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Comparative and Evolutionary Genomics of Angiosperm Trees

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Preface

Angiosperm trees display a dizzying array of diversity in morphology, anatomy, physiology and biochemistry. This diversity has been described and catalogued by various scientific disciplines, but the molecular, genetic, and evolutionary mechanisms underlying this diversity have only recently been explored.

This book, *Comparative and Evolutionary Genomics of Angiosperm Trees* marks a change in focus of tree genomics, from single species to comparative approaches. Excitingly, advances in genomic and sequencing technologies are ushering a new era of research broadly termed comparative genomics, which simultaneously exploits and describes the evolutionary origins and genetic regulation of traits of interest. Effective comparative genomic approaches for trees are enabled by an explosion in genomic data including an increasing number of complete genome sequences available for angiosperm trees, and extensive gene expression data available for a wider array of species. We believe that there is a great potential role for comparative approaches for the study of angiosperm trees, both with regards to understanding the fundamental evolution and development, as well as addressing problems of economic or ecological importance.

This book is intended as resource to provide background on the diverse biological subject areas pertaining to comparative and evolutionary genomic approaches of angiosperm trees. We elected not to make genomic technologies (e.g. the latest sequencing technologies) or computational approaches a main focus of the book, as they are already covered by other literature, and also are rapidly changing. Instead, the chapters focus on biological, genomic, and evolutionary aspects of angiosperm trees that provide information and perspectives that will support researchers broadening the focus of their research. We hope this will provide a valuable resource, and have longevity of relevance that will outlive the particulars of current-day technical approaches.

The first section of the book provides background on the evolution and diversification of angiosperm trees, as well as description of the salient features and diversity of the unique physiology and wood anatomy of angiosperm trees. The second section describes developments in the most model advanced angiosperm tree species (poplars) as well as species that are emerging models. The third section

describes the structural features and evolutionary histories of angiosperm tree genomes, followed by a fourth section focusing on the genomics of traits of biological, ecological and economic interest.

We would like to acknowledge the significant efforts of the authors of each chapter, and the overall high quality of the writing and information contained within their chapters.

Davis, CA, USA — Andrew Groover
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Introduction: Comparative Genomics of Angiosperm Trees: A New Era of Tree Biology

Quentin C.B. Cronk and Andrew T. Groover

Abstract Forest tree genomics has made enormous strides in recent years, by describing the expression and function of genes influencing tree growth and development, and even sequencing the entire genomes of select "model" tree species. We believe that the next chapter of forest tree genomics will focus on cross-species comparative approaches, which will have the ability to provide fundamental new insights into the unique biology and evolutionary history of tree species. Angiosperm trees in particular are fascinating in light of evolution. Angiosperm trees represent the extensive genome evolution, including whole genome duplications, exhibited by different angiosperm lineages. Angiosperm trees also present amazing morphological, physiological and biochemical diversity, providing the opportunity to use comparative genomic approaches to understand the evolutionary origin and diversification of traits associated with trees. This book provides background on biological, genomic, and evolutionary aspects of angiosperm trees, in support of researchers exploring the use of comparative and evolutionary genomic approaches. This introduction briefly reviews the diversity of angiosperm trees and sets out the conceptual framework for comparative and evolutionary study of angiosperm tree biology using genomic tools, and highlights individual chapters within this book.

Keywords Evolution • Wood Developmental Biology • Population Genomics • Angiosperm Trees • Comparative Genomics

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A World of Trees

The carbon dioxide monitoring station at Mauna Loa, Hawaii, has revealed a steady rise of atmospheric carbon dioxide, year-on-year. However, within this rise there is strong seasonal variation. Slightly more carbon dioxide is detectable in the northern hemisphere winter and spring and slightly less in summer and fall. Allowing for a time lag of atmospheric mixing, this is attributable to the vast areas of northern hemisphere boreal and temperate forests losing biomass and releasing carbon dioxide in the fall and taking up carbon dioxide (1.5×10^{10} tons net) with new growth in the spring (Bolin and Keeling 1963). Nothing could more clearly indicate the impact of forests on the biosphere, with trees exerting an influence on atmospheric chemistry detectable half a world away in the central Pacific.

Forests also have direct impacts on human lives. As producers of raw material for industrial processes they provide employment, directly or indirectly, for millions. Forests heat homes and cook food: 6.1 %, or 772 million tons of oil equivalent (MTOE), of primary global energy comes from wood (FAO 2014). This is mainly in the rural and developing world, where fuelwood is directly gathered from forests, but increasingly woody biomass is being grown as bioenergy crops.

As reservoirs of biodiversity, forests are important biomes for biodiversity conservation. More intangible but nevertheless important are the ecosystem services forests provide: climatic moderation, erosion and landslip prevention, and watershed protection, to name a few. Forests are also human habitat, providing food and shelter for many. Even in the urbanised West they are increasingly valued for recreation, and in terms of providing a "connection to nature" which is increasingly recognised as a necessary part of human development (FAO 2014). Additionally, forests and related disturbances (e.g. wildfire) directly affect an increasing number of people living at the wildland-urban interface.

Globally, natural (minimally exploited) and semi-natural (exploited but minimally managed) forests are under threat. This is mainly through conversion, by burning and logging, into agricultural landscapes (or "agroscapes"). However many

Table 1 Status of forestry plantations in 2000

Plantation type	Area (000 ha)
Acacia (Leguminosae)	8317
Eucalyptus (Myrtaceae)	17,860
Hevea (Euphorbiaceae)	9885
Tectona (Lamiaceae)	5716
Other angiosperm	33,556
Total angiosperm	75,334
Conifer(all)	58,134
Unspecified	53,618
TOTAL area (000 ha)	187,086
Rate of planting (000 ha/year)	4493

Source: The Global Forest Resources Assessment 2000 (FAO 2000)

of these agroscapes are subsequently used for the establishment of tree plantations in which intensively-managed, fast-growing, short-rotation trees are grown efficiently for industrial purposes (Table 1). Such plantations can, and should, take some of the pressure off natural forests and provide wide ranging socio-economic benefits, although their benefits are often disputed.

Angiosperm Trees as Genomic Subjects

This book deliberately focuses on angiosperm trees. Conifers, although important, are only dealt with here when appropriate to highlight the evolution of traits found in angiosperm trees. Angiosperm trees are much more diverse than conifers, as well as being the major component of most short-rotation plantation systems (FAO 2001; Table 1). While angiosperm trees present some challenges for biologists due to their large size and long generation times, modern genomic technology has done much to make them scientifically tractable and has allowed the exploitation of characteristics specific to trees that make them particularly attractive as biological subjects. First, wood itself is a globally important trait, not only economically valuable but also pivotal to the functioning of the biosphere. The enormous variety of angiosperm woody plants, and wood types, makes possible comparative and evolutionary approaches. Secondly, many trees have large populations and wide ranges, and show adaptation to diverse climates, and therefore make good subjects for the study of local adaptation. While putting trees in the laboratory is challenging, it has proved possible to take the laboratory to the tree and use wild populations as "natural breeding experiments". Genomic characterization of natural variation grown in common gardens has allowed the molecular basis of traits to be uncovered, for instance using genome-wide association studies (GWAS).

Angiosperm Tree Diversity

There are about 369,000 species of angiosperms (RBG Kew 2016), of which between one fifth and one quarter can be considered trees (i.e. 70,000–90,000). The angiosperms, including those that are trees, display a huge amount of morphological variation, variation that makes comparative approaches particularly rewarding. Economically, there are ca. 1575 angiosperm trees with widely traded timber (Mark et al. 2014). The majority of these are tropical, reflecting the high diversity of tropical forest biomes. The most important family of tropical timber trees by far is the Leguminosae (Fabaceae), followed by the Meliaceae. A summary of some of the most important tropical tree families for forestry is given by Cronk and Forest (chapter 1: Table 4, this volume).

In temperate regions the situation is somewhat different. There is less overall tree diversity in temperate biomes and there Fagaceae is arguably the most important forestry family (chapter 1; Table 4). When temperate forestry trees are mapped on a

summary angiosperm phylogenetic tree it can be seen that the species are highly clustered in one clade (rosid 1). When the same is done for tropical trees the spread is more even, reflecting greater tropical diversity. Genomic resources for trees are rapidly developing. Every year new genome projects are announced. Happily, any discussion in this book will quickly be out of date, which indicates the health and dynamism of the subject. Nevertheless it may be useful to make a few observations on the situation as it now exists (2016). Emerging genomes of forest trees are discussed by Sollars and Buggs (chapter 4, this volume). The best resource by far is that provided by poplar (*Populus trichocarpa*). This was the third plant genome to be completed (after *Arabidopsis* and rice) and is of high quality (Douglas, chapter 3, this volume). Many of the emerging genomes are from the same major clade (rosid 1), such as the Fagaceae genomes.

However, given the importance of the legumes in tropical forestry, the absence of a high quality tree legume genome is notable. An *Acacia* genome is nearing completion, and *Acacia* is the most important plantation legume. However a tree legume in the same clade as crop legumes like soya (*Glycine max*) would allow tremendous synergy between legume tree genomics and legume crop genomics.

The ease with which whole shot-gun sequencing can now be carried out may herald an end of the "model tree" or "model plant" paradigm. Genomic resources can now be generated for any tree, however rare or obscure. This is to be welcomed. However a note of caution should be sounded. While sequencing is easy, assembly and annotation are not. A multiplicity of poorly assembled and annotated genomes may turn out to be more trouble than they are worth.

Model Species versus Comparative Trait-Based Approaches

Woodiness is a labile trait, and it has been modified to different extents, from shrubs to forest giants, in different angiosperm lineages. It has been lost, and sometimes regained, in many lineages. The diversity of woods, from balsa to ebony and teak, differing anatomically and chemically, provide a resource for the study of the molecular basis of this trait. Trait-based approaches, i.e. choosing a trait and following it wherever it iterates in organisms, stand in opposition to the model organism-based approach in which a single organism is chosen as one in which to study many traits (or at least as many as that organism possesses). The model organism has many advantages, not least the ability to build on a growing body of organism specific protocols and resources. However the weakness of the model organism approach is the limited view of any particular trait that it offers – without examining the trait and associated regulatory mechanisms in additional species it is not possible to know if findings are unique to the model species under study, or what the evolutionary history of the trait might be. Consequently, the immense power of comparative and evolutionary approaches cannot be brought to bear when working with a single model species.

Happily the tension between the model organism approach and a comparative approach is rapidly becoming obsolete. Any organism can now be a "model" at least

in the sense that genome resources can be generated readily. Sollars and Buggs (chapter 4, this volume) detail some recent genome projects of forest trees, and, judging by the speed at which new projects are coming on stream, this is likely to be the tip of a very large iceberg. Furthermore there is the possibility of using comparisons between classic plant models such as *Arabidopsis* and emerging tree models (such as poplar). The arabidopsis-poplar comparative model approach has already proved its power in many examples (e.g. Rottmann et al. 2000; Johnson and Douglas 2007).

The huge variety of angiosperm trees provides a scientific opportunity when unleashed by genomics. Although every taxon is a potential genomics model, working with organisms that reach vast sizes and do not domesticate well in the lab has been daunting. Rather than domesticate trees to the lab, the lab has been taken to the tree. Tree biologists have learnt to use the forest as the "growth chamber" and natural populations as "breeding experiments". Common gardens, while expensive to set up, are long lasting (if land tenure issues can be solved) and have the potential to supply large quantities of data to multiple studies over many years (Fetter, Gugger & Keller, chapter 13, this volume).

Evolutionary and Comparative Genomics for Angiosperm Trees

Comparative approaches to tree biology can work at many different scales (Table 2), from the comparison of different genetic individuals in the same species, to the comparison of very different tree species in different plant families. The immense

Table 2 Comparative approaches to angiosperm tree genomics

Comparison	Questions	Examples
1. Between plant families, e.g. *Eucalyptus* (Myrtaceae) vs *Populus* (Salicaceae)	Conserved pathways in major traits, e.g. wood formation	Hefer et al. (2015), He and Groover, chapter 10, this volume
2. Between related genera, e.g. *Populus* vs *Salix*	Differences in genomic architecture, e.g. differences in sex locus architecture between willows and poplars	Olson, Hamrick and Moore, chapter 7, this volume
3. Interspecific, e.g. *Populus deltoides* vs *Populus trichocarpa*	Speciation genomics, adaptive introgression	Bawa and Holliday, chapter 8, this volume
4. Interpopulation, e.g. northern vs southern or montane vs lowland	Environmental genomics, origin of adaptations	Fetter, Gugger and Keller, chapter 13, this volume
5. Intrapopulation	Allelic variation, balancing selection, gene flow/selection balance	Fetter, Gugger and Keller, chapter 13, this volume

morphological variety of angiosperm trees lends itself in particular to comparative approaches. Top level comparisons give answers to the central question of what aspects of biology are fundamental and which are special responses of a limited clade (evolutionary lineage). This approach has been used in an attempt to define core wood genes by comparing eucalyptus and poplar (Hefer et al. 2015) for instance.

Comparative approaches can also be used in a phylogenetic context to understand how key regulators were co-opted in the origin of novel traits. The moss versions of the key wood developmental regulators, NAC transcription factors, have been shown to have a conserved function, able to act as wood regulators in angiosperms, despite mosses having no wood. This is strong evidence that it is not the primary function of this gene that has changed to facilitate the origin of wood, but the downstream modules that it regulates (Xu et al. 2014).

Environmental and Population Genomics: Exploiting Genetic Diversity

Trees are generally highly outbreeding and every wild tree is generally a genetically unique individual. Every comparison between two or more trees can therefore be very revealing of the effect of their different genetic constitutions and environmental histories on phenotypes. Trees have a number of features that makes them particularly amenable for comparative biology at the population level. Some of these features are highlighted briefly here, and detailed in chapter 7 by Olson, Hamrick and Moore, and chapter 8 by Bawa and Holliday.

First, they tend to exist as large populations with a large ranges covering more than one climatic zone. They are therefore highly suitable for landscape genomics and studies of climatic adaptation. Secondly, they are generally highly outbreeding, with effective gene flow, minimising population structure. This contrasts with inbreeding herbs like *Arabidopsis thaliana* which exist as strongly structured populations (a mosaic of inbred lines). As population structure is a confounding factor in all studies of genotype:phenotype association and local adaptation, anything that reduces it is important. Additionally, hybridization among species is common in some tree genera (e.g. *Populus*, *Salix, Eucalyptus*), presenting intriguing questions about speciation and unique ecological attributes of hybrids in forested landscapes.

Genome-wide association studies (GWAS) using natural variation in populations have begun to disentangle the molecular basis of important tree traits such as large growth, perenniality and architecture (McKown et al. 2014) as well as wood structure and the chemistry (Porth et al. 2013). Adaptation to environment is one of the most important biological phenomena, on which much of our agricultural and forestry productivity is based. With an environment changing rapidly due to CO_2 climate forcing, it becomes more important than ever to understand.

Big Questions for Angiosperm Tree Genomics

It is difficult to predict the future but a number of big questions seem to be emerging at the intersection of genomics and tree biology. These include epigenetics, structural genome variation, the genomic basis of the origin of woodiness and the genomic basis of tree traits, such as architecture and sexuality.

1. **Epigenetics**. Trees are long-lived organisms that cannot move to avoid environmental stress, so they have to endure it (Bräutigam et al. 2013). To what extent does environmentally-induced epigenetic regulation contribute to survival? As many trees can be cloned, it is possible to conduct what in human biology would be called "identical twin studies", but on a vast scale.
2. **Structural genome variation**. Angiosperm tree species display the surprising history of genome duplication and rearrangements that have occurred at various points in angiosperm evolution and lineages. Structural variation in angiosperm genomes is presented in chapter 5 by Street. As discussed in chapter 6 by Hussey, Wegrzyn and Vasquez-Gross, a closely related topic is the complex evolution of gene families in angiosperm tree lineages, including how gene families undergo selection and fractionation. Additionally, angiosperm trees exist in large populations and are known to produce, at low level, structural genome variants such as triploids (Mock et al. 2012), aneuploids (with an extra copy of a particular chromosome) and segmental variants (with translocations, inversions or duplications of parts of chromosomes). Using new genomic approaches these can now be detected within populations. What is their effect on biology, speciation and genome evolution? Discussion of these and related topics is given by Bawa and Holiday (chapter 8).
3. **Woodiness** is a globally significant trait and the molecular control of its evolution is a pressing question. It is well known that various herbaceous lineages have lost woodiness only to gain it again in some clades. These clades of recently evolved woodiness provide promising experimental systems for the investigation of the evolution of woodiness itself (Moyers and Rieseberg 2013; Davin et al. 2016). Variation in wood structure and anatomy is summarized in chapter 2 by Spicer, and a discussion of the molecular regulation of wood development is presented by He and Groover in chapter 10.
4. **Genomic basis of important traits of trees**, including comparison of these traits to similar if not homolgous traits in non-tree species. For example, tree architecture is tremendously complex. At its simplest, it is evident that some trees have narrow crowns whereas others are broad and spreading. Such traits are of great importance in commercial forestry and pomology (Segura et al. 2008) but are also important in the fundamental understanding of plant development. The regulation of tree architecture is presented in chapter 9 (this volume) by Hearn. Trees are also well known for the variety of sex expression from hermaphroditism to monoecy, dioecy or a mixture (polygamy). When studied in comparative framework, tree genomics may be expected to shed much light on the molecular pathways underlying such variation (Geraldes et al. 2015; Olson, Hamrick & Moore, chapter 7, this volume; Fetter, Gugger and

Keller, chapter 13, this volume). The regulation of perennial growth is fundamental to trees, and the regulation of phase change and phenology in trees is presented in chapter 11 (this volume) by Brunner, Varkonyi-Gasic, and Jones. Trees have also evolved additional strategies to deal with abiotic stress including drought, which is discussed in chapter 12 by Bastiaanse. Trees also have a variety of interactions with other organisms, including both pathogens and insect pests, as well as beneficial symbionts. Some of these interactions are described in chapter 14 by Plett and Plett.

These examples merely touch on some of the exciting basic science that will emerge from tree genomics over the next few years, and a bright future can be anticipated. However, as already alluded to, angiosperm dominated forests and plantations are of great ecological, economic and social importance. And many of these forests are in peril. Effective comparative genomic approaches can also provide new tools for applied forest management.

Conservation of Forest Biodiversity and Forest Genetic Resources

Forests, particularly tropical wet forests, are enormously rich in biodiversity. It is difficult to draw the line between trees and shrubs, but of the 369,000 species of flowering plants (RBG Kew 2016), between 70,000 and 90,000 can be regarded as trees. These are the largest organisms in terms of biomass on the planet, reaching, in the case of forest giants like *Eucalyptus regnans*, over 100 m in height. Many ordinary trees commonly reach 30 m or more. These are huge organisms by any standards. In turn trees support a pyramid of dependent organismal diversity. Erwin found 1143 beetle species on the tropical tree *Luhea seemannii*, of which an estimated 162 were only found on that tree species (Erwin 1982).

As well as the organismal diversity there is the genetic diversity of the trees themselves, which is essential to maintaining vigorous, well-adapted tree populations. Genomic tools are hugely powerful in characterising this genetic diversity (e.g. Geraldes et al. 2014; Fetter, Gugger and Keller, chapter 13, this volume) and in relating it to adaptation and the environment, whether through phenology (Brunner, Varkonyi-Gasic and Jones, chapter 11, this volume), abiotic stress (Bastiaanse, Theroux-Rancourt and Tixier, chapter 12, this volume) or species interactions (Plett and Platt, chapter 14, this volume).

Protection of Forests against Emerging Pests, Pathogens and Environmental Stressors Including Climate Change

Trees are large, long-lived resources that cannot move, and as such they are “sitting ducks” for pathologies of various sorts. Past epidemics such as Dutch elm disease and chestnut blight have been devastating. Plantation trees of a single genotype are

particularly vulnerable during their multi-decadal lifespan. The arrival of a new pest or pathogen, which may have multiple generations per year, is a real possibility during the relatively long rotation age. Resistance breeding, as well as planting of mixed genotypes, can mitigate risk on landscape scales. Genomics can help by permitting a gene-based understanding of resistance (Plett and Plett, chapter 14), and by providing breeding tools, as in "genomic selection" (Denis and Bouvet 2011). On the other hand, we are increasingly realising the beneficial role that fungal and bacterial endophytes in roots and leaves can play in pest and pathogen resistance. Such endophytes are very difficult to characterize by traditional means but lend themselves to metagenomic approaches.

Climate change is a major threat to forests, particularly as it now appears that the pace of change may exceed the ability of adapted genotypes to migrate to new climatic optima. Assisted migration (including planting trees that are adapted not to the present climate, but to future climate) is likely to emerge as a very important, and hotly debated, issue. Again, genomics has a role to play. Chapter 12 (Bastiaanse, Theroux-Rancourt and Aude Tixier) highlights our current understanding of how trees respond to drought stress and how genomics may aid in identifying genes and genotypes conferring resistance to drought and other abiotic stress.

The Future of Angiosperm Tree Genomics

The scientific issues highlighted in this introduction, and the other chapters in this book, indicate the health and excitement of the subject. Trees have traditionally been difficult to study and genomic tools now permit a catch-up. Much work needs to be done however. Despite the enormous importance of forests and forestry, this sector has fewer resources than the health and agriculture sectors that have already been deeply impacted by genomic technology.

The biology of trees provides many important problems that are unique to, or characteristic of, trees as opposed to other plants. For instance, most tree species are highly outbred and suffer from inbreeding depression. Hybrids, on the other hand, can show significant hybrid vigour. Indeed the highest yielding plantation crop in the world is a hybrid eucalyptus (*E. grandis* × *E. urophylla*) in Brazil. One explanation for hybrid vigour may be that trees carry a large genetic load of sublethal alleles, as suggested by genetic studies (Bradshaw and Stettler 1994). Surprisingly however, recent functional genomic work in poplar showed that most of the genome can be reduced to a haploid state without lethality (Henry et al. 2015). The interplay of inbreeding depression and hybrid vigour is just one example of many opportunities for new genomic studies in angiosperm trees. The size and genetic basis of load in trees has been a big unknown but genomic techniques offer potential solutions to this important problem. This is just one example of many opportunities for new genomic studies in angiosperm trees. As the chapters in this book will show, with powerful genomic tools at hand the solution to these and other problems in tree biology are within grasp.

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Postscript While this book was in preparation, but after the manuscript of his chapter was submitted, we received the news of the tragic death of Carl Douglas in a climbing accident in the mountains of British Columbia. We have lost a great colleague and trusted friend. Carl was a true leader in the field of the genomics of angiosperm trees and will be greatly missed. We dedicate this volume as a small tribute to his memory.

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The Evolution of Angiosperm Trees: From Palaeobotany to Genomics

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Abstract Angiosperm trees now rival the largest conifers in height and many species reach over 80 m high. The large tree life form, with extensive secondary xylem, originated with the progymnosperms and gymnosperms in the Devonian and Carboniferous. However evidence suggests that the ancestor of extant angiosperms was not a tree but either a herb or understory shrub. Angiosperm fossil woods are rare in the early Cretaceous but become common in the mid-Cretaceous. The "reinvention" of wood in the Cretaceous produced a novel xylary morphospace that has since been extensively explored by subsequent evolution. Today, large timber trees are absent in the early diverging lineages of the angiosperms, and conventional wood has been lost in the monocots. There are a few timber trees in the magnoliid clade. Most timber trees are in the rosid clade, particularly the fabids (e.g. Fabaceae) but also in the Malvids (e.g. Meliaceae). Timber trees are less common in the strongly herbaceous asterid clade but some important timbers are also found in this lineage such as teak, *Tectona grandis* (Lamiaceae). Genomic resources for angiosperm trees are developing rapidly and this, coupled with the huge variation in woody habit, make angiosperm trees a highly promising comparative system for understanding wood evolution at the molecular level.

Keywords Wood • Fossils • Evolution • Xylogenesis

Introduction

The tallest known angiosperm tree is "Centurion", a large *Eucalyptus regnans* from Tasmania measuring 99.6 m in height, 12 m around at the base, with an above ground biomass of 215 tonnes and an annual increment approaching one tonne

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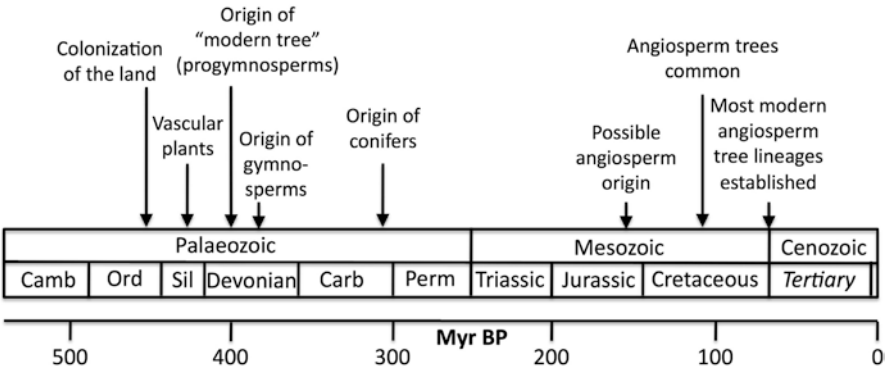

Fig. 1 Timeline of the evolution of the angiosperm tree habit (see also Table 1)

(Sillett et al. 2015). This is a big tree by any measure. *E. regnans* is only the third largest tree species after two conifers, the coast redwood and coastal Douglas† fir of western north America. However, a disputed nineteenth century record, at 132.6 m, would make *E. regnans* the world's tallest tree, if correct.

Although conifers hold most of the height records, angiosperms are notable in certain categories. Their capacity for clonal growth means that "Pando", a large aspen (*Populus tremuloides*) clone is the largest organism by biomass. It is estimated that the Pando clone covers 46.3 hectares (DeWoody et al. 2008). *Ficus benghalensis*, the Indian banyan tree, has the largest spread of a single tree. Descending prop roots stabilize branches which can grow horizontally almost indefinitely. One individual, "Thimmamma Marrimanu", is said to occupy an area of 1.9 hectares.

It was not always so. It is likely that angiosperms were once small and evolved in the shadow of gymnosperms. They took millions of years to attain ecological dominance over gymnosperms (at least in most forest biomes) and millions more to fully rival the tallest gymnosperms in size. In terms of geological history however, the rise of the angiosperms was spectacularly rapid. This chapter will follow the road to ecological dominance of the angiosperm tree. Tree genomics holds the promise of being able to understand this rise and to understand the genomic toolbox (Schrader et al. 2004; Groover 2005; Carocha et al. 2015) used to build such forest giants. The origins of this toolkit lie in the distant evolutionary past. It is therefore useful to start the journey at the earliest land plants and what genes they brought with them, from their aquatic ancestors, onto the land (Floyd and Bowman 2007), around 470 million years ago (Mya). A timeline of land plant evolution is given in Fig. 1. By comparing genomes of trees with those of the early diverging extant land plants, such as the moss *Physcomitrella patens*, we can determine the conserved developmental modules which have been reshaped, co-opted or re-used, in order to build massive organisms (Xu et al. 2014).

Early Land Plants and the Building Blocks of Complexity

Increased knowledge of the biology of *Physcomitrella patens* has allowed us a glimpse into what aspects of the molecular machinery of woodiness is

shared with early divergent clades of land plants and may be considered to have involved soon after the colonisation of the land. Three examples can be cited: (1) The KNOX proteins. These are essential for meristems function and are involved in cambial activity (Champagne and Ashton 2001; Singer and Ashton 2007; Sakakibara et al. 2008). (2) The NAC transcription factors. These are essential as regulators of xylogenesis (Xu et al. 2014). (3) The lignin pathway, including genes like 4CL (Silber et al. 2008; Souza et al. 2008). There are many examples of evolution re-using and co-opting existing genes rather than evolving novel genes de novo. It is therefore unsurprising that we can find the molecular origins tree-building in distantly related simple plants and infer their presence in the common ancestor of extant land plants.

Vascularization: The Lignin Revolution

The earliest land plants had no vascular tissue and were unable to transport water for long distances. This constrained their size and ensured dependence on external water: they were ectohydric, with water transport by capillarity along the external surface of the plant.

The endohydric condition (internal water transport) is characteristic of the more robust mosses and therefore predates vascularisation and its precursor. Endohydric mosses can absorb substrate water through basal rhizoids, and are resistant to water loss because of a cuticle-like external layer. In their stems they have well-developed conducting strands or hydroids (Zamski and Trachtenberg 1976). Examples of endohydic mosses include *Polytrichum* spp., and the largest mosses, such as *Dawsonia superba* and *Dendroligotricum dendroides* (Atala and Alfaro 2012). The hydroids together make up a conducting tissue called the hydrome (there is also a phloem-like tissue called leptome). It is debatable whether the hydrome and leptome of mosses are directly homologous to xylem and phloem or whether they represent independent evolution. Nevertheless the early stages of evolution of xylem and phloem must have been equivalent to hydrome and leptome, and therefore we can use those tissues to help us understand vascularisation (Edwards et al. 2003). Hydrome is often particularly well-developed in the sporophytes of mosses, which also have stomata. Stomata therefore predate the origin of xylem but their association with hydrome in sporophytes of mosses indicates the likely co-evolution between conducting tissues and stomata (Ligrone et al. 2012).

Plants with simple patterns of xylem and phloem (primary vasculature) occur in the fossil record (Table 1) with the appearance of the rhyniophytes (Kenrick and Crane 1991) (leafless herbs, now extinct), lycophytes (microphyllous herbs) and ferns (megaphyllous herbs). The lycophytes and ferns also evolved strategies to form tree-like organisms, which will be discussed later, but these are more akin to giant herbs that true trees.

The tracheid, eventually with its lignified cell wall (Boyce et al. 2003), represented a great improvement over the hydroid in terms of water transport and the

existence of xylem (tracheid tissue) permitted water transport over greater distances, thus allowing for greater organismal stature.

The development of primary xylem is a first step in the ontogeny of shoots, even in large extant trees, and there is no reason to suppose that primary vascularization is not fundamentally homologous throughout vascular plants. Unfortunately there are rather few simple vascular plants with completely sequenced genomes. *Selaginella* (a lycophyte) is one (Banks et al. 2011), and there are plans to sequence the model fern, *Ceratopteris richardii* (Veronica Di Stilio, pers. comm.). Further resources of this sort would be extremely valuable in studying the evolution of vascularisation.

Currently our knowledge of primary vascularisation at the molecular level comes, of course, from *Arabidopsis*. The developmental course involves, first, the differentiation and division of procambial strands (Yang and Wang 2016). Second comes differentiation into protoxylem and protophloem and thirdly the formation of metaxylem and metaphloem. Genes involved in these processes are numerous but include HD-ZIP III genes that direct xylem development. Polar auxin transport (PAT) is very important: auxin mediated transcription of MONOPTEROS (MP) leads to the expression of the HD-ZIP gene AtHB8, as well as the PIN1 auxin transporter which maintains MP transcription in a positive feedback loop (Ohashi-Ito and Fukuda 2010). A key question is how these gene modules of primary vascularization evolved in the early history of life on land (Xu et al. 2014).

Vascular Elaboration: Trees without Woody Trunks

Woody trunks, as discussed in the next section, are a feature of the progymnosperms, gymnosperms and angiosperms, together forming a single clade the lignophytes. However, tree-like organisms evolved in the lycophytes and ferns, despite less extensive development of secondary vasculature. There are no extant tree-like lycophytes, but in the fossil record of the Carboniferous, *Lepidodendron* and *Sigillaria* grew to tree-like proportions in the coal swamps (Phillips and Dimichele 1992; DiMichele and Bateman 1996). These tree lycophytes had some secondary xylem, produced from a unifacial, and more or less continuous, cambium. However, this secondary xylem did not occupy the bulk of the stem, but only a small central core. The majority of the stem was parenchymatous with structural rigidity provided by a well-developed outer bark. They could almost be described as "giant herbs", particularly as the arborescent stems were determinate and short lived. The closest living relative to these giant herbs is now the quillwort group, comprising the small aquatic *Isoetes* and its small shrubby relative *Stylites* (Karrfalt and Hunter 1980; Larsen and Rydin 2016). These extant plants would be an exciting genomic resource for the study of the origin of the tree habit in fossil lycophytes. However, no genomic resources are currently available for quillworts.

The tree habit has also evolved in ferns (monilophytes) and indeed we still have extant examples in tree ferns (Pteridophyta), such as *Dicksonia* and *Cyathea*. These

can grow several metres high but have no secondary xylem. Instead they have a complex primary vasculature (dictyostele) in a massive, mainly parenchymatous stem. Much of the structural rigidity of the stem comes from a tough outer layer of persistent leaf-bases or adventitious rootlets. These too have the aspect of a "giant herb". An extinct group of free-sporing plants, the Cladoxylales, attained considerable stature (Soria and Meyer-Berthaud 2004). The cladoxylalean *Eospermatopteris* (*Wattieza*) has left Devonian fossils in New York State that are massive trunks from organisms formerly 8 m or more high (Stein et al. 2007). Finally, a rather divergent group of ferns (in the broad sense), the horsetails or sphenophytes, are also represented by large organisms in the fossil record. The arborescent sphenophytes produced a small amount of secondary xylem in their long and narrow stems, which grew many metres high (Rossler and Noll 2006; Roessler et al. 2012). The stems are cylinders of wood around a central pith. This is a different strategy but they are still hard to fit into the modern concept of "tree" and again have something of a resemblance to "giant herbs", particularly as the aerial stems were likely mostly determinate and short-lived.

The Evolution of Woody Trunks: The Progymnosperm Legacy

The woody trunk or "hyperstele" (massive secondary development of the primary vasculature or stele) evolved first in the progymnosperms. The formation of woody trunks required the extensive production of secondary vascular tissue from a persistent and highly active continuous cambium. This permits massive stems (trunks) that are very largely composed of xylem with little or no pith (although some seed plants do have trunks with extensive pith - see below). The structural strength of such secondarily thickened stems allows for indeterminate growth into very large organisms. This contrasts with the determinate tree-like stems of arborescent lycophytes and horsetails which spring up to considerable heights to reproduce, but might not persist.

The position of the first "modern tree" is generally given to the progymnosperm *Archaeopteris* (Meyer-Berthaud et al. 1999). Progymnosperms do not have seeds but are instead free-sporing, indicating that the woody trunk evolved well before the seed. Progymnosperms appear to have had tracheids with bordered pits (Dannenhoffer and Bonamo 2003). Fossil wood of *Archaeopteris* is commonly referred to in paleontological literature as *Callixylon* (Beck 1960) and studies of well preserved *Callixylon* wood shows not only the presence of bordered pits but also of possible torus structures (Beck et al. 1982). If this interpretation is correct, then *Archaeopteris* shows advanced features of conifer wood. Wood features characteristic of conifers therefore predates conifers and even the seed habit.

When gymnosperms appear in the fossil record, they carry forward the massive wood construction of the progymnosperms (Savidge 2008). Their generally large size, together with the advantage of the seed, gave rise to ecological success. Millions of years of increasing gymnosperm dominance of the earth eventually left

only epiphytic, aquatic, marginal or forest understory niches for lycophytes and ferns. However, not all gymnosperms have the massive trunks of conifers. The solid wood and pith-free trunks of conifers is a form of construction termed "pycnoxylic", whereas the trunk of cycads is "manoxylic", with a large pith. *Ginkgo* has a mixed stem anatomy with the short shoots being manoxylic and the long shoots pycnoxylic. The final group of extant gymnosperms, Gnetales, is remarkable for its diverse habits, from lianas to small shrubs, and for the presence of vessels, independently derived from those of angiosperms. This diversity indicates the ability of this lineage to utilize different modes of woodiness in addition to the massive woodiness of conifers.

It is worth noting that these new innovations leading to massive wood partly involve the distribution and number of tracheids and partly changes to functional efficiency the tracheid itself. The basic tracheid building block is little changed from the earliest vascular plants to appear on the land, but some innovations have arisen, such as the bordered pit complete with torus (characteristic of some conifers). Many ferns and lycophytes have undifferentiated pit margins (although bordered pits have been noted in some).

Far more conspicuous, however, is the huge increase in distribution and volume of xylem. The key innovation for these lies in the persistent and continuous cambium. The challenge for genomics and development is therefore in understanding the specification and maintenance of the cambium (Groover and Robischon 2006).

Wood Reinvention: The Evolution of Angiospermous Wood

The first unequivocal angiosperm fossils are pollen grains that first appear in the early Cretaceous (from 135 Mya). At first rare, angiosperm pollen quickly increases in abundance, first in low latitudes, later in higher latitudes. By the end of the Cretaceous the angiosperms were clearly the dominant organisms of the biosphere. The first macrofossil evidence is *Archaefructus* (Sun et al. 2002), an aquatic herb from the early Cretaceous (125 Mya). However, molecular dating studies consistently suggest angiosperm origins well before this, usually some time in the Jurassic. The fossil flower *Euanthus* (Liu and Wang 2016), from the late Jurassic (160 Mya) is not universally accepted as an angiosperm. Enigmatic fossils from the Triassic such as the "monocot-like" leaf *Sanmiguelia* and some angiosperm-like triassic fossil pollen (Hochuli and Feist-Burkhardt 2013), hint at an even earlier origin. If angiosperms did originate before the Cretaceous the problem becomes how they remained so rare for so long. Darwin (1903) recognized this problem when he wrote in a letter in 1875: "the presence of even one true angiosperm in the Lower Chalk [early Cretaceous] makes [one] inclined to conjecture that plant[s] of this great division must have been largely developed in some isolated area, whence owing to geographical changes, they at last succeeded in escaping, and spread quickly over the world" (Darwin and Seward 1903).

Features of the first angiosperm may be looked for by examining the early diverging lineages *Amborella*, Nymphaeales and Austrobaileyales (Table 2). There are two growth forms here: *Amborella* and the Austrobaileyales are generally shrubs or lianas adapted to low light and high soil disturbance in humid tropical forest understory, such as stream banks in tall forest. This model of the early angiosperm is the "dark and disturbed" hypothesis (Feild et al. 2004). The Nymphaeales are very different. They are aquatic and adapted to sunny open water. This has led to the suggestion that the early angiosperms might have been aquatic: the "aquatic palaeoherb" hypothesis (Sun et al. 2002; Feild and Arens 2005).

Either way, there is no evidence that the ancestral angiosperm was a tall forest tree. Interestingly, both *Amborella* and the Austrobaileyales have a seedling phase in which they form multiple scandent shoots from a basal lignotuber (Feild and Arens 2005). Sometimes the scandent habit persists as in lianous species of *Schisandra*, *Austrobaileya* and *Trimenia*. This is interesting as the lianous habit is potentially a driver for the evolution of vessels, as may have been the case in *Gnetum* (of which many species are lianous). High hydraulic conductivity per unit area is important in the thin stems of lianas. It should be noted however that lianous species without vessels are known (Feild et al. 2012).

Waterlilies (Nymphaeaceae) have large creeping rhizomes that are often perennial. Despite the large size they have no secondary xylem and do not form a vascular cambium (although there may be a cork cambium). Instead the primary vasculature is scattered and the bulk of the rhizome is of aerenchymatous ground tissue.

Whatever specialized niche, whether understory shrub, liana or aquatic herb that early angiosperms occupied, the ecological conditions were apparently permissive to a distinctive "reinvention" of wood: now with vessels and small bordered pits with a homogeneous pit membrane (lacking a torus). Notably, if the ancestral angiosperm was an aquatic herb then woodiness, and even the vascular cambium (if this was ancestrally lost), might have had to be re-evolved.

Whatever the ecological drivers, when angiosperms increased in numbers and stature in the mid-Cretaceous to compete with gymnosperms in the forest canopy, they possessed a remarkable new vesseliferous wood. There are only a few angiosperms with only tracheids (i.e. vessels completely absent) this feature may be ancestral in *Amborella* but it is an evolutionary reversal elsewhere (Winteraceae in the magnoliids, and *Trochodendron* in the eudicots). As Feild and Arens state: "vessel origin appears to allow for the exploitation over new morphospace of xylem hydraulic design" (Feild and Arens 2005). This new morphospace has been fully exploited in subsequent angiosperm evolution.

A likely further reinvention of wood occurred in the monocot clade, which appears to have diversified from an herbaceous ancestor. The palms are monocots with an anomalous "wood", formed from extended production of fibre-capped vascular bundles distributed throughout the ground tissue. Compared to dicotyledon xylogenesis this seems bizarre and it produces "wood" unlike any other. This is not wood if that is defined as secondary xylem, but if wood is defined more generally as usable lumber then the word applies. Palm wood is functionally very effective, sup-

porting tall trees (up to 60 m in the case of the wax palm *Ceroxylon quindiuense*) and producing internationally traded and locally important hard tropical lumbers such as "red palm" lumber from *Cocos nucifera* and "black palm" lumber from *Borassus flabellifer*. The trunk is filled with functioning vascular bundles and there is no heartwood of non-functioning vessel elements. Furthermore the absence of a peripheral cambium reduces vulnerability to fire (Tomlinson 2006). The recent sequencing of the genomes of oil palm (*Elaeis guinaeensis*) (Singh et al. 2013) and date palm (*Phoenix dactylifera*) (Al-Dous et al. 2011; Al-Mssallem et al. 2013) has created opportunities for understanding the distinctive growth of palm trunks at the molecular developmental level.

There are now genomes available or soon-to-be available for several early divergent clades of the angiosperms, notably *Amborella* (Albert et al. 2013). These genomes will be of great significance for comparative work that seeks to elucidate the evolutionary developmental origin of angiosperm wood. Finally, mention should be made of a bizarre rootless aquatic dicot angiosperm, *Ceratophyllum* (Iwamoto et al. 2015), which lacks xylem, even primary xylem. As the xylogenesis pathway has been deleted in this plant, it represents a "natural knockout" experiment, which might one day be attractive to researchers.

Forest Giants: The Origin of Large Angiospermous Trees

The rapid rise and diversification of the angiosperms during the Cretaceous is well documented from fossil evidence. However much of the early differentiation appears to have been in the form of herbs (Jud 2015) and shrubs (Feild and Arens 2005). Fossil angiosperm wood does not appear until the Aptian and Albian (126–99 Mya) and does not become common until the late Cretaceous (84–65.5 Mya). At the same time, findings of fossil gymnosperm wood fall (Peralta-Medina and Falcon-Lang 2012). Recent fossil flower finds allow the identification of magnoliids as well as early diverging clades of eudicots (Proteales and Buxales) (Doyle 2015) By the end of the Cretaceous (65 Mya) the majority of eudicot lineages were well established and the abundance of fossil woods indicate that large eudicots were dominant in forests globally (Table 3).

The first diverging extant eudicot lineages include many herbaceous and shrubby clades (Bremer et al. 2009; Angiosperm Phylogeny Group 2016). However, the order Proteales includes the plane trees (*Platanus*), which are of large stature. Extinct platanoids (Maslova 2010) of various kinds may have been among the first eudicot forest dominants.

