of additive effects of a locus (e.g. the allele A from parent 1 increases a trait value as compared to the effect of the allele B from parent 2), the possible interactions between loci in driving trait variation (epistatic effects), and the effect of individual loci on multiple traits (pleiotropy). These data might be used to infer whether natural selection shaped the evolution of that trait since the divergence of the studied genotypes (Orr 1998) or whether selection on one trait could lead to indirect selection on a second, genetically correlated trait (Lande and Arnold 1983). While loci identified in these screens merely represent regions of the genome that are associated with some trait difference, additional genetic analysis can uncover the actual genetic variant driving phenotypic diversity in the trait of interest. Evolutionary questions for which determining the identity of causal variants is needed have been recently debated and enumerated (Rockman 2012, Lee et al. 2014, Rausher and Delph 2015).

Quantitative trait locus (QTL) mapping is one straightforward method to identify genetic loci associated with trait differences in a population. In its simplest instantiation, two parents that express heritable difference in a trait are first crossed to create a F1 hybrid. The F1 is then self-fertilized to create an F2 population or, in the case of self-incompatible plants, two F1s from separate biparental crosses may
be crossed to each other to generate F2s. (Many other strategies for creating a mapping population exist; for a general treatment see Falconer and Mackay, 1996). Recombination in the F1 germline results in a diversity of allelic combinations in the F2 population and it is these recombinant chromosomes that allow the researcher to identify statistical associations between genetic markers and trait values. A large panel of anonymous markers is then scored for each member of the F2 population. Historically, markers included amplified fragment length polymorphisms, microsatellite loci, or restriction fragment length polymorphisms. Today, next generation sequencing technologies are commonly used to score many thousands of single nucleotide polymorphism (SNPs) using techniques such as Restriction-site Associated DNA sequencing (RAD tags; Andrews et al. 2016)) or low coverage whole genome re-sequencing (so-called genotyping by sequencing, or GBS, approaches; Elshire et al. 2011, Huang et al. 2011). Both of these genotyping approaches are now routinely employed for diverse plant species, often without a reference genome sequence available. With a linkage map derived from these markers in hand, a researcher measures the trait of interest in each member of the mapping population and uses any of a number of statistical packages to identify associations between trait values and individual or groups of genetic markers (Van Eeuwijk et al. 2010). A key point, here, is that the genetic markers are merely genetically linked to (i.e. in linkage disequilibrium with) the genetic variant(s) which actually drive the observed phenotypic variation.

One exciting extension of QTL mapping is to identify genetic variants associated with heritable variation in transcript abundance (Kliebenstein 2009). This expression QTL (eQTL) mapping treats the abundance of each expressed transcript as a quantitative trait, and then identifies genetic markers associated with variation in transcript abundance between the parent genotypes. Making meaningful functional inference from eQTL analyses typically requires a well-annotated reference genome sequence for one of the parent genotypes; in principle, eQTL studies can be undertaken in any plant for which a linkage mapping population and reference transcriptome are available. Researchers may combine eQTL studies with QTL analysis of plant functional traits and use co-segregation between the two to identify a shortlist of possible genes involved in driving trait variation. Importantly, careful statistical consideration of environmental variation now allows for such studies to be carried out using field-grown samples (Lovell et al. 2018).

Conventional QTL mapping is typically undertaken with two (in the case of self-compatible plants) or four (in the case of self-incompatible plants) parents and therefore reflects a very limited sample of the diversity present in a natural population. Moreover, because only a few genetic crosses are made in the process of generating a mapping population, there is little opportunity for recombination to break up genetic linkages present in the parental genomes; blocks of chromosomes which show a statistical association with trait values are often quite large (i.e. there is low resolution of the genotype-
phenotype map). Association mapping using large populations of wild-collected accessions is an increasingly feasible means of identifying genetic loci associated with ecologically interesting traits (Charpentier et al. 2014). Here, hundreds to thousands of individuals are genotyped via GBS or RAD and then measured for the trait(s) of interest grown in a common garden or greenhouse setting. Considerable recent effort has improved the statistical procedures needed for these types of inference, particularly with respect to reducing the confounding effects of demography and natural selection on genotype-phenotype associations, though some challenges remain (Barton et al. 2019). Because the evolutionary history of recombination in natural populations tends to reduce linkage disequilibrium, the marker loci are typically more closely (on the chromosome) associated with the causal variant driving phenotypic variation in the trait of interest than in biparental QTL studies. Studying variation in natural populations also affords the researcher the prospect of simultaneously identifying statistical a genetic variant associated with a studied trait, the frequency of the variant in the population, and the role played by natural selection in maintaining the variant in the population (Josephs et al. 2017).

Additional methods for determining trait-marker associations are available. One commonly used approach in species for which genetic crosses are readily made is bulk segregant analysis. Here, a very large F2 population is generated and then measured for the trait of interest (Siepel et al. 2011). Pools of DNA from individuals representing the tails (high- and low-trait values) of the phenotypic distribution are then “bulked” and sequenced on a next-generation sequencing platform. Statistical associations are identified between each trait state – e.g. large or small leaves – and SNPs identified as characteristic of each bulk pool. This is a quick and relatively low-cost means of identifying marker-trait associations but is typically only possible for one trait at a time (unless traits show very high correlation).

**Genetics and the evolution of plant functional traits**

Decades of research on the model plant *Arabidopsis thaliana* (hereafter “Arabidopsis”) have provided us with a parts list of genes involved in nearly every aspect of its development, physiology, and interaction with the abiotic and biotic environment (Provant et al. 2015). To this end, we have a general idea of what types of genes and molecular pathways are involved in the expression of most herbaceous plant functional traits; it is perhaps trivial to point out that evolution likely shaped many if not most of these genes at some point in the history of life. As scientists interested in the mechanisms of evolution and how evolution shapes and is shaped by ecological interactions of plants, we are principally interested in the genetic control of phenotypic variation in plant functional traits within and between closely related species. Here, we present three broad features of leaves as exemplar traits for understanding the genetic basis of the evolution of plant functional traits: leaf structure, leaf nutrients, and stomata. Throughout, we highlight
how integration between molecular genetics, quantitative genetics, genomics, and evolutionary genetics provide novel insight into the evolution of plant functional traits in diverse species.

**Leaf structure**

Leaves are the primary structures responsible for photosynthetic carbon assimilation in plants (Lambers et al. 2008). This process, in which plants efficiently convert sunlight into chemical energy, is strongly influenced by structural features including the ratio of mass to area and the shape of the leaf, both of which show an exceptional variability among plants (Efroni et al. 2010). Canopy structure and the angle of the leaf relative to the stem are also critical determinants of photosynthesis, but here we will focus on leaves themselves.