The delimitation of the eudicot clades used here (Table 3) follows the recent APG classifications (Bremer et al. 2009; Angiosperm Phylogeny Group 2016). The asterid clade of eudicots includes rather few large trees (although many herbs, as in the predominantly herbaceous family Asteraceae). *Gmelina* and *Tectona* (teak) in the mint family Lamiaceae are notable exceptions. By contrast, the rosid clade (containing about a quarter of flowering plants) contains the majority of

Table 1 Some major plant lineages mentioned in text

Name	Origin (approx.)	Notes
Liverworts (marchantiophytes)	Mid-Ordovician (470 Mya)	Earliest land plants (mid-Ordovician) are probably referable here; or at least were similar in form to modern marchantiophytes
Mosses (bryophytes)	? Silurian (c. 440 Mya)	Fossil record poor
Hornworts (anthocerotophytes)	? Silurian (c. 435 Mya)	Fossil record poor
Rhyniophytes (extinct)	Mid-Silurian (430 Mya)	The early vascular plant, the rhyniophyte *Cooksonia* is first known from fossils in Ireland (Edwards and Feehan 1980).
Lycophytes	Late Silurian (420 Mya)	The first fossil evidence is the relatively small lycophyte *Baragwanathia* from Australia
Ferns (monilophytes)	Likely late Silurian (420 Mya)	The earliest fern (in the broad sense) fossil is generally considered to be the mid-Devonian (c. 390 Mya) *Ibyka*. (possibly representing a lineage ancestral to sphenopsids) However the fern and lycophyte lineages are likely to have split before this
Progymnosperms (extinct)	Mid Devonian (c. 400 Mya)	The mid-Devonian Aneurophytales are the first exemplars. The first "modern tree", *Archaeopteris*, first appears in the upper Devonian (380 Mya)
Gymnosperms	Late Devonian (385 Mya)	The first gymnosperms are not referable to any extant groups. The first members of extant groups, such as the first putative conifers, arose in the late Carboniferous (310 Mya)
Angiosperms	Possibly late Jurassic (160 Mya)	Unequivocal angiosperm pollen first appears in the early Cretaceous (from 135 Mya). The fossil flower *Euanthus* (Liu and Wang 2016), if accepted as angiospermous, pushes the origin back at least to the late Jurassic (160 Mya)

Table 2 Characters of the major clades of angiosperms

Clade	Life form	Xylem characters
Amborella	Shrub	Extensive bifacial vascular cambium, tracheids only
Nymphaeales	Aquatic herbs	Primary xylem only, vessels
Austrobaileyales	Shrubs, lianas	Extensive bifacial vascular cambium, vessels
Magnoliids	Shrubs, trees, lianas, herbs	Extensive bifacial vascular cambium, vessels (but tracheids in Winteraceae)
Monocots	Herbs (rarely trees, e.g. palms)	Primary xylem only (but sometimes with anomalous secondary xylogenesis, e.g. palms), vessels
Eudicots	Herbs, shrubs, lianas, trees	Extensive bifacial vascular cambium, vessels (but tracheids in *Trochodendron* and *Tetracentron*)

In addition the Chloranthaceae (a small clade of tropical shrubs of uncertain placement but near magnoliids) has characters of magnoliids. *Ceratophyllum*, an anomalous aquatic genus, has no vasculature

Table 3 Major clades of Eudicots

Group	Approx. date of origin of extant group	Tree examples
Basal eudicots	132.5–112.9	*Platanus* (Platanaceae)
Asterids	87.5	*Tectona* (Lamiaceae)
Rosids	108.7	
Rosid (fabid clade)	98.5	*Quercus* (Fagaceae), *Populus* (Salicaceae)
Rosid (malvid clade)	100.5	*Eucalyptus* (Myrtaceae), *Acer* (Sapindaceae)

Dates follow recent fossil-calibrated molecular dating studies (Magallon et al. 2013, 2015)

large trees on the planet (Wang et al. 2009). It may be divided into two groups: the fabids (the richest clade of large angiosperm trees) and the malvids. The fabids include poplar and oak among many other timbers, while the malvids contain eucalyptus and maple (see Table 4). The fabid order Malpighiales (the order that includes the largely tropical families Salicaceae and Euphorbiaceae) has diversified strongly as small trees in the tropical forest understory (Davis et al. 2005). The initial diversification of this clade (estimated in the mid-Cretaceous) has therefore been taken as a marker of the closure of the canopy of a newly angiosperm-dominated wet tropical forest (Davis et al. 2005).

The distribution of large timber trees is far from random. Figure 2 shows all the families of flowering plants with at least one species of commercial timber (Mark et al. 2014) mapped on (inner circle). The outer circle shows the distribution of the most important timber families (i.e. reported as having at least three major timber species). The concentration in the rosid clade can be readily seen (see legend to Fig. 2).

The evolution of the rosid clade enabled the dominance of angiosperms in forest ecosystems. Within the rosids, the tree morphospace has been extensively explored over the last 100 million years. The range of tree architecture and growth characteristics is staggering: from fat trunks like baobabs (*Adansonia*) to the slender growth of *Trema*; from the huge leaves of *Cecropia* to the small sclerophyllous leaves of *Myrtus*; from slow-growing oaks to fast growing poplars. The multitude of strategies in pursuit of size make angiosperm trees very promising subjects for comparative study.

Table 4 Important timber families

Family	No. of species in family with >5 sources	Order	Group	Sub-group	Example genera (genera with temperate species in bold)
Leguminosae	89	Fabales	Rosidae	Fabidae	*Millettia, Dalbergia, Hymenaea*
Fagaceae	12	Fagales	Rosidae	Fabidae	***Castanea, Fagus, Quercus***
Moraceae	9	Rosales	Rosidae	Fabidae	*Bagassa, Milicia, Antiaris*
Betulaceae	8	Fagales	Rosidae	Fabidae	***Alnus, Betula, Carpinus***
Juglandaceae	7	Fagales	Rosidae	Fabidae	***Juglans, Carya,*** *Engelhardia*
Ulmaceae	6	Rosales	Rosidae	Fabidae	***Ulmus,*** *Phyllostylon,* ***Zelkova***
Salicaceae	5	Malpighiales	Rosidae	Fabidae	***Populus****, Casearia,* ***Salix***
Clusiaceae	4	Malpighiales	Rosidae	Fabidae	*Calophyllum, Symphonia, Garcinia*
Zygophyllaceae	4	Zygophyllales	Rosidae	Fabidae	*Guiaicum, Bulnesia*
Euphorbiaceae	3	Malpighiales	Rosidae	Fabidae	*Hevea, Endospermum, Hura*
Irvingiaceae	3	Malpighiales	Rosidae	Fabidae	*Irvingia, Klainedoxa*
Rosaceae	3	Rosales	Rosidae	Fabidae	***Prunus, Malus, Pyrus***
Meliaceae	23	Sapindales	Rosidae	Malvidae	*Swietenia, Entandrophragma, Khaya*
Dipterocarpaceae	17	Malvales	Rosidae	Malvidae	*Shorea, Dipterocarpus, Dryobalanops*
Malvaceae	16	Malvales	Rosidae	Malvidae	*Ochroma, Tarrietia, Triplochiton, Ceiba*
Anacardiaceae	9	Sapindales	Rosidae	Malvidae	*Mangifera, Astronium, Campnosperma*
Myrtaceae	7	Myrtales	Rosidae	Malvidae	***Eucalyptus****, Melaleuca*
Sapindaceae	7	Sapindales	Rosidae	Malvidae	***Acer, Aesculus****, Allophylus*
Combretaceae	4	Myrtales	Rosidae	Malvidae	*Terminalia*
Rutaceae	3	Sapindales	Rosidae	Malvidae	*Euxylophora, Balfourodendron, Chloroxylon*
Vochysiaceae	3	Myrtales	Rosidae	Malvidae	*Erisma, Qualea*
Sapotaceae	9	Ericales	Asteridae		*Tieghemella, Manilkara, Baillonella*

(continued)

Table 4 (continued)

Family	No. of species in family with >5 sources	Order	Group	Sub-group	Example genera (genera with temperate species in bold)
Rubiaceae	6	Gentianales	Asteridae		*Fleroya, Nauclea, Neolamarckia*
Apocynaceae	5	Gentianales	Asteridae		*Dyera, Aspidosperma, Gonioma*
Bignoniaceae	4	Lamiales	Asteridae		*Handroanthus, Jacaranda, Roseodendron*
Ebenaceae	4	Ericales	Asteridae		*Diospyros*
Oleaceae	4	Lamiales	Asteridae		***Fraxinus***
Lamiaceae	3	Lamiales	Asteridae		*Tectona, Gmelina, Vitex*
Lecythidaceae	3	Ericales	Asteridae		*Bertholletia, Cariniana, Couratari*
Olacaceae	3	Santalales	Asteridae		*Scorodocarpus, Minquartia, Ongokea*
Lauraceae	8	Laurales	Magnoliid		*Chlorocardium, Ocotea, Eusideroxylon*
Myristicaceae	4	Magnoliales	Magnoliid		*Pycnanthus, Virola, Staudtia*

Families with at least three examples of well-known timbers (i.e. mentioned in at least five sources in Mark et al. 2014)

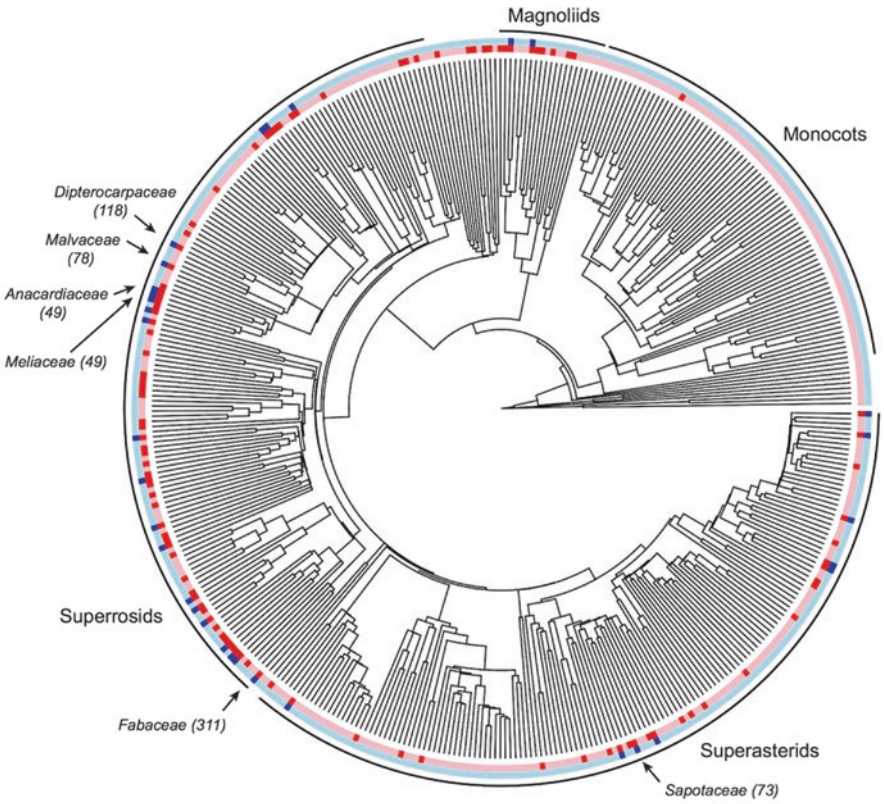

Fig. 2 Phylogenetic distribution of traded timber. Phylogenetic tree of the angiosperms (Qian and Zhang 2014) with inner ring (*red*) shows families with at least one species used as timber in trade according to a standard list (Mark et al. 2014). Timber species are defined as species reported as having traded timber by two or more of 17 sources. The outer ring shows important timber families (families with at least three timber species reported by five or more sources) in *blue*. The top six families in terms of number of species used as timber are indicated. Note that there are very few timber species in early divergent angiosperms and monocots. Asterids too have relatively few timber bearing families. Most timber species are in the Rosid clade

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Variation in Angiosperm Wood Structure and Its Physiological and Evolutionary Significance

Rachel Spicer

Abstract Angiosperms show extensive variation in wood structure that reflects their evolutionary diversification and adaptation to a wide range of environments. Evolutionary shifts between an herbaceous and woody habit are common and have produced a wide array of growth forms, including herbs, lianas, succulents, trees and shrubs. As angiosperms moved into new environments, wood structure changed to reflect a balance between functions in water transport and storage, carbohydrate and mineral nutrient storage, and mechanical support. The extent and timing of wood production were modified and new cell types were produced in varying proportions and spatial arrangements. In particular, taxonomic variation in water conducting elements and parenchyma distribution affect a plant's ability to withstand drought and freezing temperatures. In this chapter, I interpret the evolutionary significance of variation in angiosperm wood structure with references to biogeography, phylogenetics, molecular development, ecophysiology and paleobotany. Cell type-specific techniques in gene expression will continue to be key to the study of these processes.

Keywords Cambium • Xylem • Sapwood • Heartwood • Vessel • Embolism • Parenchyma • Fiber • Succulent • Liana • Tension wood

A Primer on Secondary Growth and Woody Stem Biology

So what exactly does it mean to be a woody plant? Naturally any plant that forms wood is considered a woody plant, but what is wood? Wood is, by definition, xylem produced by the vascular cambium. More specifically, wood is defined as *secondary xylem* because it is produced by a secondary meristem – one that develops from tissue that has at least partially differentiated (Fig. 1). In contrast, *primary xylem* is

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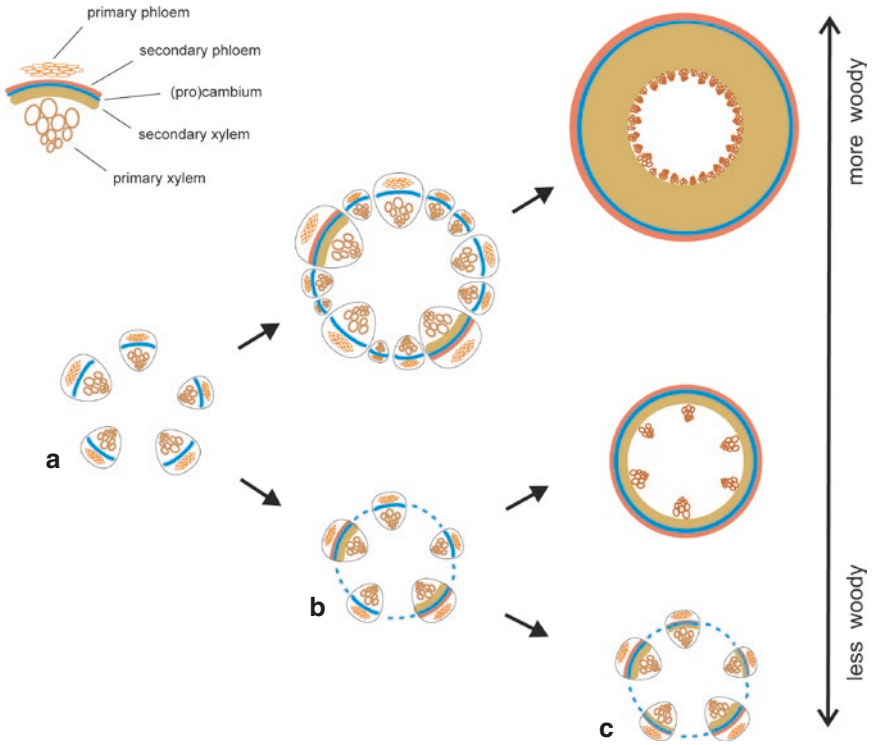


Fig. 1 The production of secondary growth via the activity of the vascular cambium. Separate vascular bundles are formed during primary growth (**a**), which serves to elongate young shoot and root axes. Primary xylem and phloem differentiate from the procambium, which is retained in most species as a narrow band of meristematic tissue located between the two vascular tissues. The isolated bands of procambium within each bundle are then gradually unified to produce the vascular cambium (**b**), although there is variation in the manner and extent to which complete unification occurs. In many trees, new vascular bundles arise in the space between existing bundles (i.e., the 'interfasicular region') such that the bundles coalesce (**b**; *upper* illustration). In other cases, especially those of less woody plants, parenchyma cells in the interfascicular region appear to dedifferentiate to form a cambium (**b**; *lower* illustration). The vascular cambium then produces secondary xylem (i.e., wood) to the inside and secondary phloem to the outside (**c**). There is considerable variation in how much wood is ultimately produced, and in some species secondary growth is restricted to the bundles (i.e., the fascicular regions) with little to no evidence of a cambium between them (Based on Eames and McDaniels (1947) and Beck (2005))

produced by the procambium, a primary meristem derived more directly from root and shoot apical meristems (Esau 1943; Esau 1965). Primary xylem is produced in all elongating roots and shoots and exists in isolated bundles, whereas secondary xylem is produced after elongation growth is complete. The process of secondary growth, which includes the production of secondary phloem in addition to xylem, serves to increase the circumference of an axis. Wood production is typically responsible for most of this increase, but the extent of wood production varies significantly among angiosperms, from essentially none in herbs to extensive in trees.

A woody stem is thus any stem with an active vascular cambium, a meristem that is in turn the source of all wood production. In most angiosperm trees, a single vascular cambium exists and produces secondary xylem toward the inside of the stem and secondary phloem toward the outside (Esau 1960).

Wood is typically specialized for the transport of water from the roots to the leaves and to provide mechanical support for foliage and reproductive organs, but it can also serve as a storage tissue for water, carbohydrates, and mineral nutrients. The structural design of wood thus represents a balance among multiple functional demands, and the great diversity of wood anatomy within angiosperms reflects the capacity for stems to specialize and develop structural and physiological adaptations to new environments. Much of the variation found in angiosperm wood derives from the production of an increasingly diverse array of cell types by the vascular cambium. The relative abundance of these cell types, their spatial arrangement within wood, the nature of physical connections among cells, and the extent of activity of the vascular cambium in different plant organs all contribute to the adaptive significance of wood. Lastly, although the production of a woody stem is an obvious route to becoming a tree, an increase in vertical stature is not the sole benefit derived from wood and it is important to not conflate secondary growth (i.e., woodiness) with an arboreal habit. Indeed, some of the most specialized forms of wood – in succulents, lianas and geophytes, for example – have evolved under complex selective pressures.

The goal of this chapter is to identify important aspects of natural variation in angiosperm wood production and to highlight the functional implications of this variation. Major themes in the structural diversity of woody stems will be framed in terms of evolutionary and developmental processes, and physiological and ecological consequences.

Variation in the Extent of Woodiness: The Woody-Herbaceous Continuum

Woodiness is thought to be ancestral in angiosperms, with the first flowering plants likely producing small to moderate amounts of wood, perhaps as shrubs (Feild and Arens 2007; Feild et al. 2004; Sinnott and Bailey 1915; APG 2016; Philippe et al. 2008; Stebbins 1965). The subsequent diversification of angiosperms shows remarkable variation in the degree of woodiness with the evolution of everything from trees, shrubs, and lianas to scrambling or creeping forms and the so-called ‘woody herbs’. This variation was achieved through shifts in the activity of the vascular cambium, such that woody and herbaceous habits exist along a continuum rather than as a dichotomy (Carlquist 2013; Lens et al. 2012a). Indeed, there is subtle variation in the developmental origin of the vascular cambium among plants with varying degrees of woodiness (Fig. 1; Beck 2005; Eames and MacDaniels 1947). Many plants considered herbaceous actually form small amounts of secondary xylem (e.g., Dickison 1996; Carlquist and Zona 1988; Carlquist 1993, 1995; Carlquist et al. 1995) and a

complete absence of the vascular cambium appears to be quite rare. Notable exceptions are the monocots (although see Arber 1925 (digitally reprinted 2010)) and the aquatic groups Nymphaeales and *Nelumbo*, which lack a vascular cambium entirely (Williamson and Schneider 1993; Philipson and Ward 1965; Philipson et al. 1971; Weidlich 1976; Borsch et al. 2008; Carlquist et al. 1995). Evolutionary transitions along this woody-herbaceous continuum have occurred in both directions (i.e., woodiness has both increased and decreased) and the labile nature of this trait has likely contributed to the diversification of angiosperms.

Shifts toward a more herbaceous habit via a decrease in wood production appear more common than the reverse (Dodd et al. 1999; Lens et al. 2013) and have been interpreted as an adaptation (or pre-adaptation) to seasonal climates, where aboveground organs dieback with the onset of freezing and survive as underground organs or as seed (Bailey and Sinnott 1914; Sinnott and Bailey 1915; Wing and Boucher 1998; Jud 2015; Isnard et al. 2012; Zanne et al. 2014). Adaptation to drought and seasonally dry climates has also been proposed (Lens et al. 2009; Li et al. 2013). In one clade of legumes, herbaceous forms have evolved from woody ancestors at least ten times, with some taxa returning to a more woody habit (Li et al. 2013). Within the order Saxifragales (sister to the subclass Rosidae) there have been at least two major shifts toward herbaceousness followed by multiple independent reversions back to being woody, with subsequent returns to an herbaceous habit (Soltis et al. 2013). Similarly complex shifts have been observed in Rubiaceae in the Gentianales (Lens et al. 2009).

The term ‘secondary woodiness’ is often applied to woody taxa that are derived from non-woody or herbaceous groups (Fig. 2). Secondarily woody plants were first observed on islands, where the descendants of herbaceous continental ancestors develop woody stems as an evolutionary adaptation following the colonization of an island (Carlquist 1974). This has been termed ‘insular woodiness’ and is a subcategory of secondary woodiness, as a return to a woody habit can occur on continents as well. Although once controversial (Mabberley 1982), the idea that an herbaceous plant could give rise to a woody plant – in some cases significantly woody (e.g., a shrub or tree) – is now well established (e.g., Carlquist 1959; Carlquist 1969; Carlquist 1970, 1985, 1992b; Baldwin 2007; Baldwin et al. 1991; Ballard and Sytsma 2000; Bohle et al. 1996; Kidner et al. 2016). Indeed, it appears to be a strikingly common phenomenon. In most cases this simply represents an increase in the activity of an existing but minimally-active cambium, often accompanying a shift from annual to perennial growth following the colonization of a region with a more moderate climate. But adaptation to more arid conditions has also been proposed for certain groups (Lens et al. 2013). The fact that an increase in woodiness has occurred in such diverse environments underscores both the multipurpose nature of wood and its developmental and evolutionary flexibility. The ease with which this shift occurs reflects the fact that herbaceous and woody plants exist along a continuum, and that this continuum is a practical consequence of development. Primary growth, which defines an herbaceous habit, is continuous with secondary growth because the procambium in elongating shoots is continuous with the vascular cambium below. Not surprisingly, there is considerable overlap in genetic modules regulating meristematic activity and xylem development during primary and secondary growth (Du and Groover 2010; Du et al. 2011; Groover et al. 2006; Robischon et al. 2011; Spicer and

Fig. 2 Examples of secondarily woody species (**a**, **b**, **e**) and 'herbaceous' relatives (**c**, **d**). *Aeonium urbicum* (**a**) on the Canary Islands, Spain [Saxifragales/Crassulaceae]; *Dubautia laxa* (**b**) on the Hawaiian Islands and closely related *Carlquistia muirii* (**c**) in California, USA [Asterales/Asteraceae]; *Echium creticum* (**d**) in Spain and closely related *Echium simplex* (**e**) on Tenerife, Canary Islands, Spain [Boraginales/Boraginaceae]. Photo credits and copyright licenses: (**a**) ©Römert, Creative Commons Attribution-Share Alike 3.0 Unported; (**b**) ©Forest and Kim Starr, Creative Commons 2.0; (**c**) ©Chris Winchell, permission to reuse granted by copyright holder, all rights reserved; (**d**) ©Javier Martin, image released to public domain; (**e**) ©Krzysztof Ziarnek, Creative Commons Attribution-Share Alike 4.0 International

Groover 2010). Emerging studies comparing gene expression in secondarily woody taxa and their herbaceous ancestors suggest a promising approach to elucidate the genetic mechanisms regulating these switches (Kidner et al. 2016; Moyers and Rieseberg 2013).

Cases of secondary woodiness should provide insight into the process by which taxa develop, or re-develop, cambial activity. Interestingly, Arabidopsis may serve as a good

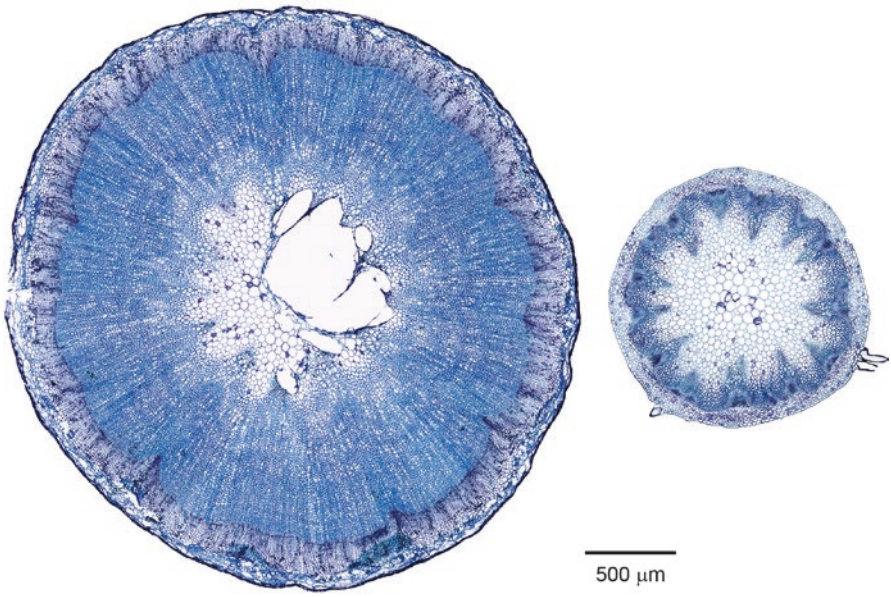


Fig. 3 Wood production in Arabidiopsis near the base of the inflorescence stem. A significant amount of wood is produced in the double mutant *soc1 ful* (*left*), in which flowering time is delayed (Melzer et al. 2008). The herbaceous wildtype Col accession (*right*, shown at same scale) produces a negligible amount of wood, but there is limited cambial activity within the vascular bundles and evidence of cambium differentiation between them (i.e., within the interfascicular region) (Adapted from Lens et al. (2012b) with permission of John Wiley & Son, Inc. Photo credit: Frederic Lens)

model for secondary woodiness (Lens et al. 2012b; Rowe and Paul-Victor 2012), especially in the context of a woody-herbaceous continuum. The inflorescence stem of Arabidopsis develops secondary growth in response to mechanical stimulation (i.e., static load bearing; Ko et al. 2004; Paul-Victor and Rowe 2011) and delay of flowering by physical (Lev-Yadun 1994; Chaffey et al. 2002) or genetic means (Melzer et al. 2008). When flowering was delayed by knocking out two MADS box genes, Arabidopsis produced a substantial cylinder of wood (Fig. 3) that bore striking anatomical similarity to other woody Brassicaceae (Melzer et al. 2008). Even wild type Arabidopsis (Columbia accession) shows limited cambial activity near the base of the inflorescence, although it is largely restricted to the vascular bundles and produces negligible amounts of wood (Fig. 3; Lens et al. 2012b). More extensive secondary growth forming a complete cylinder has also been reported (Sehr et al. 2010), but the restriction of secondary growth to the vascular bundles (i.e., the 'fascicular' region) is characteristic of other minimally woody herbs (Carlquist 1993; Carlquist et al. 1995) (Fig. 1c). In other groups characterized by frequent shifts in woodiness, the type of wood produced by the vascular cambium may differ markedly between the fascicular and interfascicular regions (Carlquist and Zona 1988; Carlquist 1993, 1995; Isnard et al. 2012). Interestingly, evolutionary shifts along the woody-herbaceous continuum appear to be more frequent when intermediate forms are present and may facilitate the

production of novel growth forms (e.g., lianas, succulents, rosette trees, etc.; Beaulieu et al. 2013; Isnard et al. 2003, 2012; Wagner et al. 2012, 2014; Rowe et al. 2004). However, while secondary woodiness does co-occur in groups with a high degree of anatomical variation, it is unclear if the relationship is causal.

In the case of taxa in which the vascular cambium is truly absent, secondary woodiness has been achieved through the origin of a completely novel meristem. This has occurred repeatedly in the monocots, including palms, 'woody' grasses like bamboo, and members of Asparagales (e.g., in the genera *Cordyline*, *Yucca* and *Dracaena*). Because these growth forms are derived from several non-homologous meristems they are not discussed here further; see Jura-Morawiec et al. 2015; Rudall 1991; Cheadle 1937; and Tomlinson and Zimmermann 1967 for reviews of secondary growth in monocots.

Variation in Cellular Composition and Arrangement: Specialization Through a Division of Labor

The first angiosperms are thought to have produced wood composed primarily of tracheids – multipurpose, dead-at-maturity, imperforate (i.e., closed at the ends but with pits in the cell wall) cells that functioned in both water conduction and mechanical support. Key to the evolution of woody angiosperms was the development of vessel elements for water conduction and fibers for mechanical support, both derived from the ancestral tracheid. Further specialization occurred within these cell types, and although the living cells in wood (i.e., parenchyma) appear to have undergone less modification, entirely new forms of living cells were added over time as well. The appearance of new cell types and new spatial arrangements is one of the primary means of evolutionary adaptation in woody tissue.

Vessels, Vessel Patterning and Xylem Water Transport

The vessel is the main functional unit of water conduction in angiosperm stems and is considered one of the most important anatomical innovations in plant evolution (Sperry 2003; Doyle and Donoghue 1986; Bailey 1944b; Bond 1989). Vessels are formed when a longitudinally aligned series of cells – the vessel elements – differentiate in a coordinated manner such that the transverse end walls are enzymatically degraded, coincident with the loss of the protoplast during programmed cell death (Butterfield 1995; Butterfield and Meylan 1982; Meylan and Butterfield 1981; Jacobsen et al. 2015). Vessels are thus long hollow tubes ranging in length from centimeters to meters, composed of many cells, through which water can flow unimpeded (Jeje and Zimmermann 1979; Zimmermann and Jeje 1981; Jacobsen et al. 2012). This is an important distinction from tracheids, where each individual cell is typically one to several millimeters long (Sperry et al. 2006; Feild et al. 2000a) and water must flow from one cell to another through pits (holes of varying shape and sizes) in the cell wall. Cell wall pits retain a porous primary cell wall

structure (termed the pit 'membrane' but unrelated to the plasma membrane) and represent a significant source of resistance to water flow (Sano 2005; Hacke et al. 2006; Lancashire and Ennos 2002; Choat et al. 2008, 2004). Vessels retain pits on their lateral walls ('intervessel' pits) to allow water to flow from one vessel to another (e.g., Bolton et al. 1987; Orians et al. 2004), but due to the greater length of vessels relative to tracheids, the contribution of pits to resistance is typically much reduced (Choat et al. 2008). Vessels also expand more laterally than tracheids prior to secondary cell wall deposition, and this larger lumen diameter combined with lack of end walls creates a low resistance pathway for highly efficient water transport (Sperry et al. 2005, 2006). This key innovation likely shaped angiosperm evolution by allowing them to colonize new environments, not only due to improved water transport capacity, but also by allowing for functional specialization in other areas like mechanical support (McCulloh et al. 2010; Doyle and Donoghue 1986; Sperry 2003; Sperry et al. 2007; Hudson et al. 2010).

Vesselless Woods

Although vessels are considered a hallmark of angiosperm evolution, several angiosperm groups are described as 'vesselless' and produce wood composed almost entirely of tracheids (Fig. 4). *Amborella*, a woody plant strongly supported as the oldest extant angiosperm, has wood composed entirely of tracheids, but in some cases there are large pores in the primary cell wall where two adjacent tracheids overlap (i.e., in nascent 'end walls'; Feild et al. 2000a; Bailey and Swamy 1948; Carlquist and Schneider 2001). Several other basal groups produce tracheid-based wood, including members of Winteraceae in the magnoliids (Bailey 1944a) and the genus *Sarcandra* in the Chloranthales (Swamy and Bailey 1950). In both of these groups, a close look at anatomy has revealed cell types that are transitional between tracheids and vessels, with extensive pitting and partial or full degradation of the primary cell wall where tracheid ends overlap (Carlquist 1983, 1989; Feild et al. 2000a; Carlquist 1987b, 1992a). Although their basal position suggests that tracheids may have been retained in these groups, it is also possible that early vessels were lost, and it is important to place the concept of loss or gain of vessels in the context of ancestors in which transitional cell types existed (Baas and Wheeler 1996; Carlquist 1996a). Trochodendraceae, a group with vesselless wood near the base of the eudicots, is even more enigmatic (Bailey and Nast 1945; Kim et al. 2004; Hacke et al. 2007; Li et al. 2011). Interestingly, early vessels and transitional cell types may not have offered much in the way of increased flow rates due to their short length, high resistance end walls and infrequent occurrence (Hacke et al. 2007; Sperry et al. 2007). Instead, early vessels may have simply provided the opportunity for specialization in the future, with wider, longer, low resistance vessels and stronger thick-walled fibers appearing later in evolution. Similarly, the retention or possible 're-invention' of tracheids in angiosperms with vessels (discussed below) underscores their utility.

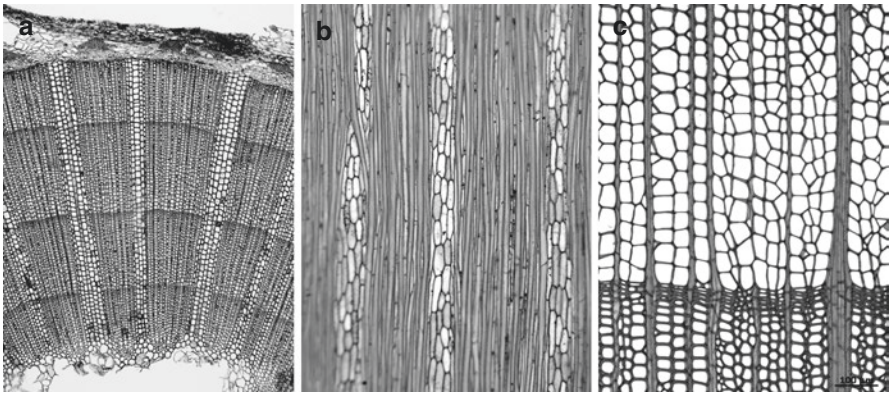


Fig. 4 Woods of angiosperms traditionally described as vesselless. *Sarcandra glabra* [Chloranthales/Chloranthaceae] stem wood in transverse (**a**) and tangential longitudinal section (**b**) shows tracheids with increasingly narrow lumen diameters at growth ring boundaries and tangential walls lacking pits. Multiseriate rays consist of a single parenchyma cell type with cell diameters larger than neighboring tracheids (scale bar not available but tracheid lumens are reported to be 10–20 μm wide). *Tetracentron sinense* [Trochodendrales/Trochodendraceae] stem wood in transverse section (**c**) showing a single growth ring boundary. Note that tracheary elements transitional between tracheids and vessel elements have been reported in both species based on remnants of degraded pit membranes in end walls (All images courtesy of InsideWood online database and reproduced with permission of the original contributors (Wheeler 2011). Photo credits and copyright: (**a, b**) ©Forestry and Forest Products Research Institute, Tsukuba, Japan; ©Peter Gasson, Kew Garden, Richmond, United Kingdom)

Vessel Distribution Patterns and Their Hydraulic Consequences

The vast majority of angiosperms produce wood with vessels that are specialized for water conduction, whose large lumens appear as 'pores' in transverse section with little to no magnification (Hoadley 1990). These pores stand out against a background of thick-walled fibers, parenchyma and in some cases, tracheids. Vessels are arranged spatially within wood in taxon-specific patterns that exhibit tremendous diversity and have great physiological significance. These patterns are achieved primarily through changes in vessel diameter throughout the growing season (i.e., in temperate or seasonally dry climates), the frequency of vessels per unit area of wood, and vessel clustering.

Changes in vessel diameter throughout the growing season – what wood anatomists describe as 'porosity' – has led to a system in which angiosperm woods are classified as ring porous, diffuse porous, or semi-ring porous (an intermediate condition, alternatively termed semi-diffuse porous; IAWA 1989; Panshin and de Zeeuw 1980). In ring porous woods, the first-formed vessels in a growth ring (the so-called 'earlywood' vessels) are significantly larger than later-formed, or 'latewood' vessels (i.e., there are two distinct size classes of vessels; Fig. 5). In contrast, in diffuse porous woods the vessels are roughly similar in diameter throughout a growth ring (Fig. 6). Intermediate cases include woods in which there is continuous,

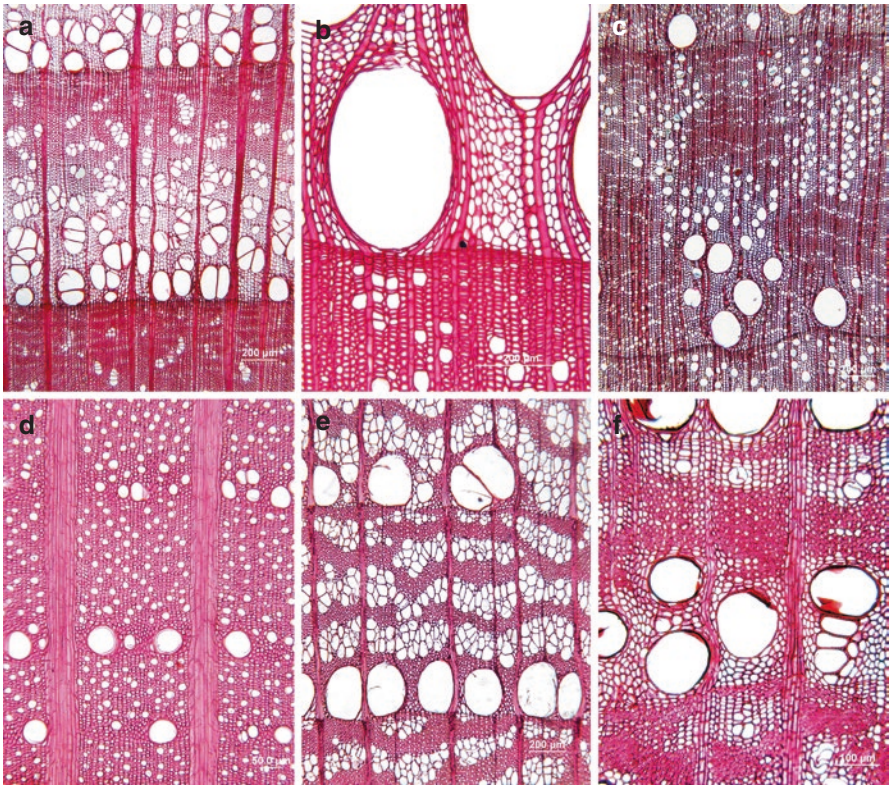


Fig. 5 Transverse sections of ring porous woods showing large earlywood vessels and a diversity of vessel spatial arrangements and clustering patterns. Species shown are (**a**) *Asimina triloba* [Magnoliales/Annonaceae], (**b**) *Castanea dentata* [Fagales/Fagaceae], (**c**) *Castanopsis fordii* [Fagales/Fagaceae], (**d**) *Koeberlinia spinosa* [Brassicales/Koeberliniaceae], (**e**) *Ulmus americana* [Rosales/Ulmaceae], (**f**) *Sophora affinis* [Fabales/Fabaceae] (All images courtesy of InsideWood (http://insidewood.lib.ncsu.edu/) and reproduced with permission of the original contributors (Wheeler 2011). Photo credits and copyright: (**a**–**f**) ©Elisabeth Wheeler, North Carolina State University, Raleigh, N.C., USA)

gradual or minor variation in vessel diameter throughout the growing season (e.g., woods in Fig. 6a, d could be classified as semi-ring porous), and although useful, these terms may not capture the full range of variation found in nature. For reasons described below, vessel distribution patterns can have a significant effect on xylem water transport. In general, water conduction is restricted to the most recently-produced wood (i.e., the outermost cylinder) in ring porous species, whereas conduction occurs throughout the sapwood in diffuse porous species, where many years of growth remain functional in water conduction (Gebauer et al. 2008; Spicer and Holbrook 2005; Phillips et al. 1996). The smaller vessels formed in the latter part of the growing season in ring porous species may remain functional for several years, but the large earlywood vessels typically only function for a single growing season (Umebayashi et al. 2008; Ellmore and Ewers 1985).

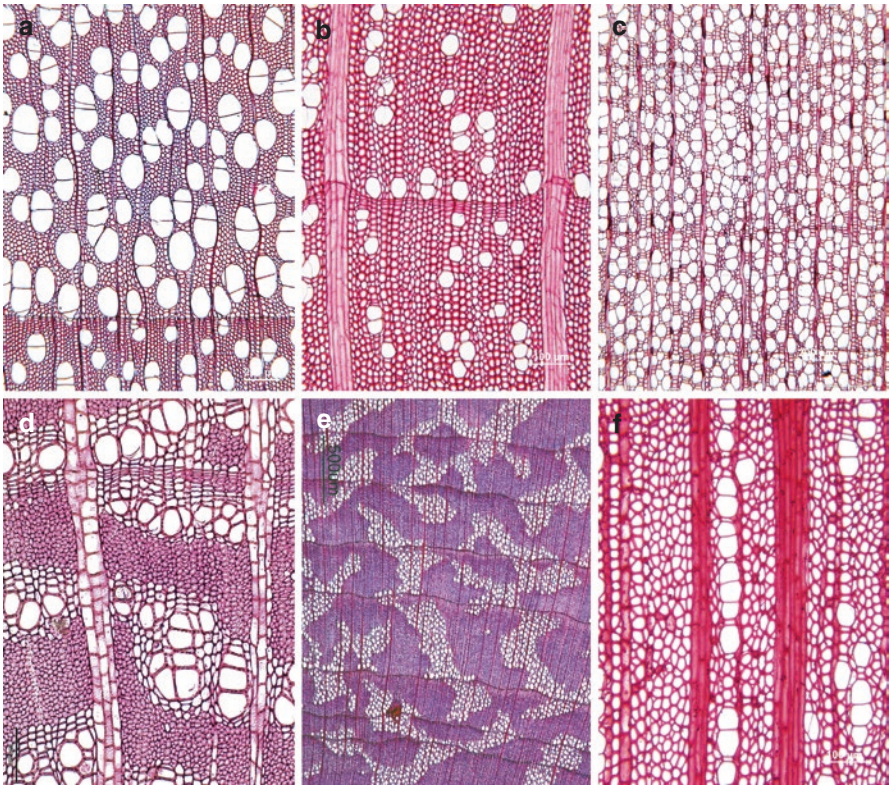


Fig. 6 Transverse sections of diffuse porous woods showing relatively uniform vessel diameter and a range of vessel density and clustering patterns. Species shown are (**a**) *Populus deltoides* [Malpighialies/Salicaceae], (**b**) *Ilex decidua* [Aquifoliales/Aquifoliaceae], (**c**) *Liquidambar styraciflua* [Saxifragales/Altingiaceae], (**d**) *Anagyris foetida* [Fabales/Fabaceae], (**e**) *Rhamnus chugokuensis*, (**f**) *Ilex cassine* [Aquifoliales/Aquifoliaceae] (All images courtesy of InsideWood (http://insidewood.lib.ncsu.edu/) and reproduced with permission of the original contributors (Wheeler 2011). Photo credits and copyright: (**a**–**d**, **f**) ©Elisabeth Wheeler, North Carolina State University, Raleigh, N.C., USA; (**e**) Forestry and Forest Products Research Institute, Tsukuba, Japan)

Vessel diameter has major functional implications in woody plants, where large diameter vessels are able to transport water more efficiently but are also more likely to become 'embolized' (McCulloh et al. 2010; Sperry et al. 2006, 2007; Hacke et al. 2016; Tyree et al. 1994). Embolized vessels are filled with air (i.e., a single air-filled vessel is said to contain an embolism) and are thus unable to conduct water, a condition that occurs following freeze-thaw events or when the tension in the xylem exceeds a certain threshold, as may occur during drought. The relationship between vessel diameter and freeze-thaw embolism is very robust, where wide vessels are vulnerable and narrow vessels are resistant to loss of function following a freeze (Davis et al. 1999; Sperry 1994; Sperry and Sullivan 1992; Tyree and Sperry 1989). This is due to the fact that vessel liquid volume, which is a function of diameter,

determines the amount of air dissolved in a vessel – air that comes out of solution as water freezes and forms an embolism upon thawing (Sevanto et al. 2012; Pittermann and Sperry 2003, 2006; Robson et al. 1988; Robson and Petty 1987). Wide vessels also tend to be more vulnerable to drought-induced embolism than narrow vessels, but this relationship is far more complex and less predictable, in part because the process of drought-induced embolism formation is not as well understood (Cochard et al. 1992; Hargrave et al. 1994). In general however, wide vessels are more likely to be found in climates with abundant water and/or little risk of freezing.

Water flows from one vessel to another through pits in the cell wall where vessels come in contact, and there is substantial variation in the degree to which vessels are interconnected (Brodersen et al. 2011; Carlquist and Zona 1988; Carlquist 2009a; Choat et al. 2008). Vessels may be solitary (Fig. 5c, d) or occur in pairs or larger groups. When vessels are grouped, they may be arranged in radial series or 'chains' (i.e., oriented from pith to bark; Fig. 6f), tangential bands (i.e., oriented parallel to the circumference of the tree; Fig. 5e), or simple clusters of a range of shapes and sizes (Figs. 5a and 6a, c, d). These patterns are highly taxon-specific and represent key features in wood species identification, but they also have functional implications (Carlquist 2009a). Although less emphasis has been placed on vessel arrangements than on vessel diameter, the extent to which vessels are interconnected affects both vulnerability to embolism and the impact of an embolism once it forms (Wheeler et al. 2005; Hacke et al. 2006, 2016; Christman et al. 2009; Loepfe et al. 2007). Air may be drawn through the pit membrane from an embolized vessel into a neighboring water-filled vessel under tension, such that highly interconnected vessels are vulnerable to embolism spread (Brodersen et al. 2013). In contrast, if vessels of varying diameters are interconnected, narrow vessels may remain functional and able to conduct water (albeit at a slower rate) once larger diameter vessels have embolized, thus providing a form of safety net (Loepfe et al. 2007). As discussed in the next section, a diversity of tracheids may serve in this role as well. Lastly, the extent to which vessels are interconnected determines 'sectoriality' of flow, where portions of the crown may be supplied by isolated regions of the xylem (Ellmore et al. 2006; Orians et al. 2004). This phenomenon has implications for pest and pathogen resistance as well as mineral nutrient and carbohydrate distribution.