*Adaptive importance of natural variation in leaf structure*

Within-species natural diversity in leaf shape, encompassing aspects of dissection, lobing, and ratio of leaf length to width, has been documented in many species of plants (e.g. Wyatt and Antonovics 1981, Andersson 1991, Atwell et al. 2010, Chitwood et al. 2012, Campitelli and Stinchcombe 2013). One remarkable morphometric analysis in wine grape identified reasonably high heritabilities (0.2-0.46) for several aspects of leaf shape. High heritabilities (0.4-0.6) have also been identified for traits such as leaf length, width, and perimeter in *Populus deltoides* (Xia et al. 2018). These high heritabilities suggest that ample genetic diversity of shape exists to allow selection to act and, while variation in leaf shape is widely interpreted as being adaptive (Brown and Lawton 1991, Schuepp 1993), we are aware of only one species for which a role for contemporary natural selection has been formally implicated in driving natural diversity of leaf shape (Bright and Rausher 2008).

A second aspect of leaf structure, leaf mass per area (LMA) and its inverse specific leaf area (SLA), has been studied extensively as it relates to plant ecological strategies because it represents a key axis of the so-called leaf economic spectrum (LES; Wright et al. 2004) and because LMA/SLA feature prominently in models of the determinants of relative growth rate (Rees et al. 2010). The LES provides a unifying framework that explains the covariation of multiple traits across vascular plant taxa, from plants with longer-lived leaves in which there is a high investment in structure and defense (and therefore have higher LMA, lower CO₂ permeability, and lower mass-based rates of photosynthesis and respiration), to plants with low-cost, short-lived leaves that photosynthesize at very high rates, but are vulnerable to herbivory and physical damage (Reich et al. 1999, Wright et al. 2004).

Much of the LES literature considers these trade-offs across a broad sampling of botanical diversity, though within-species variation in LMA and its response to environmental variation have also been well documented (Edwards et al. 2011, Des Marais et al. 2012, Donovan et al. 2014, McKown et al. 2014, Muir et al. 2014, Des Marais et al. 2017). LMA/SLA changes in response to soil drying (Des Marais et al.
2012, Donovan et al. 2014), nutrient levels (Donovan et al. 2014), to a combination of soil drying and elevated air temperature (Des Marais et al. 2017) and, in perennials, among years and seasons within years (McKown et al. 2014). LMA also changes during development of a single annual plant (Donovan et al. 2014). In line with the large role played by the environment in the expression of LMA, considerable variation in heritability within a single species has been reported for this trait (e.g. 0.121 – 0.820 in *Populus trichocarpa* (McKown et al. 2014); 0.003-0.234 in *Brachypodium distachyon* (Des Marais et al. 2017). As such, several authors have recommended caution when interpreting the ecological significance of LMA as a point estimate for a species (Poorter et al. 2009, Donovan et al. 2014).

Quantitative genetic perspectives on leaf structure

Loci driving differences in leaf dissection between two species of *Solanum* (formerly *Lycopersicon*) were identified using QTL mapping in introgression lines, revealing 22 separate loci (Holtan and Hake 2003). 22 significant QTL is an unusually large number of loci to be identified in a cross and speaks both to the number of genes describing differences in the trait between the species and the high power of detecting loci of small effect in this type of crossing design. A large number of small-effect loci affecting leaf shape traits were also identified in a *Populus deltoides* F1 mapping population (Xia et al. 2018).

A central question in the evolution of adaptive traits is whether genetic correlations among traits might constrain the efficacy of selection acting on individual traits. Working with inbred lines segregating natural genetic diversity of two closely related species of *Solanum*, Muir and colleagues found low, non-significant genetic correlations between leaf area and leaf thickness, stomatal density and the ratio of stomata on abaxial and adaxial leaf surfaces (Muir et al. 2014). Heritability of these traits was found to be small, though significantly non-zero, suggesting that natural selection could act on these traits, and the low genetic correlations among traits might allow selection to act on each trait separately. These authors also identified two QTL which together explain less than one percent of the variation observed in leaf area differences between the two species. This architecture suggests that either leaf area differences are controlled by many loci of very small (in this study, undetectable) effect, that leaf area is strongly affected by the environment, or likely a combination of the two. By contrast, analysis of genetic trait co-variance in *Populus trichocarpa* identified very high genetic correlations among leaf traits (0.6-0.9 among leaf width, area, and dry weight) and very low correlation between leaf traits and physiological parameters such as water use efficiency and leaf water potential (Chhetri et al. 2019). Working in an Arabidopsis biparental mapping population, Juenger et al. found similarly high genetic correlations among leaf traits but low, generally non-significant correlations between leaf traits and floral traits (Juenger et al. 2005).

The preceding experiments relied on biparental crosses; as such they represented a limited within-species sampling of trait diversity and had relatively low power to resolve the genomic position of
putatively causal loci. In Arabidopsis, GWAS analysis using a panel of 95 inbred natural accessions genotyped at 250,000 SNPs revealed candidate loci associated with 107 different traits, including leaf serration (Atwell et al. 2010). Although population structure – many inbred lines in the panel share recent common ancestry – significantly limits our ability to distinguish true SNP-trait associations in Arabidopsis, the authors note that many of the top SNP-trait associations are in genes with a previously demonstrated role in expression of the traits. Chitwood and colleagues present a comprehensive genetic analysis of leaf shape, identifying strong genetic correlations between major axes of variation in shape such as lobing with degree leaf hirsuteness and several fruit characters (Chitwood et al. 2014). GWAS analysis reveals just a few candidate loci associated with variation in leaf traits in this system, perhaps missing many loci of smaller effect that cannot be detected using this type of analysis.

*Molecular genetic perspectives on leaf structure*

Leaf growth is determined by two partially overlapping phases: cell division, in which the leaf primordium goes through multiple divisions and the cells proliferate while remaining relatively small, and cell expansion, in which these new cells increase in size and volume (Donnelly et al. 1999, Breuninger and Lenhard 2010). Gonzales et al. (2012) argue that leaf development is a much more complicated process, and that is the succession of five distinct yet overlapping phases (initiation, cell division, transition, cell expansion, and meristemoid division) that determine leaf size. Analyses of transgenic lines and mutants suggest that all these phases contribute, albeit to different extents, to leaf growth.

The initiation, division, and transition phases are all associated with cell proliferation. Basically, the initiation phase governs the differentiation of specific cells in the shoot apical meristem into leaf primordium and, with it, the initial number of cells available to form leaf tissues. The cell division phase determines the rate of mitotic events and the transition controls the precise duration of the latter. In the initiation phase, two possibly interconnected factors might influence leaf size: meristem size and the number of cells recruited at the leaf primordium. During this phase, several genes involved with the progression of the cell cycle, the window during which cell divisions occur, and the regulation of plant hormones involved in cell division and/or differentiation have been identified as components whose expression can affect leaf size (see Table 1 for a list of specific genes).