Co-occurrence of Vessels and Tracheids

Some angiosperms produce wood with both vessels and tracheids, defined here as imperforate cell types that function in water conduction (i.e., tracheids are closed at the ends but have substantial cell wall pitting). This condition is far more common than often recognized, with about 10 % of the species in the InsideWood database (over 7000 species described; http://insidewood.lib.ncsu.edu) recorded as having both tracheids and vessels. Terminology with respect to tracheids is problematic (see Carlquist 2001c; Rosell et al. 2007 for discussion), but two types are generally

recognized by the wood anatomy community – vascular tracheids, which are formed late in the growing season and intergrade with narrow vessel elements, and vasicentric tracheids, which are found adjacent to vessels in ring porous species, most notably around the large earlywood vessels (IAWA 1989; Panshin and de Zeeuw 1980). Phylogenetic reconstructions suggest that both vascular and vasicentric tracheids are derived, where vascular tracheids in particular are essentially just narrow latewood vessels with an intact end wall (Baas and Wheeler 1996). Indeed, vascular tracheids intermingle with latewood vessels in some species – even within a longitudinal series – and it is often impossible to distinguish them in cross section (Fig. 5e; in *Ulmus* latewood tracheids and narrow diameter vessels form wavy tangential bands (Panshin and de Zeeuw 1980)). Some researchers also recognize 'true tracheids' as those derived directly from the ancestral gymnosperm tracheid (Carlquist 2001c; Rosell et al. 2007). This distinction, while useful when comparing the tracheids in *Amborella* from those in *Quercus*, for instance, is less useful in derived groups where the evolutionary origin is less clear (e.g., in the tracheid-bearing members of Winteraceae (Doyle 2000; Doyle and Endress 2000; Feild et al. 2002; Bailey 1944a; Feild et al. 2000b; Carlquist 1983, 2000)). A careful look at tracheid development, especially with respect to cell elongation relative to the length of cambial initials (where 'true tracheids' might elongate more during differentiation than derived tracheids), would add to our understanding of these evolutionary transitions.

The spatial organization of tracheids within wood and the biogeography of taxa in which tracheids and vessels co-occur suggest functional specialization, where the addition of tracheids provides a degree of safety for water conduction in variable or extreme environments (Carlquist and Hoekman 1985a; Carlquist 1987a). Extensive pitting on tracheid lateral walls, including pits linking tracheids with neighboring vessels, support their role in water conduction and suggests that tracheids contribute to a complex hydraulic network (Sano et al. 2008; Sano et al. 2011; Carlquist 2001c). Much like small diameter vessels, tracheids may continue to conduct water when larger vessels have embolized due to drought or following freeze-thaw events (Hacke et al. 2016; Ellmore and Ewers 1986). Not surprisingly, tracheids are often found in clusters with vessels, and this condition is particularly common in Mediterranean climates, alpine environments and seasonal dry forests (Pratt et al. 2015; Carlquist and Hoekman 1985a; Carlquist 2001c). Different types of tracheids have also been associated with particular types of vessel groupings (Carlquist 1984, 1987a; Rosell et al. 2007; Yaghmaie and Catling 1984). Although work to resolve the true mechanism is scant (e.g., Hargrave et al. 1994; Pratt et al. 2015), tracheids could stabilize hydraulic capacity through redundancy or by limiting embolism spread.

Given that the ancestral gymnosperm tracheid served both mechanical and water transport functions, it is not surprising that tracheids also intergrade with fibers in angiosperm woods. 'Fiber-tracheids' are recognized as an intermediate cell type, but they are not thought to conduct water in any appreciable way based on the size and shape of cell wall pits (Sano et al. 2008, 2011) and so are not considered in the above discussion.

Fibers and Mechanical Support

Angiosperm wood is commonly composed of 30–80 % fibers by volume, such that fibers are often treated as the 'ground tissue' or bulk mass of the wood in most trees. Fibers are specialized to function in mechanical support and are generally quite long (1–2 mm on average), often doubling in length during differentiation relative to their parent cambial initial (Panshin and de Zeeuw 1980; Bailey 1920). This increase in length is achieved through 'intrusive growth' in which extension and cell wall deposition are restricted to the tips of developing fibers (Wenham and Cusick 1975; Wloch and Polap 1994; Snegireva et al. 2015; Sinnott and Bloch 1939). Fiber length contributes to the mechanical strength of a woody axis, as does wall thickness, which ranges from moderate to extreme (e.g., extremely thick-walled individual fibers can be seen in Figs. 5d, f, 6d and 7e; thinner walled fibers can be seen in Fig. 6f). Two types of fibers are generally recognized and are distinguished by their cell wall pitting – fiber-tracheids have pits with arched borders ('bordered pits'), whereas libriform fibers have pits without discernable borders ('simple pits') (Panshin and de Zeeuw 1980). Fibers are considered non-conductive owing to their narrow lumens and minimal pit connections, but they may be variably filled with air or water at different times of the year (e.g., Ehleringer and Dawson 1992; Utsumi et al. 1996). Interestingly, fibers have been proposed to contain ice under certain freezing conditions and thus contribute to the generation of positive pressure in the xylem of sugar maple during the spring tapping season (Johnson and Tyree 1992; Tyree 1983; Milburn and O'Malley 1984; O'Malley and Milburn 1983). More recently, fiber-tracheids in sugar maple have been shown to connect to vessels via pits while libriform fibers were isolated (Cirelli et al. 2008). Given the range of lumen diameters and variation in pitting connecting fibers to other cell types, it would not be surprising if some fibers had more nuanced functional roles.

A highly specialized type of fiber – the gelatinous fiber – is found in tension wood, which is formed in angiosperms in response to a gravitational stimulus (Groover 2016; Du and Yamamoto 2007; Sinnott 1952). Formed on the upper side of a leaning stem or branch, tension wood is characterized by a reduced density of vessels and a variable proportion of gelatinous fibers depending on the species and intensity of the stimulus (Ruelle 2014; Clair et al. 2006; Fang et al. 2008; Fisher and Stevenson 1981). Gelatinous fibers develop a highly modified cell wall layer – the gelatinous layer (also g-layer), named for the way it reflects light and appears jelly-like – which has an extremely high cellulose content and essentially no lignin (Andersson-Gunneras et al. 2006; Bhandari et al. 2006). Key to the mechanical role of the g-layer, its cellulose microfibrils are oriented axially (i.e., the g-layer microfibril angle is very low) and, in the absence of lignin, interact along their lengths (Mellerowicz and Gorshkova 2012; Nishikubo et al. 2007). Polysaccharides trapped between axially-aligned microfibrils are thought to be responsible for the generation of tension within these fibers (Gorshkova et al. 2015), which shorten during maturation and collectively generate a contractile force in the wood (Clair et al. 2008; Fang et al. 2008; Goswami et al. 2008; Yamamoto et al. 2005). There is thus little doubt of mechanical specialization when it comes to gelatinous fibers.

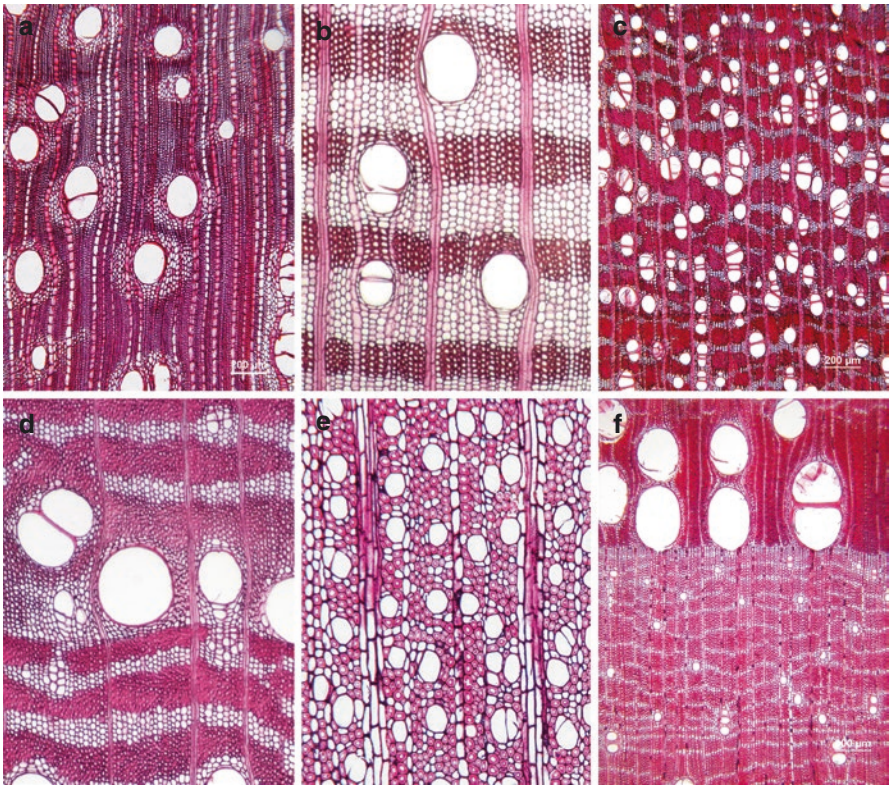


Fig. 7 Transverse sections of angiosperm woods showing a range of axial parenchyma distribution patterns. Parenchyma may ensheath vessels in wide (**a**) or thin layers (**c**, **f**), form tangential bands of varying width and position (**b**–**d**, **f**), or appear in diffuse clusters of one to several cells (**e**). Parenchyma cells are thin-walled and appear light in color against a background of thicker-walled fibers and vessels. Species shown are (**a**) *Anacardium occidentale* [Sapindales/Anacardiaceae], (**b**) *Ficus maxima* [Rosales/Moraceae], (**c**) *Celtis ehrenbergiana* [Rosales/Cannabaceae], (**d**) *Sapindus mukorossi* [Sapindales/Sapindaceae], (**e**) *Viburnum stellatotomentosum* [Dipsacales/Adoxaceae], (**f**) *Carya ovata var. australis* [Fagales/Juglandaceae] (All images courtesy of InsideWood (http://insidewood.lib.ncsu.edu/) and reproduced with permission of the original contributors (Wheeler 2011). Photo credits and copyright: (**a**, **c**, **e**, **f**) ©Elisabeth Wheeler, North Carolina State University, Raleigh, N.C., USA; (**b**, **d**) ©Els Bakker, Naturalis, Leiden, Netherlands)

At the opposite end of the functional spectrum, some fibers retain a living protoplast and thus function more like parenchyma in wood (described below). Living fibers are usually ‘septate’, where a layer of primary wall material divides a single fiber into two or more compartments (Carlquist 2014). Evidence for these cells assuming roles more akin to parenchyma include the presence of starch, often with seasonal fluctuations (Harrar 1946; Dumbroff and Elmore 1977; Plavcova et al. 2016; Yamada et al. 2011), and their positioning adjacent to or surrounding vessels and at the end of a growth ring (Sauter et al. 1973; Carlquist and Hoekman 1985b; Itabashi et al. 1999). In this sense this cell type is difficult to characterize, and may be more appropriately viewed as thick-walled parenchyma.

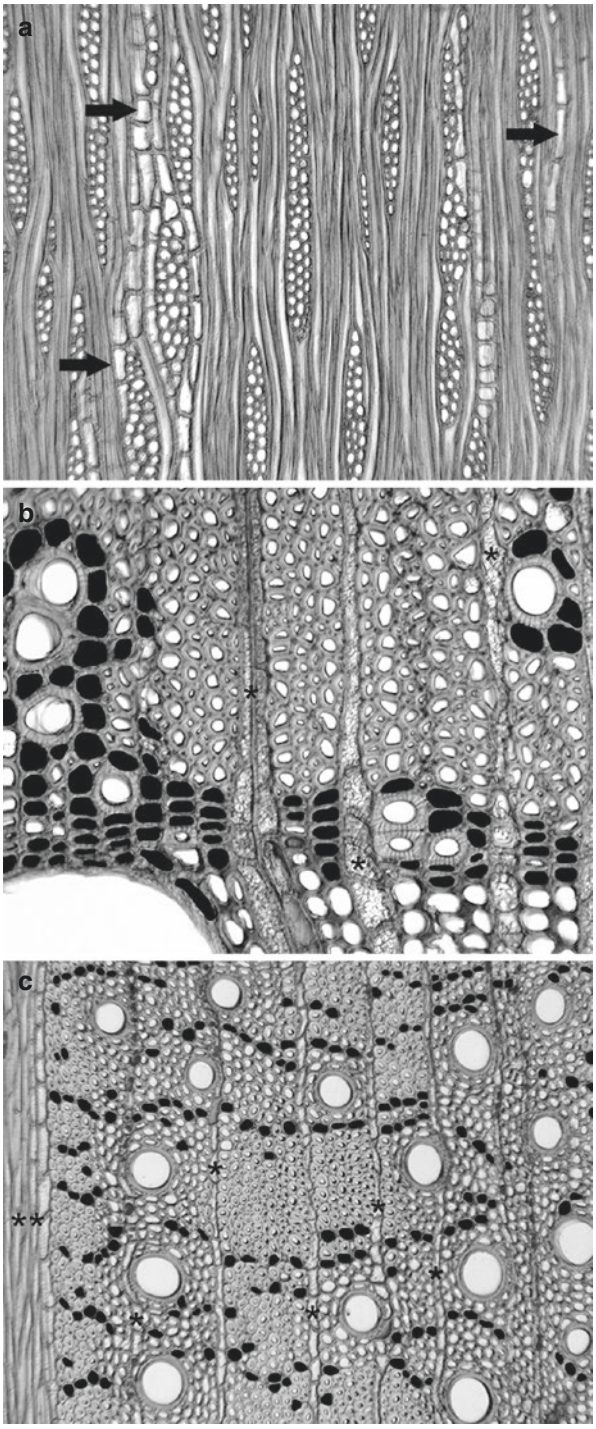
Parenchyma Abundance, Distribution and Functions in Storage, Transport and Protection

Although wood is typically dominated by dead cells – those specialized for mechanical support and water conduction – it also contains living cells, the parenchyma. Parenchyma cells are oriented both longitudinally and radially in woody stems (Fig. 8) and function primarily in carbohydrate storage and transport (Höll 2000), with additional roles in wound response, defense against pathogens, and water storage (Spicer 2014; Carlquist 2015a). There is also evidence that parenchyma cells are involved in the modification, maintenance and repair (i.e., embolism reversal via vessel refilling) of the transpiration stream (Brodersen et al. 2010; Zwieniecki et al. 2001; Lee et al. 2012; Nardini et al. 2011; Secchi and Zwieniecki 2012; Secchi et al. 2011; Secchi and Zwieniecki 2011). Although understudied relative to vessel patterning and hydraulic properties, parenchyma cells form complex three-dimensional networks in stems, frequently occupying 10–30 % of the volume in angiosperm wood, and up to 90 % or more in specialized cases (Spicer 2014). The abundance and spatial distribution of parenchyma cells in wood exhibit taxon-specific patterns, and these features together with the nature of physical connections formed between parenchyma and other cell types – particularly the conductive elements – provide insight into parenchyma function.

Rays and Rayless Woods

Parenchyma cells produced by ray initials in the vascular cambium form vertically-stacked groups that are oriented radially in the stem (i.e., they run from pith to bark) called ‘rays’ (Fig. 9). Rays are continuous through the vascular cambium such that they serve to link secondary xylem and phloem, allowing the exchange of water, carbohydrates, mineral nutrients and signaling molecules between the symplast and apoplast (Höll 1975; van Bel 1990; Pate 1975). Extensive cell wall pitting facilitates transport and exchange of materials, both between cells within a ray and between

Fig. 8 Light microscopy images illustrating the location of axial and ray parenchyma in three angiosperm woods. Axial parenchyma is shown in (**a**) tangential section of *Fraxinus americana* showing multiseriate rays and a background composed predominantly of fibers with some axial parenchyma forming strands (*arrows*); (**b**) cross section of *F. americana* showing an annual ring border with a large earlywood vessel in the lower left-hand corner and smaller latewood vessels above (axial parenchyma cells are painted black with paint tool in image analysis software based on user examination of wall thickness and presence of starch granules); (**c**) cross section of *Quercus rubra* latewood (axial parenchyma painted *black* as above). Ray parenchyma is shown in cross section (**b**, **c**) where a single star (*) indicates a narrow (uni- or biseriate) ray and a double star (**) indicates a large, multiseriate ray. Scale bar = 50 μm (Adapted from Spicer (2014) with permission of Oxford University Press)


a
b
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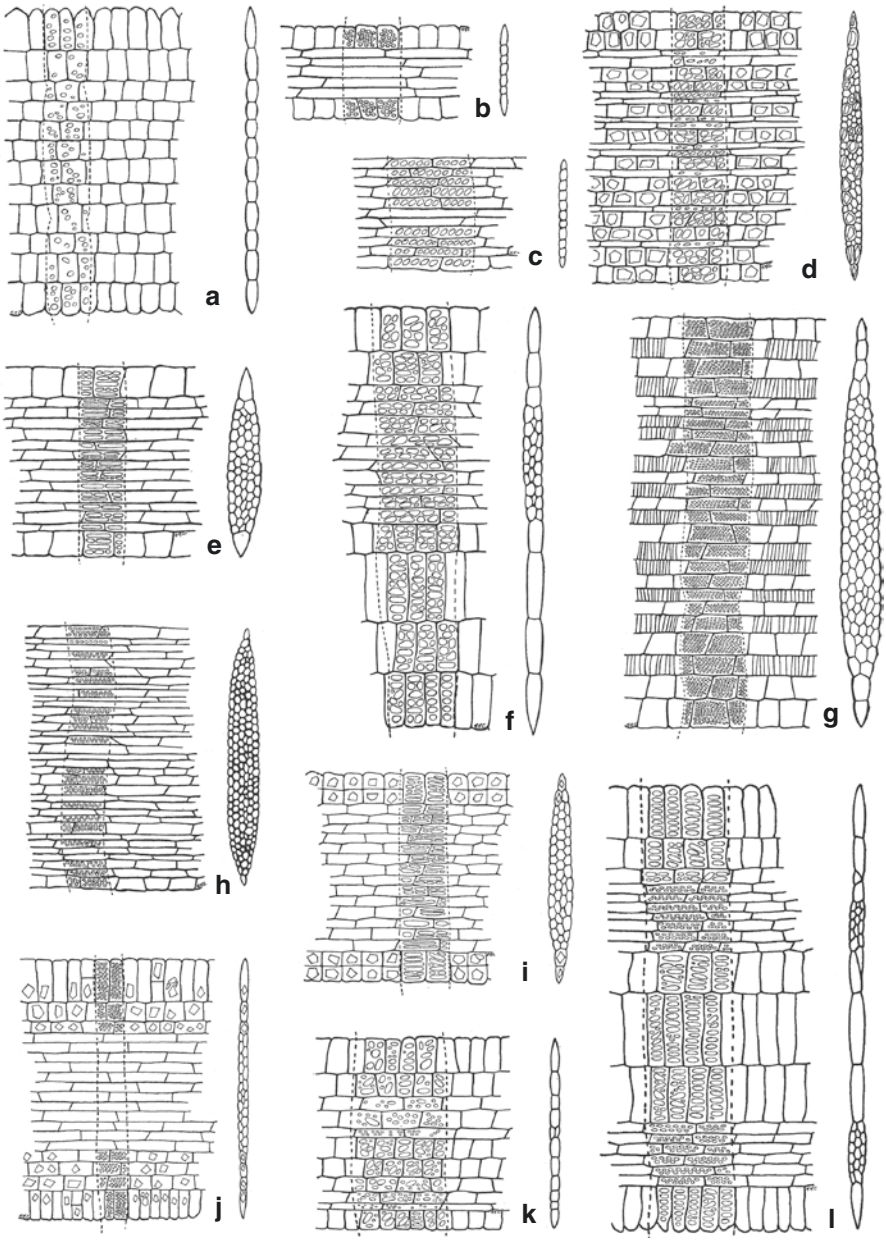
ray parenchyma and vessels (Spicer 2014; van der Schoot and van Bel 1989). Pits in the tangential walls of parenchyma cells allows for symplasmic continuity in the radial direction (Sauter and Kloth 1986); in contrast, there appears to be little to no



pitting to allow for material exchange axially or tangentially within a ray. Prominent pits are also present in the walls of ray cells where they contact vessels or other water-conducting elements (Fig. 9). This places the plasma membrane adjacent to the transpiration stream, allowing for the exchange of substances via both simple diffusion and protein-mediated transport (Alves et al. 2007; Ameglio et al. 2004; Decourteix et al. 2008; Sakr et al. 2003; Almeida-Rodriguez and Hacke 2012).

There is extensive taxonomic variation in ray size, frequency and cellular composition (Fig. 9), and some species lack rays entirely. Rays typically range from several to in some cases hundreds of cells tall when viewed in tangential section, and many taxa produce two distinct types of ray cells (i.e., the rays are said to be 'heterogeneous'). Procumbent cells are elongate in the radial direction and are thus thought to be specialized for radial transport. In contrast, upright cells are square or slightly elongate in the axial direction, typically located at the longitudinal tips of rays (e.g., Fig. 9e, f, and g). Functional specialization of upright cells is largely unknown. Rays also vary substantially in width, a feature that when measured in cell number is referred to as 'seriation' (e.g., a uniseriate ray is one cell wide). Some taxa produce rays of one or more distinct widths while others produce rays within a certain range of widths. For instance, *Quercus* species produce many uniseriate rays and infrequent rays that are 10–30+ seriate; *Acer rubrum* produces 1–5 seriate rays (Panshin and de Zeeuw 1980). Although the absence of rays in trees is extremely rare, raylessness is found in some 'woody herbs' and secondarily woody species (Carlquist 2015b), where the absence of a system for radial conduction may not be problematic in axes containing only a small cylinder of wood. Similarly, minimally woody and secondarily woody species may produce rays composed entirely or predominantly of upright cells (Carlquist 1970; Kidner et al. 2016; Carlquist 1985).

Fig. 9 Rays with varying cellular composition in angiosperm wood shown in radial (*left*) and tangential (*right*) sections. Vertical dashed lines indicate regions in contact with a vessel. Rays may be uniseriate (**a**–**c**), multiseriate (**d**, **e**, **h**, **i**), or contain both uniseriate and multiseriate regions (**f**, **g**, **j**–**l**). Erect parenchyma cells are either square or vertically elongate and occur at the upper and lower margins of rays (**a**, **b**, **e**–**j**) or in vertical bands that alternate with procumbent cells (**d**, **g**, **k**, **l**). Pits in parenchyma cell wall regions shared with vessels (delineated by a dashed outline) may occur in both types of ray parenchyma (**a**, **d**–**g**, **i**, **k**, **l**) or be absent in some (**c**, **h**) or all (**b**, **j**) procumbent cells. The degree of variation in ray structure suggests that similar variation exists in functional specialization. In some species, tile cells form via sequential radial divisions (i.e. formation of tangential walls) of developing procumbent cells (**g**). Species shown are (**a**) *Guilfoylia monostylis* [Fabales/Surianaceae], (**b**) *Salix caerulea* [Fabales/Surianiaceae], (**c**) *Eucalypus regnans*, (**d**) *Hopea nutans*, (**e**) *Panax elegans*, (**f**) *Endospermum peltatum*, (**g**) *Durio oxleyanas*, (**h**) *Acer pseudoplatanus*, (**i**) *Canarium mehenbethene*, (**j**) *Cyclostemon sp*., (**k**) *Madhuca utilis*, (**l**) *Palaquium galatoxylum* (Adapted from Chattaway (1951) with permission of CSIRO Publishing)

Variation in the cellular composition of rays traces back to the meristematic initials in the vascular cambium, and ultimately to the origin of the cambium via the unification of fascicular and interfascicular regions (Fig. 1). The dimensions of initials within these two regions differs significantly in minimally woody species (including many secondarily woody species), with fascicular procambial initials having already elongated axially (Barghoorn 1940, 1941). In contrast, the interfascicular initials are more isodiametric, and it is from these initials that the first rays are produced (Bailey 1920; Larson 1994). The production of rays with exclusively upright cells has therefore been interpreted as a form of juvenilism, where a juvenile state is expressed into adulthood, a condition common among secondarily woody taxa (Carlquist 1962, 2009b). Indeed, the production of tall rays composed of axially elongate cells (e.g., as in the secondarily woody *Begonia* (Kidner et al. 2016)) may simply reflect a juvenile form of ray ontogeny.

Axial Parenchyma

Axial parenchyma is composed of longitudinally-oriented strands of living cells (also called strand parenchyma; Figs. 7 and 8) and commonly makes up anywhere from under 1 % to over 25 % of the volume of wood in angiosperms, and up to 90 % in some extreme cases (Spicer 2014). These cells form connections with conductive elements (both vessels and tracheids) and other parenchyma via pits in the cell wall, thereby forming a three-dimensional network of living cells that connects xylem and phloem.

Axial parenchyma may surround vessels in partial or complete sheaths (Fig. 7a, d, f), and where abundant may extend out to form tangential bands of variable width (Fig. 7b–d). This configuration would clearly facilitate exchange of materials between living cells and the transpiration stream, and there is evidence for a role of parenchyma in solute deposition during embolism ‘refilling’ (Brodersen et al. 2010; Sakr et al. 2003, 2004; Salleo et al. 2009; Brodersen and McElrone 2013). There is also evidence that the ionic composition of the transpiration stream – which could be regulated at least in part by parenchyma activity – can affect the geometry of pit membranes and thus alter the resistance to water flow (Holbrook et al. 2000; Zwieniecki et al. 2001, 2003; van Doorn et al. 2011; Lee et al. 2012; Nardini et al. 2011). Interestingly, in the temperate zone, large volumes of axial parenchyma are characteristic of ring porous woods with large diameter vessels, whereas diffuse porous woods tend to have little axial parenchyma (Spicer 2014). In the tropics however, where diffuse porous woods are the norm, axial (but not ray) parenchyma volume is generally increased (Morris et al. 2016) and sheaths of axial parenchyma are often found surrounding large diameter vessels (Morris, pers. Comm.). This suggests that axial parenchyma/vessel associations are more a function of vessel diameter than they are of climate. Finally, it should be noted that the relative contribution of axial vs ray parenchyma to many of these processes is poorly understood. Most imaging appears to show ray, and not axial parenchyma, as the source of water

supplied during refilling (Brodersen et al. 2010). Ray parenchyma has also been shown to be the primary source of vessel-occluding gums and tyloses (Chattaway 1949; De Micco et al. 2016). Thus, the true functional role of extensive contact between axial parenchyma and vessel contents has yet to be demonstrated.

Although a great deal of emphasis is placed on vessel/parenchyma interactions, not all parenchyma is found in association with conductive elements. Axial parenchyma is embedded among fibers with little to no vessel contact in some taxa (Spicer 2014; Carlquist 2001a). Strands may be solitary or in small clusters (Fig. 7e) and thus difficult to detect, or they may form narrow tangential bands (Fig. 7f). Functional repercussions of this arrangement are largely unknown. In seasonal climates, axial parenchyma may also be laid down at the margin of a growth ring, where it is thought to serve as a site for carbohydrate (and/or water) storage to be used by the cambium at the start of the next growing season (Jane 1934; Chowdhury 1936; Fuchs et al. 2010; Grillos and Smith 1959; Lopez and Villalba 2016; Chowdhury et al. 2016). Note, however, that this arrangement also places parenchyma in contact with the first-formed vessels.

As noted above, living fibers may occupy similar positions to axial parenchyma (e.g., associated with vessels and/or at growth ring boundaries) and where abundant, may replace them, suggesting that these cells perform similar functions (Carlquist 2015a; Gregory 1978; Harrar 1946; Dumbroff and Elmore 1977; Plavcova et al. 2016; Yamada et al. 2011; Sauter et al. 1973; Carlquist and Hoekman 1985b; Itabashi et al. 1999). Living fibers may offer a form of carbohydrate storage with enhanced mechanical strength, as fiber wall thickness is often (although not always) greater than that of axial parenchyma (Plavcova et al. 2016; Carlquist 2014). Although living fibers may be more common in shrubs and subshrubs (which are in turn more common in arid environments) than in trees (Fahn and Leshem 1963), they are found across a diverse range of growth forms. There is clearly a great deal left to learn regarding the adaptive significance and phylogenetic distribution of living fibers.

Parenchyma Longevity and the Process of Heartwood Formation

Parenchyma in wood often demonstrate remarkable longevity, with some cells living for nearly 100 years in angiosperms and up to 200 years in some conifers (Domec et al. 2012; Domec and Gartner 2002; Spicer 2014). The transition from sapwood to heartwood in trees and shrubs is defined by the death of parenchyma (and living fibers, where present) such that no living cells exist in heartwood, and there is considerable taxonomic variation in parenchyma longevity (Chattaway 1952; Spicer 2005). This process does not occur in minimally woody axes, but is instead characteristic of large, long-lived and arborescent growth forms. The coordination of cell death among different living cell types is not well documented, but in some cases axial parenchyma surrounding vessels die long before ray parenchyma (e.g., in the highly parenchymatous *Adansonia*; Chapotin et al. 2006). Within

rays, cells located at the tips often die before those located in the center of the ray (Nakaba et al. 2012; Spicer and Holbrook 2007b). Parenchyma may also live for 5–25 years or more after water transport through the sapwood has ceased, as in ring porous *Fraxinus* (Spicer and Holbrook 2005), suggesting a role in carbohydrate storage that is independent of the transpiration stream.

Considerable evidence suggests that parenchyma cell death is a developmentally regulated process and thus a form of senescence (Chattaway 1952; Spicer and Holbrook 2005, 2007a, b), during which the innermost cylinder of wood is progressively compartmentalized and protected from microbial degradation. Parenchyma activities prior to cell death include the synthesis of phenolic compounds (Chattaway 1952; Bamber and Fukazawa 1985; Magel 2000), gums and resins (De Micco et al. 2016; Chattaway 1949), and the occlusion of vessels by newly produced cell wall material (i.e., tyloses; De Micco et al. 2016; Clerivet et al. 2000; Saitoh et al. 1993; Chattaway 1949; Cochard and Tyree 1990). The biosynthesis of 'extractives' prior to parenchyma cell death gives rise to the taxonomic variation in commercially important heartwood properties (e.g., decay resistance, coloration, and fragrance). As a consequence, there is a great deal of research on the genetic and biochemical pathways leading to extractive synthesis and deposition in wood (e.g., Patten et al. 2008; Kuroda et al. 2014; Nakaba et al. 2015, 2016; Moniodis et al. 2015; Celedon et al. 2016).

Expansion of Parenchyma in Woody Succulents and Lianas

Some woody plants have adapted to extreme aridity through the development of highly specialized growth forms dominated by parenchyma. Because parenchyma cells by definition remain alive at maturity, they are able to store water within their protoplasts by maintaining a favorable osmotic gradient (Ogburn and Edwards 2012). There have been many independent origins of so-called succulent growth forms in woody angiosperms, and a body plan with extensive parenchyma has been achieved via multiple routes (Fig. 10; Ogburn and Edwards 2010; Olson 2003; Carlquist 2016; Hearn et al. 2013). Both axial and ray parenchyma are greatly expanded in *Adansonia* (the baobabs; Chapotin et al. 2006), *Adenia* (Hearn 2009a) and *Moringa* (Olson and Carlquist 2001) to form the swollen stems characteristic of pachycauls or 'bottle trees'. In some of these stems, parenchyma de-differentiates and produces even more parenchyma through localized meristematic activity (Mauseth 2004; Hearn 2009b). In other groups, the formation of successive cambia (again relying on the latent meristematic potential of parenchyma; described below) produces highly parenchymatous organs through the production of large amounts of conjunctive tissue. In other species described as 'geophytes' this storage tissue is largely underground, a mechanism to survive drought and/or fire (Davies et al. 2016; Maurin et al. 2014; Simon and Pennington 2012). Given the diversity of developmental means to achieve the same end, it is clear that producing wood with abundant parenchyma is a successful adaptation to aridity.

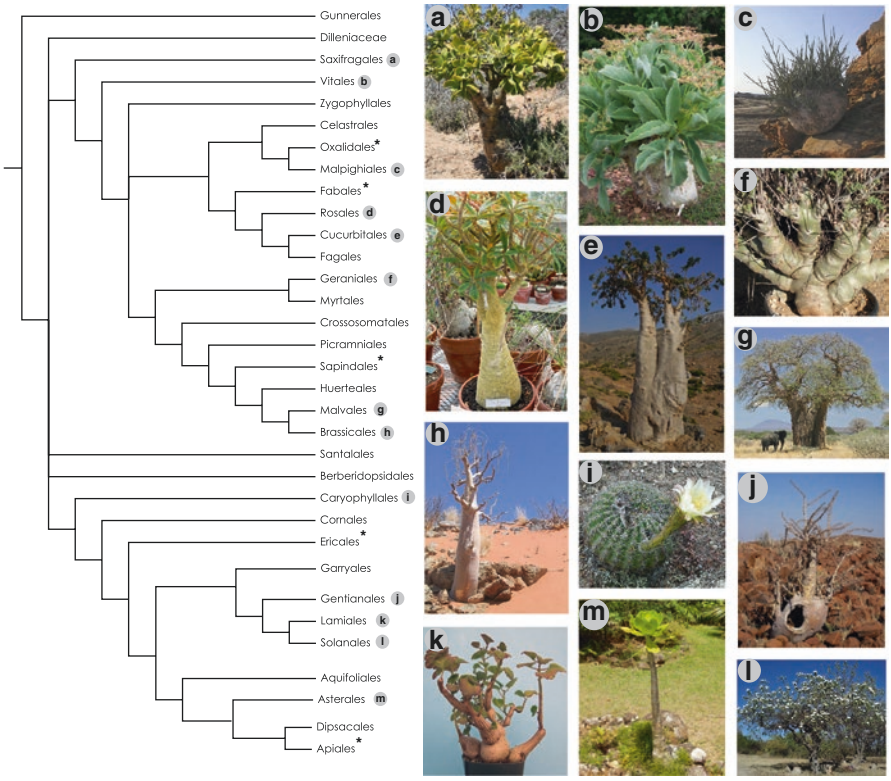


Fig. 10 A phylogeny of core eudicots illustrating multiple origins and the broad phylogenetic distribution of stem succulents. Within the majority of the indicated lineages, relatively few taxa are succulent. Asterisks indicate additional lineages with stem succulent taxa that are not illustrated. Species shown are (**a**) *Tylecodon paniculatus* [Crassulaceae], (**b**) *Cyphostemma juttae* [Vitaceae], (**c**) *Adenia pechuelii* [Passifloraceae], (**d**) *Dorstenia gigas* [Moraceae], (**e**) *Dendrosicyos socotrana* [Cucurbitaceae], (**f**) *Pelargonium carnosum* [Geraniaceae], (**g**) *Adansonia digitata* [Malvaceae], (**h**) *Moringa ovalifolia* [Moringaceae], (**i**) *Echinopsis mamillosa* [Cactaceae], (**j**) *Pachypodium lealii* [Apocynaceae], (**k**) *Plectranthus ernstii* [Lamiaceae], (**l**) *Ipomoea arborescens* var. pachylutea [Convolvulaceae], (**m**) *Brighamia insignis* [Campanulaceae]. Photo credits: (**a**, **b**) Marco Schmidt, (**c**) public domain, (**d**, **i**) Stan Shebs, (**e**) Edson Gentile, (**f**) Juergen Menzel, (**g**) Ferdinand Reus, (**h**) Violet Gottrop, (**j**) Hans Hillewaert (**k**) no copyright information, (**l**) Ruddy Benezet, (**m**) Daderot (Adapted from Hearn et al. (2013) with permission of University of Chicago Press)

Many tropical lianas also develop large volumes of parenchyma through a variety of means, including expanded ray and axial parenchyma production and the development of successive cambia (described below) (Caballé 1993; Carlquist 1991; Gasson and Dobbins 1991; Olson 2003; Ewers and Fisher 1991; Fisher and Ewers 1991). In some liana species the interfascicular region of the cambium produces axial parenchyma while the fascicular region produce more 'normal' wood with vessels and fibers (Carlquist 1993; Wagner et al. 2014; Wagner et al. 2012). In many cases the

proliferation of parenchyma is thought to be an adaptation to a climbing habit, which requires flexibility at later stages of development (Isnard et al. 2012; Isnard et al. 2003; Rowe et al. 2004). Parenchyma can also add torsional resistance to stems, and, because of their latent meristematic activity, can repair stems that have become fractured or split (Dobbins and Fisher 1986; Fisher and Ewers 1989; Fisher 1981). The contribution of parenchyma to wood biomechanics has received very little attention compared to its roles in water and carbohydrate storage.

Variation in the Production of Xylem and Phloem Cylinders: 'Anomalous' Cambial Activity

In most woody dicots – especially trees and shrubs that are ancestrally (i.e. 'primarily') woody – a single vascular cambium produces xylem to the inside and phloem to the outside. The volume of xylem produced is typically much greater than that of phloem, but the ratio of the two vascular tissues is constant such that the stem is roughly cylindrical (Larson 1994). Although this framework dominates our understanding of wood development in commercially important tree species, significant variation in cambial activity exists when considering woody dicots as a whole, including alterations in the relative rates and position of xylem and phloem production, and the development of multiple cambia in a single stem (Carlquist 2001b). Interestingly, these variants are most common in groups that include frequent shifts in the degree of woodiness. However, these same groups are also characterized by a diversity of growth forms (e.g., lianas and succulents in addition to trees, shrubs and herbs) and/or evidence of rapid radiations. Lianas in particular are known for producing a range of unusual stem forms through altered cambial activity (Fig. 11; Angyalossy et al. 2012; Carlquist 1991). It is not clear whether significant variation in wood traits is simply a byproduct of diversification and niche exploitation or whether evolutionary lability in wood production is a driver of diversification.

Shifts in the Relative Rates and Direction of Xylem and Phloem Production

Changes in the ratio of xylem to phloem production are one source of variation in the macroscopic layout of a woody stem. If xylem production is increased relative to phloem in specific regions of the cambium, an irregular stem shape results, a condition most commonly seen at the base of large tropical trees in the form of buttressed roots (He et al. 2013; Smith 1972) and in tropical lianas with flattened or elliptical cross sections (Basson and Bierhorst 1967; Angyalossy et al. 2012; Caballé 1993; Carlquist 1991). Fluted, buttressed and non-cylindrical stems provide mechanical advantages (Christensen-Dalsgaard et al. 2008a, b; Crook et al. 1997),

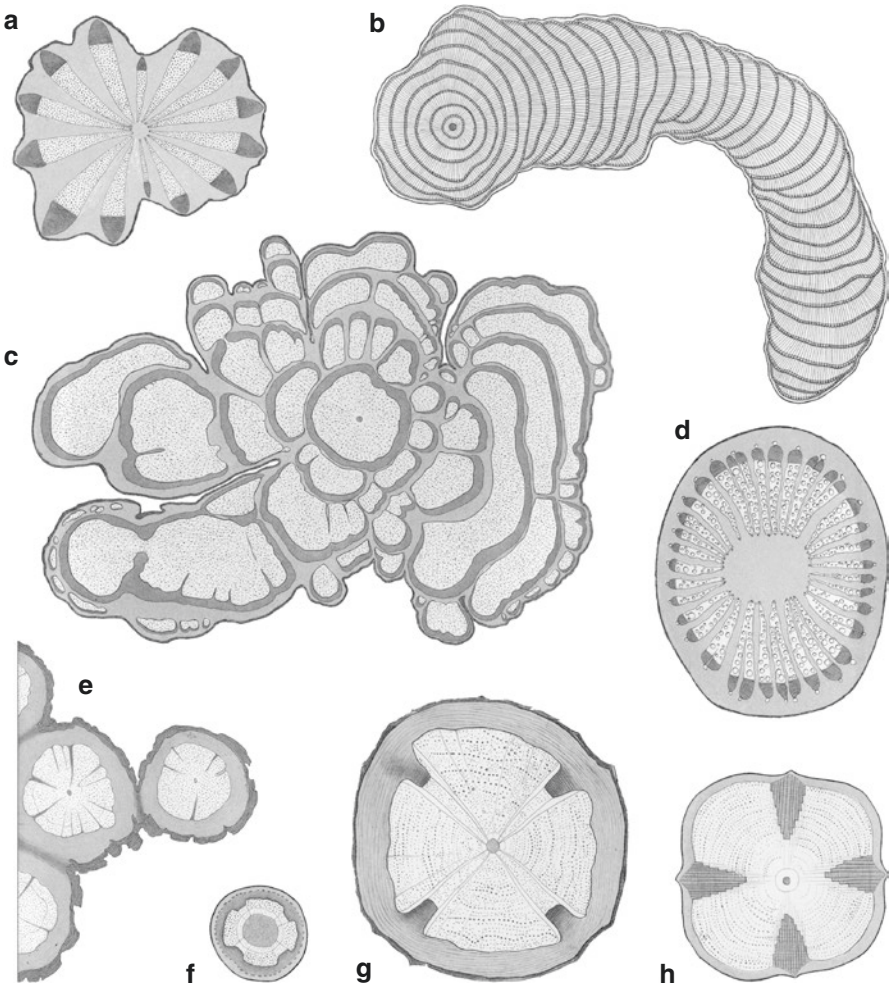


Fig. 11 Variation in cambial activity in liana stems from Brazil as illustrated in a classic text by Schenck (1893). In most stems the lighter stippled or dotted areas represent xylem and the darker cross-hatched or striated areas represent phloem. Successive cambia active on only one side of the stem forms the flattened shape in (**b**). Wedges of phloem found penetrating into the xylem are shown in (**f**, **g**, **h**). Isolated fascicular regions of xylem and phloem separated by parenchymatous tissue are shown in (**a**, **d**). Species shown are (**a**) *Begonia fruticosa* [Curcurbitales/Begoniaceae], (**b**) *Chondodendron platyphylla* [Ranunculales/Menispermaceae], (**c**) *Paullinia pinnata* [Sapindales/Sapindaceae], (**d**) *Cissus sulcicaulis* [Vitales/Vitaceae], (**e**) *Serjania ichthyoctona* [Sapindales/Sapindaceae], (**f**, **g**, **h**) unidentified stems of two Bignoniee stems, where f and g represent a developmental series of one individual [Lamilaes/Bignoniaceae]

and in the case of some buttressed tree roots may be accompanied by mechanically-specialized cell types (terSteege et al. 1997). Whereas large and often shallow-rooted tropical trees develop asymmetric stems for stability, development of

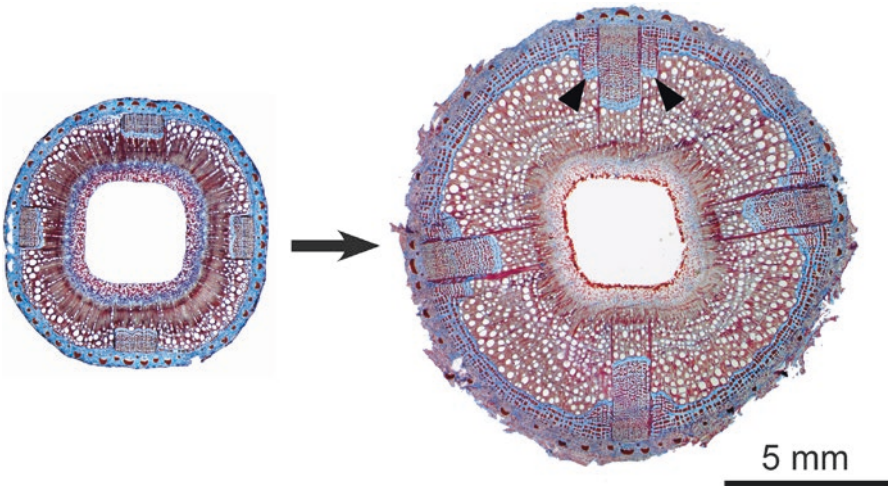


Fig. 12 Developmental origin of phloem wedges in the liana *Stizophyllum riparium* [Lamilaes/Bignoniaceae]. Lateral steps (*arrowheads*) develop along the margins of each wedge as new regions of the cambium begin to produce a higher ratio of phloem:xylem tissue. While young stems contain phloem bands with straight margins, older stems contain triangular phloem wedges with stepped margins (Adapted from Pace et al. (2015a) with permission of Oxford University Press. Photo credit: Marcelo Pace)

asymmetry in lianas – especially a ribbon-like stem form – may instead aid in the climbing habit by providing flexibility (Putz and Holbrook 1991).