The following phase, cell division, also has a significant impact on leaf size. The idea is that, given a constant period in which new cells are formed, higher division rates will invariably lead to a higher number of cells and, consequently, a larger leaf area. Several genes have been identified to regulate this process, e.g. the ANAPHASE PROMOTING COMPLEX 10 (APC10), a subunit of the APC/C protein complex that coordinates mitotic events and leads to higher cell division rates when overexpressed (Capron et al. 2003, Eloy et al. 2011). (Again, see Table 1 for a more comprehensive list of genes involved in this process). The transition phase will also affect leaf size due to its control over the arrest of
cell proliferation and the developmental shift from cell division to expansion. It has been shown, for
example, that the overexpression of GRF-INTERACTING FACTORS (GIFs) leads to larger leaves due
to both an increase in cell division rates and a later exit from this phase (Lee et al. 2009). Multiple factors
ranging from miRNAs (Liu et al. 2009, Wang et al. 2011) to transcription factors (Palatnik et al. 2003)
have been associated with cell division timing and its effect on leaf size.

Once cell division ceases, growth is maintained primarily through cell expansion: an increase in cell
turgor leads to the relaxation of the cell wall that is immediately accompanied by the synthesis of new
wall components. Genes associated with wall loosening and wall synthesis have all been identified as
regulators of the expansion process (Scheible and Pauly 2004, Cosgrove 2005, Mansoori et al. 2015, Hu
et al. 2018; see Table 2). During the expansion process, stem-like cells known as meristemoids are also
undergoing division and differentiation into highly specialized cells such as stomatal guard cells. We
discuss the molecular mechanisms associated with stomata control later in this review, but it is important
to point out that some genes involved in stomata development, such as PEAPOD (PPD; White 2006,
Wang et al. 2016), have been shown to significantly affect leaf size.

Because plants are modular organisms, the overlap of leaves along the canopy is not uncommon and
the form of a leaf – more specifically the leaf index (the ratio of leaf length to leaf width) – might have a
significant effect on leaf overlap (Takenaka 1994). Four regulating systems govern leaf index, two
associated with the polarized growth in the leaf length and two associated with leaf width (Tsukaya
2006). On the leaf length axis, ROTUNDIFOLIA4 (Narita et al. 2004, Ikeuchi et al. 2011) controls the
number of cells along the longitudinal plane by affecting positional cues and cell proliferation rates whilst
ROT3 (Tsuge et al. 1996, Kim et al. 1999) regulates the growth of these cells. In terms of growth in the
leaf-width plane, ANGUSTIFOLIA3 (Tsuge et al. 1996, Kim et al. 2002), which is thought to be
associated with microtubule arrangement regulation, and both the SPIKE1 (Qiu et al. 2002) and AtHB13
(Hanson et al. 2001) which are related to cytoskeletal organization, have been described as having
essential roles in cell expansion. The precise genetic mechanisms behind cell proliferation at the lateral
axis remain unclear, yet there is evidence from comparative studies that such a control does exist
(Kuwabara et al. 2001). In addition to these mechanisms governing polarized growth, there are several
genes involved with leaf initiation and cell commitment that also play a critical role in leaf shape
development (see Table 3 for details). Variation in leaf dissection observed between two species of
Solanum (wild tomatoes) is controlled by a KNOX ortholog (Kimura et al. 2008), suggesting one way in
which these canonical leaf patterning genes might be deployed in different plant species to generate the
spectacular diversity of leaf form observed in nature.

Beyond the two-dimensional realm of leaf size/shape, leaf thickness and leaf-tissue density are also
important aspects determining LMA. The ANGUSTIFOLIA (AN) gene, for example, is associated with the
control of LMA because of its effect on leaf thickness. Leaves of *angustifolia* mutants are narrower but thicker than the wild-types ones, a consequence of the improper control of polarity-specific cell elongation. Consequently, *an* mutants have a much higher LMA than wild-type plants (Tsukaya 2003); a similar phenomenon is observed in *rot3* (Tsuge et al. 1996) and *curly-leaf* (Kim et al. 1998) mutants. QTL studies have also helped us to identify candidate genes involved with the control of leaf thickness, including *ERECTA* (*ER*). Interestingly, *ER* was identified in a genetic screen for factors associated with drought-resistance in cotton, even though water limitation is not usually correlated with leaf thickness (Levi et al. 2009). Still, it has been suggested that water limitation may have an impact on LMA, largely because of its effect of leaf-tissue density. Several studies point towards water stress as a factor leading to leaves with smaller mesophyll cells, thicker walls (Utrillas and Alegre 1997), and higher contents of carbon and sugars (Fredeen et al. 1991).

Much of the preceding work relates generally to the developmental control of leaf architecture and, indeed, few molecular genetic studies specifically measure traits such as LMA/SLA that so dominate the ecological literature. We aimed to bridge this gap using a systems biology approach and a panel of natural accession of Arabidopsis to identify molecular determinants of SLA (Des Marais et al. 2012). By assessing genetic correlations between SLA and transcriptional modules we generated a list of candidate genes associated with natural genetic diversity in SLA. As compared to earlier work in Arabidopsis which largely relies on synthetic loss of function mutants and transgenic overexpression lines, described above, our study was able to reveal natural variants affecting SLA; additional work is needed to causally link these variants to SLA (e.g, by swapping putatively causal alleles among accessions via transgenics) and to assess whether such variation has an adaptive role in the field.

Priorities and future work on the genetics and evolution of leaf structure

In summary, there is an extensive literature on the genetic basis of leaf development and, more specifically, on the control of leaf size. Still, most of these studies focus simply on the mechanism through which specific genes affect cell and tissue function, with virtually none assessing adaptive value or even genetic variation within populations. Whilst these studies are extremely important in clarifying the identity, and underlying mechanisms, of genes associated with a particular trait, they leave major gaps. First, the studied genotypes are usually extreme, arising from mutants showing either overexpression or complete loss-of-function, which makes it difficult to predict whether small differences in expression rates, natural allelic variants, or point mutations would be reflected in ecologically relevant phenotypic differences. Second, it is unclear whether variation in leaf traits that affect photosynthetic performance (generally estimated from single, isolated measurements) ultimately affect fitness and, therefore, are subject to selection. GWAS studies, as conducted in Arabidopsis or poplar (Atwell et al. 2009).
Leaf nutrient relations

Nitrogen (N) is arguably the most important element in the context of plant functional ecology. It is a key component of Rubisco (D-ribulose 1,5-bisphosphate carboxylase/oxygenase), the enzyme responsible for carbon reduction in plants and, as such, it plays a significant role in the coordination of the LES (Spreitzer and Salvucci 2002). Estimates suggest that > 50% of the leaf N may be invested in Rubisco and other components of the photosynthetic machinery (Makino et al. 1994, Yin et al. 2018) and, not surprisingly, several studies show that photosynthetic rates scale linearly with leaf N concentration (in either an area or a mass basis (Reich et al. 1999, Wright et al. 2004).

Phosphorus (P) is also an essential plant nutrient that can limit productivity in a range of terrestrial ecosystems (Vitousek et al. 2010, Veneklaas et al. 2012). The nature of this limitation, however, is very different than that of N. Soil P is derived primarily from rock weathering, meaning that the P concentration in the soil is highly dependent on the rock parent material and, once exhausted, this P cannot be readily replenished (Walker and Syers 1976). Because of this, P limitation is usually pervasive in the old, climatically buffered infertile landscapes of the southern hemisphere (Hopper 2009). In these regions, the long-term absence of glaciation events, associated with the geological stability, allowed the deep weathering of the soils that have become severely P impoverished (Hopper 2009). The gradual depletion of P sources through geological time and the relative absence of catastrophic events that could lead to extinction favoured the selection of nutritional and other biological traits associated with coping with P-limiting conditions, and plants that evolved in these regions display a number of adaptations related to functioning at low leaf P concentration.

Adaptive importance of natural variation in leaf nutrient relations

Patterns of within-species variation in leaf N have been studied extensively in both natural and crop species. Heritability for leaf N content for plants grown in either controlled or natural environments are typically moderate: 0.4 for leaf nitrate and 0.6 for leaf free amino acids in Arabidopsis (Loudet et al. 2003), 0.19-0.35 for total leaf N on a mass basis among several environmental treatments in Brachypodium distachyon (Des Marais et al. 2017), 0.4 for leaf % N in Pinus taeda (Cumbie et al. 2011), and 0.21 on either area or mass basis in Populus trichocarpa (McKown et al. 2014).

Proteaceae species that naturally occur at P-poor soils of South-Western Australia, for example, extensively replace phospholipids with galactolipids and sulfolipids during leaf development (Lambers et al. 2012). They also preferentially allocate P to photosynthetically active cells (Guilherme Pereira et al. 2010, Chhetri et al. 2019), or systems biology studies exploiting natural diversity (e.g. Des Marais et al. 2012), may offer a bridge between molecular and ecological studies in this regard.
2018, Hayes et al. 2018) and function at extremely low levels of ribosomal RNA (rRNA) (Sulpice et al. 2014), the largest organic P fraction in leaves (Veneklaas et al. 2012). Combined, such traits allow them to function at lower leaf P concentration, decreasing these species’ requirement for P. Whilst most studies have focused on the physiological processes associated with these traits, there is evidence that at least a few of these may be under genetic control. It has been shown, for example, that the number of rRNA genes show variation among species, among individuals of the same species, and even among different tissues within the same individual (Rogers and Bendich 1987). These authors proposed that this variation is the result of differential rates of unequal recombination events in the rRNA region of the genome. Interestingly, stress may affect recombination rates and, therefore, rRNA gene copy number as was observed among wild populations of wheat (Rogers and Bendich 1987), with changes in temperature and rainfall regime (Flavell et al. 1986), and in flax, with changes in fertilizer treatment (Cullis 1977). Whilst a high copy number of rRNA genes might be associated the production of large amounts of rRNA, which can represent up to ~50% of the organic P pool in the leaves (Veneklaas et al. 2012), the correlation between gene copy number and leaf P is merely speculative and further studies are required to elucidate if such relationship exists.

Quantitative genetic perspectives on leaf nutrient relations

Genetic studies on the control of leaf N published on the last 15 years reveal two trends. First, that QTL associated with leaf N concentration and content, as well as those involved in the control of NO₃⁻ accumulation (the largest fraction of total leaf N), have generally large effects on phenotypic variation, typically explaining more than 20% of the phenotypic variation observed. Second, QTL x environment interactions were frequently observed.

Genome-wide association studies (GWAS) have also been conducted to identify the genetic components associated with the control of leaf N. Zhang et al. (2015), for example, identified several genes involved with NO₃⁻ accumulation in maize (namely a chlorophyll a,b-binding protein, a glutamate synthetase, an NADP-malate dehydrogenase, and a phosphoenolpyruvate carboxylase kinase) through the use of Nested Association Mapping, a technique in which QTL mapping is combined with the very high resolution of GWAS. In a different study, Yang et al. (2015) identified several loci related to leaf color ratios in rice, parameters that have been shown – in this and previous studies – to significantly correlate with leaf NO₃⁻ content. In fact, one of the identified loci was co-located with the rice NITRATE TRANSPORTER 1 gene, supporting the relationship between leaf color ratio and N metabolism.

Loudet et al. (2003) identified several QTLs representing at least 18 genes associated with total N, nitrate (NO₃⁻), and free amino acid content in Arabidopsis. Interestingly, Loudat et al. (2003) suggests that, because of the co-localization of several QTLs explaining variations in total N and NO₃⁻, it is
possible that the leaf N variation could be essentially explained by variations in NO$_3$- content. Similarly, Mickelson et al. (2003) identified 8 QTLs that explained 29% of the variation on total leaf N concentration in barley, with the support interval of several of them overlapping with those of QTLs relevant for NO$_3$- content and remobilization. Hirel et al. (2001) found 5 QTLs which collectively explain 28% of phenotypic variation in NO$_3$- content in maize, with at least two being putatively involved in the control of NO$_3$- accumulation. Curiously, whilst Gallais and Hirel (2004) describe co-localization between several QTLs and maize genes, which might suggest association, the gene(s) regulating NO$_3$- content in maize - like in Arabidopsis and barley - remain largely unknown. Identifying these genes would provide invaluable information on the molecular mechanisms underpinning NO$_3$- storage and leaf N regulation.

Using a different model system, Brouillette et al. (2007) identified several QTLs for leaf nutrient concentration in early-generation hybrids between *Helianthus annuus* and *Helianthus petiolaris*. In this study, they found that a large number of QTL with small effect explained most of the variation in leaf chemistry, with the notable exception of leaf N, where two QTL explained ~25% of phenotypic variation. In a similar study, Rönnberg-Wästljung et al. (2005) found eight QTL that explained over 20% of the variation in leaf N content in a *Salix dasyclados* and *Salix viminalis* hybrid population grown under contrasting water regimes. It is important to note that the interspecific genetic crossing strategy used by Brouillette et al. (2007) and Rönnberg-Wästljung et al. (2005) likely underestimates the effect of these QTL in natural populations of the parental *Helianthus* and *Salix* species.

There are only a few quantitative genetic studies on factors controlling leaf P concentration, content, and remobilization, with most of them focusing on model species. Bentsink *et al.* (2003) for example, identified 5 QTLs that collectively explained 33% of the variation in inorganic phosphorus (P$_i$) accumulation in Arabidopsis leaves, with a single QTL accounting for ~88% of the this variation alone. In fact, the same QTL mapping has been identified in several other RIL populations of Arabidopsis with the Ler accession as a parent (Vreugdenhil et al. 2004, Waters and Grusak 2008, Ghandilyan et al. 2009, Prinzenberg et al. 2010); a vacuolar membrane ATPase subunit is considered the most likely candidate gene underlying its large effect (Prinzenberg et al. 2010). In *Brassica rapa*, Zhao et al. identified two QTL that collectively explained ~38% of the variation in leaf P among a RIL population (Zhao et al. 2008). Similarly, Norton *et al.* dentified two QTL for leaf P concentration in rice, each of them explaining about ~10% of the variation in leaf [P] in the studied RIL population (Norton et al. 2010).