Other variants of cambial activity alter the relative positions of xylem and phloem while maintaining a roughly cylindrical stem shape. If xylem production inwards is slowed or stopped and phloem production outwards is accelerated in localized regions of the cambium, the stem remains cylindrical but radial arcs, wedges, or strips of phloem appear to penetrate into the xylem (Fig. 12; Pool 2008; Pace and Angyalossy 2013; Pace et al. 2009). This condition has arisen in at least five different eudicot orders independently but is especially common in the Bignoniaceae (Lamiales), where a series of studies illustrate the developmental and evolutionary shifts leading to a diversity of related stem forms (Lima et al. 2010; Pace et al. 2009, 2011, 2015a, b; Pace and Angyalossy 2013). Islands of phloem may even become embedded in the xylem if the cambium breaks and later re-establishes within the phloem, bisecting it (Scott and Brebner 1889; van Veenendaal and Denouter 1993; Chalk and Chattaway 1937; Pace et al. 2009). Although less common, smaller pockets of phloem may become embedded within the xylem when isolated regions of the cambium produce phloem (or parenchyma that later differentiates into phloem) to the inside, rather than to the outside of the stem, and later return to normal xylem production (den Outer and van Veenendaal 1995; Ramesh Rao and Dyal 1992; Carlquist 2002; Singh 1943, 1944). Functional studies on most of these anomalous stem forms are lacking but enhanced juxtaposition of xylem and phloem may facilitate the exchange of water and carbohydrates, in some ways mimicking ray activity.

Similarly, because phloem includes a significant amount of parenchyma and parenchyma retains latent meristematic activity (i.e., it can readily de-differentiate and undergo mitotic divisions), these stems may be able to withstand or recover from physical damage more easily.

Successive Cambia

Concentric rings or crescents of alternating xylem, phloem and parenchyma are formed when successive cambia develop within a single stem. This condition has arisen multiple times independently in as many as 14 orders within the eudicots and includes many variants, but a few features appear common among them (Spicer and Groover 2010; Carlquist 2001b). A single, bidirectional (i.e., producing xylem to the inside and phloem to the outside) cambium forms initially, but after it has functioned for some time – years, in some cases – a new cambium develops via de-differentiation of parenchyma cells in an outer region of the stem, typically from cortical or phloem parenchyma (Esau and Cheadle 1969; Carlquist 1996b). Additional cambia form over time, always to the outside of the existing cambium, and produce 'conjunctive tissue' in addition to xylem and phloem. Conjunctive tissue is composed largely of parenchyma but may include bands or clusters of thick-walled fibers as well. Interpretation of the nature of the tissue that gives rise to each new cambium – i.e., whether it is parenchyma (e.g., Rajput et al. 2012b; Rajput and Rao 1998) or a long-lived meristem itself (e.g., Carlquist 2007; Rajput et al. 2008) – varies. The net result is a stem with alternating cylinders of conjunctive tissue, xylem, and phloem, such that the volume occupied by living cells is quite high. In some species each cambium forms what are effectively bundles of secondary xylem and phloem separated by broad radial wedges of parenchyma and/or living fibers (e.g., as in *Cocculus*; Rajput and Rao 2003), further increasing the volume of living tissue in the stem. In others, cambia may be incomplete and anastomose with each other to produce crescent-shaped rings of vascular tissue (Robert et al. 2011; Rajput et al. 2012a; Rajput and Rao 2000; Schmitz et al. 2008). As with other cambial variants the functional benefits of this design have yet to be demonstrated, but the added parenchyma (or living fiber) volume could provide enhanced carbohydrate and/or water storage, extensive routes for carbohydrate transport, and improved flexibility and wound response.

Conclusions and Future Directions

The developmental flexibility of the vascular cambium has allowed for an exceptional degree of diversity among angiosperm growth forms and wood anatomy, which has in turn contributed to their successful colonization of a wide range of natural environments. Variation in the activity of the cambium, the production of a

variety of novel cell types with highly specialized forms, and the ability to arrange these cell types into complex three-dimensional structures all provide the foundation for this flexibility. The challenge now is to get at the molecular underpinnings of some of the most important traits in wood structure and function.

First, the lability of many of these traits (e.g., that herbaceous plants retain the ability to produce wood; that individuals produce both tracheids and vessels) suggests that taxonomic and environmental variation are a product of the regulatory networks governing gene expression, such that comparative transcriptomics will be an important approach. Second, much of this work will require spatially explicit, cell- and tissue-scale techniques. Key knowledge gaps in this area include early cell fate markers, including markers to distinguish highly differentiated cell types (e.g., vessels and fibers) as well as relatively undifferentiated types (e.g., parenchyma and meristematic initials). While the former distinction would allow one to ask questions about how different spatial patterning of cell types are achieved (e.g., through differences in hormonal signaling, environmental cues, or the interaction of the two), the latter would allow one to ask questions about the developmental origin of the cambium, both in its 'normal' role as well as in species with 'anomalous' cambia.

There are some promising examples. Traditional cell microdissection of ray and fusiform initials has been used to show differences in polysaccharide metabolism between these cell populations (Goue et al. 2008; Goue et al. 2012), and a novel cambial regeneration system in Arabidopsis combined with laser capture microdissection (LCM) was used to identify a repressor and activator of cambial activity from the interfascicular region of the nascent cambium (Agusti et al. 2011). LCM has also been used to detect gene expression differences in dormant and active cambia (Zheng et al. 2013). In contrast, serial cryosectioning in the tangential plane – a spatially-explicit technique but one that lacks cellular-level resolution – has been used to map the expression of auxin transport genes (Schrader et al. 2003; Carraro et al. 2012), genes underlying growth and dormancy (Schrader et al. 2004a), and markers for progressive stages of xylem and phloem development (Schrader et al. 2004b). While informative and producing large datasets, this technique does not allow one to ask many of the questions at the foundation of wood structural variation due to the lack of cell-type-specific resolution.

As investigators continue to develop and refine new tissue collecting and handling techniques for improved cellular scale resolution, an increasing number of woody (and minimally and/or secondarily woody) angiosperm genomes are being made available. These advances, combined with new techniques for rapid analysis of large transcript volumes, leaves us well positioned to tackle some of the big questions in angiosperm wood evolution.

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Populus as a Model Tree

Carl J. Douglas

Abstract Model organisms are important in providing tractable experimental systems, tools, and resources to investigate conserved biological processes. Progress in understanding such processes in model organisms can, in many cases, then be used to inform the biology of other organisms closely or distantly related, thereby illuminating the nature of conserved processes as well as taxon-specific variations. Many aspects tree biology are difficult or impossible to study in herbaceous model plants, including a perennial habit and long life span, secondary growth from a vascular cambium, phenology including winter dormancy and re-activation of growth in spring, and mechanisms of adaptation to local environment over large geoclimatic ranges. For this reason, it has long been considered desirable to develop a model tree species. Trees of the genus *Populus* (poplars) are prominent forest tree species in temperate regions of the northern hemisphere and have been used as experimental organisms to understand various aspects of tree biology for over three decades. Rapid advances in generating genomic, bioinformatic, and functional genomic information and tools for poplars in the last 15–20 years, as well as specific aspects of poplar biology, have led to the widespread adoption of poplars as model tree. In this chapter, I review aspects of poplar biology that are relevant to its status a model tree, including some of the advantages and challenges in working with poplars compared to other plant model systems. I then briefly review the history of poplar as model tree, and touch upon on major advances in poplar genomics that have been crucial in its widespread recognition as a model tree species. Finally, I highlight some of the recent insights into selected biological processes important for tree biology gained from use of the poplar model system, such as secondary growth and wood formation, sexual maturation and seasonality, and adaptation to local environment.

Keywords Salicaceae • *Populus trichocarpa* • *Populus euphratica* • model organism • genomics • gene networks

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Populus as a Model: Introduction

Model organisms play key roles in modern biology by enabling complex biological processes to be studied in ways that are difficult or even impossible in related non-model organisms. In successful models, collective efforts of the research community enable the development powerful research tools and resources that make experiments and data analysis feasible, where they would otherwise be impractical or time consuming. In addition to pushing the boundaries of biological understanding using tractable experimental systems, a promise of model system biology is that this information can be then used to inform the biology of other organisms closely or distantly related to the model species, often with practical goals in mind. In the era of genomics, in which model system reference genomes, but also the genomes of hundreds of other species, are available, model systems also enable comparative genomic and evolutionary approaches to biological questions. Basic evolutionary principles suggest that there is a core set of conserved genes that are required for conserved processes common to all eukaryotes. Indeed, Benchmarking Universal Single-Copy Orthologs (BUSCO) sets representing such genes (e.g., 3023 for vertebrates and 429 for all eukaryotes) have been identified (Simão et al. 2015) and used to assess genome assembly and annotation. Genomes are dynamic and gene gains and losses are common over evolutionary time. This is especially true in plant genomes, where polyploidization, and segmental and tandem gene duplications are common. Regulatory networks and modules governing basic and fundamental processes, such as apical meristem development and photoperiodic control of development in plants, are evolutionarily conserved (Nardmann and Werr 2007; Song et al. 2015). Comparison of the well annotated reference genomes of model organisms, which represent single highly evolved species, to the genomes of related organisms can identify patterns of gene family expansion and contraction, and help to differentiate between ancient and core regulatory components and components that have diversified due to selection over the course of speciation.

Prominent examples of eukaryotic models include the budding yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, and the fruit fly *Drosophila melanogaster*. Examples of the power of this approach in contemporary biology are too numerous to list, but one example is the use of genetic (and later genomic and computational) approaches to unravel the molecular control of the cell cycle in budding yeast. A premise of this work was that mechanisms controlling the cell cycle and progression through the cell cycle would be conserved in eukaryotic cells. That this premise was correct became abundantly clear by the mid-1990s, with the elucidation that all of the key cell cycle regulatory proteins perform similar functions in yeast and humans (Nasmyth 1996), and this information was translated from yeast to humans for understanding and treating cancer and other diseases. Of course the control of the cell cycle and cell division is extremely important in plants, since control of these processes at apical and other meristems is critical in generating plant form, and are critical to many agriculturally important traits. It is inconceivable that our current detailed knowledge about cell cycle control in plants

(Polyn et al. 2015) would have been possible to elucidate without using prior knowledge from the yeast model system. Cell cycle and cell division regulation is especially important in the cylindrical vascular cambium meristem found in trees, since this stem cell population gives rise to the wood (secondary xylem) produced during secondary growth. The formation and regulation of vascular cambium is an area of intense research interest (Du and Groover 2010; Ye and Zhong 2015) for which prior knowledge of cell cycle control, originally from the budding yeast model system, serves as a foundation for research into this key aspect of tree biology.

In the context of model organism biology as discussed above, do we need a model tree and if so, why poplar? Fundamentally, the arguments for adopting a model tree revolve around the aspects of tree biology that are difficult or impossible to study in herbaceous models, or can be studied more appropriately in a tree. Biological processes of fundamental importance to trees that are difficult to study in non-tree plant models such as Arabidopsis, rice, and *Brachypodium* include a perennial habit and long life span, secondary growth from a vascular cambium, wood formation and massive commitment to secondary cell wall biosynthesis, phenology including winter dormancy and re-activation of growth in spring (for trees in temperate regions), and mechanisms of adaptation to local environment over large geoclimatic ranges that are typical of many trees. In the face of predicted massive, human-influenced climate change and the key roles that trees play in many ecosystems, as well as the future need for sustainable biomass production as a source of bioenergy and biochemical feedstock, understanding tree biology will be of increasing importance. While Arabidopsis can be considered a useful model for fundamental processes underlying tree biology such as wood formation (Nieminen et al. 2004; Zhang et al. 2011; Strabala and MacMillan 2013; Zhang et al. 2014), the complex physiology, biochemistry, and specialized regulatory networks underlying perennial growth, dormancy and generation of the tree habit suggest that model tree species are required. In this spirit, this chapter discusses the role of poplar as a model system for tree biology, over 15 years since this was first proposed in the literature (Bradshaw et al. 2000).

Novel Aspects of Poplar Systematics, Biogeography, and Cultivation

"Poplar" is used here to refer to poplars, cottonwoods, and aspens of the genus *Populus. Populus* is one of two major genera in the Salicaceae, together with *Salix* (willows), and contains 32–40 species, depending on taxonomic treatment (see Cronk 2005; Dickmann and Kuzovkina 2015 for recent summaries and proposals for *Populus* taxonomy). The genus is subdivided into sections, each exclusively trees that share much in common but have distinct habits, morphologies and ecologies. These include the cottonwoods (section *Aigeiros*), balsam poplars (section *Tacamahaca*), aspens (section *Populus*), Afro-Asian poplars (section *Turanga*), and large-leaf or swamp poplars (section *Leucoides*). Readers are referred to Dickmann

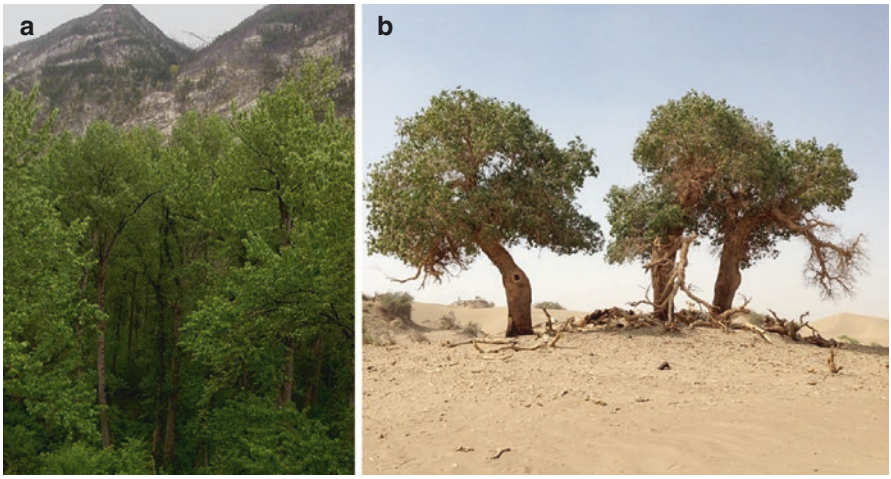

Fig. 1 Contrasting habitats of poplars. (**a**) *Populus trichocarpa* (*black cottonwood*) in Stein River valley, Coast Range, British Columbia, Canada. (**b**) *Populus euphratica* (Euphrates poplar) near Ejin, Inner Mongolia, China

and Kuzovkina (2015) for a recent detailed summary of the biology and systematics of the genus *Populus*.

Poplar species have widespread distributions throughout the Northern Hemisphere (Dickmann and Kuzovkina 2015). Many poplar species have very broad continental and pan-continental ranges over which there is substantial variation in climate and environment, including temperature, precipitation, and seasonal variation in day length – suggesting the presence of substantial intraspecific genetic and phenotypic variation between populations that are adapted to local conditions. Across their ranges, poplars are generally found in riparian habitats and are common along river drainages and flood plains (Fig. 1a). However, adaptations in some poplar species have allowed them to colonize extensive drier upland habitats (notably aspens, section *Populus*) and river systems and ephemeral water sources in arid regions of the Middle East and central and eastern Asia where water and salt stress are common (e.g., *P. euphratica*, section *Turanga*) (Fig. 1b).

An interesting aspect of poplar biology is the relative porosity of barriers to gene flow between species, leading to frequent interspecific hybridization between species of the same section and sometimes between species in different sections. Hybridization occurs naturally in zones of sympatry and has been exploited commercially for decades in poplar breeding. Two well characterized examples of complex natural hybrid zones in which pure species co-exist with recent (F1) or advanced generation hybrids include *P. alba* x *P. tremula* (section *Populus*) hybrids in European river floodplains including the Danube river in Lower Austria (Lexer et al. 2007), and the *P. balsamifera* (*Tacamahaca*) × *P. angustifolia* (*Tacamahaca*) × *P. deltoides* (*Aigeiros*) hybrid swarm in southern Alberta, Canada, where all three species are sympatric (Rood et al. 1986). Recent results from studies using genomic

and population genomics approaches have shed light on the evolutionary, ecological, and adaptive consequences of naturally occurring hybrids and the role of introgression in providing adaptive genetic variation (Christe et al. 2016; Floate et al. 2016; Suarez-Gonzalez et al. 2016).

Poplar cultivation is ancient, and for millennia plantings of poplars have graced human influenced landscapes and ecosystems, primarily in Europe and Asia, but more recently in North American and in regions far from the native ranges of poplar species including the temperate Southern Hemisphere (e.g., Chile, Australia and New Zealand). Contemporary poplar plantations are common in suitable locations in Europe, South and North America, and Asia (particularly China), where they provide efficiently produced woody biomass for solid wood, pulp and paper, and bioenergy applications. For such commercial applications, most poplar genotypes deployed are interspecific hybrids that outperform pure species due to heterosis (Berguson et al. 2010). A case in point regards intersectional *P. trichocarpa* x *P. deltoides* (x *generosa* or x *interamericana*) hybrids of two major North American species developed at the University of Washington and Washington State University in the 1980s (Heilman and Stettler 1985; Stettler et al. 1988). In optimal sites and conditions along the Columbia River that separates the US states of Oregon and Washington, these F1 hybrids exhibit extremely high biomass yields (up to 19 t ha^{-1} $year^{-1}$, Burguson et al. 2010). Similarly x *euromerica* hybrids between *P. trichocarpa* and *P. nigra*, both in section *Tacamahaca*, provide high performing genotypes of commercial value in North America and Europe (Berguson et al. 2010). Many early and classic molecular genetic and biochemical studies using poplars as a model employed *P.* x *generosa* and *P.* x *euramerica* clones such as H11-11 and Dode cv 'I214', respectively, due to their wide availability, ease of vegetative propagation from hardwood cuttings, and in some cases availability of cell cultures (for example, Sarni et al. 1984; Parsons et al. 1989; Allina et al. 1998; Moniz de Sa 1992).

Populus species and hybrids most relevant to discussion of poplars as a model tree include those that have been used in breeding and plantation forestry, those that have been used as subjects for molecular genetic, physiological and other experimental studies, those for which substantial genomic information is available, and those for which substantial information on phenotypic and/or genotypic variation is available. Table 1 lists a selection of these poplar species and hybrids, organized by section. It is likely that as the poplar model continues to expand in the tree and plant research community, these species will play the most prominent roles as models for understanding genetic, physiological, and evolutionary processes that are conserved in trees.

Defining Biological Characters of *Populus* as Model Species

Several aspects of poplar biology highlight processes that are either lacking or poorly understood in herbaceous annual model plants, or which affect the range of available experimental approaches. Like its sister genus *Salix*, all *Populus* species are dioecious, with separate male and female individuals that produce male and

Table 1 Representative *Populus* species used in model systems biology

Section (English name)	Representative species	Common English name	Relevance to model system biology	Key or recent references
Turanga (Afro-Asian poplars)	*P. euphratica*	Euphrates poplar	Salt and drought tolerance	Ma et al. (2013)
Aigeiros (Cottonwoods)	*P. deltoides*	Eastern cottonwood	Breeding; transformation	Bryan et al. (2016)
	P. nigra	Black poplar	Major European poplar; breeding; genomic resources	Faivre-Rampant et al. (2016)
Tacamahaca (Balsam poplars)	*P. balsamifera*	Balsam poplar	Major NA species; genomic resources	Olson et al. (2010) Suarez-Gonzalez et al. (2016) Menon et al. (2015)
	P. trichocarpa	Black cottonwood	Breeding; major NA species, *Populus* reference genome; genomic resource	Tuskan et al. (2006) Evans et al. (2014) Geraldes et al. (2014) McKown et al.(2014)
Populus (Aspens)	*P. alba*	White poplar	Hybrids; used in transformation; includes *P. tomentosa*, a major Chinese experimental species	Smith et al. (2015) Christe et al. (2016)
	P. grandidentata	Bigtooth aspen	Hybrid use for transformation	Smith et al. (2015)
	P. tremula	European aspen	Major European species; genomics resources; hybrids used in transformation	Obudulu et al. (2016) Wang et al. (2016) Sundell et al. (2015) Sterky et al. (2004)
	P. tremuloides	Quaking (NA) aspen	Major NA species	Wang et al. (2016)

female flowers lacking gynoecia and stamens, respectively. Thus, poplars are exclusively outbreeding. Generation of inbred lines is not possible, and advanced generations require crossing of F1 progeny to siblings of the opposite sex, or backcrossing to the male or female parent. Field observations indicate sex ratios of approximately 1:1 in *P. balsamifera* (Richardson et al. 2015), and range-wide collections of several hundred *P. trichocarpa* and *P. balsamifera* made without knowledge of the sex of the accessions exhibit approximately 1:1 ratios of males and females (Geraldes et al. 2015). Morphologically and physiologically, there is little or no evidence for sexual dimorphism aside from flowers. However, scattered reports suggest differences in the ecology and growth rates of males and females, for example in the North American aspen *P. tremuloides* (Grant and Mitton 1979). Further complicating use of genetic approaches in poplars, trees do not flower until reaching ages of 4–10 years, depending on species and growth conditions (Richardson et al. 2015). Floral primordia are initiated in the previous summer and overwinter in dormant floral buds. Flowering occurs early in early spring before leaf flush, when flowers emerge from pre-formed floral buds. Controlled breeding experiments typically utilize dormant branches, which are detached from large, mature trees of known sex. The branches are brought to greenhouses or growth chambers where male and female flowers flush out and can be used for genetic crosses. Given the annual flowering cycle, then, breeding and genetic experiments are confined to one time per year, using mature trees of known sex. In nature, following wind pollination by males, female poplar trees produce huge amounts of tiny, wind-blown cottony seeds. While seed production can be massive, seeds in nature remain viable for only a few weeks (Dickmann and Kuzovkina 2015).

Recently, the sex determining locus on chromosome 19 of *P. trichocarpa* and *P. balsamifera* was identified and shown to be conserved in other species of sections *Tacamahaca* and *Aigeiros* (Geraldes et al. 2015). Surprisingly, the sex-determining locus discovered in parallel studies in aspens (section *Populus*), also on chromosome 19, is distinct in position and gene content (Kersten et al. 2014; Robinson et al. 2014). The development of molecular markers that can reliably and easily distinguish sexes using leaf DNA from sections *Tacamahaca* and *Aigeiros* (Geraldes et al. 2015) and section *Populus* (Kersten et al. 2014; Robinson et al. 2014), should facilitate ecological and physiological experiments aimed at revealing potential differences between poplar sexes at the ecological, landscape, and physiological levels. Initial analyses in *P. tremula*, however, failed to reveal any evidence for sexual dimorphism in physiological or vegetative gene expression phenotypes (Robinson et al. 2014).

Given the complexities of sexual reproduction in poplar, genotypes are usually maintained and propagated asexually, using shoot or root cuttings. Indeed, a remarkable aspect of poplar biology is the ease of vegetative reproduction of most species (Dickmann and Kuzovkina 2015), both in nature and in experimental/commercial applications. In some sections, for example section *Populus* (aspens), vegetative reproduction by root suckering is common, which can lead to large genetically identical clonal blocks on the landscape. In other sections, particularly *Tacamahaca* and *Aigeiros*, rooting of hardwood cuttings or detached branches occurs readily, which

Fig. 2 Clonal propagation of *Populus* genotypes for experimental use. (**a**) Clonally propagated *Populus trichocarpa* genotypes in a greenhouse, University of British Columbia, Vancouver Canada. (**b**) Field testing of a clonally propagated *Populus deltoides* × *P. nigra* F1 gene dosage variation population, USDA Forest Service, Placerville, CA, USA

can lead to clonal structural in natural populations, for example along river drainages where vegetative propagules (branches) can move some distance from mother trees. From a practical perspective, the ease of vegetative propagation, commonly from 1-year old branches in clonal archives for balsam poplars and cottonwoods such as *P. trichocarpa, P. balsamifera, P. deltoides,* and *P. nigra* or suckering roots of aspens such as *P. tremula,* allows identical genotypes to be easily propagated at the appropriate scales for experimental or commercial applications in growth chambers, greenhouses, or in the field (Fig. 2). While facile vegetative propagation makes poplars experimentally tractable, for reasons outlined above, seed production is not a feasible option for maintaining or propagating genetically defined accessions. Thus, archival clone banks are required for long-term maintenance of genotypes of interest, which can require substantial resources depending on the number of genetic accessions archived.

The ease of vegetative propagation also facilitates the collecting of large numbers of individuals from wild populations for common garden experiments that are the basis for contemporary genotype-phenotype association studies using population genetic and population genomic tools. Collections of hundreds of genetically distinct individuals from across the ranges of *P. trichocarpa* (Xie et al. 2009), *P. balsamifera* (Soolanayakanahally et al. 2009), *P. nigra* (Storme et al. 2004; Faivre-Rampant et al. 2016), and *P. tremula* (Luquez et al. 2008) have been reported. Replicated common garden trials of these collections, coupled with extensive phenotyping and genome-wide calling of Single Nucleotide Polymorphisms (SNPs) of each individual have brought poplars to the forefront of the plant systems used for population genomic studies evolution and for probing the molecular basis of plant adaptation (Weigel and Nordborg 2015).

A final aspect of poplar biology relevant to its model system status is the ability to generate transgenic lines for experimental purposes. Regardless of taxon, the

ability to test gene function using transgenic approaches, in which genes or gene variants generated by recombinant DNA technology are introduced to alter gene expression levels or to express gene variants, is of paramount important in functional biology. *Agrobacterium tumefaciens*-mediated transformation of poplar is well established, and regeneration of transgenic trees has been shown to be possible for number of species and hybrids (see Maheshwari and Kovalchuk (2016) for a recent example, and a review of successfully transformed and regenerated species). In this attribute, poplars have an advantage over its sister genus, *Salix*, which has proved recalcitrant to date to genetic transformation. Although a number of poplar genotypes have been transformed, the ease of transformation varies markedly, and most work is carried out on a limited number of easily transformed and regenerated genotypes and clones. Prominent among these is a hybrid aspen clone, *P. alba* x *P. tremula* clone "INRA 717-1-B4", sometimes referred to as "INRA 717-1B4" or "INRA 717". In 1992, this clone was identified by Leple and colleagues at the Institut National de la Recherche Agronomique in France (Leple et al. 1992) as being particularly amenable to transformation and regeneration following infection by *Agrobacterium*. Subsequently, it has been widely adopted in the poplar community as a workhorse for genetic transformation, and a Google Scholar search using the term "INRA 717" yields 246 results (March, 2016). A genome sequence for this individual has been completed and us available at Popgenie (http://popgenie.org/).

Populus as a Model Tree: Rationale and History

With a huge diversity of tree species, choosing a appropriate a model trees presents challenges. However, in the early 2000s poplar was proposed as such a model by several tree biologists (Bradshaw et al. 2000; Taylor 2002; Brunner et al. 2004; Cronk 2005), and a few years poplar later was well established as a model tree species (Jansson and Douglas 2007; Ellis et al. 2009). The reader is referred to these reviews for detailed discussions of why poplar was considered the most appropriate model for tree biology. Briefly, among these attributes are ease of experimentation (vegetative and clonal propagation, genetic manipulation), rapid growth, a history of breeding and genetics, and use in plantation forestry.

Poplars were one of the first trees used by plant molecular biologists to study processes such as lignin biosynthesis and response to stress (for example, Sarni et al. 1984; Parsons et al. 1989; Moniz de Sa et al. 1992; Bugos et al. 1991; Subramaniam et al. 1993; Allina et al. 1998; Baucher et al. 1996). In parallel, the power of quantitative genetics using structured populations (e.g., F2 populations derived from backcrossing *P. trichocarpa* x *P. deltoides* F1 hybrids) for investigating genotype-phenotype relationships in poplar became evident (Bradshaw and Stettler 1995). In this and a series of subsequent pioneering publications (e.g., Villar et al 1996; Wu et al. 1997; Frewen et al. 2000; Ferris et al. 2002) phenotyping of such populations in replicated clonal trials for traits such as bud set and flush, rust resistance, leaf shape and physiology, and stem growth and form was combined

with extensive genotyping for segregating molecular markers on relatively dense molecular marker-derived genetic maps. These studies used these data to identify Quantitative Trait Loci (QTL) explaining significant fractions of the variation in these multi-genic traits, foreshadowing contemporary QTL and association mapping approaches that can now be carried out at higher resolution using dense markers derived from genome sequencing efforts (Muchero et al. 2013, 2015 and below).

These studies illustrated the power of combining genetic and genome-wide marker information with population-wide tree physiological and other phenotypic data, and made it evident that by the ability to combine molecular biology, genetics and physiology poplar could make poplar into a powerful model tree (Bradshaw et al. 2000). Indeed, it was the development of the first genomic resources for poplar at about the same time, followed by a poplar reference genome sequence (first tree genome, and third plant genome after Arabidopsis and rice to be sequenced and assembled), and attendant resources developed by many groups, that firmly cemented poplar as a tree model, propelling it past other potential forest tree model species for which similar genomic resources were slower to be developed. Subsequently, the genomics of forest trees has blossomed, with notable achievements being, for example, sequencing of Norway spruce (Nysted et al. 2013), loblolly pine (Neale et al. 2014), *Eucalyptus* (Myburg et al. 2015), and oak genomes (Plomion et al. 2016). Like the poplar genome, the *Eucalyptus* genome has facilitated numerous lines of research into tree biology and comparative genomics (Strauss and Myburg 2015), nicely illustrating the importance of genomic resources in establishing selected tree species as models. In this spirit, some of the key events in poplar genomics that helped establish it as a model for tree biology are discussed below.

Poplar Genomics: Milestones and Resources

The genomics of poplar began with the report of over 5000 expressed sequence tags (ESTs) from genes expressed in developing xylem (Sterky et al. 1998), which allowed for the first time a global view of tree genes expressed during wood formation. This development was soon followed by larger studies in poplars, in which ESTs corresponding to over 20,000 genes were identified in libraries made from different stages of xylem development and several other tissues and organs (Sterky et al. 2004), and ESTs corresponding to over 35,000 genes from leaves, herbivore affected leaves, and developing xylem were reported (Ralph et al. 2006). These and other EST studies in poplar were important in facilitating gene discovery and comparative genomics, but also helped inaugurate functional genomic approaches for global gene expression profiling using microarrays. Such microarrays were originally based on several thousand cDNAs annotated as ESTs. Classic early gene expression profiling studies employing such arrays allowed, for the first time, biological processes important for tree biology including wood formation (Hertzberg et al. 2001), autumnal leaf senescence (Andersson et al. 2004), and response to

insect herbivory (Ralph et al. 2006), to be analyzed at a genome-wide basis, with the identification of gene sets important in these processes. These and many similar studies too numerous to cite here, firmly established poplar as a model tree, with new functional genomic tools building on the biology and genetic work of previous decades.

The assembly, annotation, and interpretation of the genome of an individual female *P. trichocarpa* tree ("Nisqually1") was published in 2006 (Tuskan et al. 2006). This classic and highly cited paper, a high point in plant as well as tree genomics, provided a critical resource for tree biology. As the first tree and third plant genome to be sequenced, considerable effort was put into generating a polished reference genome. This included using genetic and genomic resources (full-length cDNAs, BAC-based physical map, thousands of full-length cDNA sequences) to aid chromosome-based assembly of contigs, and the anchoring of the sequence to genetic and physical maps. Extensive efforts by multiple bioinformatics groups employed different gene model prediction algorithms, a community-based gene annotation effort (Tuskan et al. 2006; Kelleher et al. 2007; Ralph et al. 2008) and ongoing annotation have contributed to a high quality reference genome that still ranks as one of the best in plant biology in terms of assembly and annotation.

However, even a high quality reference genome is an approximation of the true genome, and will necessarily contain some level of error in assembly and gene model prediction. The poplar genome assembly and annotation has undergone two major rounds of revision by the Joint Genome Institute (JGI) from the originally published version 1.1, and the current v3.0 contains 422.9 Mb of assembled sequence (out of a total genome size estimated at 485 Mb), with 41,335 annotated loci with protein-coding transcripts, which are systematically designated by chromosome and chromosomal location (e.g., Potri.001G000900; see *Populus trichocarpa* v3.0 at the JGI plant genome portal Phytozome 11, https://phytozome.jgi.doe.gov/pz/portal.html for details). It should also be pointed out that the genome is from a wild individual that is highly heterozygous. Thus, the assembled genome is largely a mosaic of haplotypes. A striking aspect of poplar biology revealed by the genome is the highly duplicated nature of the genome, reflecting a Salicaceae-specific whole genome duplication event dated at 60 MYA (Tuskan et al. 2006). Most regions of the 19 chromosomes contain syntenous blocks of paralagous genes found on one or more other chromosomes (Tuskan et al. 2006), and around 8000 duplicated gene pairs have been retained, most with likely neo- or sub-functionalization (Rodgers-Melnick et al. 2012).

The poplar genome provided a springboard for further development of genomic tools and resources. Fabricated microarrays for every annotated poplar gene, based on Affymetrix or Nimblegen technology became widely used for genome-wide gene expression profiling provide functional insights into processes important for trees – for example, transition to secondary growth (Dharmawardhana et al. 2010) and response to drought (Wilkins et al. 2009). The era of microarray analysis of transcriptome dynamics in poplar has been well reviewed by Tsai et al. (2011). More recently, RNA sequencing (RNAseq) as became the favored approach for transcriptome profiling, and the reference genome greatly simplifies this approach

as Illumina-based short RNAseq reads can be readily mapped to the reference, and expression levels expressed quantitatively as Fragments Per Kilobase of transcript per Million mapped reads (FPKM) (see for example, Bao et al. 2013; Hefer et al. 2015; Gerttula et al. 2015).

As in other model organisms, genomic efforts in poplars have moved towards obtaining sequence data from many individuals of the same species to catalog their genomic and genetic diversity. This approach is more feasible in poplar than most other tree species due the relatively small genome, and the robust reference genome that allows facile mapping of short next generation sequencing reads to the reference. This information, most commonly in the form of genome-wide single nucleotide polymorphism (SNP) data, supports evolutionary genomics, population genomics of local adaptation, landscape genomics (distribution of allelic variants on the landscape) and phenotype-genotype correlations (e.g., genome-wide association studies, GWAS) to reveal genetic variants underlying phenotypic variation. Genome-wide SNP discovery based on transcriptome and whole genome resequencing of individuals in ascertainment populations has been used to develop Illumina Infinium SNP genotyping arrays for *P. trichocarpa* and *P. nigra* (Geraldes et al. 2011, 2013; Faivre-Rampant et al. 2016), allowing facile SNP genotyping of hundreds or thousands of individuals for thousands of SNPs. Whole genome or reduced representation (exome) resequencing at the population level (i.e., hundreds of individuals) has become feasible in the last 5 years based on reduced costs for next generation sequencing, allowing SNP variants to be called in hundreds of individuals on a genome-wide basis. These data can be used for downstream population genomics, evolutionary genomics and/or GWAS studies without the need to employ genotyping arrays (see for example Slavov et al. 2012; Evans et al. 2014; Geraldes et al. 2015; Holliday et al. 2016; Suarez-Gonzalez et al. 2016). It is likely that, in the near future, the genomes of thousands of individual poplar individuals from many species will become available, making poplar an extremely promising tree species to study local adaptation and phenotype-genotype association.

Many model species have extensive genetic resources and genome-wide reverse genetic resources to support functional studies. The biology of trees makes them less amenable to such approaches. While there is a high degree of natural variation in a wide array of phenotypes that can be revealed in common garden experiments (e.g., Porth et al. 2013c; McKown et al. 2013), genetically defined mutants are almost unheard of. However, the reverse genetic tool of "TILLING", in which a population of individuals is screened by molecular methods for sequence variants that potentially affect gene function, has been shown to be feasible at the population level in poplar (Gilchrist et al. 2006), and this approach coupled with next generation sequencing can be effective in identifying rare major affect SNP alleles or mutations (e.g., premature stop codons; Marroni et al. 2011) in poplar tree populations. Such individuals could be propagated vegetatively or used in breeding to study the phenotypic effects of potential loss of function mutants. However, community resources based on this approach have not yet been developed.

Another functional genomics approach that has been effectively applied to poplar is "activation tagging", in which populations of transgenic trees are generated with

random integrations of a strong and constitutively active promoter such as the cauliflower mosaic virus *35S* promoter are generated, potentially activating ectopic expression of flanking genes. This is followed by screening of such populations for novel phenotypes. Association of such phenotypes with mis-expression of a particular gene can be inferred from the genomic position of the integrated *35S* promoter, and substantiated by recapitulation experiments. A recent example of the success of this approach is the identification of the *EARLY BUD-BREAK 1* (*EBB1*) gene, encoding a APETALA2/Ethylene Responsive Factor (AP2/ERF) transcription factor regulating the reactivation of cell divisions in meristem cells after winter dormancy (Yordanov et al. 2014). In principle, locations of *35S* promoters in 100 or 1000 of individuals in activation tagged poplar populations could be determined, and made available to the community as a reverse genetic resource for potentially finding phenotypes associated with a gene of interest, analogous to widely used Arabidopsis T-DNA insertion lines. However, logistical, regulatory, and financial issues involved in the long-term maintenance of transgenic populations are daunting.

Recently, a novel functional genomics approach has been developed in poplar based on generation of random gene dosage variants in population of over 500 F1 hybrid clones derived from pollination of a female *P. deltoides* individual with gamma-irradiated pollen from *P. nigra* (Henry et al. 2015). Gamma-irradiation induces a high rate of insertion/deletion (indel) mutations, such that the pollen population contained a high proportion of dosage lesions in the form of chromosomal deletions and insertions. Next generation sequencing of the F1 population was used to map regions of aneuploidy (e.g., haploidy or triploidy) onto the genome in each F1 individual by identifying regions of increased or decreased sequence coverage. Here, the ease of clonal propagation of poplar was exploited, circumventing the need to sexually propagate variants that may be meiotically unstable, and facilitating deployment of the population in field trials (Fig. 2b). Since gene dosage differences are expected generate gene expression and other variation, leading to phenotypic variation (Henry et al. 2015), this phenotypic analysis of this population and correlation of phenotypes with gene dosage-induced changes in gene expression provides a new way of identifying novel genes and regulatory modules associated with traits of interest to tree biologists, such as wood formation, gravitropism, and phenology.

Web interfaces that integrate structural and functional genomic information for the community are critical to support research in model system biology. Poplar as a model tree is fairly advanced in this regard. A key community resource is the JGI Phytozome 11 site, which hosts the latest version of the *P. trichocarpa* reference genome, along with RNAseq expression data and gene variant (SNP) data. Phytozome hosts the genomes and annotations of 60 additional plant species, making it especially useful for comparative genomics. A second key community resource is the Poplar Genome Integrative Explorer (PopGenIE), hosted by the Umea Plant Science Center in Sweden and now integrated into the The Plant Genome Integrative Explorer Resource (PlantGenIE, www.PlantGenIE.org; Sundell et al. 2015). The site contains a wealth of query-able poplar structural and functional genomics data a resource for tree and plant biologists.

Poplar as a Model System: What Have We Learned About Tree Biology?

Poplar was first proposed as a tree model system over 15 years ago. Research by many groups in the years following the publishing of the poplar genome (Tuskan et al. 2006) has exploited the power of the poplar reference genome and genomic resources to address problems in tree biology, physiology, trait variation, and adaptation to environment (see Wullschleger et al. 2013 for a recent perspective). Such studies clearly suggest that poplar is living up to expectations as a system to provide insights into fundamental processes important in tree biology, such as wood development, dormancy and perenniality, and adaptation to environment. Also important has been the use of poplar genomic information in comparative studies, particularly in light of the recently published *Eucalyptus* genome (Myburg et al. 2015) which, like poplar is a fast growing tree used in plantation forestry with multiple species adapted to different environments. A few selected highlights of recent and future developments illustrating the contributions of the poplar model to selected areas of tree biology are discussed below.

Networks in Wood Formation

Secondary growth and the massive metabolic commitment to wood and secondary cell wall formation are defining characteristics of trees, making these processes and their regulation of primary interest to tree biologists. The outlines of a feed-forward transcriptional regulatory network regulating secondary wall formation and deposition of cell involving NAC domain, MYB, and KNOTTED-LIKE transcription factors has been well described Arabidopsis, and many of the corresponding genes identified in poplar (Zhang et al. 2014). The annotation of the key NAC domain master regulators in Norway spruce (Nysted et al. 2013) and *Eucalyptus* (Hussey et al. 2015) now allows comparative studies on this network in diverse tree species during wood formation.

As highlighted by Mizrachi and Myburg (2016), it is now possible in model tree species to go beyond traditional gene-by-gene studies and use systems biology and systems genetics approaches to study the complex process of wood formation. Such approaches use which use multi-level data (e.g., metabolite, expression, protein-protein interaction, protein-DNA interaction, genetic diversity) about the global behavior of systems following experimental perturbation (systems biology) or in genetically diverse populations of individuals (systems genetics). Examples of the systems biology approach in poplar are metabolic flux analysis in lignin biosynthesis (Chen et al. 2014) and transcriptional network analysis of secondary growth and wood formation (Liu et al. 2014). As a start towards systems genetics in poplar, integrative networks for SNP variation underlying phenotypic variation in wood chemistry, density, and gene expression in 16 different *P. trichocarpa* genotypes

were generated using a Bayesian network learning procedure (Porth et al. 2013a), suggesting the predictive power of such approaches. The systems genetics approach relies on modeling the large-scale genetic variation that underlies quantitative phenotypic variation (Mizrachi and Myburg 2016). Common gardens of range-wide collections of poplar species for which extensive wood phenotyping data are available (Porth et al. 2013b; Faivre-Rampant et al. 2016), and analogous information from *Eucalyptus* pedigrees (van Dyk et al. 2011), should allow effective application of systems genetics approaches to inform the biology and quantitative genetics of wood formation in trees in general, while the experimental tractability and genomic resources of poplar make the systems biology approaches (including modeling of transcriptional networks in wood formation) very promising (Liu et al. 2014).

A ubiquitous phenomenon in angiosperm trees is the formation of tension wood upon gravitropic stimulation (e.g., when trees are placed or forced into a horizontal vs. vertical position) that allows mature tree stems to right themselves and resume vertical growth. Both the mechanisms of sensing and signal transduction following gravi-stimulation and the formation of the biochemically distinct wood are amenable to experimental manipulation and systems biology analysis in poplar (see Groover 2016 for a recent review). A powerful example of the use of systems biology and network analysis to model the gravitropic response in the poplar model system comes from Gerttula et al. (2015), who developed an experimental system to study gravi-stimulation and its biochemical and physiological consequences for tension wood formation in poplar. After perturbing the system in different ways by varying gravi-stimulation, gibberellic acid (GA) levels, and expression of the key poplar *ARK2* (*KNOX*) transcription factor that regulates activity of the vascular cambium, the authors employed genome-wide transcriptome and computational analyses to generate a model for gravitropism and tension wood formation in woody stems. This model can now be further tested not only in poplar, but also other angiosperm trees, where it should provide the basis for greater understanding of this process in trees in general. This is a powerful illustration of the model system approach applied to trees.

Flowering, Maturation, and Seasonality

Flowering strategies in trees vary significantly from those in other angiosperm models. In contrast to annual plants, perennial trees flower seasonally for many years after reaching sexual maturity, and transition from the juvenile non-reproductive phase to the mature, reproductive phase can often take years. Poplar has been proposed as a useful model to address the process of maturation and floral initiation in trees (Brunner and Nilsson 2004). Since flowering in poplar is also tied to seasonal growth and dormancy, there is also a strong connection to these processes. In Arabidopsis, the FLOWERING LOCUS T (FT) protein was identified as a key, mobile protein that regulates flowering in response to day length (see Turck et al. 2008 for a review). Two poplar *FT* orthologs (*FT1* and *FT2*), paralogs derived from

the salicoid whole genome duplication, were identified as regulators of flowering and the transition from the juvenile to reproductive phases. *FT1*, in addition, controls short-day induced bud set and consequent cessation of growth, a key adaptation to the perennial life style of temperate trees (Böhlenius et al. 2006; Hsu et al. 2006). More recent work in poplar has unravelled the partially overlapping but complementary roles of poplar *FT1* and *FT2* in seasonal cold-induced reproductive onset in the winter and promotion of vegetative growth and inhibition of bud set in the spring (Hsu et al. 2010). This work has stimulated further work on the transitions between bud set and bud release (Howe et al. 2015), and these and other regulatory circuits identified in poplar are likely conserved in a multitude of angiosperm trees (Shim et al. 2014).

The poplar model system has also contributed significantly to our understanding of sex determination and dioecy in trees and plants in general. Using genomic resources (genome-wide SNPs from over 50 re-sequenced P. *trichocarpa* and *P. balsamifera* genomes) and phenotypic data (the sex of the trees grown for over 5 years in common gardens), Geraldes at al. (2015) used GWAS to identify a sex-determining locus that is conserved in *Populus* sections *Tacamahaca* and *Aigeiros*. This surprisingly small (100 kb) region in which recombination is strongly repressed contains 13 annotated genes, some of which are promising candidates for regulating floral dimorphism. It is anticipated that these results will stimulate further work on mechanisms underlying dioecy in poplars, and reproductive development in general in tree species.