*Molecular genetic perspectives on leaf nutrient relations*

In contrast with a robust literature using QTL mapping approaches to identify loci associated with variation in leaf N and P concentrations, there are relatively few examples of forward-and-reverse genetic studies on the mechanisms controlling leaf N concentration per se. Coschigano et al. (1998) suggested that *FERREDOXIN-DEPENDENT GLUTAMATE SYNTHASE 1* (*GLU1*) might play an important role in
primary N assimilation in leaves of Arabidopsis, a conclusion based on the differences between wild-type and glu1 mutants in chlorophyll concentration. Whilst this study only provides indirect evidence of GLU1 involvement in N assimilation, given that the authors never analyzed leaf N concentration and/or content per se, the results are consistent with other studies. GLU1 is intimately associated with photorespiration in Arabidopsis, and glu1 mutants showing 5% of the wild-type levels of GLU1 activity develop serious N-deficiency symptoms when grown under conditions conducing to photorespiration, but not when growing at 1% CO$_2$ (Somerville and Ogren 1980). In fact, the relationship between N assimilation and photorespiration have been both demonstrated experimentally – with Arabidopsis and wheat shoots (Rachmilevitch et al. 2004) – and through mathematical modelling (Busch et al. 2018). The identification of GLU1 and other genes involved in the coordination of leaf N assimilation via the photorespiratory pathway is particularly important in the context of climate change, where increasing atmospheric [CO$_2$] might affect the photosynthesis/photorespiration balance and lead to N limitation even in N-rich soils.

Moving on towards forward-and-reverse genetic studies on the mechanisms governing leaf [P], these are even more scarce than those on quantitative genetics, and almost exclusively limited to Arabidopsis. Poirier et al. (1991), for example, isolated an Arabidopsis mutant that accumulated only 5% of the P, and between 24–44% of the total leaf P of wild-type plants (Poirier et al. 1991). This deficiency was caused by a mutation at a locus designated pho1, which controlled the xylem loading of P, even though the P-uptake rates were similar between pho1 and wild-type plants at a wide range of P supplies. Later on, Delhaize and Randall (Delhaize and Randall 1995) identified a new mutation at a locus designated pho2. In this case, mutants showed three times more P in their leaves than wild-type plants, mostly as P$_i$, apparently due to a deregulation over the amount of P$_i$ that is normally accumulated at shoots of Arabidopsis. Finally, Duan et al. (2008) showed that AtSPX3 might significantly influence leaf P concentration. In their study, partial repression of AtSPX3 by RNA interference under P-limiting conditions led to an increase of ~12.5% and ~65% in total leaf [P] and leaf [P$_i$], respectively.

**Stomata**

Leaf stomata are the primary structures that facilitate gas exchange between plants and the environment and the most direct means through which water may leave above-ground plant tissues. Accordingly, their structure, function, and evolution has received considerable interest (Franks and Beerling 2009, Kim et al. 2010, Dow and Bergmann 2014, Muir 2015). The rate of CO$_2$, O$_2$, and water vapor exchange between the atmosphere and the leaf interior is determined developmentally via the size of individual stomatal pores and their density on the leaf surface and dynamically through changes in the turgor pressure of the two guard cells which flank the stomatal opening.

*Adaptive significance of natural variation in stomatal traits*
There exists considerable variation in stomata size, shape, density, location, and behavior both within and between species (Tardieu and Simonneau 1998, Oren et al. 1999, Franks and Farquhar 2007, Muir 2015, Dittberner et al. 2018) which is frequently associated with aspects of plant ecology and evolution (Franks and Beerling 2009, Bartlett et al. 2016). Here we focus on within-species variation as this variation is the raw substrate for evolution by natural selection. In Arabidopsis, stomatal density and size show very high heritability (0.59 and 0.56, respectively; Dittberner et al. 2018) which is reflected in clear response of these and physiologically related traits such as water use efficiency to natural selection (e.g. Dudley 1996). Stomatal density and stomatal length also have moderate heritability in Populus trichocarpa, in the range 0.34-0.41 depending on which sides of the leaf are measured (McKown et al. 2014). Interestingly, stomatal density was strongly correlated with many climate parameters at the site of origin of the studied P. trichocarpa varieties. While stomatal density and size show strong heritability under constant environments, these characters do also show considerable plasticity in response to environmental cues such as atmospheric CO$_2$ concentration (Lake and Woodward 2008). Developing a better understanding of mechanisms driving genetic diversity in stomatal response to the environment – and the ecological relevance of this GxE -- may allow us to better model and mitigate the response of plant populations to climate change (Ainsworth and Rogers 2007, Monroe et al. 2018).

Quantitative genetic perspectives on stomata

Muir et al. identified 23 QTL associated with differences in stomatal density between two Solanum species (Muir et al. 2014). This is a remarkable number of QTL to be detected in a single mapping population and speaks both to the high heritability of stomatal density in this cross, as well as the high statistical power of their mapping strategy. These authors were also able to ascertain that natural selection was likely driving the phenotypic divergence in stomatal density as well as stomatal ratio (the proportion of stomata found on the abaxial and adaxial surfaces of leaves) between the two parents.

Despite the high heritability of stomatal characters in Arabidopsis, no loci were associated with natural genetic variation in density and just two were associated with size in a large Arabidopsis GWAS panel (Dittberner et al. 2018). Two processes might explain the challenge with identifying loci describing variation in stomatal size and density. First, many loci of independently small effect might be responsible for phenotypic variation in these traits. The fairly simple genetic basis of the stomatal patterning in Arabidopsis, described below, suggests that this is likely not the case. Alternatively, many independent mutations affecting stomatal patterning may segregate in Arabidopsis, thus occurring at very low frequencies and thereby limiting the power of GWAS to identify statistical associations between SNPs and trait values. Such recurrent mutations in a small set of causal loci have been observed in other traits in Arabidopsis (e.g. Monroe et al. 2016) and have, more generally, been identified as a likely cause of “missing heritability” (i.e. that it can be difficult to account for all of the loci which contribute to the
observed heritability in a trait (Manolio et al. 2009). By contrast, McKown and colleagues identified 18 genes in *P. trichocarpa* with significant associations to at least one stomatal character using a GWAS approach (McKown et al. 2014). GWAS analysis suggests a strong role for the *P. trichocarpa* ortholog of the stomatal patterning gene SPEECHLESS (see below) in determining the density and location of stomata in this species, with striking geographical correspondence between allelic diversity at SPEECHLESS and climate (McKown et al. 2019).

The ecological genetics of stomatal size and behavior have been studied extensively as they relate to Water Use Efficiency (WUE), which measures the amount fixed carbon per unit of water used. WUE is widely studied from a quantitative genetic perspective because proxies of WUE such as the ratio of $^{12}$C to $^{13}$C isotopes are fairly easy to measure (Farquhar and Richards 1984). As such, natural diversity in WUE is well documented in many plant species (Hubick and Farquhar 1989, Quisenberry and McMichael 1991, Geber and Dawson 1997, Van den Boogard et al. 1997) and, in some cases, QTL describing the genetic basis of these traits are known (Juenger et al. 2005, Brendel et al. 2007, Des Marais et al. 2016). Building on earlier QTL analyses which identified five QTL describing genetic variation in WUE between two *A. thaliana* ecotypes (Juenger et al. 2005), we identified a SNP which causes an amino acid substitution affecting WUE (Des Marais et al. 2014). This study demonstrated that a natural variant in a signaling protein, AtMPK12, alters the size and hormonal response of guard cells, increasing stomatal conductance and thereby reducing both WUE and whole plant transpiration efficiency. Further work on this system demonstrated that the AtMPK12 allele conferring low water use efficiency experiences a selective advantage when grown with competitors, likely because the allele causes plants to be greedy with water use, though it suffers a fitness penalty when water is limiting (Campitelli et al. 2016).

*Molecular genetic perspectives on stomata*

The development of guard cells is best understood in the model plants *A. thaliana* and *Brachypodium distachyon*. In Arabidopsis, guard cells arise from epidermal precursor cells during leaf development through a series of cell divisions regulated by three transcription factors, SPEECHLESS (SPCH), MUTE, and FAMA (reviewed by Lau and Bergmann 2012). Each of these transcription factors is itself under tight regulatory control, ensuring proper size for each stomate, proper spacing between stomata, and, thus, genetic control the ideal rate stomatal conductance, $g_{\text{max}}$, for the present environment. An interesting feature of guard cell development is that the precursor of mature guard cells, known as meristemoid cell, is able both to control its own fate and regulate the fate of adjacent cells, thus ensuring that its neighbors do not also develop into guard cells (Robinson et al. 2011). In the model grass *B. distachyon*, orthologs of several genes involved in Arabidopsis stomatal development also regulate guard cell fate, though some of these genes have unique functions or interact with one another differently than
Guard cell development is a plastic trait; plants can adjust the density of stomata on a leaf cell to match maximum stomatal conductance to water availability, light level or intrinsic variation in the demand for photosynthetic carbon reduction (e.g. Lampard et al. 2008). Remarkably, changes in guard cell size and spacing – and thus stomatal conductance – are accompanied by changes in the internal structure of leaves in an apparent effort to coordinate gas exchange and water loss to optimize both photosynthetic carbon reduction and plant water status (Dow et al. 2017).

Guard cells are also a model system for understanding environmentally responsive plant cell signaling. Guard cell turgor pressure, and thus stomatal aperture, responds to environmental parameters including water availability, light level and quality, atmospheric CO$_2$ concentration, ozone, and pathogens, as well as a large number of internal cues (Nilson and Assmann 2007, Kim et al. 2010). The cascade of cell signaling processes resulting in stomatal closure in response to abscisic acid (ABA) is exceptionally well-characterized in Arabidopsis (Kim et al. 2010). ABA is perceived in cells by a group of PYR/PYL receptor proteins which act to suppress the activity of PP2CA protein phosphatases (Park et al. 2009). In the absence of PP2C inhibition (Mustilli 2002), the OST1 protein positively regulates the production of reactive oxygen species (Pei et al. 2000) which begins a cascade of cellular events ultimately resulting in depolarization of guard cell membrane (reviewed by Ward et al. 2009).

**Synthesis and Future Directions**

Several interesting themes emerge from the preceding discussion. First, the types of studies which contribute to our understanding of the genetic basis of each trait vary considerably (Table 4). In the case of leaf structure, dozens of specific genes which control the thickness, density, and overall area of leaves have been isolated and characterized through molecular genetics analysis (Tables 1&2), though nearly all of this work relies on Arabidopsis as a model plant and few studies have dissected the genetic control of within-species phenotypic diversity in these traits. There is likewise detailed understanding of the molecular control of stomatal development as well as the physiological response of stomatal aperture to environmental cues. Phenotypic diversity in stomatal size and density has been well-documented but few studies have investigated the genetic basis of this diversity either within or between species or determined whether this diversity has any selective effect. Molecular control of plant nitrogen status has been studied largely in the context of uptake and assimilation and, to some degree, resorption during senescence. The molecular control of leaf nitrogen concentration and content *per se* – a central component of the LES – is less well understood. Similarly, there are very few studies on the molecular control of leaf P concentration and content. Of these four traits, we have the most comprehensive picture of the genetic architecture of leaf N, perhaps because it is readily scored in large mapping populations.
Writing in this journal in 2003, Remington and Purugganan noted the paucity of research on the genetic architecture of traits in wild plant populations and outlined a strong case for studying “the extent and molecular basis of evolutionary genetic correlations between plant growth measures … and ecophysiological traits such as efficiency of photosynthesis and resource use” (Remington and Purugganan 2003). At that time, evolutionary quantitative genetics in non-model species was largely restricted to low-resolution QTL analyses which could assess how many loci were associated with phenotypic differences between two parents and the relative effect size and direction of loci. In 2003, there was little prospect for identifying the specific genes and variants underlying detected QTL or driving genetic correlations between traits of non-model species. Their prediction that association mapping would become an essential tool for understanding the genotype to phenotype map was prescient, and subsequent advances in genome sequencing and GWAS methods now allow for fine chromosomal resolution of the statistical association between genetic variants and traits of interest, as discussed above.

With these advances in generating genotypic marker data and GWAS methodology, collecting and analysing phenotypic data in high throughput is now the primary limitation for most studies. An additional, related limitation is annotating the functions of genes and other features in genome sequences. As a sobering point of reference, in our best-studied plant species Arabidopsis thaliana, just 16% of 27,655 genes annotated in the reference genome have been annotated through the efforts of direct experimental analysis (The Arabidopsis Information Resource). We anticipate that progress on improving genome annotation and genotype-phenotype mapping will come from a suite of new phenomic tools (e.g. Chen et al. 2014, Fahlgren et al. 2015) that facilitate standardized measurement of ecologically relevant traits in high throughput. Of particular interest are tools for measuring traits directly related to ecophysiology, including parameters of photosynthesis (e.g. Meacham-Hensold et al. 2019, Rungrat et al. in press). An enduring challenge will be accurately measuring and analysing parameters related to growth and biomass partitioning, especially for root traits. Some novel methods for measuring roots in situ are coming online but most still remain labor intensive and expensive (Araus and Cairns 2014). Completely realizing the ecological and evolutionary context of plant growth regulation will require considerable advances in these types of phenomic approaches.