Genetic Basis of Phenotypic Variation and Adaptation to Local Environment

Like many tree species, the natural ranges of poplar species span large geographical and climatic ranges. Consequently, adaptation to local environments is critical in the ability of these species to thrive in these environments, many of which were recolonized in interglacial periods, the most recent following the last glacial maximum 21 K years ago (for example see Levsen et al. 2012). Natural populations of *Populus* species harbor large reservoirs of genetic and phenotypic diversity, making them amenable to GWAS, as well as local adaptation and landscape genomics studies. The richness of the genomic and biological resources available to the *Populus* community, especially population-wide resequencing using the *P. trichocarpa* reference genome and extensive phenotyping have made poplar an especially powerful system for understanding the origins of genetic variation and how it relates to phenotypic diversity in the natural environment.

Parallel studies in *P. trichocarpa* employing 1000 of SNPs in 435 accessions from a range-wide collection grown in common gardens (Fig. 3) that were genotyped using either a SNP array (McKown et al. 2014; Geraldes et al. 2014) or by whole genome resequencing (Evans et al. 2014) have revealed candidate loci associated with a broad range of adaptive, phenology, and ecophysiological traits (Evans et al. 2014; McKown et al. 2014; Geraldes et al. 2014). Importantly, in these studies, allelic

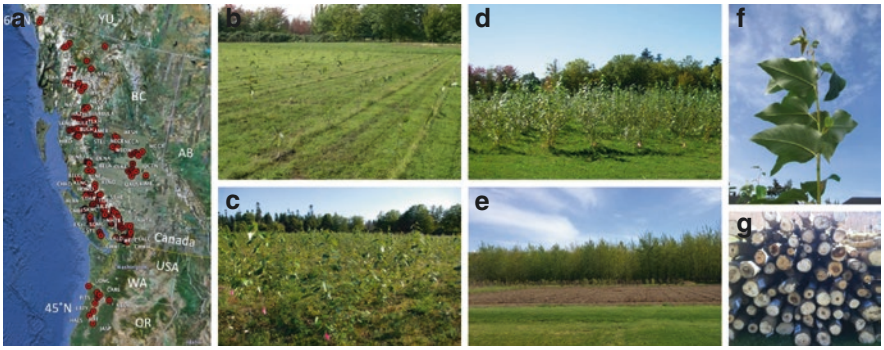

Fig. 3 *Populus trichocarpa* range-wide collection and common garden. A collection of wild P. trichocarpa accessions from the British Columbia Ministry of Forests, Lands, and Natural Resource Operations (MOF) was established in a replicated common garden trial at Totem Field, University of British Columbia in 2008. The collection contains 435 unique accessions (**a**) Provenance of the collections, from 60° N to 45° N (*red dots* indicate river drainages from which populations were sampled). (**b**) Establishment of Totem Field trial from clonal cuttings, 2008. (**c**) Totem Field trial in 2009. (**d**) Totem Field trial in 2010. (**e**) Totem Field trial in 2014. (**f**) Current year apical shoot from the trial. (**g**) Partial destructive harvest of Totem trial showing phenotypic diversity of 4-year old stems

variants identified by GWAS were also found in many cases to also be under selection and/or correlated with adaptation to local environment. In one striking case, several loci in a 600-kb block of genes on chromosome 15 that were identified by GWAS as contributing to variation in phenology and ecophysiology traits also showed allele frequency differences among accessions from different river drainages, and allelic variants were strongly correlated with climatic and geographical variables (McKown et al. 2013; Geraldes et al. 2014) indicating the action of diversifying selection. Interestingly, differentiated individuals in the drier and colder northern and eastern parts of the *P. trichocarpa* range also showed significant admixture with *P. balsamifera* (Geraldes et al. 2014), which can freely hybridize with *P. trichocarpa* in zones of contact. Recent work employing ancestry analysis revealed that the 600-kb region identified by GWAS has introgressed from *P. balsamifera*, and that introgressed *P. balsamifera* alleles of some genes in the block show evidence of functional differentiation from *P. trichocarpa* alleles (Suarez-Gonzales et al. 2016), supporting adaptive introgression as a source of adaptive alleles as *P. trichocarpa* expanded its range into transitional environments.

In a complementary approach, Holliday et al. (2016) used exome capture resequencing of 391 individuals from collections across the *P. trichocarpa* range including transects of varying latitude and elevation to identify differentiated SNPs correlated with these variables, where local adaptation to maximize growth while minimizing frost damage over the growing season can be expected. This study identified candidate loci for local adaptation to latitude and altitude, and many loci from altitude transects clustered into genomics islands of divergence that may include co-adapted alleles and hitchhiking neutral loci.

Recent work in population-wide SNP calling in *P. nigra* based on whole genome resequencing, development of a 12 K SNP genotyping array, and genotyping of 888 *P. nigra* individuals (Faivre-Rampant et al. 2016) suggests that similar population genomic and association studies will soon be extended to this species. At the moment, the dense genomic data, wide-ranging spatial sampling of accessions, and intensive phenotyping efforts, coupled with the power of the poplar reference genome, have put poplar into the forefront of studies of adaptation (Weigel and Nordborg 2015) and genomic variation at the landscape level (Bragg et al. 2015), fulfilling its promise as a model tree species for such studies. Similar studies are well advanced in other trees.

Conclusion

Trees, both in natural and managed settings, are of central importance to many of the earth's ecosystems and to human wellbeing. As well as supporting much of the earth's biodiversity and providing environmental services such as erosion control, phytoremediation, and carbon sequestration, forest trees efficiently produce woody biomass for a range of uses (e.g., solid wood for structural and fuel applications, pulp and paper, bioenergy, and biochemically complex feedstocks as alternative sources of fossil fuel-based organic compounds). In the face of human population expansion and climate change, natural and managed forests are likely to play increasingly important roles, yet are also increasingly threatened. As a model species, information from poplar will be instrumental as it is translated to other species and used to address these issues.

Globally, forest products are increasingly generated from highly productive plantations rather than wild forests, and such managed forests can help relieve pressure on wild forests and contribute to their preservation. Genomics-based breeding strategies developed in poplar, using its attributes as a model system, to target traits such as wood formation, wood structure, drought tolerance, productivity, tree form, and adaptation to local environments are potentially powerful in domestication of many forest trees (Harfouche et al. 2012; Porth and El-Kassaby 2015; Allwright and Taylor 2016). As discussed above, poplars provide superb systems for understanding adaptation to environment, and this information will contribute to strategies for adapting future forests to threats from rapidly changing climate (Aitken and Bemmels 2016). Finally, given the rapid advances in genomics of other select gymnosperm and angiosperm trees (Nysted et al. 2013; Neale et al. 2014; Myburg et al. 2015; Plomion et al. 2016) rapid expansion of comparative studies will help in translation of results from poplar to a wide range of other tree species, both at the systems biology and ecosystem levels, and at the level of understanding the roles of individual genes in tree physiology, development, and ecological adaptation.

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Emerging Genomics of Angiosperm Trees

Elizabeth Sollars and Richard Buggs

Abstract Genome sequence assemblies of many angiosperm trees used in forestry are now emerging, in addition to the well-characterised genomes of black poplar and eucalyptus reviewed in previous chapters of this book. Whilst the number of published genomes of angiosperm forest trees lags behind that of angiosperm trees grown commercially for fruit or nuts, many new projects are underway. This is aided by the ever-decreasing cost of DNA sequencing technologies and has diverse motivations including tree improvement, ecological and evolutionary studies. In this chapter, we briefly review a number of recent whole genome projects including Chinese chestnut, European ash, dwarf birch, pedunculate oak, purple willow and shrub willow. We also describe new projects not yet in the public domain or with non-genomic data, and list various online resources where data and information can be accessed. We discuss potential future steps in improving genome assemblies, and the uses of such information in fields such as genomic selection to assist tree breeding.

Keywords Genome sequencing • Angiosperm • Tree • Breeding • Transcriptomics

Introduction

The past decade has seen the emergence of genome projects on angiosperm forest tree species that have never before been viewed as model organisms. In many cases, the only previous genetic research upon these species was the study of population genetic diversity at a handful of loci. Unlike poplar (see Chap. 5) and eucalyptus (see Chap.6), most of these species have not yet been, and in some cases will never be, subject to intensive selection and breeding programmes. For many, their economic importance would scarcely justify a multi-million dollar genome project. But the rapid fall in the cost of sequencing since 2006 has permitted their genome sequencing at low expense, while the increasing availability of bioinformatics tools and high performance computers has made genome assembly possible even for small research groups.

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Biologists with specific interests from evolution to epidemiology have been able to sequence the genomes of their study species as foundational data for their research.

Such projects bring with them prospects of using genetic markers ascertained from a reference genome sequence to breed improved genotypes. Whether it be for desirable traits such as yield and wood quality, or defensive traits such as disease resistance, utilising genetic information will speed up breeding in tree species with typical generation times of above 10 years (Neale and Kremer 2011). Even when breeding is not in view, genomic data can greatly enhance our understanding of local adaptation and ecological genetics in native tree species (Plomion et al. 2016; Neale and Kremer 2011).

In this chapter, we review the emerging genome sequences for six tree species of which we are aware to have genome assemblies (excluding fruit and nut trees): Chinese chestnut (*Castanea mollissima*), pedunculate oak (*Quercus robur*), European ash (*Fraxinus excelsior*), purple willow (*Salix purpurea*), shrub willow (*Salix suchowensis*), and dwarf birch (*Betula nana*) (See Table 1). Several of these have been placed in the public domain before publication of an associated paper, to the benefit of the research community (Neale et al. 2013). We mention other genome sequencing projects of which we are aware, but are not yet in the public domain. We also highlight other species for which transcriptomes, genetic maps or genome-wide marker data are available, and which are likely candidates for future whole genome sequencing projects. We mention only in passing the numerous genome projects on fruit and nut trees, as the main foci of these projects are agronomic rather than forestry or wood product-related. Inevitably, there is likely to be interesting research on some forest tree species that we are unaware of, and which will therefore be missed in this review.

Chinese Chestnut (*Castanea mollissima*)

The Chinese chestnut has received particular attention as a genomic resource because the species is resistant to chestnut blight, a disease caused by the pathogenic fungus *Cryphonectria parasitica (*Anagnostakis 1987*)*. This fungus has devastated American chestnuts, which are highly susceptible, since its introduction to the USA around 1904 (Anagnostakis 1987). Considerable effort has gone into breeding American chestnut trees with resistance to the fungus either through hybridising American with Japanese or Chinese chestnuts, or by using transgenics to introduce resistance genes into the American chestnut genome (Hebard et al. 2014).

Genetic and physical maps of the *Castanea mollissima* (~800 Mbp) genome were published in 2013 (Kubisiak et al. 2013; Fang et al. 2013). A consortium led by John Carlson at Penn State University has assembled a genome sequence of *C. mollissima* using a combination of 454 and Illumina MiSeq reads, and BAC paired-end Sanger sequences (Carlson 2014), with scaffolds anchored into pseudo-chromosomes using the physical map. They also annotated the genome with over 36,000 gene models, which are available on the Hardwood Genomics webpage (www.hardwoodgenomics.org). The consortium has sequenced additional genotypes of Chinese and American chestnut to obtain variant data (Carlson 2014). A research group based at Purdue University has resequenced 16 Chinese chestnuts and hybrids

with variable blight resistance, in order to investigate variation at loci implicated in resistance (LaBonte and Woeste 2016). In addition, transcriptomic data are available for American chestnut (*C. dentata*), Japanese chestnut (*C. crenata*), and European chestnut (*C. sativa*), generated under The Fagaceae Project in the USA (http://www.fagaceae.org/).

Pedunculate Oak (*Quercus robur*)

Quercus robur is one of Europe's most widespread trees, with considerable timber and ecological value. Due to its long generation time, the focus of genomic research on this species has been upon ecological and speciation genetics, rather than selection and breeding for trait improvement (Plomion et al. 2016). A genome sequence, based on Sanger, 454, and Illumina read data, is being assembled by a French collaboration led by Christophe Plomion at INRA (the French National Institute for Agricultural Research) in Bordeaux (Plomion et al. 2016). The 1C-genome size of pedunculate oak is ~740 Mbp (Lesur et al. 2011). A tree was chosen for sequencing for which genetic maps (Durand et al. 2010; Bodénès et al. 2012) and a small amount of exploratory genomic sequence data (Lesur et al. 2011; Faivre-Rampant et al. 2011) had already been generated (Lesur et al. 2011; Faivre-Rampant et al. 2011). This tree had been used as a parent in crosses to study quantitative traits (Plomion et al. 2016). An initial assembly was released in November 2014 on the project's website, http://www.oakgenome.fr, consisting of 17,910 scaffolds (Plomion et al. 2016). The second, improved version consists of approximately 1400 scaffolds ordered into 12 pseudomolecules with the help of a linkage map (C. Plomion, pers. comm.). The consortium has also generated genome sequence data for four other European oak species (Plomion et al. 2016). In addition, transcriptomic data are available for red oak (*Quercus rubra*) and white oak (*Quercus alba*) generated under The Fagaceae Project in the USA (http://www.fagaceae.org/).

European Ash (*Fraxinus excelsior*)

The European ash, *Fraxinus excelsior*, is a widespread woodland tree in Europe of ecological and economic consequence. Though it had previously been subject to little molecular genetic research, apart from investigations of population structure and mating systems, it became the subject of genome sequencing in 2012 due to the rapid spread of the fungal pathogen *Hymenoscyphus fraxineus* across Europe (Pautasso et al. 2013). The ~880 Mbp genome of a low heterozygosity British tree produced by self-pollination was sequenced using 454 and Illumina technologies by a collaboration between Queen Mary University of London and Eurofins MWG GmbH with assemblies released at www.ashgenome.org. Meanwhile, a Danish tree that had been shown to have low susceptibility to *H. fraxineus* (McKinney et al. 2011) was Illumina sequenced at The Genome Analysis Centre, Norwich and released at https://geefu.

oadb.tsl.ac.uk/. Collaboration between the two institutions led to the annotation of the reference genome from the low heterozygosity tree, which proved easier to assemble, and the low coverage sequencing of 37 further *F. excelsior* trees representing provenances from across Europe to study natural variation (E. Sollars et al., in press). The reference genome has facilitated associative transcriptomic studies, identifying gene expression markers associated with reduced susceptibility to *H. fraxineus* in Denmark (Harper et al. 2016). In the USA, where ash populations are being devastated by the emerald ash borer, transcriptome sequencing has been conducted on green ash (*F. pennsylvanica*) and white ash (*F. americana*) within the Hardwood Genomics Project (http://hardwoodgenomics.org/). Low coverage genome sequencing of green ash has also been generated under this project.

Purple Willow (*Salix purpurea*)

Salix purpurea has become a key model species in genetic improvement for shrub willows, for use as a biomass crop. A genome project is led by Larry Smart's group at Cornell University and involves researchers from Oak Ridge National Laboratory and the J. Craig Venter Genome Institute. The ~450 Mbp genome has been sequenced using the Illumina platform and assembled into scaffolds which have been annotated using RNA-seq data and anchored to a genetic map (Carlson et al. 2014). This has been released at http://phytozome.jgi.doe.gov/. The genome has already been used as a reference for genotyping by sequencing (GBS) of 100 of further individuals, leading to the identification of genetic markers associated with growth and biomass yield (Carlson et al. 2016; Gouker et al. 2016).

Shrub Willow (*Salix suchowensis*)

The genome of *Salix suchowensis* was published in 2014 by research institutes in China, USA, and the UK (Dai et al. 2014). It consists of ~304 Mbp of sequence in 103,144 scaffolds, on which 26,599 putative protein-coding genes were annotated. They also compare the genome to that of poplar (*Populus trichocarpa*), and investigate divergence, substitution rates, and whole genome duplications in the two species. Since the publication of the genome sequence, it has been used in a genome-wide study of heat shock proteins (Zhang et al. 2015). The researchers identified 27 HSPs and studied their expression profiles during development and in response to abiotic and biotic stresses such as heat, drought, and salt.

Dwarf Birch (*Betula nana*)

Dwarf birch is a small tree found in boreal scrub communities; one of the most northerly distributed woody angiosperms. Though of little economic importance, it is a keystone species to the ecology of the sub-arctic. It also holds promise as a

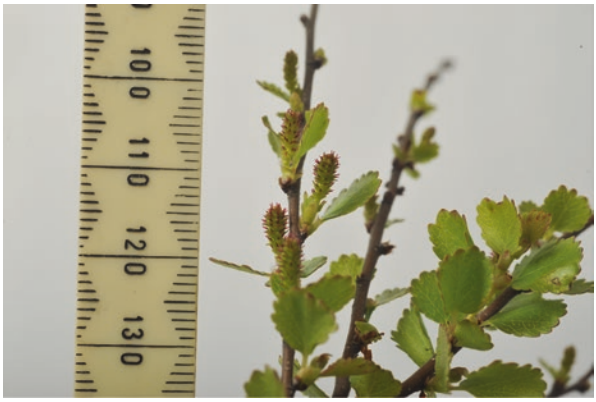

Fig. 1 Sequenced individual of dwarf birch (*Betula nana*), a species found in boreal regions. The small size of dwarf birch allows it to be cultivated in limited space. Scale in mm

Table 1 Assembly statistics for six selected emerging angiosperm tree genomes

Species	1C genome size (mbp)	Assembly version	No. scaffolds	Assembly size	Scaffold N50 (kbp)	No. genes annotated
C. mollissima	794	v1.1	41,260	724 mbp	39.6	36,478
Q. robur	740	v1.0	18,000 > 2000 bp	1350 mbp	257	54,000
F. excelsior	877	v0.5	89,487 nuclear ≥ 500 bp 26 mitochondrial 1 plastid	868 mbp	104.0	38,852
S. purpurea	450	v1.0	7528	392 mbp	17,359.0	37,865
S. suchowensis	429		103,144 ≥ 100 bp 7516 ≥ 2000 bp	304 mbp	925	26,599
B. nana	450		551,923 75,763 ≥ 500 bp	564 mbp	18.79	None

model organism, being small in size and short in generation time, with a ~450 Mbp 1C-genome size. A draft genome was published in 2013 (Wang et al. 2013), of the individual shown in Fig. 1, based on Illumina sequencing. Though fragmented and preliminary, this assembly was a useful reference for the restriction amplified digest (RAD) sequencing of other individuals of *B. nana*, and also *B. pubescens* and *B. pendula* (Wang et al. 2013). An improved assembly using SMRT sequencing (Pacific Biosciences, CA, USA) is underway).

Genomes of Angiosperm Fruit and Nut Trees

Whilst this chapter is mainly focused on angiosperm trees related to forestry or biomass production, it must be noted that a wealth of genomic data is being generated on other angiosperm trees used for agronomic purposes. Genome sequences have been assembled for species such as: walnut (*Juglans regia*) (Martínez-García et al. 2015), European

hazelnut (*Corylus avellana*) (Rowley et al. 2012; Rowley 2016), several citrus fruit (*Citrus*) genomes (Wu et al. 2014; Xu et al. 2013), apple (*Malus domestica*) (Velasco et al. 2010), peach (*Prunus persica*) (Verde et al. 2013), Chinese white pear (*Pyrus* x *bretschneideri*) (Wu et al. 2013), European pear (*Pyrus communis*) (Chagné et al. 2014), pistachio (*Pistachia vera*) (Kafkas 2016), cacao (*Theobroma cacao*) (Argout et al. 2011), coffee (*Coffea canephora*) (Denoeud et al. 2014), papaya (*Carica papaya*) (Ming et al. 2008), date palm (*Phoenix dactylifera*) (Al-Dous et al. 2011; Al-Mssallem et al. 2013; Mathew et al. 2014), and oil palm (*Elaeis guineensis*) (Singh et al. 2013). The genome of the rubber tree (*Hevea brasiliensis*), used for its latex production, has also been assembled and published (Rahman et al. 2013). In addition there are several projects assembling the olive (*Olea europaea*) genome; the International Olive Genome Consortium (http://olivegenome.karatekin.edu.tr/), the OLEA consortium (http://www.oleagenome.org/), and the Olive Tree Genome Project (http://olive.crg.eu, Cruz et al. 2016).

These datasets are important in informing comparative studies of tree genomes generally and in some cases provide useful reference genomes for timber trees, such as walnut cultivars grown for forestry rather than nut production, and rubber trees used for timber once latex productivity has declined.

Projects Not Yet in the Public Domain

There are also angiosperm tree genome projects that we know to be underway but which have at the time of writing not yet released data into the public domain. By their nature, such projects are little publicised. The authors are aware of a project in Finland on *Betula pendula* (silver birch) (Rajaraman and Salojärvi 2015) and another in China on *Betula platyphylla* (Japanese white birch) (C. Yang pers. Comm., see http://birch.genomics.cn/). There are no doubt other projects on genera that we do not work on and thus are unaware of.

Species with Genome-Wide Data

In addition to the whole genome sequence assemblies outlined above, genome-wide data is now available for many other trees. The Dendrome (http://dendrome.ucdavis.edu/) portal provides comprehensive and continually updated access to these rapidly growing resources, many of which have not yet been accompanied by a published paper (Wegrzyn et al. 2008). In several cases, these datasets have been collected to accompany and aid the interpretation of the reference genomes we have already described, such as the population datasets mentioned above for oak species in Europe, European ash, and American chestnut and hybrids. Here, we will not catalogue everything that is available, but mention a selection of tree species which are the first in their genus to be subject to sequencing, and may therefore emerge as reference sequences for their genus as and when funding becomes available. The USA-based Fagaceae Project has released 454 transcriptomic data for American beech (*Fagus grandifolia*) (http://www.fagaceae.org/), as well as generating data for oaks and chestnuts. European

groups have also released transcriptomic data for *Fagus sylvatica* on Dendrome. Low coverage genome sequencing was recently reported for ten native hardwood tree species from the eastern United States including: blackgum (*Nyssa sylvatica*), redbay (*Persea borbonia*), sugar maple (*Acer saccharum*), sweetgum (*Liquidambar styraciflua*), and honeylocust (*Gleditisia triacanthos*) (Staton et al. 2015).

Future Steps

Rapid progress has been made in angiosperm forest tree genomes over the past few years due to the rise of next generation sequencing, and the pace of progress is set only to increase as new technologies such as Oxford Nanopore (Oxford Nanopore Technologies, Oxford, UK) sequencing, SMRT sequencing (Pacific Biosciences, CA, USA), and optical mapping continue to improve (Howe and Wood 2015; VanBuren et al. 2015). This raises the question as to where researchers need to focus their future efforts. To some extent, research programmes on poplar (see Chap. 5) and eucalyptus (see Chap. 6) provide good exemplars, though less funding may be available for emerging genomes than was available for those species. Several possibilities are available to researchers, which we outline below.

Firstly, improvement of current reference genome assemblies is needed. Most of the genome sequences reviewed above are still in fragmented states and far from being assembled at a chromosomal level. Some are lacking genetic maps to anchor scaffolds to, and where maps are available, a high proportion of scaffolds remain unanchored. Furthermore it is well known that *de novo* genome assemblies often miss genes, spuriously duplicate them, or join contigs erroneously (Denton et al. 2014; Elsik et al. 2014; Alkan et al 2011). Difficult decisions need to be taken regarding how much time and effort to devote to improving reference genomes. A better genome assembly may lead to more powerful genome-wide analyses of, for example, trait-associated loci or patterns of introgression. However, in some cases, especially where the reference tree selected is highly heterozygous, the genome contains many repetitive elements, or is polyploid, a chromosomal-level genome assembly may be almost impossible with current technologies. In these cases, and perhaps more widely, the wisest course may be to do the best possible assembly with 200× Illumina coverage and as much longer read data that can be afforded (such as 454 or PacBio reads), and then wait for new technologies to develop or improve (such as Oxford Nanopore technology at the time of writing (Goodwin et al. 2015)) before attempting to make substantial improvements to the assembly. Enhancements of sufficient magnitude are likely to stem from technologies that focus on joining and ordering scaffolds, such as optical mapping and BioNano Irys, or filling in assembly gaps that the usual technologies are unable to sequence, rather than from simply obtaining additional sequencing data.

Secondly, researchers can focus on sequencing more individuals from the same species. This has been done in many of the species mentioned above, such as ash, chestnut, oak, and willow, where a focal sequence now has a penumbra of additional genomes, often sequenced at lower coverage and assembled using the focal individual

as a reference. There is a danger that differences in gene content among individuals, where such variation exists, may be undiscovered by this approach, but it does allow the characterisation of much genome-wide variation within species, which may aid the development of SNP panels for larger studies of population variation. Without population-level studies, bottom-up inferences of the functional significance of loci within the genome are impossible, and all we can rely on are homology searches to better functionally-characterised plant genomes. These are of course useful for initial annotation of plant genomes, but rely heavily on the assumption that similar sequences will have similar functions. Such approaches are of limited value for the characterisation of taxonomically restricted genes (Khalturin et al. 2009).

Thirdly, sequencing of other species of the same genus can be undertaken, as this may allow the characterisation of genes responsible for key differences between species, such as ecological adaptations in *Q. robur* versus *Q. petraea* or blight resistance in *C. mollissima* versus *C. dentata* (see above). Here, the independent assembly of the different genomes may be particularly important, in order to take account of species-specific genes and gene-family expansions. Reference-guided assembly approaches have been developed which assemble genome sequences independently of a related reference so as to retain any variation, but still use the reference to aid the placing of scaffolds into longer contiguous sequences (Bao et al 2014; Kim et al. 2013). On the other hand, mapping of reads from closely related species to a single reference genome can identify population dynamics such as hybridisation and introgression (Suarez-Gonzalez et al. 2016).

Fourthly, research could focus on functional characterisation of genes within the reference genome using experimental approaches. However, such approaches, which have worked well for *Arabidopsis*, are highly challenging in tree species. Generation of inbred lines, knock-out lines, or multiple mapping populations are seldom feasible for long-lived trees which outgrow laboratory growth cabinets long before reproduction is a possibility. In some cases, experiments can be carried out using orthologues in a model plant species (Salmon et al. 2014). However, newly developed, targeted methods could easily achieve what would take years with conventional approaches. For example, targeted mutagenesis by CRISPR/Cas9 has been used to create knockout mutations in *Populus tomentosa* (Chinese white poplar) (Fan et al. 2015), and the expression of genes was inhibited using virus-induced gene silencing in two other *Populus* species (Shen et al. 2015).

A related consideration is at what point in time research groups should publish their genome assemblies. In recent years there has been a growing willingness to release data early (Neale et al. 2013), but it is notable that four of the six major reference genomes reviewed above do not have a final peer-reviewed publication associated with them, even though some of the assemblies have now been available online for years. This may be due to a lack of funding meaning that personnel are not available for the higher-level analyses and manuscript writing that is needed. It may be due to consortia waiting for further improvements to the assembly and annotation before they attempt a high-impact publication: the presubmission paper for *Q. robur* (Plomion et al. 2016) outlines such a strategy. It may be due to lengthy review processes by journals. Indeed, the only genomes of those reviewed here with a final publication at present are *B. nana* (which follows the opposite extreme of publishing a very preliminary and highly

fragmented genome with no annotation (Wang et al. 2013)) and *S. suchowensis* (Dai et al. 2014). It would seem that there are currently as many different publishing strategies for tree genomes as there are reference assemblies.

Decisions about when to stop improving a genome, or when to publish can be informed by quality metrics. Various statistics are often used to compare assemblies and allow optimisation of a series of variable parameters used during assembly, or to look for notable improvement upon receiving additional data. Simple measurement statistics such as the length of assembly, number of scaffolds, and N50 give an indication of contiguity, but cannot describe the quality of the sequence. Computational methods that search the assembly for conserved genes (Simão et al. 2015; Parra et al 2007), or use the mapping of DNA reads can be used for this purpose (Vezzi et al 2012; Clark et al. 2013). However, given that some genomes are harder to assemble than others, and that different uses of genome assemblies require different levels of quality, there is no single standard for when a genome assembly is considered publishable.

Broader Implications

Angiosperm forest trees in the past may have appeared to be less promising species for genetic improvement than their gymnosperm counterparts. However, emerging genomes show that there is an important place for genomic research on angiosperm forest trees. Because their genome sizes are much smaller than the multi-gigabase genomes of conifers (Kelly et al. 2012), they are much quicker and cheaper to sequence. The sequencing of multiple individuals is also feasible. Genomic research is thus more likely to yield rapid benefits for angiosperm trees than for gymnosperms.

Herbaceous angiosperms have tended to dominate genomic research, as they are far more amenable to experimentation and breeding than angiosperm trees. Recent years have seen the successful application of genomic selection to annual crop species (Heffner et al 2009), which has allowed time and money to be saved in breeding due to selection of seedlings at early, pre-reproductive ages. If such approaches are economically viable because they can save a few weeks per generation in annual crop breeding, they may have a huge impact on tree selection and breeding (Denis and Bouvet 2012; Resende et al. 2012), where they can allow selection to take place many years before a tree is of reproductive age. Therefore, genomic selection may ultimately have a bigger impact on angiosperm tree breeding than on angiosperm crops.

As well as holding great promise, genomic research on a wide range of angiosperm trees is necessary and timely (Neale and Kremer 2011). Global trade and lax biosecurity measures have resulted in unprecedented spread of pests and diseases around the globe in the past decades, causing grave damage to tree populations (Brasier 2008; Boyd et al. 2013). Moreover, the value of natural capital and the need for renewable energy sources and carbon fixation is now appreciated more than ever (Helm 2015). It has recently been suggested that the replacement of angiosperm woodland with coniferous plantations may have contributed to, rather than mitigating, climate change (Naudts et al. 2016). As an emerging field, the genomics of angiosperm trees has much to offer our planet (Table 2).

Table 2 A selection of emerging tree genome projects

Tree	Species	Lead Researcher(s)/group	Data available	URL
Ash	*Fraxinus excelsior*	The British Ash Tree Genome Project, Richard Buggs, QMUL	WGS, genome assembly, SSRs, RNA-seq, gene annotation, bisulphite-seq (ongoing)	www.ashgenome.org
		The Nornex Consortium, Allan Downie, John Innes Centre	WGS, genome assembly, RNA-seq, gene annotation	https://geefu.oadb.tsl.ac.uk
	Fraxinus pennsylvanica	Hardwood Genomics Project	WGS, SSRs, RNA-seq	www.hardwoodgenomics.org
	Fraxinus americana		WGS, SSRs	
Beech	*Fagus grandifolia*	The Fagaceae Genomics Project	RNA-seq, EST assembly	www.fagaceae.org
Birch	*Betula nana*	Richard Buggs, QMUL	WGS, genome assembly, RAD-seq	www.birchgenome.org
	Betula platyphylla	Chunping Yang, Northeastern Forestry University, China	WGS, genome assembly, gene annotation	http://birch.genomics.cn
Black Cherry	*Prunus serotina*	Hardwood Genomics Project	WGS, SSRs	www.hardwoodgenomics.org
Black Walnut	*Juglans nigra*	Hardwood Genomics Project	WGS, SSRs, RNA-seq, ddRADtag (ongoing)	www.hardwoodgenomics.org
Blackgum	*Nyssa sylvatica*	Hardwood Genomics Project	WGS, SSRS, RNA-seq	www.hardwoodgenomics.org
Chestnut	*Castanea crenata*	The Fagaceae Genomics Project	EST assembly	www.fagaceae.org
	Castanea dentata		EST assembly	
	Castanea mollissima		WGS, EST assembly, Physical map	
	Castanea sativa		EST assembly	

Honeylocust	*Gleditsia triacanthos*	Hardwood Genomics Project	WGS, SSRs, RNA-seq,GBS (ongoing)	www.hardwoodgenomics.org
Oak	*Quercus alba*	Hardwood Genomics Project	WGS, SSRs, EST assembly	www.hardwoodgenomics.org
	Quercus robur	Christophe Plomion, INRA	WGS, genome assembly, SNPs, transcriptome assembly, genetic map	https://w3.pierroton.inra.fr/QuercusPortal/index.php www.oakgenome.fr
	Quercus rubra	Hardwood Genomics Project	WGS, RNA-seq, ddRADtag (ongoing)	www.hardwoodgenomics.org
Redbay	*Persea borbonia*	Hardwood Genomics Project	WGS, SSRs	www.hardwoodgenomics.org
Sugar Maple	*Acer saccharum*	Hardwood Genomics Project	WGS, SSRs, RNA-seq	www.hardwoodgenomics.org
Sweetgum	*Liquidambar styraciflua*	Hardwood Genomics Project	WGS, SSRs, RNA-seq	www.hardwoodgenomics.org
Tulip poplar	*Liriodendron tulipifera*	Hardwood Genomics Project	RNA-seq, GBS (ongoing)	www.hardwoodgenomics.org
Willow	*Salix purpurea*	Larry Smart, Cornell University	WGS, genome assembly, gene annotation	https://phytozome.jgi.doe.gov/pz/portal.html
	Salix suchowensis	Tongming Yin, Nanjing Forestry University	WGS, genome assembly, EST assembly	http://www.ncbi.nlm.nih.gov/bioproject/203514

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Structural Genomics of Angiosperm Trees: Genome Duplications, Ploidy, and Repeat Sequences

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Abstract Angiosperm genomes frequently undergo polyploidy, which results in a genome doubling event, followed by genome rearrangement and organisation. Such whole genome duplication events often result in immediate speciation, contributing to the spectacular radiation of the angiosperm lineage, while also creating two copies of each gene, which subsequently diverge, further fuelling species evolution. In addition to the pronounced role that polyploidy plays in shaping plant genomes, repetitive elements also serve to drive smaller scale patterns of duplication, rearrangement and novel variation. The action of repetitive elements is a pivotal source of evolutionary novelty, creating novel structural rearrangements, modulating gene expression and driving epigenetic variation.

Recent advances in sequencing technologies are revolutionising our ability to explore ever more genomes, enabling new insight into the pronounced role that whole genome and local patterns of duplication have played in shaping tree genomes, including the prevalent signature of genome duplication in the *Populus trichocarpa* genome and the striking prevalence of gene duplication in *Eucalyptus grandis.*

Keywords Polyploidy • whole genome duplication • repeat • transposable element • genome assembly • evolution

The availability of an ever-increasing number of sequenced plant genomes (Michael and Jackson 2013) has shown that the genomes of plants are far more dynamic than those of their animal counterparts (Hettiarachchi et al. 2014). Indeed, vertebrate genomes separated by hundreds of millions of years of evolution and divergence can be relatively well aligned, with conserved large-scale synteny (Smith et al. 2002; Hillier et al. 2004; Dehal and Boore 2005). Additionally, conserved non-coding DNA sequences (CNS, also referred to as conserved non-coding elements, CNE) can be identified across the entire vertebrate lineage (Woolfe et al. 2005; Lee et al.

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2011). A number of studies have identified CNEs in plants at varying phylogenetic depths (Kritsas et al. 2012; Hettiarachchi et al. 2014), however these are far fewer, less conserved and shorter on average than those identiied in vertebrate species. Likewise, plant genomes that diverged far more recently than vertabrate genomes show distinctly lower rates of macro colinearity (Barker et al. 2012).

One salient feature of plant genomes is their long-known propensity for the formation of polyploids (Fawcett and Peer 2010; Jiao et al. 2011; Soltis et al. 2014; Wendel 2015; Soltis et al. 2015), which results in a whole genome duplication (WGD) event (genome doubling), typically followed by a burst of genome shuffling and reorganisation (Otto 2007) that terminates with the restoration of a diploid state (Wolfe 2001). The importance of WGD, which create duplicated gene copies, have been long recognised, with their evolutionary importance formalised in the pivotal work of Ohno (1970). Indeed, it is likely that the prevelence of WGD and smaller-scale segmental gene duplication events was a key source of evolutionary raw material fueling the dramatic radiation of flowering angiosperm species (Darwin's "abominable mystery"). In addition to the ubiquitous role that genome duplications have played in shaping plant genomes, repetitive elements are also instrumental. While duplication creates exact copies at the point of the duplication event, the activity of repeats serves to shuffle, disrupt and rearrange genomes, creating structural variants that can affect organism function, either through direct effects on protein coding genes or via epigenetic effects, such as changes to chromatin structure.

The past decade has seen an explosion in the availability of genome assemblies as a result of next generation sequencing (NGS) methods. Comparative analyses of these assemblies is enabling a far more comprehensive picture of plant genomes. In addition to the genomes of model plant species and plants of agricultural importance, the array of available genomes has expanded to include examples of species of cultural or scientific interest in addition to those facing imminent threat from biotic attack or rapid climate change. These new genomes have included a number of tree species (Table 1, see Resources below), from which it is apparent that tree genomes *per se* resemble those of all other plants, with a wide range of genome sizes, repeat content and the presence of WGD events. As such, the features that make a tree a tree lie not in any gross scale characteristics of tree genomes but rather in the detailed patterns of gene duplication, loss and retention, as well as differences in gene family expansion and the regulatory control of genes.

Here, I briefly overview the processes of polyploid formation, WGD events, the major classes of repetitive elements active in plant genomes, and the current challenges and future directions for research. I draw particular attention to examples from published tree genomes and direct readers to current resources.

Polyploidy

The availability of multiple genome sequences has now firmly established that polyploidy events are found in the lineages of all extant angiosperms (Vision et al. 2000; Tang et al. 2008; Paterson et al. 2010; Jiao et al. 2011). Comparative analyses of

Table 1 Details of selected sequenced angiosperm tree genomes

Species	Reference	Available in Phytozome (PH), PLAZA (PZ), EnsemblPlants (EP), PlantGenIE (PL), Plant genome duplication database (PD).
Betula nana	Wang et al. (2013a)	
Carica papaya	Ming et al. (2008)	PH, PZ, PD
Castanea mollissima	http://www.hardwoodgenomics.org/chinese-chestnut-genome	
Citrus clementina	Wu et al. (2014)	PH
Citrus sinensis	Xu et al. (2013)	PH, PZ, PD
Coffea	Denoeud et al. (2014)	
Eucalyptus grandis	Myburg et al. (2014)	PH, PZ
Fraxinus excelsior	http://www.ashgenome.org	
Hevea brasiliensis	Rahman et al. (2013)	
Malus x domestica	Velasco et al. (2010)	PH, PZ, PD
Populus trichocarpa	Tuskan et al. (2006)	PH, PZ, PL, PD, EP
Populus euphratica	Ma et al. (2013)	
Populus tremula	http://popgenie.org	PL
Populus tremuloides	http://popgenie.org	PL
Populus tremula x tremuloides 'T89'	http://popgenie.org	PL
Populus tremula x alba '717-1B4'	Zhou et al. (2015)	PL
Prunus persica	Verde et al. (2013)	PH, PZ, PD, EP
Prunus mume	Zhang et al. (2012)	PD
Pyrus communis	Chagné et al. (2014)	
Pyrus bretschneideri	Wu et al. (2013)	PD
Quercus robur	Plomion et al. (2016)	
Salix purpurea	https://phytozome.jgi.doe.gov	PH
Salix suchowensis	Dai et al. (2014)	PL
Theobroma cacao	Motamayor et al. (2013)	PH, PZ, PD, EP
Ziziphus jujuba	Liu et al. (2014)	

available genomes also allows attempts to reconstruct the ancestral angiosperm genome and gene set, in addition to identifiying lineage and species-specific genes (Paterson et al. 2010). Polyploid species are far more prevalent in angiosperms than in other eukaryote lineages, with an abundance of extant angiosperm polyploid species (Wood et al. 2009). However, these remain the exception rather than the rule and polyploid species have been shown to experience higher rates of extinction than diploids (Mayrose et al. 2011). Opinion regarding the importance of polylpoidy in angiosperm speciation has shifted significantly throughout the past century. Much of the seminal work in the field was produced by Stebbins in the first half of the twentieth century, and is reviewed in Soltis et al. (2014). Many of Stebbins' concepts have stood the test of time, most notably his classification of the various types

of polyploidy events (see below), while others have been refined or initially countered and later refuted, such as his view that polyploids represented evolutionary dead-ends (Levin 1983). Nonetheless, the study of polylpoidy remains as relevant today as ever for furthering our understanding of the drivers of angiosperm diversity and the evolution of traits of adaptive and agronomic importance.

With the increasing availabaility of genome resources, higher resolution for determining the past timing of polyploidy events has made it possible to link these events to the emergence of key morphological and physiological adaptations of plants (Jiao and Paterson 2014). Such repeated phases of polylploidy and subsequent diploidization have been pivatal in shaping plant genomes (Adams and Wendel 2005; Wendel 2015; Wendel et al. 2016), resulting in the creation of novel genes, the expansion of specific gene families and, more generally, are thought to be the driving force behind the accelerated rates of land plant evolution compared to other crown group eukaryotes (Jiao and Paterson 2014). A WGD event typically results in a speciation event, which leads to an increase in biodiversity (potentially thus explaining, at least in part, the spectacular explosion in diversity of the angiosperms and the lack thereof in gymnosperms, which rarely form polyploids) as well as representing new genetic material for natural selection to act upon (Levin 1983; Adams and Wendel 2005; Leitch and Leitch 2008; Van de Peer et al. 2009). Interestingly, Lee et al. (2011) showed that the rate of divergence and loss of CNEs in teleost fish is higher than that of the bony vertebrate species considered, with only the teleost fish lineage having undergone WGD events since divergence from their last common ancestor, suggesting that the more dynamic and rapid rates of CNE divergence and loss in plants may be a more general phenomena associated with genomes tolerant of WGD events.

Types of Polylpoidy

Two distinct mechanisms of polyploid formation are recognised: autopolyploidy and allopolyploidy (reviewed in Ramsey and Schemske 1998). An autopolyploid is formed as the result of an intraspecific WGD in contrast to allopolyploidy, which results from the hybridization of two species with an associated genome doubling. Evidence as to the origin of observed polyploidy traditionally relied on cytological examination, with autopolyploids more likely to form multivalent pairings during meiosis as a result of higher homology than for allopolyploids, where bivalents are more likely (De Storme and Mason 2014). However, these are not clear-cut distinctions, and current understanding is that these two possibilities do not occur as absolute states but that there is, rather, a continuum between the two.

There are three primary mechanisms resulting in ploidy increases: meiotic non-reduction; somatic genome duplication; karyotype changes resulting from aneuploidy or dysploidy. In the case of meiotic non-reduction an error during the process of meiosis results in a mitotic-like replication during the process of meiosis and the

resultant formation of diploid gametes – a process termed 'sexual polyploidisation'. In the majority of such cases, these gametes will fuse with a reduced, haploid gamete thus creating a triploid (unilateral sexual polyploidisation). Most typically the seed of such triploids is non-viable, a phenomenon referred to as the triploid block (Schatlowski and Köhler 2012). However, in rare cases a viable triploid lineage can be formed and, most typically, at this point a new species is effectively produced as the successful offspring become immediately reproductively isolated (Jiao and Paterson 2014). Alternatively, a diploid gamete can fuse with a second diploid gamete to form a tetraploid (bidirectional sexual polyploidisation), a process that is far more likely to yield a stable and viable tetraploid lineage. Readers are referred to De Storme and Mason (2014) for a recent, comprehensive review of the cellular processes that can lead to polyploidisation resulting from both intra- and inter-specific hybridisations. The relative importance of these different mechanisms is unknown, however an intriguing clue may arise from the fact that the few gymnosperm species with reported cases of polyploidy are also those in which endoreduplication is more common (Leitch and Leitch 2012). Endoreduplication is far more, prevalent in angiosperms than gymnosperms, and somatic genome duplication and mitotic errors may, therefore, be of particular importance to angiosperm evolution. However this remains unexplored and there are a number of other differences between gymnosperms and angiosperms that may also underlie to lack of polyploidy in gymnosperms.

Whole Genome Duplications

Over the past decade, a number of phylogenetically important genome sequences became available, with each additional genome allowing refinement of our understanding of the evolutionary history of the angiosperm lineage. Most recently, new insights into the basal angiosperm genome were made possible with the release of the *Amborella trichopoda* genome (Chamala et al. 2013; Rice et al. 2013). Preliminary analysis using BAC (bacterial artificial chromosome) end sequences of Chinese chestnut (*Castenea mollissima*; Staton et al. 2015) and walnut (*Juglans regia*; Luo et al. 2015) suggests that shared life history traits also influence genomic divergence rates in addition to phylogenetic distance, an observation in line with that presented by Zuccolo et al. (2011) where more rapid divergence from *Amborella* was observed for the lineages leading to *Arabidopsis* and *Oryza* than to *Populus* or *Vitis*. Updated analysis making use of the increase in resolution afforded by these genomes has provided new insight into the timing of paleopolyploidy events, revealing periods of high polyploid formation, most strikingly at the Cretaceous-Palaeogene boundary (Vanneste et al. 2014a, b). This was a remarkable period of mass extinctions, representing an acutely stressful situation for survival. A number of observations (reviewed in Vanneste et al. 2014b and Ramsey and Schemske 1998) suggest a link between rates of polyploidy and stress with, for example, greater production of unreduced gametes occurring in conditions of abiotic

perturbation or fluctuation. There is also evidence that gene duplication may represent a mechanisms of increasing genome adaptability under fluctuating, stressful conditions (Kondrashov 2012).