Despite these challenges, we foresee increasing conceptual synergy and convergence of methods between the fields of molecular genetics, physiology and development, and ecological genetics. Multiple studies have leveraged our rich understanding of Arabidopsis molecular genetics in field studies which assess the environmental dependence of traits such as flowering time and fitness (Agren et al. 2013), and infer the history of natural selection acting on putatively functional variants (Fournier-Level et al. 2011, Exposito-Alonso et al. 2018) or the association between climate and variants (Hancock et al. 2011, Lasky et al. 2012, Lasky et al. 2014). In some cases, specific genetic variants in genes of known function have
been experimentally demonstrated to underlie loci identified in the field (e.g. Park et al. 2018). While Arabidopsis is a powerful system for these types of integrated studies, its short life cycle, self-fertile reproductive system, and extremely broad ecological distribution are not likely representative of most plants. Several emerging ecological genetic systems should allow us to explore the molecular, ecophysiological, and developmental basis of plant functional traits in plants with diverse ecologies and life histories. Key aspects of these models will be good genomic resources, a tractability of experimental validation of gene function in vivo, and prospects for ecological analysis under realistic field settings. Studies of wild relatives of well-studied crop species, such as rice, sunflower, tomato, will likely be of great value in this regard, as will several emerging model wild species including Brachypodium (Vogel 2016), Mimulus (Wu et al. 2008), Panicum (Lowry et al. 2014), Populus (Douglas 2017), and Setaria (Brutnell et al. 2015).

Considering the significant challenges with identifying and validating the genetic basis of ecologically plant traits, and in linking these genetic mechanisms to the action of natural selection, we advocate a careful consideration of what traits are of specific interest to a given research question and a clear hypothesis that requires linking genotype to phenotype. Probably the most essential message in this regard is for researchers to ask first what trait they are studying and whether existing literature can help deconstruct the trait into readily assayed constituent traits. As one example cited above, Water Use Efficiency is a highly complex trait affected by leaf and stomatal architecture, the rate of carbon assimilation, plant hydraulics, and the response of guard cells to environmental cues. Each of these traits very likely impact plant ecology in ways beyond their effect on WUE and any one of them may well be the target of contemporary or past selection in nature. Advancing our mechanistic understanding of the evolution of plant functional traits will require careful consideration of the complexity of plant form and function and continue synthesis of molecular, ecological, and quantitative genetics.

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| Symbol | Gene name | Control of leaf size | Reference(s) |
|--------|-----------|----------------------|--------------|
| CKX1-6 | CYTOKININ OXIDASE/DEHYDROGENASE 1-6 | Overexpression leads to a decrease in number and size of meristematic cells, as well as a decrease in leaf expansion rate/duration | Werner et al. (2001; 2003) Holst et al. (2011) Brenner (2012) |
| CKS1 | CYCLIN-DEPENDENT KINASE SUBUNIT 1 | Overexpression causes inhibition of cell-cycle progression, leading to reduced meristem size | De Veylder et al. (2001) |
| SWP | STRUWWELPETER | *struwwelpeter* mutants shows a shorter window of cell-proliferation at the leaf primordium stage | Autran et al. (2002) Clay & Nelson (2005) |
| REV1 | REVOLUTA | rev-1 mutants cannot properly limit cell divisions at the leaf meristem, which leads to larger leaves | Talbert et al. (1995) |
| APC10 | ANAPHASE PROMOTING COMPLEX 10 | Overexpression leads to increased cell division rates at the early stages of leaf development | Capron et al. (2003) Eloy et al. (2011) |
| CDC27a | CELL DIVISION CYCLE PROTEIN 27 HOMOLOG A | Overexpression leads to increased cell-division rates during the entire life cycle of the plant | Rojas et al. (2009) Lima et al. (2013) |
| GIF1-3 | GRF-INTERACTING FACTORS 1-3 | *gif1/2/3* triple mutants show reductions in cell proliferation rates at the leaf primordium stage | Lee et al. (2009) |
| CDK | CYCLIN-DEPENDENT KINASES | Downregulation leads to problems with cell-cycle progression, causing a reduction in cell number | Rymen et al. (2007) |
| KRP | KIP-RELATED PROTEIN | Expression leads to a negative regulation of CDK and, consequently, a decrease in final leaf area | Rymen et al. (2007) |
| DELLA | DELLA PROTEINS | Overexpression inhibits both cell proliferation and expansion, leading to reduced final organ size | Alvey & Harberd (2005) Yoshida et al. (2014) |
| SLY1 | SLEEPY1 | Involved with the gibberellic acid (GA)-mediated degradation of growth repressing DELLA proteins | McGinnis et al. (2003) |
| TCP4 | TEOSINTEBRANCHED1/ CYCLOIDEA/PCF4 | Coordinates cell division/differentiation in leaves through transcriptional regulation of miR319 | Palatnik et al. (2003) |
| GRF1-4/7-9 | GROWTH-REGULATING FACTORS 1-4/7-9 | Coordinates window during which cell divisions occurs through interactions with miR396 | Kim et al. (2003) Liu et al. (2009) Wang et al. (2011) |
| ANT | AINTEGUMENTA | Overexpression leads to increased organ size due to increased cell division during late development | Krizek (1999) Mizukami & Fischer (2000) |
| AIL6 | ANT-LIKE PROTEIN 6 | AIL6 is suggested to act alongside ANT to control leaf development; *ail6* mutants show small leaves | Krizek (1999) |
| ARGOS | AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE | Overexpression leads to a prolonged cell division period, which leads to organs with more cells | Hu et al. (2003) |
| DA1 | UBIQUITIN-ACTIVATED PEPTIDASE | *da1*-1 allele affects both DA1 and DAR, leading to longer cell proliferation period and larger leaves | Li et al. (2008) |
| DAR | DA1-RELATED PROTEIN | DAR is suggested to act redundantly with DA1 in controlling cell proliferation during organogenesis | Li et al. (2008) |
| EOD1 | ENHANCER OF DA1-1; BIG BROTHER | EOD1 acts as a repressor of plant growth; down-regulation leads to increased leaf and petal size | Disch et al. (2006) |
| KLU | KLUH; CYP78A5 | Overexpression of KLU leads to a longer window of cell proliferation, thus producing larger organs | Anastasiou et al. (2007) |
| **ARF2** | AUXIN RESPONSE FACTOR 2 | ARF2 regulate the expression of genes associated with cell division/expansion in response to auxin | Schruff et al. (2006) |
| **EBP1** | ErbB-3 BINDING PROTEIN 1 | Promotes cell proliferation and influences cell-size threshold for division during early development | Horváth et al. (2006) |
Table 2 - Genes involved in the control of leaf size through regulation of cell expansion and meristemoid division phases, with mechanisms and references.

| Symbol | Gene name                        | Control of leaf size                                                                                           | Reference(s)                      |
|--------|----------------------------------|---------------------------------------------------------------------------------------------------------------|-----------------------------------|
| EXP    | EXPANSIN                         | Involved in the dissociation of microfibril that is necessary for wall-loosening and cell expansion           | Cosgrove (2005)                   |
| CES A  | CELLULOSE SYNTHASE               | Encode components of the cellulose-synthesizing complex necessary for production of the cell wall             | Cosgrove (2005)                   |
| CSL    | CESA-LIKE PROTEINS               | Involved in the synthesis of xyl glucan, xy lan, mannan and other β-D-glycans of the cell wall               | Richmond & Somerville (2000)      |
|        |                                  |                                                                                                               | Cosgrove (2005)                   |
| KOR1-3 | KORRIGAN 1-3                     | Encodes three functionally different membrane-bound endo-1,4-β-D-glucanases                                  | Vain et al. (2014)                |
|        |                                  |                                                                                                               | Mansoori et al. (2014)            |
| GT     | GLYCOSYLTRANSFERASES             | Involved in the biosynthesis of multiple cell-wall polysaccharides                                           | Keegstra & Raikhel (2001)         |
|        |                                  |                                                                                                               | Scheible & Pauly (2004)           |
| ARL    | ARGOS-LIKE PROTEIN               | Overexpression leads to larger cotyledons and leaves through mediation of hormone signaling                   | Hu et al. (2006)                  |
|        |                                  |                                                                                                               | Feng et al. (2011)                |
| TOR    | TARGET OF RAPAMYCIN KINASE       | Involved in the promotion of cell expansion in response to favorable environmental conditions                  | Deprost et al. (2007)            |
|        |                                  |                                                                                                               | Barrada et al. (2019)             |
| ZHD5   | ZINC FINGER HOMEODOMAIN 5        | Overexpression leads to plants with high growth rates and large leaves; the mechanism is unclear              | Hong et al. (2011)                |
| RTP2a  | REGULATORY PARTICLE AAA-ATPase 2a| Involved with endoreplication, increased ploidy levels, and larger cell volume in Arabidopsis                 | Kurepa et al. (2009)              |
|        |                                  |                                                                                                               | Sonoda et al. (2009)              |
| SPCH   | SPEECHLESS                       | Controls asymmetric divisions which are needed for the establishment of stomatal lineage cells                 | MacAlister et al. (2007)          |
| TMM    | TOO MANY MOUTHS                  | Involved in the signalling pathway that regulates the differentiation of stomatal lineage cells               | Nadeau & Sack (2002)             |
|        |                                  |                                                                                                               | Dow et al. (2017)                 |
| SDD1   | STOMATAL DENSITY AND DISTRIBUTION 1| Negative regulator of the differentiation process that lead to stomatal guard cell formation                   | Berger & Altmann (2002)          |
| PPD    | PEAPOD                           | Negative regulator of meristemoid proliferation                                                               | White (2006)                      |
|        |                                  |                                                                                                               | Wang et al. (2016)                |
Table 3 - Genes involved in the control of leaf shape, with mechanisms and references.

| Symbol | Gene name | Control of leaf shape | Reference(s) |
|--------|-----------|-----------------------|--------------|
| ROT3   | ROTUNDIFOLIA 3 | Encodes a plant-type cytochrome P450 involved in the control of cell expansion in the leaf-length direction | Tsuge et al. (1996) Kim et al. (1999) |
| ROT4   | ROTUNDIFOLIA 4 | Involved in the control of cell number along the body axis by affecting positional cues and proliferation rates | Narita et al. (2004) Ikeuchi et al. (2011) |
| AN3    | ANGUSTIFOLIA3 | Involved in the expansion of cells along the leaf-width direction via regulation of microtubule arrangement | Tsuge et al. (1996) Kim et al. (2002) |
| SKP1   | SPIKE1    | Involved in the control of leaf cell expansion through regulation of cytoskeletal organization | Qiu et al. (2002) |
| HB13   | HOMEODOMAIN LEUCINE ZIPPER CLASS I PROTEIN (HD-Zip I) | Encodes a transcription factor involved in the sucrose-mediated control of leaf lateral expansion | Hanson et al. (2001) |
| CLV1-3 | CLAVATA 1-3 | Encodes putative receptor kinases associated with shoot apical meristem activity and maintenance | Clark et al. (1997) Kessler & Sinha (2004) |
| WUS    | WUSCHEL   | Involved in the organization of undifferentiated cell population and shoot apical meristem maintenance | Laux et al. (1996) Kessler & Sinha (2004) |
| KN1    | KNOTTED1  | Involved in the maintenance of the shoot apical meristem identity; member of the KNOX family | Jackson et al. (1994) Kessler & Sinha (2004) |
| STM1   | SHOOTMERISTEMLESS1 | Encodes a gene required for shoot apical meristem formation during embryogenesis | Jackson et al. (1994) Kessler & Sinha (2004) |
| RS1    | ROUGHSHEATH1 | Involved in the maintenance of the shoot apical meristem identity | Kessler & Sinha (2004) |
| PHAN   | PHANTASTICA | PHAN is involved in the development of the adaxial domain of leaves; phan mutants lack adaxial cell types | Kessler & Sinha (2004) |
| AS1    | ASYMETRIC LEAVES1 | Involved in the negative regulation of KNOX genes; as1 mutants have prominent lateral outgrowths or lobes | Sun et al. (2002) Kessler & Sinha (2004) |
| CLF    | CURLY LEAF | CLF affects cell division at earlier stages and elongation throughout the development of leaf primordia. | Kim et al. (1998) |
| ER     | ERECTA    | Encodes a putative protein kinase that participates in the coordination of cell growth patterns | Torii et al. (1996) Levi et al. (2009) |
Table 4 – Leaf functional traits analysed in this review with a summary of their ecological relevance, the research focus over the previous 15 years, a list of the most studied taxa, and some of the outstanding questions.

|                         | Leaf structure                      | Leaf nutrients                           | Stomata                                 |
|-------------------------|-------------------------------------|------------------------------------------|-----------------------------------------|
| **Ecological relevance**| Plant growth, productivity          | Photosynthesis, plant growth, productivity| Photosynthesis                          |
| **Focus in the last 15 years** | Molecular                           | QTL & GWAS                              | Physiology / Molecular                   |
| **Most widely studied taxa** | *Arabidopsis thaliana*              | *Arabidopsis thaliana,* barley, maize, rice, wheat | *Arabidopsis thaliana,* *Brachypodium distachyon* |
| **Outstanding questions** | Adaptive value; genetic variation within populations | Identity of genes and mechanisms that drive phenotypic variation | Integration of physiology and population genetics |