Restoration of Diploidy

Despite the fact that all angiosperm species are paleopolyploids, the majority of extant species are diploids, suggesting that there is an active mechanism that restores the diploid state in the majority of species that avoid extinction following a polyploidy event – a process termed diploidization (Wolfe 2001). The process(es) leading to the restoration of diploidy are not fully understood, and recent analyses of genomic sequences have revealed that there can be distinct bias in which of the two copies of a genome are preferentially retained during the genomic shuffling that occurs as diploidy is restored (Barker et al. 2012; Renny-Byfield et al. 2015; Wendel 2015).

The Fate of Duplicated Genes

Following a WGD event, two copies of each genes are present in the genome – initialy two identical copies (paralogs). In the generations immediately following a WGD, severe genomic instability is observed, with a rapid burst of structural changes at the chromosomal level including fusions and fissions, duplications, inversions, translocations and deletions. These changes are accompanied by rapid transcriptional alterations (Adams and Wendel 2005; Barker et al. 2012) and epigenetic remodulation (Chen 2007), the outcome of which is clearly represented in the case of *Populus trichohcarpa* (Tuskan et al. 2006), where a range of near exact chromosomal duplicates and complex fusions (for example chromosome 1) were revealed by analysis of the genome sequence (Fig. 1). The availability of plant genomes has shown that the most common outcome after such an event is the loss of one of the two copies (Otto 2007). The impact of this process of post-duplication gene loss is made clear by the finding that *Arabidopsis thaliana* has undergone three rounds of polyploidy on the path to its current genome, yet is a diploid with one of the smallest angiosperm genomes (Adams and Wendel 2005). It was originally thought that which of the two copies eliminated would be largely stochastic, however recent analysis has revealed cases of distinct bias (see above). Although the majority of duplicated genes will be lost, a large number of paralogous pairs can, and do, remain. For example Tuskan et al. (2006) reproted ~8000 gene pairs remaining in the *Populus trichocarpa* genome that result from the Salicaceae WGD, while analysis of the draft *Salix suchowensis* genome suggets significantly greater loss of paralogs resulting from the WGD (Dai et al. 2014).

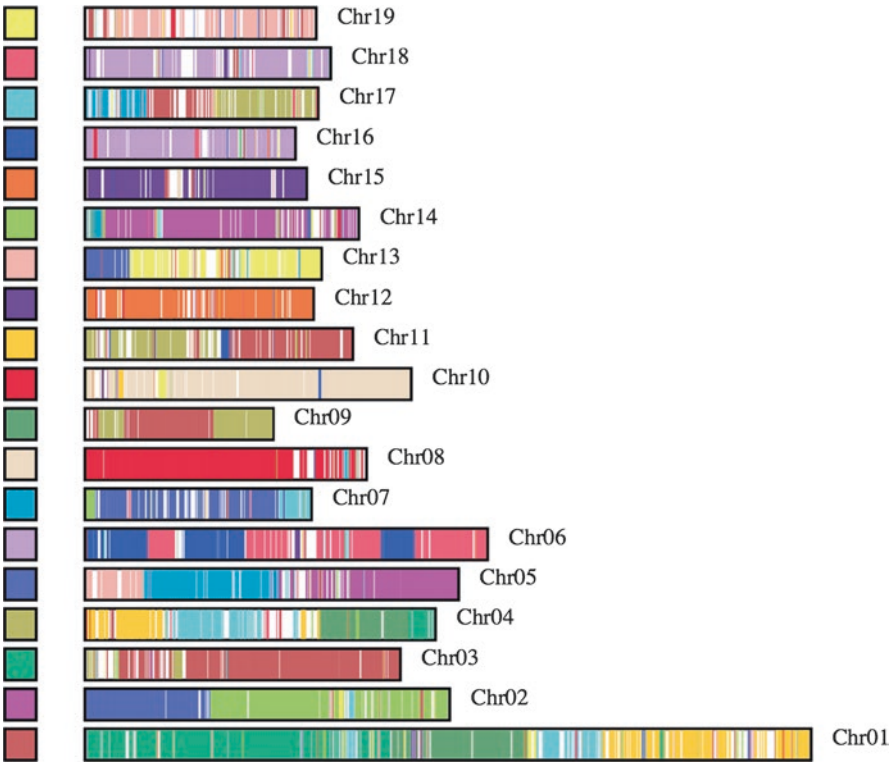

Fig. 1 Visualisation of the *Populus trichocarpa* genome (v3.0), showing paralogous regions as identified by performing a self-self alignment using the sensitive Cactus nucleotide alignment tool (Paten et al. 2011) and following the methodology presented in (Zamani et al. 2013). A range of complexity in chromosome shuffling following the Salicacaeae whole genome duplication can be seen from the highly similar chromsome pairs of Chr12 and Chr15 through the the complex case of Chr01, which contains fusions of regions from a number of chromosomes

Following duplication, genes can undergo four fates: psedogenisation of one gene copy and subsequent degradation over time; concerted evolution to maintain both copies; sub-functionalisation, whereby the two copies either develop distinct expression domains or regimes (*e.g.* becoming restricted to specific tissues, conditions or developmental stages); or neo-functionalisation, whereby one or both copies develop novel functions (Conant and Wolfe 2008; Innan and Kondrashov 2010; Levasseur and Pontarotti 2011; Barker et al. 2012). It is thought that sub- and neo-functionalisation have been particualrly important processes providing material for evolutionary novelty, and that thus the repeated rounds of polyploidisation and diploidization may represent the most important mechanism underlying the rapid diversification of flowering plants. Indeed, this can result in polyploid species rapidly diverging from their diploid parents (Van de Peer et al. 2009; Fawcett and Peer 2010) and the discovery of differences in retention rates among functional gene classes suggest biological significance to loss and retention patterns (Charon et al. 2012).

Analysis of 20 available plant genomes has revelaed a set of genes that are resistant to duplication and that appear to always be rapidly returned to a single copy state (De Smet et al. 2013). As is the case for genes retained after duplication, these strictly (and mostly) single copy genes are enriched for specific biological functions and represent many housekeeping or essential functions (De Smet et al. 2013). While this set of genes display deep sequence conservation, this alone is not a signature predictive of whether a gene will resist duplication as there are also numerous duplicated genes of high sequence conservation. A number of hypotheses have been proposed as to why certain genes may resist duplication. Perhaps the most commonly stated is that of dosage balance, whereby it is important for some genes to be retained in the correct copy number relative to another gene (Barker et al. 2012; Schnable et al. 2012; Birchler and Veitia 2012; Conant et al. 2014). However, the obvious problem with this is that balance of maintained following a WGD event as all genes are duplicated. De Smet et al. (2013) observed that many single copy genes encode proteins involved in organelle funciton. As the chloroplast genome is not duplicated by a WGD event, stoichiomaetric balance will remain important for any protein that must be present in balance with a chloroplast-encoded partner. Alternatively, any gene where a mutation leads to a detrimental dominant-negative phenotype will likely be maintained at low copy number as additional copies increase mutational exposure (Levasseur and Pontarotti 2011).

Experimental systems to explore the effects of changes in gene copy number are few and far between for tree species. However, Henry et al. (2015) recently described a system in *Populus* where copy number of genomic regions has been altered by gamma-irradiation of pollen. Exploring the effects of the represented copy number variants at all levels from genome to phenotype represents a powerful resource for understanding the effects of copy number changes. This is also a unique resource for exploring the effects of changes in gene balance and whether detrimental phenotypes are observed for lines containing altered copy number for the duplication resistent genes identified by De Smet et al. (2013).

Other Mechanisms of Gene Duplication

The discovery of a substantial number of arrays of tandemly duplicated genes in the *Eucalyptus grandis* genome (Myburg et al. 2014) provides a clear example that gene duplication mechanisms other than WGD can also have important effects for the evolution of a species. A number of mechanisms other than WGD can result in the creation of complete or partial copies of genes. Any duplication affecting a unit smaller than an entire chromosome is referred to as a segmental duplication, which typically results from replication errors during meiosis (Kaessmann 2010; Barker et al. 2012), and that include tandem, interspersed, intra-chromosomal and inter-chromosomal subtypes (Lynch 2007). Segmental duplications can range from a few base pairs up to mega base pair regions, spanning a range from micro INDELs (INsertion DELetion) through to large structural variants (SVs), respectively.

Mechanisms leading to such segmental duplications include non-homologous end joining (NHEJ) nonallelic homologous recombination (NAHR), unequal crossover and retroposition (Lynch 2007; Kaessmann 2010; Levasseur and Pontarotti 2011; Chandan and Indra 2014), with each mechanisms having distinct consequences, such as a lack of introns for gene duplications arising from retroposition, for example.

Repeat Content and Genome Structure

One remarkable feature of eukaryotic genomes is their relatively similar gene content despite vastly variable genome sizes – the so-called C-value paradox. Although it is clear that polyploidy and WGD are of significant importance to plant genome evolution and genome size variation, by far the most variable and dynamic part of all plant genomes is represented by transposable elements (TEs) – DNA fragments that can move from one location to another in a genome, of which long-terminal repeat TEs (LTR-TEs) are the most abundant in plant genomes (Feschotte et al. 2002; Lisch 2013; Bennetzen and Wang 2014). TEs are classified on the basis of their mode of replication, which either operates via a copy-and-paste (class I) or cut-and-paste (class II) mechanism (Bennetzen and Wang 2014). TE classes are further sub-divided into families based on characteristics such as whether they are autonomous (encoding all of the components needed for their replication), semi-autonomous or non-autonomous, with, for example, short interspersed (retro)elements (SINEs) relying on the *trans*-acting function of autonomous long interspersed (retro)elements (LINEs). Different TE families display distinctly contrasting patterns of where copies are likely to insert within the genome. For example, in maize it has been observed the high copy number LTR-TEs do not insert into genes whereas low copy number elements do. Similarly, miniature inverted-repeat TEs (MITEs), a relatively abundant class of non-autonomous DNA elements, preferentially insert close to, but not inside of, genes (Bennetzen and Wang 2014).

Despite the number of plant genomes that are now available for comparative analyses, no rules or patterns explaining the highly variable patterns of repeat expansion and deletion have yet been uncovered. If anything, findings to date most probably highlight the importance that random chance plays, with not only the extent of TE expansion and subsequent retraction but also the TE families involved appearing to be largely stochastic. There are still relatively few examples where genome sequences are available for highly related species, but these have uncovered that even species that diverged recently can experience vastly different rates of change in genome size and in the distribution of repeat activity throughout the genome. A recent review by Bennetzen and Wang (2014) makes many excellent observations relating to these processes, including that life history, effective population size, migration and selection are all significant contributors to the dynamics of repeat retention or loss at the species level.

Although the activity of TEs is generally perceived to have negative effects on a genome, a number of potentially beneficial effects can also arise from repeat activity. For example, following a polyploidy event, the differential insertion of repeats in the two genome copies can enable proper, and differentiated, pairing during meiosis (Soltis and Soltis 1999; 2000; Jannoo et al. 2004; Chen and Ni 2006). Contrasting patterns of repeat silencing between the two genome copies can result in further differentiation, for example leading to changes in gene activity via the spreading of epigenetic effects and, eventually, changes to the heterochromatin landscape (Hollister and Gaut 2009; Hollister et al. 2011). Within diploid genomes, if a TE insertion occurs within the coding or regulatory region of a gene, the resulting mutation will often be detrimental. As such, polyploidy may actually enable increased TE activity due to a relaxation of purifying selection against detrimental TE insertions as a result of genome doubling increasing redundancy and, consequently, increasing the number of potentially neutral selective sites (Matzke and Matzke 1998).

While WGD events create large scale macro-synteny, the activity of repeats subsequent to the duplication event then causes breaks in the macro-synteny, resulting in a loss of micro-synteny. As insertion of TEs into genes is highly mutagenic, disruption to the order of genes as a result of TE activity is relatively rare. However, the action of TEs will result in the insertion of novel elements between genes, tandem duplications and, less frequently, translocations or inversions with repeated rounds of activity leading to extensive disruption of synteny over time. Analysis of angiosperm genomes has revealed that repeat activity appears to occur in bursts that are often, but not exclusively, relatively short lived and that are followed by a subsequent round of elimination. Variation in the speed and extent of elimination has been observed, although the reasons underlying this variation remain unresolved. Plant genomes are armed with two protective strategies to prevent uncontrolled TE-driven genome size expansion: An active defence system that transcriptionally silences repeats (Lisch 2009; Lisch and Slotkin 2011) therefore preventing their spread and expansion, and the ability to delete repeats by unequal intra-strand homologous recombination (UR), non-homologous end joining (NHEJ) repair of DNA double strand breaks (DSB) or by illegitimate recombination (IR) (Devos et al. 2002; Gaut et al. 2007; Wang et al. 2013b). The presence of repeats in all eukaryotic genomes shows that these defence mechanisms are leaky or imperfect resulting in an evolutionary arms race between repeats, which must adapt and develop methods to escape the host genome silencing and removal defence mechanisms, and the consequent overcoming of that evolved escape mechanism by the host (Nosaka et al. 2012). Without repeat removal by UR/IR, genomes would expand uncontrollably in what has been termed a "one way ticket to genome obesity" (Bennetzen and Kellogg 1997). Plants utilise the evolutionary ancient mechanism of RNA interference common to all eukaryotes (Mukherjee et al. 2013) as a highly efficient method of silencing active repeats, thereby preventing further expansion. Much of what we know about the mechanism of silencing derives from studies of *A. thaliana*, where almost all TEs are silenced in nearly every cell (Slotkin et al. 2009). This silencing is maintained across mitotic divisions via the maintenance of symmetric methylation

(Martínez and Slotkin 2012) with methylation status being reinforced by continual re-targeting of TEs by 24 nt siRNAs (Feng et al. 2010) such that 24 nt siRNA presence correlates well with methylation rates. In all plant genomes examined to date, silencing of TEs via methylation has been observed with the majority of TEs being highly methylated (Lisch and Slotkin 2011; Simon and Meyers 2011).

The biogenesis of 24 nt siRNAs involves transcription of *RNA Polymerase IV* (*POLIV*), which is copied into double-stranded RNA by RNA-dependent RNA Polymerase 2 (RDR2) and cleaved into siRNAs by Dicer-like 3 (DCL3). siRNAs are then incorporated into Argonaute 4 (AGO4), AGO6 or AGO9, subsequently guiding sequence-specific DNA and histone methylation through a pathway termed RNA-directed DNA methylation (RdDM) that establishes methylation by Domains Rearranged Methyltransferase 2 (DRM2) (Henderson and Jacobsen 2007). Recently, an alternative pathway for establishing *de novo* DNA methylation by 21 nt siRNAs was identified although the mechanism remains ill-defined (Wu et al. 2012). Primarily using evidence from *A. thaliana,* it has been shown that TE silencing is inherited, resulting in *trans*-generational epigenetic suppression of TE activity via a process whereby gamete companion cells (the male vegetative cell and female endosperm) become hypo-methylated resulting in re-activation of TEs (Slotkin et al. 2009; Gehring et al. 2009). Transposition in these cells has no consequence on the progeny but results in large-scale production of siRNAs that are exported into the germ cell causing hyper-methylation of TEs, both perpetuating the established "immune system" memory against transposons and preventing new transpositions during this essential developmental stage (Ito et al. 2011).

Repeats are deleted by unequal and illegitimate recombination. UR and IR occur between two highly similar regions such as the terminal short inverted repeats of LTRs. Illegitimate recombination is defined by not requiring involvement of a recA protein or large (>50 bp) regions of homology and can result from different mechanisms including slipped strand repair and double-strand break repair (Devos et al. 2002). Mispairing between either the inverted repeats of a single LTR or between different LTRs and subsequent crossing-over then leads to deletion of the intermediate DNA. In the case of intra-chromosomal UR, very large stretches of DNA can be deleted whereas IR deletes short regions of up to tens of base pairs. UR creates a solo LTR signature due to the removal of one of the terminal repeat units of the LTR. A consequence is that such LTRs can no longer be dated, as dating is based on comparison of the two termini, which are identical at the point of insertion. As such if UR or IR are active enough that many or the majority of LTRs are solo, repeats will appear to be very young as only recent repeats can be dated. Evidence for the importance of these mechanisms has been examined in very few plant species to date, with those studies finding clear differences in the relative importance of IR and UR among species (Bennetzen et al. 2005), although in all cases evidence of both mechanisms was found and concluded to be adequately active and sufficiently efficient at repeat removal to account for the failure to detect repeats older than a few million years. In *A. thaliana* and rice a high proportion of solo LTRs were discovered (accounting for the young age of identified repeats) in contrast to maize, where solo LTRs are rare but identified LTRs are also young (Devos et al. 2002).

Population genetics are also an important factor influencing the process of genome size change. Although UR can rapidly shrink genomes by the removal of large block of DNA between LTRs, it is hard to do so without deleting genes leading to negative fitness and selection. Although mutational effects are less problematic for small deletions, it is unlikely that natural selection could act at the level of genome size to distinguish between individuals with such small resultant differences in genome size. Indeed, if even very small increases in genome size were adequate to measurably reduce fitness, genome expansion would be exceedingly rare. In organisms with smaller effective population sizes, natural selection will be less effective at purging slightly deleterious genome size increases (Lynch et al. 2011).

Analysis of the angiosperm tree genomes available to date shows that they resemble the genomes of all other angiosperm species in gross characteristics, with no tree-specific defining features. Comparison of, for example, the genomes of eucalyptus, poplar and rubber trees (Tuskan et al. 2006; Rahman et al. 2013; Myburg et al. 2014) reveals a range of genome sizes, repeat content and variable histories of WGD and tandem gene duplications. However, the recent availability of additional genomics data from tree species has indicated that rates of divergence and synteny degradation are lower for tree species, probably as a result of their longer generation times (Staton et al. 2015; Luo et al. 2015). Many trees species also have large effective population sizes, are out breeding and have multiple generations contributing alleles. Thus alleles are reintroduced for extensive periods of time, particularly for species such as poplars and aspen where clonal propagation is common (Ingvarsson and Street 2011). Comparison of a willow and poplar genomes also suggested that contrasting life histories can have pronounced effects on rates of gene loss and gain, even among closely related species (Dai et al. 2014), although it remains unclear as to what extent the varying quality of genome assemblies affects such findings.

Methods and Challenges to Analysing Genomes

The highly dynamic and variable nature of plant genomes presents challenges to the comparative analysis of genomes and has required the development of specific analysis tools or pipelines, as those developed for mammalian genomes are not able to cope with the extensive rearrangements and genomic shuffling resulting from the repeated cycles of polyploidisation and diploidization recorded in extant plant genomes. Almost universally, the first step of this analysis is to perform an all-against-all BLASTP (protein BLAST) search. These results are either directly analysed to identify pairs of maximal homology, either intra- or inter-specifically, or as input to methods designed specifically to infer ortholog-paralog relationships – of which orthoMCL (Li et al. 2003) is currently the most commonly used tool. After ortholog or maximal homology inference there are two commonly used approaches to identify evidence of a WGD event. The first is to calculate the synonymous substitution rate (K_s) of paralogs and to then plot the frequency distribution of K_s, whereby a WGD will be identified as a clear peak in the distribution and where the

age of the duplication event can be inferred given a known or assumed mutation rate. A major caveat of such an approach is that such peaks become increasingly less pronounced over time as paralogs diverge beyond the point where they can be identified as such due to saturation effects (Rabier et al. 2014). An alternative approach is to identify blocks of synteny around paralogs, for which ADHoRe (Vandepoele et al. 2002) is a currently popular choice. There are also a number of other methods that are designed more generally to perform inter-specific genome comparisons, which can also detect intra-specific patterns of duplication. For example Saguaro (Zamani et al. 2013) applied complex statistical analyses to explore patterns of genome similarity/divergence. Such analyses allow recreation of the ancestral angiosperm genome, including the identification of the core shared set of genes inherited from the common ancestor (Paterson et al. 2010).

Although there are now a number of plant genomes available, the quality of the genome assemblies can significantly affect the feasibility and robustness of genome-scale analyses. For example, many of the most recent plant genome assemblies produced using next generation short read sequencing platforms have very low contiguity (De La Torre et al. 2014) making synteny inference impossible or of highly limited value, often with any regions that are assembled into longer contiguous sections being highly biased. Similarly, gene annotation quality is significantly affected by genome assembly quality, which can significantly impact gene family inference and K_s based approaches. Although these limitations are commonly known, their importance is often overlooked or under-stated and it remains to be seen how many of the results examining gene duplication and loss will hold up as improved genome assemblies are produced. Although the latest generation of single molecule sequencing platforms, which can produce kilobase pair long sequence reads, will make genome assembly substantially easier and of higher quality, they also present as yet unresolved challenges that are of particular importance to many tree genomes. For example, longer sequencing reads lead to increased haplotype splitting – a feature that requires existing analysis software tools to be adjusted to take this into account.

Available Resources

There are now a number of data resources hosting plant genomes, of which two of the most widely used are Phytozome (https://phytozome.jgi.doe.gov, Goodstein et al. 2012) and PLAZA (http://bioinformatics.psb.ugent.be/plaza/, Proost et al. 2015). Relatively recently, the Ensembl project also introduced a plant focused resource (EnsemblPlants; http://plants.ensembl.org/; Bolser et al. 2016). Of particular relevance to WGD, the Plant Genome Duplication Database (PGDD; http://chibba.agtec.uga.edu/duplication/; Lee et al. 2013) provides tools for exploring chromosome synteny and colinearity. The Comparative Genome resource (CoGe; https://genomevolution.org/coge/; Lyons et al. 2008) also provides extensive web tools for comparing sequenced genomes, including plants. There are additionally

tools aimed more specifically at identifying conserved and diverged gene expression regulation, which can be used to explore divergence of paralogs following WGD events. The PlaNet resource (http://aranet.mpimp-golm.mpg.de, Mutwil et al. 2011) enables comparison of co-expression networks for eight plant species, of which *Populus trichocarpa* is the only tree species currently included, on the basis of Pfam domain or PLAZA gene families sharing co-expressed genes in one or more species. PlantGenIE (http://plantgenie.org, Sundell et al. 2015) contains dedicated genome portals for *Arabidopsis thaliana, Populus* spp. and *Picea abies* that are integrated via the ComPlEx tool (Netotea et al. 2014) that allows direct and interactive comparison of co-expression networks between two species. Future updates to these resources will hopefully integrate additional tree species, although for many of the currently available genomes this would first require assembly quality improvement and, for comparative expression resources, suitable and adequately comprehensive expression data.

Future Directions

There have been a number of methods presented recently that utilise next generation sequencing to provide information on the three dimensional conformation of chromosomes, for example Hi-C (Belton et al. 2012). Application of these methods to plant genomes is currently limited, although available examples include *Arabidopsis thaliana* (Wang et al. 2015). Application of such methods in a range of species, spanning variable timescales of WGD events, will offer further insights into the dynamics of genome structure and function following duplication. Sequencing based approaches to profile the epigenome, including BS-Seq to assay methylation status and ChIP-Seq for chromatin marks, will additionally complement such knowledge. Again, such data exists for a limited number of plant species and more extensive phylogenetic coverage will be needed to render these truly informative from a comparative perspective.

Conclusions

Availability of the first plant genomes has provided extensive new insights into the evolutionary history of angiosperm genomes – most prominently that all extant angiosperms are paleopolyploids. There have similarly been extensive insights into the repeat structure and history of plant genomes and the interplay between repeat activity and silencing and the prevalence of epigenetic modifications. Currently many of these insights remain isolated and a future challenge will be their integration to provide a more comprehensive understanding of the implications and outcomes of WGD and polyploidisation. This improved understanding should also integrate knowledge of population genetics, recombination effects, life history and

selection and should aim to provide understanding spanning temporal scales from the short-term, immediate effects of WGD through to the long-term implications and outcomes. In addition to improving our understanding of plant genome structure and evolution, these approaches will also provide new insights into the divergence of gene regulation among species and, importantly, should link these aspects to phenotypes, evolutionary adaptations and future ability to adapt to climate change and cope with environmental perturbation.

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Evolutionary Histories of Gene Families in Angiosperm Trees

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Abstract Genes can be grouped into families based on either the presence of conserved domains or by parameter-based clustering of pairwise alignments of the proteins they encode. The vast majority of gene families found in angiosperm trees have existed before the origin of seed plants, while the lineage-specific adaptations of trees have depended on highly dynamic but selective patterns of gene family gain and loss. The mechanisms governing the diversification of gene families, among them various types of gene duplication, horizontal gene transfer, protein domain re-arrangement and de novo evolution, each play distinct roles in expanding the functional repertoire of the core proteome of land plants. In this chapter we reconstructed a parsimonious evolutionary history of gene family gain and loss in angiosperm tree lineages relative to close herbaceous relatives, gymnosperms and nonvascular plants, revealing considerable variation in the frequency and functional enrichment of gain and loss events across lineages. Throughout the chapter, we highlight general and tree-specific examples of gene family adaptations that have contributed to the remarkable success of these organisms.

Keywords Gene family • Evolution • Protein domain • Functional enrichment • Genomics • Angiosperm

Introduction

The production of vascular tissues (lignified xylem and phloem) from a vascular cambium, a process known as secondary growth that gives most tree stems their characteristic girth, has evolved multiple times: the woodiness of trees is neither unique to them, nor is it a major hurdle for adaptation from a herbaceous predecessor (Groover

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2005). Despite the ancient emergence of secondary growth in vascular land plants – preceding early gymnosperms – there is tremendous variation in the anatomical and developmental characteristics that secondary growth has manifested in angiosperms. Monocot trees such as palms, for example, have lost the vascular cambium entirely (Philipson and Ward 1965; Rudall 1991), and some non-woody dicot trees (among them the papaya, *Carica*) have evolved alternative strategies to support vertical growth without relying on the strength of lignified wood (Kempe et al. 2014). Central to the evolution of vascular cambiums, however, is the co-option of gene families with conserved functions in developmentally similar tissues, such as those regulating stem cell proliferation and differentiation in the shoot apical meristem (Spicer and Groover 2010). Further evidence that angiosperm trees have employed ancient gene "toolkits" during the evolution of secondary growth development programs is the finding that key transcription factors regulating secondary cell wall formation, an essential process in wood development that also takes place in herbaceous angiosperms, are evolutionarily conserved as far back as nonvascular bryophytes (Xu et al. 2014). In addition to their woodiness, trees exhibit a kaleidoscope of species- and genus-specific innovations in gene families that have contributed to their success.

Proteins can be classified into homologous families based on significant sequence similarities that reflect their descent from a common ancestral gene following species divergence or gene duplication. Families may further be united into superfamilies that may be comprised of hundreds of genes, or alternatively distinguished into subfamilies sharing highly similar sequence features (Dayhoff 1976; Punta et al. 2012). The classification of proteins, and their encoding genes, into families encompasses two main approaches that yield varying family sizes and membership: profile-based protein databases define families on predefined structural features such as sequence signatures typically described by hidden Markov models, among them Pfam (Punta et al. 2012), SUPERFAMILY (Gough et al. 2001) and TIGRFAMs (Haft et al. 2003), while comparative genomics resources such as PLAZA (Proost et al. 2014) define families according to parameter-based clustering of pairwise alignments, for example those based on BLAST (Altschul et al. 1997) or TRIBE-MCL (Enright et al. 2002). The latter approach, which typically produces smaller families of closely related orthologs and paralogs, is adopted in this chapter because of its utility in detecting subtler changes in protein families across lineages, but it is stressed that the heavy influence of clustering methods and parameters produces variable family number estimates across studies (see Demuth and Hahn 2009 for review).

Angiosperm trees, like all land plants, share a subset of core gene families with other vascular and nonvascular plants, as well as a minority of lineage-specific genes without known homologs in currently available genomes. While gene families have been lost from some lineages, others have been shaped by dynamic rearrangements and extensive gene duplications and losses, a non-stochastic process that appears to be adaptive in many instances (De Smet et al. 2013; Maere et al. 2005; Qian and Zhang 2014). A rapidly increasing compendium of annotated tree reference genomes (Table 1) is facilitating the study of gene family evolution through comparative genomics approaches, providing valuable insight into the genomic basis of lineage-specific traits and adaptations. We indicate here notable

Table 1 Published, annotated reference genomes of angiosperm and gymnosperm tree species

	Tree species (order)	Notable gene family adaptations	References
Dicot angiosperms	*Carica papaya* L. (Brassicales) **Papaya**	Smallest number of genes per conserved gene family known in angiosperms	Ming et al. (2008)
	Citris sinensis cv. Valencia L. (Sapindales) **Sweet orange**	Largest known number of *GalUR* genes, catalysing rate-limiting step in vitamin C biosynthesis	Xu et al. (2013), Wu et al. (2014)
	Eucalyptus grandis L'Hér (Myrtales) **Rose gum**	Largest known number of terpene synthase genes	Myburg et al. (2014)
	Malus × *domestica* Borkh. (Rosales) **Apple**	Unique pome fruit associated with expansion of Type II MADS-box family	Velasco et al. (2010)
	Populus trichocarpa Torr. & Gray (Malpighiales) **Black cottonwood**	Expansion of disease- and insect-associated domains (LRR, NB-ARC, thaumatin)	Tuskan et al. (2006)
	Prunus persica L. (Rosales) **Peach**	Tandem duplication of C3H and HCT enzymes associated with stone fruit development	Verde et al. (2013)
	Theobroma cacao L. (Malvales) **Cacao**	Large-scale expansion of LRR-RLKs and flavonoid, lipid and terpene biosynthesis gene families	Motamayor et al. (2013), Argout et al. (2011)
Monocot angiosperms	*Musa acuminata* Colla (Zingiberales) **Banana**	Largest number of transcription factor genes, second to *Glycine max*	D'Hont et al. (2012), Droc et al. (2013)
	Phoenix dactylifera L. (Arecales) **Date palm**	Expansion of LEA gene family linked to drought and heat tolerance	Al-Dous et al. (2011) Al-Mssallem et al. (2013)
Gymnosperms	*Picea abies* L., *P. glauca* (Moench) Voss (Pinales) **Spruce**	Smallest known proportion of Type I MADS-box transcription factors, but expansion of some Type II MADS-box clades	Nystedt et al. (2013), Birol et al. (2013), Warren et al. (2015)
	Pinus taeda L. (Pinales) **Pine**	Dramatic expansion of distinct class of disease resistance-associated TIR-NBS-LRR genes	Neale et al. (2014), Wegryzyn et al. (2014), Zimin et al. (2014)

Woody and non-woody taxa are included. Notable gene family adaptations, in many cases linked to the unique biology of the lineage, are described for each genome

protein family innovations described in the literature that in many cases appear to have played an adaptive role in the distinct biology of each species.

In this chapter, we discuss the general histories and origins of protein-coding angiosperm gene families. We reflect on the mechanisms by which gene families evolve, including a number of recently described examples in angiosperm trees. Finally, we reconstruct the evolutionary dynamics and identify functional enrichments of lineage-specific gene family gains and losses over the last 400 million years, with emphasis on sequenced tree species relative to their herbaceous cousins.

Origins and Evolution of Plant Protein Families and Functional Domains

When, and how, did tree gene families originate? How have they diversified during the course of angiosperm evolution? Studies of dozens of annotated plant genomes paint a picture of lineage-specific modulation of ancient gene families inherent to the vast majority of plants, accompanied by the evolution of a non-negligible complement of novel "orphan" genes, in any given species. The vast majority of the approximately 56,000 protein families known in embryophytes (land plants) appeared by the mid-Ordovician some 450 million years ago (MYA) (Guo 2013), with the embryophytic core proteome estimated at between 6,820 (Banks et al. 2011) and 7,100 (Jiao and Paterson 2014) families. Ancestrally, the estimated core proteome of Viridiplantae (all green plants) is substantially less at roughly 2,745 families, with a large number of unique families in the algal outgroup *Chlamydomonas reinhardtii* (Guo 2013). Despite the general utilization of common ancestral gene families across land plants, each plant genome contains only a fraction of the total gene family repertoire due to substantial gene family gain and loss in different lineages. Gene families are further subjected to evolutionary mechanisms of diversification including (1) whole genome duplication (WGD) events, (2) small-scale duplications such as segmental, dispersed, retro and tandem duplications, (3) horizontal gene transfer, (4) protein domain re-arrangements and (5) *de novo* evolution. In this section, we explore these diversification mechanisms in more detail.

Angiosperms are renowned for their history of WGDs arising from autopolyploidy (intraspecific genome doubling) and allopolyploidy (hybridisation) events (Van de Peer et al. 2009). Documented WGD events in land plants are indicated in Fig. 1. While WGD itself merely duplicates gene copies within gene families, the resulting functional redundancy between the nascent homeologs relaxes the per-copy strength of purifying selection, allowing them to diverge and undergo neo- and subfunctionalization (Zhang 2003), or a combination of both (He and Zhang 2005). The fate of homeologs is well known to depend on their biological functions: according to the gene dosage hypothesis (Birchler et al. 2005; Birchler and Veitia 2007), stoichiometrically constrained proteins such as those in multi-protein complexes cause inefficient complex assembly when the abundance of any one component is altered. Extensive comparative genomics studies in plants have shown

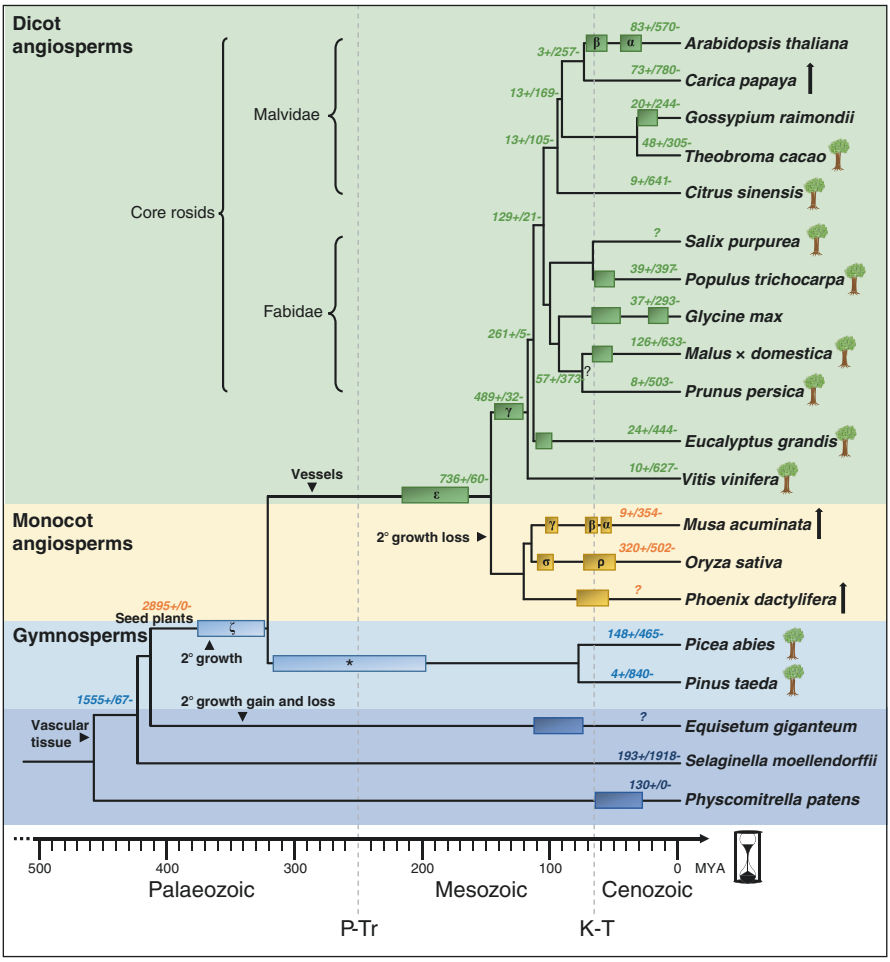

Fig. 1 Phylogeny of sequenced tree genomes and key herbaceous angiosperms, showing WGD events and inferred gene family gains and losses**.** Tree symbols indicate woody taxa (containing lignified secondary xylem) with a tree-like habit; non-woody plants with a tree-like habit are indicated by vertical arrows. The number of gene families gained or lost along selected branches of the phylogeny are indicated by positive and negative integers, respectively. *P-Tr* Permian-Triassic extinction event, *K-T* Cretaceous-Tertiary extinction event, *MYA* million years ago (Divergence dates were inferred from Wikström et al. (2001), Chaw et al. (2004), Janssen and Bremer (2004), Tuskan et al. (2006), Willyard et al. (2007), Jiao et al. (2011), Magallón et al. (2013) and Li et al. (2014b). WGD events were taken from Rensing et al. (2007), Van de Peer et al. (2009), Fawcett et al. (2010), Schmutz et al. (2010), Velasco et al. (2010), D'Hont et al. (2012), Vekemans et al. (2012), Myburg et al. (2014) and Vanneste et al. (2014, 2015). *Conflicting data for this WGD has been reported by Nystedt et al. (2013) and Li et al. (2015b))

that essential "housekeeping" genes revert to single-copy status rapidly after WGD events, while gene dosage-sensitive regulatory proteins such as transcription factors and chromatin-associated factors have an intermediate copy number (De Smet et al. 2013; Li et al. 2016). Transcription factors are overrepresented among WGDs, which maintain stoichiometry, as opposed to small-scale duplications that lead to unbalanced dosage effects (Freeling 2009). The functional diversification of transcription factors preferentially retained after many rounds of WGDs in angiosperms in particular is thought to have contributed significantly to their morphological evolution (Rensing 2014). Expression divergence between homeologs also contributes to phenotypic divergence (Allario et al. 2011) and is correlated with nonsynonymous substitutions associated with positive selection (Ganko et al. 2007). Almost half of the homeolog pairs arising from the *Populus trichocarpa* salicoid WGD event have diverged in their expression patterns (Rodgers-Melnick et al. 2012). For example, duplicated members of secondary cell wall-related cellulose synthase genes in hybrid aspen have contrasting absolute and tissue-specific expression patterns, with evidence of transcriptional network re-wiring of their promoters (Takata and Taniguchi 2015). Similar observations were reported among GT43 family members, associated with xylan biosynthesis during secondary cell wall formation (Ratke et al. 2015).

Segmental and single-gene duplications are characterized by small-scale duplications of individual chromosomal regions or genes, respectively. Single-gene duplications encompass tandem (that is, adjacent to the parental copy), proximal (near to the parental copy), dispersed (far-removed from the parental copy) and retro duplications (via reverse transcription of mRNA transcripts derived from the parental copy). While segmental duplications tend to conserve *cis*-regulatory architecture at promoters, single-gene duplications may not include the parent gene's regulatory elements (Wang et al. 2012). This is especially true of dispersed duplications which diverge in expression from the parental copy faster than tandem or segmental duplications (Casneuf et al. 2006; Li et al. 2009b). Interestingly, while plant gene families undergo all modes of duplication, many families show a strong bias for either tandem or segmental duplication (Cannon et al. 2004; Wang et al. 2012). Apparently tandem duplications, which tend to share a similar chromatin and regulatory environment with the adjacent parental copy, tend to amplify existing functions while segmental duplications may facilitate functional diversification (Flagel and Wendel 2009).

Protein families are generally characterised by a distinct pattern of one or more conserved functional domains. Existing protein domains within gene families have undergone considerable re-arrangement and re-distribution of these basic building blocks within and across families to create novel protein functions (Bornberg-Bauer and Albà 2013; Jiao and Paterson 2014; Kersting et al. 2012; Yue et al. 2011). While ancient domains have been co-opted for plant-specific regulatory functions, such as the likely modification of microbial homing endonucleases to form the DNA-binding domains of plant AP2/ERF and WRKY transcription factors (Yamasaki et al. 2013), around 545 domains, most of them with unknown functions, have emerged relatively recently during green plant evolution (114 of them in

angiosperms) (Kersting et al. 2012). Among angiosperm tree lineages, apple (*Malus* × *domestica*) and sweet orange (*Citrus sinensis*) have higher than average rates of net domain gain, in contrast to the high net loss rate in date palm (*Phoenix dactylifera*), since their divergence from sequenced relatives (Kersting et al. 2012). Domain re-arrangements and domain expansions (that is, an increase in a domain's relative frequency in the genome) feature more prominently along terminal phylogenetic branches than more ancestral lineages. For example, a detailed analysis in *Eucalyptus grandis* showed that genes containing expanded domains were overrepresented among its large repertoire of tandemly duplicated genes, enriched for innate immune responses, protein signalling and pollen recognition, and significantly associated with flower- and root-preferential expression (Kersting et al. 2015). These results suggest that tandem duplication is a mechanism by which this Myrtales representative has expanded protein domains related to stress response and floral development.

Horizontal gene transfer (HGT) is the acquisition of foreign DNA that does not involve vertical transmission through sexual reproduction. Compared to prokaryotes and single-celled eukaryotes, HGT is rare in multicellular organisms. A famous exception is that of pathogenic *Agrobacterium* species, which routinely transfer their virulence genes on T-DNA carriers to host plant somatic cells during infection, which in rare cases become fixed in plant genomes as was recently discovered in sweet potato cultivars (Kyndt et al. 2015). Despite its paucity, it is thought that HGT has played an underappreciated role not only in plant proteome diversification (Bock 2009), but also early land plant evolution (Wang et al. 2014). For example, the first enzyme in the phenylpropanoid pathway, PAL, was horizontally acquired by pioneer land plants from soil microbes (Emiliani et al. 2009), and transaldolase-like proteins, which have been shown to affect vascular development in rice, were derived from Actinobacteria during early land plant evolution (Yang et al. 2015). Similarly, bacteria and fungi have acquired and modified plant expansin genes, in some cases enabling pathogen-mediated manipulation of the host plant cell wall (Nikolaidis et al. 2013). Close contact between distantly related species of plants, such as parasitic or epiphytic plants and their hosts (Mower et al. 2010; Xi et al. 2013) or natural grafting, can facilitate much rarer events of plant-to-plant HGT. Remarkably, even entire nuclear genomes may be transferred between naturally grafted plants to create allopolyploid hybrids, as deduced from artificial grafts between herbaceous and woody tobacco species (Fuentes et al. 2014). Few examples of lineage-specific genes transferred horizontally are known in vascular plants. However, in gymnosperms it was recently revealed that Canary Island Pine (*Pinus canariensis*) has two mitochondrial NADH dehydrogenase subunit 5 intron 1 (*nad*5–1) fragments, one of which was acquired from an unknown angiosperm donor (Wang et al. 2015). The basal angiosperm *Amborella trichopoda* has undergone substantial HGT of mitochondrial genes, and in some cases entire mitochondrial genomes, from green algae, moss and angiosperm donors (Bergthorsson et al. 2004; Rice et al. 2013; Taylor et al. 2014). Finally, the parasitic dicot *Striga hermonthica* is known to have acquired a nuclear gene of unknown function from sorghum or a related monocot host (Yoshida et al. 2010). These three examples lack

proof of adaptive value of the donor genes, but a recent publication documents one of the first adaptive HGT events between land plants: the neochrome photoreceptor, which allows many ferns to respond to low light levels, was acquired from hornworts approximately 179 MYA (Li et al. 2014a). As deeper taxonomic sampling, increased genomics resources and enhanced phylogenetic methods advance, the impact of HGT on angiosperm tree gene family evolution will be better understood.

How do entirely new gene families evolve? While lineage-specific domain rearrangements, gene fusions, or partial duplications of existing gene sequences are the foundation for many novel families, most species also have a sizeable minority of unique genes with no conserved domains and no significant homology to other organisms (that is, their origins cannot be explained by HGT). These "orphan" genes evolve from two principal mechanisms: de novo from noncoding sequences, and via duplication from existing genes, often via transposable element insertion events, followed by extensive sequence substitutions to point incognito (Neme and Tautz 2013; Rutter et al. 2012). In some cases, partially duplicated genes may recruit novel open reading frames from neighbouring noncoding sequence (Katju 2012). The emergence of orphans, which can be considered founder genes if they diversify into novel families, is not uniform across evolution. Phylostratigraphy, an approach used to date the emergence of founder genes, reveals a spike in the rate of founder gene formation during the emergence of early land plants and a particularly pronounced increase during the radiation of Rosids (Tautz and Domatez-Lošo 2011). Orphans tend to be shorter than ancestral genes, show reduced absolute but more tissue-specific expression, lack selection-driven refinement of GC content and codon usage, and are more likely to play a role in regulating species-specific environmental responses than metabolic catalysis (Arendsee et al. 2014; Donoghue et al. 2011). One of the most famous examples of a functionally characterised plant orphan protein is Qua-Qine Starch (QQS) in *Arabidopsis thaliana*, first identified as a protein of unknown function that was upregulated in a starch synthesis-deficient mutant and found to modulate starch and protein accumulation (Li et al. 2009a). Interestingly, despite only being known in *A. thaliana*, the introduction of QQS into distantly related dicot and monocot backgrounds recapitulates its *A. thaliana* gain-of-function phenotypes due to its interaction with a conserved subunit in the transcriptional regulatory complex NF-YC (Li and Wurtele 2015; Li et al. 2015a). The function of QQS is, however, a rare find among plant orphan genes. Regarding angiosperm trees, Hefer et al. (2015) identified 15,791 (38 %) and 14,231 (39 %) genes in *Populus trichocarpa* and *E. grandis*, respectively, that were absent from the reciprocal species. Of these, 10,626 (67 %) *Populus trichocarpa* and 4,539 (32 %) *E. grandis* genes also did not have significant hits in *A. thaliana*, suggesting they may be orphan genes. The singleton genes in *Populus trichocarpa* and *E. grandis* were enriched for a multitude of biological functions, especially chromatin and chromosome organisation in both lineages, and the regulation of gene expression and other cellular processes in *E. grandis* (Hefer et al. 2011). The elucidation of the functions of orphan genes is an exciting prospect in the post-genomics era and may allow us to better understand their obscure evolution.

Gene Family Gains and Losses and Their Functions Across Angiosperm Trees

How are gene families distributed across angiosperm lineages? While gene family gains and losses have been investigated in representative monocot, dicot, gymnosperm and primitive plants (e.g. D'Hont et al. 2012; Guo 2013; Jiao and Paterson 2014; Nystedt et al. 2013; Wegrzyn et al. 2014), the significance of this process in the lineage-specific evolution of trees is not yet well understood. In this section, we explore the extent and functional enrichments of gene family gains and losses for sequenced angiosperm and gymnosperm tree lineages, relative to key herbaceous taxa and nonvascular plants. To avoid misleading comparisons between taxon-specific gene family estimates in previous studies due to methodological differences, we re-constructed an updated parsimonious evolutionary history of protein family gains and losses from publicly available proteomes.

Briefly, full-length protein sequences collected from 17 model plants were accessed from the comparative plant genomics platform, PLAZA (Proost et al. 2014), and other public resources (Neale et al. 2014; Nystedt et al. 2013). The sequences were subjected to an all-vs-all BLASTp search (E-value $\leq 10^{-5}$) and the alignments were passed to TRIBE-MCL (Enright et al. 2002) to create a clustered network used for protein family determination. The families were then processed through InterProScan (Quevillon et al. 2005) and assigned protein domains as well as Molecular Function and Biological Process terms from the Gene Ontology (GO) (Ashburner et al. 2000). For species with limited annotation resources, orthologous BLAST hits were mapped to the ontology to provide functional context. To contend with inaccuracies from incomplete annotations and fragmented gene models, only protein families with at least five members were considered. The resulting matrix of protein families served as input into the Dollop program available through PHYLIP (Felsenstein 1989). The assigned protein domains also served as a filter (Finn et al. 2016) to remove retroelements. Overrepresented molecular function and biological process terms for gene families gained or lost in particular lineages were identified using a Z-score > 6.0.

Figure 1 illustrates the evolutionary relationships of major land plant taxa, including sequenced tree genomes, showing approximate divergence times and known WGD events. The inferred gene family gain and loss events are superimposed along each branch of the phylogenetic tree. We represent the protein family dynamics for nine tree or tree-like woody taxa (as well as two nonwoody tree-like taxa, *Carica papaya* and *Musa acuminata* genotype DH-Pahang) relative to four well-described herbaceous angiosperms (*A. thaliana*, *Gossypium raimondii*, *Glycine max* and *Oryza sativa*), the vascular lycophyte *Selaginella moellendorffii* and the nonvascular bryophyte *Physcomitrella patens*. Gain and loss estimates are lacking for the non-sequenced lycophyte representative *Equisetum giganteum*, the date palm (*Phoenix dactylifera*) (Al-Dous et al. 2011; Al-Mssallem et al. 2013) due to unavailable annotation data, and willow (*Salix purpurea*) (JGI-DOE, http://phytozome.jgi.doe.gov) due to data restrictions. A total of 10,761 gene families were

generated with at least 5 members, where 1,469 protein families were shared across all 17 species while 1,281 were species-specific. A set of 21 families were unique to monocots, 54 to dicots, five to gymnosperms, and none identified as specific to the basal bryophyte and lycophyte representatives.

Our analysis showed that two major gene family gain events occurred during the evolution of vascular plants: a net gain of 1,488 families during the rise of primitive vascular plants, and a subsequent net gain of 2,895 leading to the common ancestor of all seed plants, likely facilitated by a common ζ WGD event approx. 319 MYA (Jiao et al. 2011) (Fig. 1). We identified a number of significantly overrepresented molecular functions and biological processes among gained and lost families for the major plant lineages, as listed in Table 2. In some cases the enriched terms are unexpected, for example terms associated with flowering plants such as inflorescence development and pollen germination in the non-flowering lineage *Physcomitrella patens* (Table 2). The biases and deficiencies in the functional evidence currently supporting these ontologies are apparent in such cases, and taxon-specific GO analysis may help to refine these annotations (Huntley et al. 2014). Nonetheless, a large diversity of significantly enriched functions can be found in gained and lost families that may suggest known and novel adaptations specific to each lineage. The enrichment of mannose biosynthesis among gained families in the gymnosperms, for example (Table 2), is congruent with the high galactoglucomannan content of softwoods (Timell 1967). Similarly, we can speculate that the enrichment of root morphogenesis-associated genes in families gained by *Selaginella moellendorffii* (Table 2) may be related to the unique root-forming rhizophore organ of this early vascular plant (Lu and Jernstedt 1996). A pronounced net gain of gene families is evident in the early radiation of angiosperms, with 736 gained and 60 lost in the common ancestor of the monocot and dicot groups indicated, 489 families gained and 32 lost in the dicot ancestor following the γ WGD event (Jiao et al. 2012; Vekemans et al. 2012), and a net gain of 256 families in the common ancestor of the core rosids (Fig. 1). Notably, more derived angiosperm lineages showed an overwhelming net *loss* of gene families in our analysis, in many cases following independent WGD events within the last 100 million years (Fig. 1).

Among the most broadly distributed protein families, the largest family contained 5,750 proteins, represented by at least five members in all of the 17 species. This family, which all contained the Pentatricopeptide repeat (PPR) and a protein tyrosine kinase domain, is broadly associated with protein binding, endonuclease, and oxidoreductase activities. The next largest family, with 2,851 proteins, was also annotated with a PPR domain as well as the small MutS-related (Smr) domain (Moreira and Philippe 1999). These genes are associated with protein binding, adenylate cyclase, and RNA binding activities. The third largest, with 2,335 proteins, featured members containing Leucine Rich Repeat (LRR) and protein kinase domains. These genes are associated with protein kinase activity especially in the context of biotic stress response, ATP binding and purine ribonucleotide binding. Following this, a family of 2,179 proteins across all species was annotated as cytochrome P450. Subsequent broadly distributed families were missing

Table 2 Significantly enriched molecular functions and biological processes associated with gained and lost protein families in selected plant lineages

Lineage	Gained families	Lost families
Bryophytes (*Physcomitrella patens*)	Light-independent DNA repair Amidase activity Allantoinase activity Guard cell differentiation Inflorescence development Pollen germination	None
Lycophytes (*Selaginella moellendorffii*)	siRNA and miRNA binding Sulfate assimilation Root morphogenesis	Salicylic acid-mediated signaling Response to hypoxia
Gymnosperms (*Pinus taeda*, *Picea abies*)	Lipoic acid synthase activity Mannose biosynthesis Starch synthase activity Virus-induced gene silencing	Phosphoadenylyl sulfate reduction Adenylyl-sulfate reductase activity Photosystem II antenna complex
Monocots	Nicotinamidase activity NAD metabolism Glutathione biosynthesis Histone deacetylase activity Pyridoxal phosphate-dependent decarboxylase activity	MYB transcription factor activity Cytochrome P450 activity Heat shock protein (hsp40) activity Voltage-gated chloride channel activity
Rosids	Methyltransferase activity Cysteine-type endopeptidase activity Ubiquitin-dependent protein catabolism Asparagine biosynthesis Isoprenoid biosynthesis Thymidylate synthase biosynthesis (not caps) Mucilage biosynthesis during seed coat development	Hexose metabolism Spermidine biosynthesis Nucleotidyltransferase activity Glutamine biosynthesis
Fabids	CTP biosynthesis Leucine catabolism Dihydropyrimidinase activity	Uroporphyrinogen decarboxylase activity Chlorophyll synthetase activity
Malvids	Sterol 5-alpha reductase activity Mitochondrial genome maintenance Biotin binding	Pigment binding Allantoinase activity Chaperone activator activity

from one or more of the 17 species compared. The fifth largest, absent from two species, had 2,037 proteins and contained domains annotated as Toll/interleukin-1 receptor (TIR), the NB-ARC (Nucleotide-Binding Adaptor shared by APAF-1, R proteins, CED-4) domain, and Leucine-rich repeat (LRR) domains. Genes in this family are frequently associated with disease resistance and activities related to protein binding and transmembrane receptor signaling activity (Biezen and Jones 1998; Jones and Dangl 2006).

In addition to the major groups represented in Table 2, inspection of lineage-specific protein families revealed enrichment for molecular functions that may be important for particular lineages. Of these, eight families were moderately confined to five or fewer species but contained at least 100 members each. The largest had 349 proteins across four non-angiosperm species (*Physcomitrella patens*, *Picea abies*, *Selaginella moellendorffii* and *Pinus taeda*). They contained a TIR domain and are functionally associated with transmembrane signaling receptor activity and numerous binding activities (protein, ribonucleotide, anion, and ion binding). The second largest of this class had 262 genes and was found in *Glycine max*, *Populus trichocarpa*, *Theobroma cacao* and *Gossypium raimondii*. This protein family is annotated with the reverse transcriptase zinc binding domain and RNase H activity, which hydrolyses the RNA strand of a DNA/RNA hybrid. The third largest, with 205 proteins confined only to the Rosaceae members *Prunus persica* and *Malus* × *domestica*, had little annotation information but is broadly associated with amino transferase-like activity. A number of species-specific protein families were also identified. The largest single species family belonged to *A. thaliana*, with 110 proteins containing F-Box associated, and phospholipase C domains. *Selaginella moellendorffii* has the second largest single species family with 103 members, however specific annotation information was not identified. *Populus trichocarpa* had the third largest single species family with 82 proteins. The annotated domains included the C-terminal catalytic domain of Ubiquitin-like-specific protease 1, and functional associations derived from GO terms included cysteine-type peptidase activity and RNA-directed RNA polymerase activity.

Conclusion

Gene family diversification is a crucial means by which plants have adapted to their diverse habitats. In this chapter, we've seen that most protein families in angiosperm trees are ancient and were present in primitive nonvascular land plants. WGDs and small-scale duplications followed by sequence and expression divergence, as well as the re-arrangement of existing domains, comprise the main mechanisms by which these ancient families have generated functional novelty. In some cases, HGT and de novo evolution have also created new gene families in some angiosperm lineages, but the degree to which this mechanism contributes to adaptation has been poorly studied in trees.

Parsimonious reconstructions of protein family gain and loss events in sequenced tree genomes and their closest sequenced herbaceous lineages show a highly dynamic process of protein family gain and loss events, even where genera have diverged relatively recently. Although a vast diversity of significantly enriched functions and biological processes can be identified among these gained and lost families through automatic annotation using a limited gene ontology, the biological significance and meaning of these enrichments to particular lineages, especially in trees, remains obscure. It is hoped that the lineage-specific functional enrichments reported here will stimulate new questions into the genetic basis of adaptation of angiosperm trees.

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Breeding Systems, Mating Systems, and Genomics of Gender Determination in Angiosperm Trees

Matthew S. Olson, J.L. Hamrick, and Richard Moore

Abstract Angiosperm trees and non-woody plants differ in the prevalence of different genetic transmission systems with the potential to result in categorical differences in adaptive capacity and genomic structure. Decades of careful investigations have revealed that although most tree species are hermaphroditic (perfect flowers), dioecy (unisexual individuals) is more common than throughout the angiosperms, and self-fertilization is exceedingly rare. These patterns indicate that the benefits of outcrossing are strong drivers of the evolution of angiosperm trees. Moreover, patterns of the evolution of genetic sex determination regions in dioecious trees are highly dynamic, including differences between closely related species and genera in the genetic characteristics and location of sex determination regions and which sex is heterogametic (XY vs. ZW). This short review emphasizes that investigations of factors influencing genetic transmission are likely to provide answers to fundamental questions regarding genomic and phenotypic evolution in angiosperm trees.

Keywords Sex chromosomes • Dioecy • Outcrossing • Selfing • Carica • Populus • Salix

Introduction

Transitions among mating and breeding systems are among the most common evolutionary pathways in angiosperms (Wright et al. 2013), yet their importance for the evolution of angiosperm tree genomes is not fully appreciated. Breeding and mating systems have strong impacts on the genomic architecture of trees through their influence on genetic transmission, genome diversity, population structure,

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population migration, and levels of inbreeding and inbreeding depression (Arunkumar et al. 2015). In this chapter, we focus on our current understanding of angiosperm tree mating and breeding systems, discuss their influence on tree genomes, and review recent advances towards understanding the evolution of sex chromosomes in angiosperm trees. Plant mating systems are defined as the placement along the continuum of sexual reproduction from primarily self-fertilization (or selfing) to overwhelmingly outcrossing (Lande and Schemske 1985; Schemske and Lande 1985), whereas plant breeding systems are defined as the morphological and/or genetic factors that regulate which individuals are compatible for mating (Richards 1997). Trees pose unique challenges for studies of breeding and mating systems. They are large, long-lived, and usually have numerous reproductive events (iteroparous), creating difficulties for collecting detailed information across generations (Bawa 1974). Experimental pollinations are often required to definitively determine breeding systems because either stamens or pistils may be non-functional, even when their flowers appear perfect (Bawa and Opler 1975). Finally, genetic resources for mapping genes influencing sex determination, such as inbred lines, are unavailable for most tree species, so creative alternative strategies must be devised. Nonetheless, angiosperm trees offer some of the most interesting evolutionary systems in which to address the evolution of breeding systems, and understanding tree mating systems is essential for understanding the patterns and processes driving genome evolution in trees.

Breeding Systems

Tree breeding systems influence genome structure and diversity through their influence on mating systems (see below) and through the potential for development of genomic associations between mating types and genomic regions (e.g. sex chromosomes). Angiosperm trees exhibit a similar range of breeding systems as non-woody plants, with studies identifying hermaphroditism, monoecy, dioecy, andromonoecy, gynodioecy, hermaphroditic self-incompatibility, and complex mixtures of these systems (sometimes termed polygamodioecy or polygamonoecy; Bawa 1974; Gibson and Diggle 1998; Machado et al. 2006; Vamosi et al. 2015). Angiosperm trees, or more generally, woody flowering plants, have long been known to exhibit a high incidence of dioecy (Darwin 1876; Stebbins 1950). Several tropical flora exhibit frequencies of dioecious trees higher than 20% (Bawa and Opler 1975; Machado et al. 2006; Chen and Li 2008), with a Venezuelan cloud forest exhibiting dioecy in over 30% of tree or dominant woody species (Sobrevila and Arroyo 1982; Matallana et al. 2005). Relative to all angiosperm species (mean ~6% dioecious species; Renner 2014) trees are an outlier, suggesting that the biology associated with the arborescent growth form favors the evolution of dioecy. Tropical dioecious trees, however, tend to be within the same genera, indicating that speciation often occurred after the evolution of dioecy, and raw number of species likely overestimate the numbers of transitions from hermaphroditism to dioecy in the history of tree diversification. In an attempt to compare phylogenetically independent

lineages, Renner and Ricklefs (1995) tested for associations between biological characteristics and dioecy at the level of plant families and found no strong association between tree growth form and dioecy, but their analysis identified abiotic pollination and longevity as characteristics that may favor the evolution of separate sexes in trees (Renner and Ricklefs 1995; Renner 2014).

Among all angiosperms monoecy is slightly more common than dioecy (Renner 2014), but dioecy may be more common than monoecy in angiosperm trees, although no comprehensive survey has been published. Bawa (1974) found that monoecy was more common than dioecy in a temperate deciduous forest, but that dioecy was more prevalent in a tropical semideciduous forest. Other studies of tropical forests also found a higher incidence of dioecy than monoecy (Bawa and Opler 1975; Tanner 1982; Bawa et al. 1985; Ibarra-Manriquez and Oyama 1992; Chen and Li 2008). Since tree diversity in the tropics is higher than in temperate forests, dioecy is likely more common than monoecy among angiosperm tree species. Outside of dioecy and monoecy, however, relatively little is known about tree breeding systems, likely due to the difficulty of conducting experiments or observations to definitively determine the breeding system. These difficulties may be one reason that breeding systems like hermaphroditic self-incompatibility, andromonoecy, gynomonoecy, androdioecy, and gynodioecy are under-represented in research reports of tree breeding systems (Bawa and Opler 1975; Renner and Ricklefs 1995). Another possibility is that these breeding systems are less common in trees. For instance, a recent review found that gynodioecy was less common in non-herbaceous and tropical plants (Caruso et al. 2016). A more thorough understanding of the diversity of tree breeding systems will provide a better foundation for making sense of the range of patterns of genomic diversity and architecture in trees.

Plant Mating Systems

A species' mating system has a strong influence on patterns of genomic diversity, and its characterization is necessary to place studies of population genetics and genome evolution in their proper context. Evolutionary transitions in mating systems influence genome structure and diversity through changes in effective population size, the frequency of homozygosity, and alterations in linkage disequilibrium (Wright et al. 2013). For instance, increased self-fertilization results in lower effective population sizes (N_e) by decreasing the independence of chromosome segregation and sampling (Charlesworth and Wright 2001; Wright et al. 2008). In turn, lower N_e decreases the effectiveness of selection to drive both positive selection on slightly beneficial alleles and background selection on slightly deleterious alleles (Charlesworth 2009). Higher selfing rates also may lead to increased inbreeding depression through exposure of deleterious recessive genotypes to selection and decreased expression of genotypes exhibiting overdominance for fitness (Wright et al. 2013). Finally, selfing results in higher levels of non-random association among loci (gametic phase disequilibrium), resulting from decreased N_e, and lower population level recombination rates (ρ), as well as from the maintenance of

associations because random chromosomal associations are not generated during mating (Charlesworth and Wright 2001; Wright et al. 2013). Thus, species with increased levels of selfing are expected to exhibit less diversity resulting from lower N_e, higher frequencies of effectively neutral loci, large linkage blocks, and limited population-scaled recombination.

Determination of the mating system does not require large numbers of loci, and general patterns of tree mating systems have been discerned from analyses of datasets compiled prior to the genomic era. Plant mating systems are typically defined by the proportion of selfing (s) and outcrossing (t), where $t = 1 - s$; the proportion of outcrossing ranges from 0.0 (complete selfing) to 1.0 (complete outcrossing). A plant species' or population's mating system is arbitrarily classified as predominantly selfing ($t \leq 0.2$), mixed-mating ($0.2 \leq t \leq 0.8$) or predominantly outcrossing ($t \geq 0.8$; Schemske and Lande 1985; Goodwillie et al. 2005). Plant mating systems can be inferred or estimated in at least three ways. The first and least quantitatively accurate method is to infer the mating system based on floral structures. A second approach involves the manipulation of flowers in some way (bagging flowers to prevent pollination) or by controlled crosses (applying different frequencies of self and outcross pollen on receptive stigmas and monitoring seed set). The most accurate and straightforward method to obtain quantitative estimates of outcrossing is to use genetic markers (loci) to identify outcrossed individuals in progeny arrays.

The mixed-mating model, which is applied to estimates of outcrossing using genetic markers, was first proposed by Hayman (1953) and modified by Workman and Allard (1962) and Brown and Allard (1970) and divides the mating process into two components: random mating (i.e. outcrossing) and self-fertilization. If allele frequencies in the pollen pool are known, an estimator for the rate of outcrossing is given by $t = h/p$, where h is the frequency of heterozygotes among the progeny of homozygous maternal plants and p is the population-level frequency of an allele that is different from that of the homozygous maternal plant. Although there are several procedures available to estimate t, by far the most commonly used is the MLTR program developed by Ritland and Jain (1981) and Ritland (1990). This procedure not only provides estimates of t and s (selfing rate; $= 1 - s$) but also estimates the proportion of biparental inbreeding (the mating of two related individuals) which is calculated by $t_m - t_s$, where t_m is the multilocus estimate of outcrossing and t_s is the mean single locus estimate of outcrossing (Brown 1989).

Assumptions of the Mixed-Mating Model The mixed-mating model is based on four assumptions that apply to both single or multi-locus approaches, and a fifth assumption is only pertinent for multi-locus analyses (Clegg 1980):

1. *All matings are either selfing or random outcrosses*. This assumption indicates that no other type of mating occurs in the population (e.g. biparental inbreeding). This assumption is routinely tested by the MLTR procedure since the proportion of biparental inbreeding ($t_m - t_s$) can be determined (Brown 1989).
2. *Maternal plants sample from the same pollen pool*, i.e. allele frequencies in the pollen pools of each maternal individual should not be significantly different. This assumption is rather routinely violated, but rarely tested. Murawski and Hamrick (1991) found that pollen pools from the progeny arrays of several

maternal individuals exhibited significant heterogeneity in pollen allele frequencies for nine different neotropical angiosperm tree species located on Barro Colorado Island, Panama as measured by F_{ST} (the proportion of among tree to total genetic diversity across maternal trees). Mean F_{ST} values across all nine species were related to flowering tree densities with low density species having higher F_{ST} values than species with higher flowering tree densities. Fortunately, such heterogeneity has little effect on overall population level estimates of t_m when sufficient numbers of maternal individuals are analyzed (>10).

3. *The genotype of the maternal individual has no effect on the mating system estimate*. This assumption has rarely been tested since the marker loci used to estimate the mating system are assumed to be selectively neutral. For morphological loci, such as flower color, this assumption may take on greater importance due to pollinator biases (Levin and Kerster 1972; Levin and Watkins 1984).
4. *No selection occurs between fertilization and the point in the life cycle that the mating system is estimated*. Typically, estimates of outcrossing are conducted on germinated seedlings. As a result, if there is selection acting against selfed embryos and seedlings, there may be significant differences in the estimates of outcrossing rates between the initial or primary rate of outcrossing at fertilization and the point in the life cycle at which the mating system estimate is made. This assumption has been infrequently tested due to difficulty in sampling the earliest stages of embryo development. Studies that sampled cohorts at different life cycle stages have found increases in the rates of outcrossing associated with older life history stages (Moran and Brown 1980; Hufford and Hamrick 2003; see below).
5. *All loci are independent*, i.e. they are not genetically linked. This assumption is only important for multi-locus estimates. It is rarely tested when mating system estimates are made, but generally when linkage disequilibrium is tested most marker loci are found to segregate independently of one another.

Estimates of the mating system can vary among species, but can also vary among populations within species (e.g. Schoen 1982) in response to a variety of environmental variables (e.g. density of flowering individuals, pollinator densities, and climatic factors; Adams and Allard 1982). Estimates of outcrossing can also vary among maternal plants within populations due to genetic differences among individuals, physical locations, phenological differences, etc., and vary between fruits or inflorescences on individual plants.

Distribution of mating systems in angiosperm trees We compared the distribution of Angiosperm tree species in the three mating system categories to all plant species, all tree species and all angiosperm species (Table 1). Data from Goodwillie et al. (2005) is based on mating system estimates made from progeny arrays (i.e. MLTR) and as a result should be considered the most reliable. Mating system classifications for all plant species, all tree species, all angiosperm plants, and angiosperm trees was derived from the databases of Hamrick and Godt (1996) and Hamrick et al. (1992). Classification of the mating system for these two datasets was made from formal mating system analyses, estimates of inbreeding within populations, statements made by the authors of the original papers and/or descriptions of the floral structure from pertinent floras.

Table 1 The distribution of plant species in three mating system categories

	All plant species		All trees[b]	All angiosperms[c]	Angiosperm trees[c]
	A[a]	B[c]			
No. of species	345	714	192	616	229
Outcrossing	0.44	0.67	0.93	0.59	0.85
Mixed mating	0.42	0.14	0.06	0.18	0.13
Selfing	0.14	0.19	0.01	0.23	0.03

Data are compiled from two estimates for all plant species, for all trees, for all angiosperms and for angiosperm trees
[a]Data from Goodwillie et al. (2005)
[b]Data from Hamrick et al. (1992)
[c]Data from Hamrick and Godt (1996)

There were two significant results from these comparisons (Table 1). First, the proportion of mixed mating species in the Goodwillie et al. (2005) analysis (0.42) is much higher than the proportion of mixed-mating species based on the classification of species used for the allozyme database (0.14). Pooled values of outcrossing and mixed mating, however, are similar for the two datasets (0.86 and 0.81) indicating that estimates of the proportion of selfing species by the two methods are more reliable than the proportions of mixed-mating and outcrossed species (see also below). Angiosperms follow the same pattern as all seed plants with 77 % of all angiosperms being either outcrossed or mixed-mating and 23 % selfing species. The higher proportion of selfing in the angiosperms is due to the high proportion of selfing annuals (44.5 %; Hamrick and Godt 1996). Angiosperm trees, however, have a much higher proportion of mixed-mating and outcrossed species (98 %) than do all plants or all angiosperms. Only six tree species were classified as selfing (Table 1): *Prunus persica* and five palm species in the genus *Pinanga* from Borneo. This result is consistent with that of the gymnosperms for which none of the 115 species examined (Hamrick et al. 1992) were predominantly selfing. Thus, long-lived woody plants are predominantly outcrossing or mixed mating. Also, most mixed mating trees tend to fall in the highest group of mixed mating species ($0.6 \leq t \leq 0.8$). In Goodwillie et al. (2005) this mixed mating category made up 54 % of the mixed mating species. An exception is *Magnolia obovata* ($t_m = 0.39$–0.42) which falls within the lower category of mixed mating (Ishida et al. 2003). However, there are at least two caveats in regards to the proportion of mixed mating vs. outcrossing angiosperm species. First, a few angiosperm families are represented multiple times in the database. The four families with the highest representation constitute 59 % of the angiosperm tree species in the database (Fabaceae = 39 spp., Fagaceae = 37 spp. [29 *Quercus*], Mytraceae = 22 spp. [19 *Eucalyptus*] and Arecaceae = 20 spp.). Also, many self-compatible species probably have estimated outcrossing rates that are higher than the primary outcrossing rate due to selection against inbred progeny. Studies that estimate outcrossing at different life history stages generally demonstrate systematic selection against selfed progeny, i.e. t_m increases with cohort age. Moran and Brown (Moran and Brown 1980) sampled seeds from serotinous capsules from 30 maternal

trees of *Eucalyptus delegatensis* over three continuous years. Seeds from the oldest fruits had higher outcrossing rates (t_m=0.85) than seeds from the youngest fruits (t_m=0.66) suggesting that selection against selfed progeny had occurred while the seeds were stored on the tree branches. Hufford and Hamrick (2003) sampled fruits of *Platypodium elegans* at three stages of a single cohort for 12 mature trees located on Barro Colorado Island. Fruits aborted 6 months prior to fruit maturation (August 1997) had a t_m value of 0.79, whereas embryos from mature fruits (March 1998; t_m=0.82) and established seedlings (May 1998; t_m=0.91) had higher outcrossing estimates, indicating that selection against selfed progeny occurred primarily between the fruit dispersal stage and the established seedling stage.

There are at least two advantages to outcrossing. The first advantage to outcrossing is avoidance of inbreeding depression, whereas the primary advantage of selfing is reproductive assurance (Baker 1955). The low incidence of selfing and the high incidence of obligate outcrossing (dioecious species) in angiosperm trees suggests that reproductive assurance has not been a strong factor influencing the evolution of their mating systems and underscores the importance of reproductive assurance in herbaceous plants. It is possible that the long lived iteroparous nature of trees obviates the need for reproductive assurance through selfing. Understanding the factors favoring selfing in the few trees with this mating system will provide insight into the validity of the reproductive assurance hypothesis. The second advantage is that the progeny of an outcrossing species are more genetically diverse. This is due to two factors: outcrossed derived maternal individuals will be heterozygous at a higher proportion of their loci and the number of paternal individuals will be much greater.

Effects of mating system variation on genetic diversity within and among angiosperm tree populations Previous reviews (Hamrick and Godt 1989; 1996; Table 2A) of the plant allozyme literature demonstrate that selfing species have less genetic diversity than species with mixed mating or outcrossing mating systems. This pattern of lower diversity at the nucleotide level has also been supported for genome-wide comparisons of selfing and outcrossing species (Arunkumar et al. 2015). This trend is more pronounced at the within-population level and for genetic differentiation among populations (F_{ST}). The mean F_{ST} value for selfing species (0.51) is five times higher than the mean F_{ST} value for outcrossing wind-pollinated species (0.099). Furthermore, genetic diversity for mixed-mating and outcrossed species appear to be dependent on their pollination vectors, with wind-pollinated species having higher genetic diversity within their populations and less genetic diversity among populations.

Data for all tree species (gymnosperms and angiosperms, Table 2B) lacked sufficient representation in the selfing and mixed-wind categories (see comments above) for valid comparisons with the other tree categories. In particular, the mixed-animal category only had 11 species, so inferences based on statistical analyses should viewed with caution. Unlike the analyses for all plant species, only H_{es} has a significant difference between the animal and wind outcrossed categories, with animal pollinated species harboring higher species-wide genetic diversity.

Table 2 The influence of mating systems on the levels and distribution of genetic diversity

A. All plant species[1]

Mating system	Species level			Population level			Among populations
	P_s	A_s	H_{es}	P_p	A_p	H_{ep}	F_{ST}
Selfing	41.8[b]	1.69[b]	0.124[b]	20.0[c]	1.31[b]	0.074[d]	0.510[a]
Mixed – Animal	40.0[b]	1.68[b]	0.120[b]	29.2[bc]	1.43[b]	0.090[cd]	0.216[b]
Mixed – Wind	73.5[a]	2.18[b]	0.194[a]	54.4[a]	1.99[a]	0.198[a]	0.100[c]
Outcrossed – Animal	50.1[b]	1.99[ab]	0.167[ab]	35.9[b]	1.54[b]	0.124[bc]	0.197[b]
Outcrossed – Wind	66.1[a]	2.40[a]	0.162[ab]	49.7[a]	1.79[a]	0.148[b]	0.099[c]

B. Tree species[2]

Mating system	Species level			Population level			Among populations
	P_s	A_s	H_{es}	P_p	A_p	H_{ep}	F_{ST}
Selfing	ND	ND	ND	ND	ND	ND	ND
Mixed – Animal	29.9[b]	1.51[b]	0.075[c]	17.2[b]	1.21[b]	0.035[b]	0.122[a]
Mixed – Wind	ND	ND	ND	ND	ND	ND	ND
Outcrossed – Animal	63.2[a]	2.18[a]	0.211[a]	47.6[a]	1.72[a]	0.163[a]	0.099[a]
Outcrossed – Wind	69.1[a]	2.31[a]	0.173[b]	53.0[a]	1.84[a]	0.154[a]	0.077[a]

C. Angiosperm tree species[3]

Mating system	Species level			Population level			Among populations
	P_s	A_s	H_{es}	P_p	A_p	H_{ep}	G_{ST}
Selfing	ND	ND	ND	ND	ND	ND	ND
Mixed – Animal	42.2[a]	1.66[a]	0.165[a]	26.9[a]	1.38[a]	0.157[a]	0.185[b]
Mixed – Wind	ND	ND	ND	ND	ND	ND	ND
Outcrossed – Animal	68.1[b]	2.34[a]	0.233[a]	48.4[b]	1.75[a]	0.174[a]	0.112[a]
Outcrossed – Wind	67.1[b]	2.18[a]	0.214[a]	50.9[b]	1.80[a]	0.214[a]	0.093[a]
Gymnosperms[2]	71.1	2.38	0.169	53.4	1.83	0.151	0.073

[a,b,c] Means followed by the same letter in a column are not significantly different at the 0.05 probability level

Genetic diversity values are given at the level of the species, within populations and among populations P_s *and* P_p percent polymorphic loci at the species and population levels, A_s *and* A_p number of alleles per locus at the species and population levels, H_{es} *and* H_{ep} genetic diversity at the species and population levels [H_{es} is calculated as the average allele frequencies from all pooled individuals, and then calculating an expected heterozygosity value; H_{ep} is the mean of the expected heterozygosity for each population]; F_{ST} proportion of the total genetic diversity among populations, *ND* no data

[1]Parameter values based on the data from Hamrick and Godt (1989)

[2]Parameter values based on the data from Hamrick et al (1992)

[3]Parameter values based on the data from Hamrick and Godt (1996)

Analyses for the angiosperm tree database (Table 2C) indicates that outcrossing species have significantly higher frequencies of polymorphic loci ($P<0.011$) at both the species and within-population levels than mixed-mating animal pollinated species. Values of A or H_e at both the species and population levels were not significant, but outcrossing species had consistently higher levels of genetic diversity for these parameters. Outcrossing species also have significantly ($P<0.005$) less genetic differentiation among their populations ($F_{ST}=0.11$ and 0.09) than mixed mating species (0.18). For the most part, outcrossing animal pollinated angiosperm species and outcrossing wind-pollinated angiosperm species have similar levels of genetic diversity at both the species and population levels. An exception is their H_{ep} values where outcrossing wind-pollinated species have higher but non-significant ($P<0.220$) mean H_{ep} values (0.214) than the outcrossing animal pollinated species (0.174). Also, outcrossing wind-pollinated angiosperm species have somewhat lower mean F_{ST} value (0.093 vs. 0.112). The trends seen for angiosperm trees are generally consistent with those seen for all trees and all plant species.

Genetics and Genomics of Sex Determination in Angiosperm Trees

To our knowledge, the molecular genetics of flower sex expression have not been studied in any monoecious tree species, so we must look to herbaceous systems for insights. Sex determination genes have been cloned in both maize (*Zea mays*; Bensen et al. 1995; Acosta et al. 2009) and cucurbits (Boualem et al. 2008; Martin et al. 2009; Boualem et al. 2015). In both systems two separate loci are required to block stamen or carpel production, but the molecular pathways differ. In cucurbits, ethylene signaling influences both stamen and carpel development through expression of ACS-7 and ACS-11 (Boualem et al. 2015), whereas in maize gibberellins regulate stamen production, and genes regulating carpel production involve either jasmonic acid, Brassinosteroids, or miRNAs (Aryal and Ming 2014). Unfortunately, this variety of sex determination mechanisms provides no clear clues for initiating investigations of sex determination in monoecious trees, but it does provide a two-locus framework from which we can begin to understand the evolution of sex chromosomes in dioecious trees evolved from monoecious ancestors (Boualem et al. 2015, but see Golenberg and West 2013).

Sex determination in dioecious trees has received much more attention than in monoecious trees. Dioecy is arguably the most common breeding system in angiosperm trees outside of hermaphroditism (see above), and its evolution and maintenance has dramatic effects of genome structure and diversity, which we are only beginning to elucidate. Dioecious species are characterized by sex chromosomes, which we are broadly defining as the chromosome harboring the sex determination genes even if recombination occurs across a large proportion of the chromosome. Sex determination genes release a cascade of different molecular pathways leading

to the eventual differentiation of male and female flowers. In *Populus balsamifera* these pathways result in over 11,000 genes being differentially expressed in male and female flowers (Wang, Sanderson, Wu, Tiffin, & Olson, unpublished manuscript). Sex determination genes are notoriously difficult to identify because they are embedded in large non-recombining segments of the chromosome, termed the sex determination region (SDR), and do not segregate independently from other genes in the linkage group. Sex determination regions have been mapped in only a handful of species, with those of agronomic importance such as poplar, willow, papaya, kiwifruit, and persimmon receiving the most interest (Liu et al. 2004; Fraser et al. 2009; Akagi et al. 2014; Kersten et al. 2014; Geraldes et al. 2015; Pucholt et al. 2015b). Complicating the discovery of sex chromosomes in angiosperm trees, all tree sex chromosomes studied to date are homomorphic and cannot be differentiated cytologically. Moreover, the SDR in trees typically comprises only a small percentage (less than 10 %) of an otherwise recombining pair of autosomes. Thus, it is only after the recent development of inexpensive low-density genetic markers such as genotyping-by-sequencing (GBS; Elshire et al. 2011) that SDRs have been successfully mapped in a variety of dioecious tree species.

Genetic theory of the evolution of sex chromosomes posits that natural selection favors the permanent linkage of two sex-determining factors, one suppressing female expression and one promoting male-expression, in the non-recombining SDR (Westergaard 1958; Charlesworth and Charlesworth 1978; Charlesworth 2006; Charlesworth 2015). Selection favors the repression of recombination between the two sex determining factors to eliminate the production of sexual neuters and hermaphrodites, with lower overall reproductive output than pure males or females (Charlesworth and Charlesworth 1978; Charlesworth 2002; Bergero and Charlesworth 2009). Because of the association between monoecy and dioecy within angiosperm tree families, the evolutionary pathway from monoecy to dioecy is hypothesized as the most common pathway in angiosperm trees (Renner and Ricklefs 1995). Moreover, a two locus pathway to the SDR was recently experimentally validated in cucurbits (Boualem et al. 2015), although alternative hypotheses exist (Golenberg and West 2013). Along with the non-recombining SDR, all currently mapped tree sex chromosomes also have regions that recombine normally, termed pseudoautosomal regions (PAR). In *Populus* and *Salix* the majority of the sex chromosome (chr19 and chr15 respectively) is comprised of PAR, with a relatively small SDR (Geraldes et al. 2015; Pucholt et al. 2015a), and in papaya and kiwifruit the SDR comprises less than 10 % and 15 %, respectively, of the sex chromosomes (He et al. 2003; Liu et al. 2004; Wang et al. 2012). The small SDRs are found in both pericentromeric (*Populus tremuloides*, *Salix*, papaya) and telomeric (*Populus trichocarpa*, kiwifruit) chromosomal regions (Fraser et al. 2009; Wang et al. 2012; Geraldes et al. 2015; Pucholt et al. 2015a). Pericentromeric regions in particular are characterized by low gene density and low rates of recombination, and these properties may facilitate the formation of the non-recombining SDR.

Expansion of the size of the non-recombining region surrounding the SDR is thought to result from resolving the gender conflict caused by alleles with sexually antagonistic (SA) fitness effects (Rice 1987). Recombination suppression is favored

between the SDR and a nearby loci with SA alleles to eliminate expression of the wrong allele or gene in the wrong gender (Charlesworth and Charlesworth 1980; Rice 1987; Lahn and Page 1999). Over time, additional SA loci are "captured" by the non-recombining region, resulting in divergence "strata" of different ages along the sex chromosomes. Such strata have been identified in humans (Lahn and Page 1999), birds (Handley et al. 2004) and papaya (Wang et al. 2012). Sequencing of the approximately 8 Mb non-recombining SDR of the *Carica papaya* Y (also known as the male specific region of the Y, or MSY), has identified at least three evolutionary strata. These strata are defined by different divergence times, and the oldest two strata, roughly 7 Myr and 1.9 Myr old, are defined by large chromosomal inversions (Wang et al. 2012). Identification of strata in additional angiosperm trees will provide insight into generalities in the evolution and turnover of SDRs and their impacts on genomic evolution.

The genomic location of the SDR can be highly labile in trees. This is most clearly evident in the SDR in the Salicaceae. Take for example the sex chromosomes of the sister genera *Salix* and *Populus*. All species in both genera and outgroups are dioecious, indicating that dioecy is the ancestral condition. However, recent studies have found that shrub willows (subgenus Vetrix) are ZW, with the SDR located near the centromeric region of chromosome 15 (Fig. 1a; Hou et al. 2015; Pucholt et al. 2015a); in contrast *Populus tremuloides* is XY, with the SDR near the centromeric region on chromosome 19 (Kersten et al. 2014), and *P. trichocarpa* is also XY, but with the SDR at the telomeric region of chr19 (Fig. 1a; Geraldes et al. 2015). Together even with this sparse phylogenetic sampling, SDR evolution in *Salix* and *Populus* appears to express diversity in sex determination that rivals the most dynamic animal systems (Woram et al. 2003; Mank et al. 2006; Li et al. 2011; Gamble et al. 2015). Theoretical studies also implicate SA loci as driving shifts in the location of the SDR (van Doorn and Kirkpatrick 2007), and may underlie changes in which sex carries heterogametic sex chromosomes (XY↔ZW; van Doorn and Kirkpatrick 2010).

Dioecy also appears to be the ancestral condition in the Caricaceae, with 32 of 35 species being dioecious (Carvalho and Renner 2012). The handful of species with alternative reproductive mechanisms, including one monoecious species and two polygamous species, are likely derived conditions (Carvalho and Renner 2012). Here too, there is evidence for the independent origin of the SDR subsequent to the origin of dioecy; however, unlike the case in poplar, the case in the Caricaceae may represent an outstanding case of parallelism. First, the age of the oldest evolutionary stratum in *Carica papaya* is estimated only as 7 Myr, which is well after the approximately 27 Myr divergence from the sister genus *Vasconcellea* (Carvalho and Renner 2012). However, when sex-linked genes are analyzed in a phylogenetic context, we find evidence of independently arisen X and Y alleles at those loci among sister taxa (Fig 1b; Wu et al. 2010). It could be that in the case of the Caricaceae, we are witnessing an extreme case of parallelism, where the same genomic region is co-opted by the SDR multiple independent times. Testing of this hypothesis awaits further comparative genomic analyses between sister taxa.

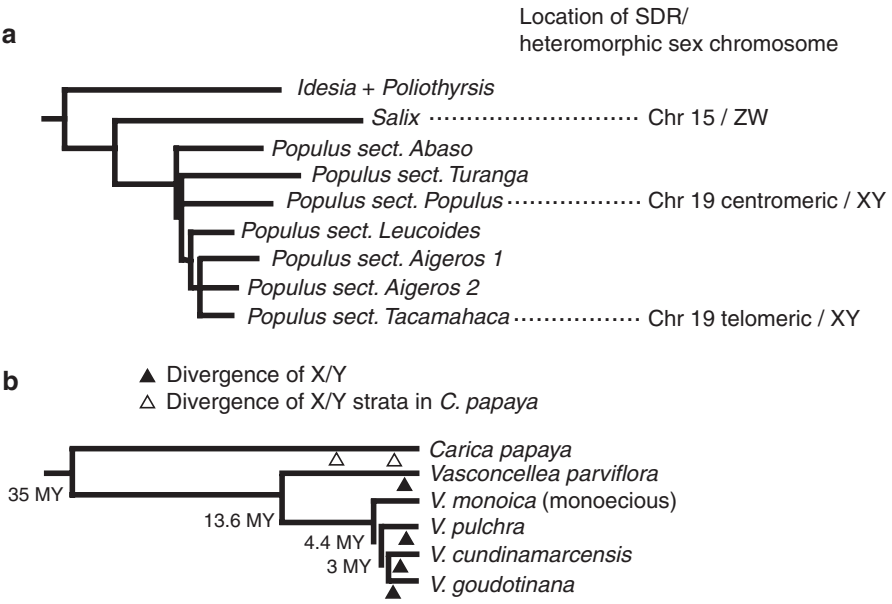

Fig. 1 The locations and ages of sex determination regions and the heterogametic sex (XY male or ZW female) are dynamic within the Salicaceae and *Carica* the taxonomic groups with the two most well-studied sex chromosomes in angiosperm trees. (**a**) Dioecy evolved prior to the divergence of Salix and *Populus* and all members of these genera are dioecious. Sex determination is ZW and the sex determination region (*SDR*) is on chromosome 15 in *Salix purpurea*. The SDR has not been mapped in any of the other six subgenera of Salix, which together comprise >1000 species. In contrast, sex determination is XY in Populus, with the SDR located on the centromeric region of chromosome 19 in section *Populus* (aspens) and on the telomeric end of chromosome 19 in section *Tacamahaca* (balsam poplars). The SDR is probably in a similar in sections Aigeros and Tacamahaca, but the location in sections Leucoides, Turanga and Abaso are unknown. (**b**) Estimates of divergence times for sex-linked loci in Caricaceae species post-date speciation events. The divergence time estimates (*open arrows* for *Carica papaya* strata 1 and strata 2; *closed arrows* for *Vasconcellea* spp.) are younger than the estimated divergence times (at nodes) for these species. This supports the multiple, independent origins of the sex determining region in these taxa despite the fact that dioecy is an ancestral character for the Caricaceae (monoecy in *V. monoica* is a derived state). Divergence time estimates of species are based on Carvalho and Renner (2012) and for the sex-linked loci from Wu et al. (2010)

The absence of recombination in the SDR also has deleterious influences because of the accumulation of weakly deleterious mutations and/or repetitive elements ultimately leading to gene loss and chromosomal degeneration (Bachtrog 2013). This degeneration is thought to be driven by the Hill-Robertson effect caused by genetic interference of selection at linked sites in the SDR (Hill and Robertson 1966; McVean and Charlesworth 2000; Charlesworth 2012). In the absence of recombination, stochastic deleterious mutations cannot be effectively purged leading to their irreversible accumulation (a process known as Mueller's ratchet). Furthermore, the effects of genetic hitchhiking are exacerbated, such that fixation of beneficial mutations also leads to the fixation of linked, weakly deleterious alleles (Bachtrog 2013).

The Hill-Robertson effect has been documented most closely in the SDR of papaya. Here, signs of degeneration of Y-linked coding sequence, including elevated rates of nonsynonymous mutations relative to synonymous mutations, are found primarily in the oldest evolutionary stratum (Wu and Moore 2015). Furthermore, nonsynonymous polymorphism is increased relative to synonymous polymorphism, consistent with the ineffective purging of weakly deleterious alleles in the non-recombining Y (Wu and Moore 2015). Interestingly, because of its pericentromeric location, evidence of coding sequence degeneration also is found in the corresponding X-region, as recombination is restricted in this region relative to the rest of the genome (Wu and Moore 2015). Because signs of degeneration are attenuated in younger evolutionary strata, it is possible to gauge the evolutionary tempo of degeneration, as the two youngest strata, at 1.9 Myr and 0.3 Myr do not show strong signs of degeneration. This suggests that sex chromosome degeneration may be slower in plant systems than animal systems, possibly because deleterious recessive alleles are exposed to stronger selection in plants because their haploid gametophytic stage is more independent than in animals (Charlesworth and Charlesworth 1992). Recent work in the perennial herbaceous plant, *Silene latifolia*, however, suggests that slower degeneration may not be a universal feature for plant sex chromosomes (Papadopulos et al. 2015).

Even as advances in genomic resources have allowed researchers to identify cryptic SDRs in a number of dioecious tree species, in many cases the identity of the underlying sex-determining genes remains elusive. Although the genomic regions containing the sex-determining loci may be relatively small and gene poor, such as the 100 kb region of *P. trichocarpa* with an estimated 35 genes (Geraldes et al. 2015), identification and functional verification of the genes regulating sex expression in these regions can be complicated. For example, in papaya, there are only 92 genes in the MSY; however, due to genetic hitchhiking, selection for favorable alleles also extends to neighboring neutral loci, and traditional population genetic methods for identifying targets of selection cannot be used. Functional verification of such genes may be hindered not so much by the lack of gene transformation techniques than by the difficulty in working with long-lived perennial systems. Furthermore, it is likely that different genes are involved, perhaps even in closely related systems, ruling out the use of functional homology to screen for likely candidates. That being said, promising progress in the identification of the genes underlying sex expression is being made. For example, in persimmon, a Y-linked small RNA that suppresses female expression has been identified (Akagi et al. 2014). This raises the likelihood that other small RNAs may be responsible for sex-determination in other tree species as well.

Conclusions

This brief overview of angiosperm tree breeding and mating systems highlights the prominence of outbreeding and dioecy. Although angiosperm trees exhibit a range of breeding systems, there is a trend towards an excess of dioecy relative to

herbaceous species. This may result partially from selection for outbreeding, but also may result from adaptive benefits of gender dimorphism (Geber 1999), or increased speciation/decreased extinction rates in lineages of dioecious trees; all hypotheses that remain to be tested (and see also Bawa and Holliday, this volume). Breeding systems such as gynodioecy are rare and, when found, may deserve attention for factors that have influenced their evolution and maintenance (Gibson and Diggle 1998). Although not reviewed above, many tree species classified as dioecious actually harbor small frequencies of individuals that exhibit monoecy, hermaphroditic flowers, or a mixture of both, and species classified as monoecious may exhibit andromonoecy or gynomonoecy. Increased attention into whether these rare individuals arise because of the lack of strong suppression of recombination across sex determination regions, from environmental influences that effect phytohormonal control of flower development (Golenberg and West 2013), or from a lack of developmental canalization would provide insight into factors influencing the evolution of gender and sex chromosomes in angiosperm trees.

The results of Hamrick and Godt's (1996) review indicate that long- and short-lived perennials (including trees) have much lower frequencies of selfing (<2 % and 14.5 %, respectively) than annuals (44.5 %), suggesting that factors selecting for selfing (e.g. ephemeral habitats with short growing seasons) are rare in angiosperm trees. Trees tend to inhabit more mesic environments and, unlike annuals, their reproductive success is not dependent on a single reproductive event.

Another question is whether a woody habit coupled with a predominantly outcrossing mating system directly affects the higher levels of genetic diversity and lower among-population differentiation observed for forest trees. Hamrick and Godt (1996) argued that the height of trees and their comparatively lower population densities, relative to herbaceous plants with similar combinations of life-history traits, should lead to greater pollen and seed dispersal distances. The greater dispersal potential of trees relative to herbaceous plants influences the scale at which individuals randomly mate and likely results in larger effective population sizes, thus decreasing the probability that novel alleles are lost due to genetic drift. Nonetheless, compared to other angiosperms, angiosperm trees tend to exhibit low levels of nucleotide diversity, consistent with low effective population sizes (Wright and Gaut 2005; Savolainen and Pyhajarvi 2007). Comparisons between herbaceous sister species that exhibit selfing and outcrossing showed that outcrossing species harbor larger effective population sizes and higher levels of nucleotide diversity (Charlesworth and Wright 2001); thus, only rare angiosperm trees with reduced outcrossing rates are expected to have reduced species-wide nucleotide diversity resulting from their mating systems. As more angiosperm tree species from more diverse habitats (e.g. tropical communities) and geographical locations (e.g. Africa, Asia, and South America) are added to the existing databases, future reviews of the genetic diversity literature will determine whether trends identified herein are more broadly supported. Future studies will focus on nucleotide diversity data from SNP studies, and thus may provide additional insight into factors influencing diversity such as effective population size and its influence on the rate of adaptive evolution (Wright and Andolfatto 2008) and the strength of purifying selection (Ohta and Kimura 1971; Eyre-Walker et al. 2002; Charlesworth 2009;

Wang et al. 2016). For these comparisons to be made, however, tree biologists must be careful to collect data that is comparable across species. It is well known that different classes of nucleotide positions evolve at different rates (Wright and Gaut 2005), because some are more strongly influenced by selection than others. For instance, the third-base codon sites evolve more quickly than first or second positions, and sometimes more quickly than introns (Ingvarsson 2005; Kim et al. 2007; Olson et al. 2010). Calculating and reporting a breakdown of π and Watterson's θ for different types of sites, especially synonymous site diversity because of its near-neutrality, provides some of the most useful estimates of diversity for determining the influence of breeding and mating systems on N_e and patterns of genomic diversity. Unfortunately, inexpensive screening methods such as RAD-seq and GBS target arbitrary regions and the functional classification of the SNP positions often is unknown, reducing their utility for cross-species comparisons.

When we consider the evolutionary genomics underlying sex expression and breeding systems in tree species, we are left with a number of outstanding questions. For one, genetic theories for the origin of dioecy from hermaphroditism typically involve gynodioecious or androdioecious intermediates, yet many dioecious tree species lack close relatives with these breeding systems. Instead, a more likely path in trees may be from a monoecious intermediate (Renner and Ricklefs 1995). The prospect for this pathway has been validated in herbaceous cucumber (Boualem et al. 2015); however, the experimental evidence is lacking in tree species. While comparative genomics of related taxa with different breeding systems may allow for the inference of the pathway to evolving dioecy in tree species, such comparative tests are complicated by the clustering of dioecy in particular lineages, such as in the Salicaceae and Caricaceae. In these taxa, it is most likely that dioecy evolved once in the common ancestor of the lineage, which may be as many as 75 Myr for *Populus* and *Salix* and 35 Myr ago for the Caricaceae.

If dioecy evolved only once in the Salicaceae and Caricaceae, it is logical to question how subsequent evolution of the sex determination region (SDR) has impacted the genome. In the case of *Populus* and *Salix*, there clearly has been evolutionary turnover of SDRs, such that its genomic location varies among taxa. This may be simply a case of chromosomal rearrangements or transposition of an SDR to a different chromosome, perhaps driven by the acquisition of SA loci; and indeed, this seems most parsimonious for *Populus* species. This hypothesis is challenged, however, by evidence that the sex determination genes differ between the balsam poplars (*Populus* section Tacamahaca, including *P. trichocarpa*) and the aspens (*Populus* section Populus; Geraldes et al. 2015). Given this result, it seems even less likely that the underlying genes will be conserved between the ZW system of *Salix* species and the XY system of Poplar (but see Hou et al. 2015). In contrast, in the Caricaceae, homologous sex-linked loci are found in *Carica papaya* and *Vasconcellea* species; however, the age at which the SDR formed is predicted to post-date speciation (Wu et al. 2010). This would suggest that recombination suppression, and the formation of the SDR is localized on a core genomic region that may contain homologous sex determining genes. Confirmation of this hypothesis awaits comparative genomic analyses of the SDR and their genomic positions from different Caricaceae species.

One glaring deficiency in the study of the genetics of dioecy and sex determination in tree species, though, is the lack of identified sex determination regions and sex chromosomes in many species. Little is known about the extent to which alternative sex determination systems, such as environmental sex determination, might regulate sex expression in dioecious angiosperm trees. Also, given the prevalence of dioecy among tree species, it is likely that additional types of sex chromosomes, including heteromorphic sex chromosomes, will be discovered. Understanding patterns of diversity of sex chromosomes in angiosperm trees provides a wealth of untapped information about their influences on genomic evolution and adaptive sexual dimorphism. Finally, the genomic structure and/or gene composition of the sex chromosomes may be affected by ecology; for example, sexually antagonistic alleles underlying differences in male and female responses to environmental stress may be preferentially localized to the SDR and/or PAR of the sex chromosomes (Delph et al. 2010; Spigler et al. 2011). Because agronomic species are often grown in conditions divorced from their ecology, it may be of merit to identify an ecological tree model with sex chromosomes to address such questions. Fortunately, there is an opportunity for such studies in natural populations of *Populus* and *Salix*, and even to some extent in wild populations of *Carica papaya*.

Because of the immense ecological and evolutionary importance of trees, understanding trends in botanical, ecological, and genomic diversity requires that we overcome their challenges for addressing biological questions. Angiosperm trees have always been recalcitrant systems for evolutionary investigations because of their long lives and large sizes (McClure et al. 2014), and genomics offers new tools for questions ranging from identifying genes under selection, to mapping genetic factors contributing to quantitative traits, inferring the past demographic histories, and understanding the influences of mating systems on genomic architecture. Breeding and mating systems are the primary mechanisms by which trees regulate genetic transmission, and understanding the breadth of breeding and mating system diversity in trees is crucial for proper interpretations of patterns of genomic architecture and diversity. Most trees are primarily outcrossing; a larger proportion of trees are dioecious than any other life history category of plants, and most of these dioecious trees likely harbor sex chromosomes. Future investigations targeted at better understanding of adaptive factors influencing tree mating systems and the impacts of mating systems on population and genomic diversity will provide key information for understanding the basic biology of these organisms that are foundations of many ecosystems worldwide.

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Genomics of Speciation in Temperate and Boreal Angiosperm Trees

R. Bawa and J.A. Holliday

Abstract Understanding how species form and persist has been a central objective of evolutionary biology since the time of Darwin. Species emerge where two or more populations develop reproductive barriers yielding phenotypic and genetic divergence. This definition has led some to question the reality of species boundaries in plants. In contrast to animals, in which behavioral traits may allow sympatric groups to establish and maintain reproductive isolation (RI), plants do not benefit from mate selection in quite the same way. As a result, the definition of plant species has long confounded evolutionary biologists, as have the ecological and genetic processes that underlie their formation. Temperate and boreal angiosperm trees make an interesting case study in this regard, where a lack of RI often leads to difficulties in defining closely related species. With widespread and overlapping distributions, many tree species must resist gene flow from congeners, often in the absence of pre- and post-zygotic barriers, in order to maintain adaptations to their particular niche. While high rates of intraspecific gene flow likely promote species cohesion, angiosperm trees also show extensive interspecific hybridization in contact zones. In this chapter we highlight recent work to understand the pattern and process of speciation in angiosperm trees, and how contemporary sequencing technologies are providing insights into the genomic mechanisms that underlie genomic porosity on the one hand, and isolation on the other.

> The principle of benefit derived from divergence of character…will lead to most divergent variations…being preserved and accumulated by natural selection
>
> – Charles Darwin

Modes of Speciation

Geography drives local adaptation and is therefore one of the major forces leading to speciation. Differences in altitude, latitude, edaphic factors, and biotic interactions can trigger the development of reproductive barriers, but some underlying

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level of reproductive isolation (RI) is usually necessary to establish local adaptation. The extent of local adaptation depends on the balance between gene-flow and selection (Savolainen et al. 2007), and this interplay is therefore key to speciation. Wind dispersal of pollen is extensive in trees, ranging from 100 of meters to several kilometers (Austerlitz et al. 2004; Smouse and Sork 2004), and animal-mediated pollen flow, while less common, can also extend several kilometers (Savolainen et al. 2007; Hamrick 2000). In wind-pollinated trees, seed dispersal can be 20–200 times lower than pollen dispersal (Ennos 1994; Bacles et al. 2006), but fossil based seed dispersal estimates, ranging from 50 to 500 m/year (Hewitt 1999), suggest that seed migration may have a significant impact on overall population connectivity. For example, in tulip poplar (*Liriodendron tulipifera*) seed dispersal up to 10 km has been reported (Nathan et al. 2002). The degree of effective migration between populations distinguishes speciation in terms of allopatry, which is purely geographic isolation with very low number of migrants per generation ($N_m \sim 0$), parapatry, in which populations occupy adjoining regions with low to intermediate level of gene flow ($0 < N_m < 0.5$), and sympatry, where the effective migration is high ($N_m > 0.5$) (Coyne and Orr 2004; Mallet et al. 2009).

Allopatric speciation occurs when there is no geographical contact between populations and thus very low or non-existent gene-flow (Coyne and Orr 2004). In this scenario, selection and drift can act freely and independently (Nosil 2012). Geological processes have led to RI and diversification across many tree genera. For example, divergence of three closely related *Populus* species (*P. tremuloides*, *P. davidiana* and *P. tremula*) coincided with the tectonic split of the Bering land bridge and the rapid uplift of the Tibetan plateau, which generated a geographical divide (Du et al. 2015). Allopatric speciation may also have been a consequence of post-glacial history. Many northern trees migrated out of glacial refugia to colonize higher latitudes since the last glacial maxima, and associated long distance dispersal could have been followed by decreased gene flow from the ancestral population, resulting in geographical isolation that facilitated formation of independent evolutionary lineages. An interesting example is the sister species *Populus trichocarpa* and *P. balsamifera*, which diverged ~75 ka. This time period corresponded with the last glacial maxima (LGM) and modeling of the paleoecological niches for these species during the LGM suggests a scenario of allopatric speciation, with *P. trichocarpa* restricted to the coast, and refugial *P. balsamifera* populations to central Alaska (which was ice-free due to low precipitation) and the intermountain western United States (Levsen et al. 2012).

While allopatric speciation has generated much extant diversity within tree genera, parapatric and sympatric speciation (collectively referred to as non-allopatric speciation) have seen a greater research focus in recent years due to their more complex genetic and ecological underpinnings. Non-allopatric divergence is characterized by high gene flow, and its interplay with selection determines the pace of speciation (Coyne and Orr 2004; Savolainen et al. 2007). Parapatric speciation, where there is balance between selection and gene flow, is perhaps the most common mode of non-allopatric divergence. In parapatric speciation, newly arisen adaptive mutations can become fixed in the face of gene flow provided the strength

of selection is greater than that of migration (Haldane 1948; Slatkin 1973). Parapatric divergence may be clinal, where populations are continuously distributed across a heterogeneous environment, or involve discrete populations (i.e., the stepping-stone model). Theoretical studies have shown that it is easier to achieve parapatric speciation in the context of discrete rather than continuous populations, but additional factors (e.g., assortative mating, pre- or post-zygotic isolation) are usually required. Cain et al. (1999) studied reinforcement dynamics in mosaic and clinal hybrid zones, and showed that the stepping-stone model is characterized by selection against both unfit homozygotes and heterozygotes, but clinal hybrid zones involve reinforcement selection only against heterozygotes, leading to faster divergence in the stepping-stone model. However, in trees, clinal variation between species across contact zones is more common (Lexer et al. 2007; Penaloza-Ramirez et al. 2010; Roe et al. 2014; Zeng et al. 2011). Hence, both pre- and post-zygotic barriers to gene flow are expected to contribute to establishment and maintenance of RI where tree species diverge in the absence of geographical isolation.

Pre-zygotic Barriers to Gene Flow

Reproductive isolation (RI) involves the development of barriers to gene flow among populations, which may be due to ecological or genetic factors. These barriers are key to the emergence of species, and normally are a by-product of diversifying selection and genetic drift (Rieseberg and Willis 2007). Theory and empirical studies suggest RI is the most common way for species to form, as it does not require complete geographic isolation for genetic differences to accumulate (Agrawal et al. 2011; Lowry et al. 2008). RI causes an overall reduction in the effective migration rate, which leads to both neutral and adaptive divergence among populations over time (Baack et al. 2015). These reproductive barriers can be differentiated based on their time of occurrence and can be caused both by external (ecological) forces or internal (genetic) forces. The first line of barriers are pre-zygotic (pre-pollination). Pre-pollination barriers restrict the transfer of pollen of one species to the stigma of another, and can be classified further as (i) eco-geographic isolation arising from differential habitat preference; (ii), mechanical isolation, caused by differences in reproductive structures; (iii) temporal isolation, such as differences in reproductive phenology; (iv) or competition based isolators (Baack et al. 2015).

Habitat preference/Eco-geographic isolation Habitat isolation is defined as geographic isolation between populations, and arises due to local adaptation. This mode of RI is normally characterized by low fitness of individuals in the alternative habitat, reducing the likelihood of successful mating with individuals from different populations. Habitat isolation can be described in two forms: macrospatial and microspatial isolation. Macrospatial isolation is a form of allopatric speciation, where there is no gene flow between populations due to geographical barriers. Microspatial isolation, on the other hand, is mostly driven by local adaptation, where populations occur in the same general area, but their reproductive encounters

are reduced due to adaptations and preferences to different ecological niches (Coyne and Orr 2004). Both macrospatial and microspatial isolation occur commonly in angiosperm trees. For example, a study in white oaks (*Quercus gambelii* and *Q. grisea*) showed that even though there is overlap in geographical distribution of both of these species, hybrids are rare even in areas of sympatry, and each of these lineages remains genetically distinct (Howard et al. 1997). Similarly, *Populus* species in the intermountain western United States (*P. angustifolia*, *P. fremontii* and *P. deltoides*) have overlapping geographical distributions and can freely hybridize in nature, but all the species maintain distinct niches, with few natural hybrids outside of contact zones. This could be attributed to distinct local adaptation of these three lineages to different geographical environments that contribute to some level of reproductive isolation (Stettler et al. 1996).

Temporal isolation Divergent developmental schedules among populations may facilitate RI, though in continuously distributed tree populations this mechanism may also contribute to a pattern of isolation-by-distance or isolation-by-adaptation. Temporal isolation in trees may arise due to climatic differentiation among populations that yields divergence in optimal timing of flowering. Among annual plants, extensive divergence in flowering time has been documented along environmental clines. For example, coastal and inland ecotypes of *Mimulus guttatus* are temporally isolated as a result of very little overlap in flowering time (Lowry et al. 2008). In the perennial sunflower *Helianthus maximilani*, strong differentiation was observed among populations for flowering time and other growth related traits. Due to their extended juvenile periods, coupled with the difficulty of studying flower phenology in reproductively mature (large) individuals, few studies of intra- or interspecific variation in flowering have been undertaken in trees. In *Eucalyptus globulus*, dwarf coastal ecotypes evolved from tall inland ecotypes and differ in their flowering time. No introgression was found between these species in spite of their close proximity, which suggests temporal RI between them (Foster et al. 2007). Similar studies in other angiosperm trees, including *Platanus acerifolia*, *Ulmus minor*, and *Carpinus betulus*, showed divergence in flowering time in populations from different latitudes (Chuine et al. 2000), which suggests that RI as a function of differences in reproductive phenology may be an important route to RI in trees, but more research is needed in this area. While studies of vegetative phenology in trees has yielded extensive evidence for local adaptation in the timing of these transitions (Savolainen et al. 2007), more work is needed to understand the role of variation in the timing of flowering as a potential factor in the development of RI.

Immigrant inviability This type of isolation normally arises when populations have reduced survival in the non-native habitat, and should not be confused with the habitat isolation where non-native populations are excluded in certain geographic regions or ecological niches. Immigrant inviability reduces effective gene flow and fosters selection for habitat preferences through fitness trade-offs (Nosil 2012). These trade-offs are associated with poor adaptation to non-native habitats and are caused by local adaptation to heterogeneous environments. In angiosperm trees, where pollen is usually wind-dispersed, distant populations subject to

divergent selection frequently have lower fitness in their non-native habitat (Hardy et al. 2006; Kawecki and Ebert 2004; Ducousso et al. 1996). Various factors – biophysical, physiological, and climatic – contribute to selection against immigrants. Many studies of immigrant inviability in trees have been conducted within species due to an interest in finding well-adapted provenances for reforestation (Morgenstern 1996). Traits of particular importance to temperate and boreal trees are related to climatic adaptation, and include vegetative bud phenology (Luquez et al. 2008), fall, winter and spring cold hardiness (Howe et al. 2003), and drought tolerance (Street et al. 2006).

Pollinator preference and conspecific pollen precedence While insect pollination is uncommon in temperate and boreal trees, sexual isolation in a few cases may be due to differential pollinator preferences that arise from co-evolution and/or habitat isolation. These barriers have been shown to be very effective mode to enforce RI among populations growing in close proximity (Coyne and Orr 2004; Kirkpatrick and Ryan 1991). While flower color is the most commonly identified pollinator divergence pattern in plants, other modes of pollinator divergence may be more important in angiosperm trees. In Joshua tree (*Yucca brevifolia*), a tree-like yucca endemic to the southwest United States, eastern and western subspecies are pollinated by different species of yucca moth (*Tegeticula synthetica* in the west and *T. antithetica* in the east) (Pellmyr and Segraves 2003), which appears to be due to differences in floral morphology (Godsoe et al. 2008). Abiotic factors also play a role in RI among populations. In European oaks, pollen fitness differed significantly for mesic and dry sites, leading to reduced cross-pollination among these two site types (Williams et al. 2001). While pollen-pistil interactions and differences in pollen fitness can act as absolute barriers, RI can be escalated by competition between inter- and intraspecific pollen, also known as conspecific pollen precedence (CPP). CPP is normally characterized by decreased fertilization rates interspecific pollen, and therefore is also referred to as a post-pollination pre-zygotic barrier. In oaks, fertilization and fruiting was reduced with inter-specific compared with intraspecific pollen (Varela et al. 2008), indicating pollen competition as a potential mode of RI (Rieseberg et al. 1995).

Pollinator preference and CPP can result from both ecological and genetic factors. While habitat preference or local adaptation might force plants to adapt to a new set of pollinators and pollination based morphological changes, an alternative reason for the development of pre-zygotic barriers is reinforcement (Hopkins 2013). Recent studies in *Phlox spp*. and fireweed (*Chamerion sp.*) have suggested development of these pollinator divergences as selection against hybridization (Hopkins and Rausher 2012; Baldwin and Husband 2011). Development of some of these pre-pollination barriers might have happened after development and establishment of post-zygotic barriers (Wallace 1912). Since, most post-zygotic barriers involve wasted resources in terms of fewer gametes with lower fitness, populations might use pre-pollination barriers like flower color or conspecific pollen precedence to reduce interspecific pollination (Baack et al. 2015). Having said that, not many studies in angiosperm trees show this type of mating isolation, and other forms of

RI (e.g., habitat preference or temporal isolation) that evolve early in the speciation process and reduce the need for sexual or mating isolation, may be more important (Nosil and Yukilevich 2008).

Post-zygotic Barriers to Gene Flow

Post-zygotic barriers are a second line of defense against interspecific pollination, as they normally evolve after the establishment of pre-pollination barriers. These barriers can be divided into two forms. First, extrinsic or ecological barriers are caused by divergent selection or local adaptation and are dependent on genotype x environment interactions (Nosil 2012). Extrinsic post-zygotic barriers include reduced hybrid viability, hybrid incompatibility, and sexual selection against hybrids. By contrast, intrinsic or genetic barriers are independent of the environment and are caused by genetic factors (Seehausen et al. 2014). Intrinsic post-zygotic barriers can take the form of Bateson-Dobzhansky-Muller incompatibilities, gene-duplications, mutations, genomic conflict, or chromosomal rearrangements.

Hybrid incompatibility Post-mating isolation caused by incompatible genetic differences between loci of parental individuals is referred to as hybrid incompatibility. These incompatibilities are genetic in nature, but can be initiated by local adaptation. Such incompatibilities arise due to divergent selection that favors different combination of alleles in different geographical areas. When parents from different locally adaptive regions form a hybrid, the alleles from the different environments may not be compatible with each other. Various theoretical and empirical studies have shown that intrinsic genetic incompatibilities can be driven by ecological forces. A simulation study by Agrawal et al. (2011) showed that intrinsic hybrid incompatibility can originate via divergent selection and high linkage disequilibrium among adaptive loci, and suggested that these mechanisms could drive populations to RI even in the face of high gene flow. This was corroborated by an experimental study in monkeyflower (*Mimulus* sp.) where adaptation to copper tolerance was linked to various loci responsible for hybrid incompatibility (Wright et al. 2013). Another interesting example where ecology drives hybrid incompatibility is cyto-nuclear interactions (Whittemore and Schaal 1991; Fishman and Willis 2006). These interactions are relatively common in plants, and transplant experiments have shown lower hybrid fitness caused by a mismatch of the organelle and nuclear genomes from differentially adapted parents (Fields et al. 2014; Moison et al. 2010; Stoll et al. 2015). Macaya-Sanz et al. (2011) showed extensive segregation distortion in backcross progeny arising from a cross between a female *Populus tremula x alba* F_1 hybrid and a pure *P. alba* father. This distortion favored *P. tremula* haplotypes in the offspring, which may have been due to genomic conflict between interspecific cyto-nuclear combinations.

Ecological and sexual selection against hybrids Similar to immigrant inviability, this mode of RI is characterized by lower fitness of the hybrid in either of its parents'

home environments. The basic premise behind such isolation is that hybrids formed by parents from two different environments will have an intermediate phenotype and highest fitness in the intermediate of the two 'extreme' niches. These intermediate niches are not very common in nature, leading to lower overall hybrid fitness (Nosil 2012). In addition, intermediate hybrids may not have appropriate floral or reproductive traits, which could cause pollinator-based discrimination, leading to sexual isolation (Baack et al. 2015). This type of post-zygotic isolation should be very common among the trees, because of high gene flow among populations. In the early stages of the speciation process, intraspecific gene-flow might lead to fertilization and formation of hybrids between ecotypes, but the net effective gene flow is substantially reduced by ecological and sexual selection against hybrids, leading to RI (Varela et al. 2008; Roe et al. 2014). In European aspen (*Populus tremula*) and white poplar (*Populus alba*), preferential gene flow was observed from European aspen to white poplar, even though these species can introgress with each other in either direction (Lexer et al. 2005). Similar studies of hybrid zones in the sympatric oaks *Quercus gambelii* and *Q. grisea*, showed preferential pollen movement and better fruit survival from *Quercus gambelii* to *Q. grisea* than in the other direction (Williams et al. 2001). This suggests ecological and sexual selection against hybrids as a form of RI in these systems.

Bateson-Dobzhansky-Muller incompatibilities (BDMI) BDMIs are an intrinsic post-zygotic barrier arising from epistatic interactions between two or more loci, which reduce fitness in the hybrids (Bateson 1909; Muller 1940; Dobzhansky et al. 1937). BDMIs were at first thought to be caused by genetic drift, but drift may be too slow and dependent on N_e to explain BDMIs, and meiotic drive, transposons, or selfish genetic elements are the more likely causes of such incompatibilities (Crespi and Nosil 2013; Nei et al. 1983). No studies in angiosperm trees have empirically demonstrated the role of BDMIs in RI, but various types of admixture and hybrid isolation behaviors have been attributed to such epistatic interactions among loci. A review on admixture mapping and hybrid zones highlighted the importance of epistatic interactions in RI (Buerkle and Lexer 2008). Since RI is normally effective when there is a synergistic relationship between multiple phenotypes, there is a good chance that epistatic interactions such as BDMI might play an important role in determining these effects (Buerkle and Lexer 2008). Moreover, the phenotypes observed in hybrid zones might not be due to additive effects, but rather to epistatic interactions. Larcombe et al. (2015) performed cross-pollination experiments among eucalyptus species and fitted three different models of the relationship between hybrid fitness and genetic distance. The results suggest a decrease in hybrid fitness with increase in genetic distance and that the relationship between genetic distance and hybrid compatibility was exponential in nature. This result supports the snowball hypothesis of BDMI in generating RI.

Chromosomal speciation Rearrangements, inversions, and translocations can cause disruptions in the meiotic paring of chromosomes, leading to sterility or incompatible hybrids (Livingstone and Rieseberg 2004). In addition, these rearrangements decrease the frequency of recombination due to mechanical interference with cross-

ing over, and therefore can lead to accumulation of hybrid incompatibilities in a relatively short period of time (Noor et al. 2001). The impact of these arrangements could also be due to the genes harbored in rearranged regions (Navarro and Barton 2003). A study in monkeyflower (Lowry and Willis 2010) supports a role for chromosomal inversion in flowering time and other traits related to RI. Different ecotypes of monkeyflower were found to have two inversions, which when introgressed into different ecotypic backgrounds led to significant differences in adaptive traits. Recent studies in *Populus* and *Salix* have revealed the presence of chromosomal rearrangements in sex chromosomes, and their role in divergence. Hou et al. (2015) studied the evolution of sex chromosomes from autosomes in the *Salicaceae*, and found that in *Populus* sex chromosomes have recently evolved, following divergence from *Salix*, and that these chromosomes contain highly divergent haplotypes with severe suppression in recombination, suggesting the importance of sex chromosomes in RI between species. Coyne and Orr (2004) suggest that while chromosomal speciation may cause RI, it can only be effective in the presence of meiotic drive and small effective population size. More studies are needed to test the importance of chromosomal speciation in trees to determine if it can alone be an effective mode of RI, and this topic is further discussed in the section entitled 'Genomics of Speciation'.

Genic speciation Locus-based incompatibilities originating by mutations, including duplications and deletions, fixed through selection or drift, are referred to as genic speciation. While mutations in a single gene could enable speciation, provided those mutations have a strong effect on RI (Nosil and Schluter 2011), it is unlikely that most speciation events are caused by single mutations. The effect of single or multiple genes can be manifested either in the form of pleiotropy or high linkage disequilibrium with the genes causing RI (Coyne and Orr 2004). Various empirical studies have demonstrated the role of genic speciation in RI. For example, the *yup* flower colour gene leads to pollinator-based isolation in monkeyflower (Moyle et al. 2012; Bradshaw and Schemske 2003). While the *yup* case is a striking example, characterizing the genetic architecture of local adaptation may more generally lead to a better understanding of the loci that underlie interspecific adaptive divergence. Recent population genomic studies have highlighted the role of divergent selection in speciation.

Role of Polyploidy and Hybridization in Speciation

Speciation until now has been mostly defined as divergence between populations, which has progressed long enough for the populations to diverge into species. Having said that, homoploid divergence is not the only way speciation progresses: polyploidization and hybridization are additional routes, very common in plants, that are known to play role in speciation (Coyne and Orr 2004).

Polyploid speciation Polyploidy is defined as the condition where the individual carries three or more set of complete chromosomes, and polyploidization is very

important in context to speciation because it can be a rapid route to reproductive isolation (Ramsey and Schemske 1998). Polyploidy may persist as stable sets of homeologous pairs of chromosomes, or go through a process of re-diploidization (i.e., paleopolyploidy). Being far more common in plants than animals, polyploidy is one of the major distinguishing features of plant speciation (Wood et al. 2009). Polyploids can be classified into three different types: autopolyploids, which arise from intraspecific crosses, allopolyploids, which arise from interspecific crosses, and autoallopolyploids, which combine the characteristics of both the auto and allopolyploids. Polyploids can be formed through somatic doubling, meiotic non-reduction, or polyspermy (Grant 1981; Ramsey and Schemske 1998). Somatic doubling involves failure of the chromosomes to separate during anaphase; meiotic non-reduction occurs when the cell membrane fails to form during gametogenesis, giving rise to diploid gametes; and polyspermy occurs when two male gametes (sperms) fertilize an egg (Grant 1981; Levin 2002). Polyspermy is uncommon in plants as compared to somatic doubling and meitic non-reduction, with the latter thought to be the most common mechanism of polyploidization (Ramsey and Schemske 1998; Thompson and Lumaret 1992). Meiotic non-reduction can be triggered by the presence of major genes, which include deleterious mutations at low frequency (Levin 2002). Moreover, polyploidization may be involve an intermediate stage where a triploid individual backcrosses with one of the diploid parent to form the stable polyploid (Ramsey and Schemske 1998). The frequency of polyploidy varies significantly among plants, with ~31 % of ferns being polyploid, but only ~15 % of angiosperms (Wood et al. 2009). Polyploidy is common in some tree genera (Levin and Wilson 1976; Stebbins et al. 1950), which has been attributed to the greater degree of clonal/vegetative propagation compared with other taxa, which increases the incidence of somatic doubling (Grant 1981). Self-fertilization, which is common in plants, increases the chances of polyploid establishment as compared to outcrossing species, where polyploid individuals may remain at low frequency for a long period of time (Grant 1981; Stebbins et al. 1950). However, in trees, while selfing does occur, inbreeding depression is a significant factor (Petit and Hampe 2006) and may limit the persistence of polyploids arising from selfing.

The role of polyploidy in speciation depends on the frequency with which different kinds of polyploidization occur. Autopolyploidy occurs at a much higher rate than allopolyploidy: 10^{-5} events per generation, which is similar to the genic mutation rate (Ramsey and Schemske 1998). Ramsey and Schemske (1998) highlighted various factors that could favor one type of polyploidy over another in terms of speciation. First, autopolyploids are not easy to identify because they are morphologically similar to their parents, which suggests that in general autopolyploidy may not lead to diversification. In addition, autopolyploids are mostly sterile, so although common, autopolyploids are not persistent (Ramsey and Schemske 1998). This is consistent with the rarity of in situ allopolyploidization on oceanic islands, wherein autopolyploidy is the primary route to polyploidization due to the higher level of divergent species found on such islands (Schluter and William 2000). While the rate of formation of allopolyploids may be similar to that of autopolyploids on a per meiosis basis, the former also depends on the rate of interspecific hybridization,

which may be rare, and hence the overall number of allopolyploidization events is lower (Ramsey and Schemske 1998).

There are many cases of diversification in plants resulting from polyploidization. For example, auto-tetraploid fireweed (*Chamerion* sp.) flowers 1 week later compared to its diploid progenitors, causing reproductive isolation, and although there was some overlap in flowering between parental (diploid) and tetraploid fireweed, this asynchrony decreased hybridization by at least 50 % (Husband and Schemske 2000). In the perennial herb *Heuchera grossularifolia*, autotetraploid plants larger floral parts compared to the diploid parents, which altered the number and type of pollinators on the polyploid plant, leading to reproductive isolation (Segraves and Thompson 1999). Angiosperm tree taxa harboring polyploid species include *Alnus* (alder), *Betula* (birch), *Fraxinus* (ash), *Tilia* (basswood or linden), *Acer* (maple), *Salix* (willow) and *Magnolia* (Wright 1976). Phylogenetic analysis of birch, maple, ash, and magnolia reveals multiple polyploid lineages in each of these genera, which suggests independent polyploidization events led to speciation and diversification in these genera (Grimm et al. 2006; Jarvinen et al. 2004; Jeandroz et al. 1997; Parris et al. 2010). In addition to extant polyploids, there is evidence for paleopolyploidy in angiosperm trees, the best example of which being the salicoid duplication revealed by sequencing of the *Populus trichocarpa* genome (Tuskan et al. 2006).

When gene flow promotes divergence Gene flow is one of the most important factors determining the pace of adaptive divergence and potential for speciation in trees. While it usually acts as a homogenizing force, decreasing phenotypic diversity among populations, an increase in geographical contact can also trigger speciation in some scenarios. Regions where two species geographical ranges overlap (i.e., hybrid zones) are characterized by strong selection against maladapted hybrids. This selection against hybrids can cause asymmetric hybridization, and lead to assortative mating (Nosil 2012; Lexer et al. 2007). If subsequent chromosomal rearrangements lead to sterility between the hybrid offspring and the parent species, recombinational speciation may ensue (Rieseberg 1997). The most extensively studied example of potentially nascent hybrid speciation is in central Europe, where the ranges of *Populus alba* and *P. tremula* overlap. *P. alba* and *P. tremula* are morphologically distinct in their leaf characteristics, and occur in very different habitats, with the former mostly occurring in lowland flood plain forests and latter occurring in mixed upland habitats (Lexer et al. 2007). Extensive hybridization between the two parental species suggests weak reproductive isolation, and hybrids mostly occur in the contact zone and have an intermediate leaf phenotype (Lexer et al. 2009). Interestingly, substantial differences in ancestry have been observed between the seedling and adult stages, with the former comprised of the full range of hybrid classes, while the latter are mostly comprised of intermediate (especially F1) individuals. A defined genotypic discontinuity between the hybrids and parental species suggests this may be an example of incipient hybrid speciation (Lindtke et al. 2012). However, range shifts in the parental species may be necessary to reinforce RI with the hybrids for speciation to be realized (Lexer et al. 2010). Natural hybrid zones between tree species are common, and future work in these settings should shed new light on the role of gene flow in promoting divergence.

Genomics of Speciation

The advent of next generation sequencing methods has led to a recent focus on the genomic regions underlying speciation (Hohenlohe et al. 2010; Hudson 2008; Ellegren 2008). Genetic differentiation is highly heterogeneous across the genome both within- and among-species, and potential factors that affect interspecific genome-wide divergence include selection, drift, structural variation (Noor et al. 2001), variable mutation and recombination rates (Noor and Feder 2006), and genomic conflict (Rice and Holland 1997). The genomics of divergence are expected to differ between allopatric and sympatric speciation. In allopatric divergence, disruptive gene flow is not an important factor – all regions of the genome therefore have an equal chance of differentiation, and even though this differentiation can be heterogeneous, clustering is not favored by migration-selection dynamics (Noor and Bennett 2010; Turner and Hahn 2010). Therefore, divergence in allopatry is expected to result smaller regions of differentiation, which are much more dispersed across the genome as compared to in the cases with gene-flow. For example, a study of genomic divergence in allopatric populations of ferns reported small, diffuse regions of differentiation (Nakazato et al. 2007). In contrast, gene flow does impede both neutral and adaptive divergence in cases of sympatric speciation. Due to this homogenizing effect, tight linkage among adaptive genes should be favored (Gavrilets 2004). Theoretical studies corroborate this expectation. Yeaman and Whitlock (2011) used individual-based simulations to examine the effects of low, intermediate, and high migration rates on the genetic architecture of adaptation. They found that as migration rates approach a critical threshold, above which local adaptation collapses, increasingly concentrated genetic architectures are favored. When migration was just below the critical threshold the effect was maximized, and divergence could be explained by a single large-effect haplotype. The magnitude of this effect depended on the mutation rate, effect size of individual mutations, and strength of selection, and was most pronounced when effect sizes of individual alleles within the 'island of divergence' was small. Yeaman (2013) further showed that these islands may be the result of genomic rearrangements that bring adaptive alleles into close physical proximity, rather than divergence hitchhiking alone.

Where species diverge in sympatry, a particular focus in recent years has been identifying these regions of the genome subject to divergent selection, which may resist recombination between nascent or recently diverged species and therefore contribute to RI. F_{ST} is commonly used in this context as a measure of genetic differentiation, and unusual loci or regions are identified by comparison with a model-based or empirical null distribution (Beaumont and Balding 2004; Foll and Gaggiotti 2008). Another framework that has been employed in the context of natural hybrid zones uses multinomial regression to identify genomic clines that deviate from the genome-wide pattern of introgression, and are indicative of selection (Gompert and Buerkle 2009). Regions of exceptional differentiation have been referred to as genomic islands or genomic continents of divergence, depending on their size (Nosil et al. 2009; Via 2012).

Studies in animal taxa provided the first evidence of genomic islands of divergence (Harr 2006; Nadeau et al. 2012; Turner et al. 2005). Among plants, Andrew and Rieseberg (2013) studied wild, hybridizing sunflowers (*Helianthus* sp.) and reported varied levels of genetic differentiation and size of genomic islands between ecotypes within and between species. In contrast to interspecific comparisons between *H. annuus* and *H. petiolaris*, fewer and more clustered genomic islands were identified within species when ecologically divergent populations that occupy sand dune and non-sand dune habitats were compared. This result supports the work of Yeaman and Whitlock (2011) and suggests that early in the speciation process, when gene flow is a significant impediment to genomic divergence, clustering of ecologically relevant loci promotes local adaptation. The findings of Andrew and Rieseberg are consistent with a recent study in black cottonwood (*Populus trichocarpa*), both altitudinal and latitudinal clines were sampled. The altitudinal clines, which entail higher migration relative to selection, had more numerous and larger genomic islands of divergence compared with the latitudinal case, which reinforces the idea that gene flow promotes clustering of adaptive loci that resist the deleterious effects of recombination with mal-adapted migrants (Holliday et al. 2016). A genome scans of two red oak species (*Quercus robus* and *Quercus petraea*) revealed multiple loci on different linkage groups as targets of divergence, and some of these targets were closely clustered and showed high correlation within distances <2 cM (Scotti-Saintagne et al. 2004). This suggests the presence of multiple islands of divergence across the genome and the importance of linkage disequilibrium (genetic hitchhiking) in elevating the levels of divergence.

These studies and various others (Chapman et al. 2013; Horton et al. 2012, 2015) highlight mechanisms through which divergence and RI can be achieved at the genomic level. One question of interest is the importance of the size of differentiated region in sympatric speciation. Larger genomic islands may be due to stronger divergence hitchhiking, but also have a higher probability of harboring additional adaptive alleles that promote divergence and resist gene flow. However, it is difficult to tease apart the effects of linked selection from direct selection on multiple adaptive alleles (Buerger and Akerman 2011). Nosil and Sandoval (2008) showed that reduced gene flow within islands of divergence could increase the role of genetic drift, causing additional neutral differentiation and increasing the overall genetic divergence. Large genomic islands could also evolve due to their location in an area of the genome with naturally has a low recombination, e.g. centromeres or chromosome inversion, and such regions have been shown to promote divergence in some cases (Noor et al. 2007; Feder et al. 2003; Kirkpatrick and Barton 2006). Finally, genome-wide patterns of divergence could also be affected by the origin of adaptive genetic variation, whether new mutations or standing variation. While the theory above assumes adaptation from *de novo* mutation, adaptation probably frequently proceeds from already segregating variants, which may be conditionally neutral (Barrett and Schluter 2008; Savolainen et al. 2013). Because standing genetic variation has no wait period for mutation establishment, such variants rise in frequency

much more rapidly than new mutations (Barrett and Schluter 2008). However, detecting such regions is difficult, as historical recombination has broken linkage disequilibrium with other genomic regions, and selection produces a less pronounced signature of hitchhiking, a phenomenon known as a soft sweep (Przeworski 2003). Various studies have highlighted the role of pre-existing variation in divergence. For example, a genome-wide scan of *Arabidopsis thaliana* showed partial and soft selective sweep between populations (Horton et al. 2012). Trees in particular harbor a great deal of standing variation, and it is likely that adaptation and speciation proceeds at least in part from standing variation (Holliday et al. 2010; Holliday et al. 2016).

Conclusions

We have discussed both ecological and genetic factors that impinge on the potential for speciation. Which factor is most critical to the buildup of RI in angiosperm trees? Nosil et al. (2005) and Lowry et al. (2008) suggest that pre-pollination barriers are most common in general as compared to post-pollination and post-zygotic barriers, with habitat isolation and immigrant inviability having the strongest effect. These factors clearly contribute to the formation and maintenance of angiosperm tree species. Perhaps the most important factor for the maintenance of species is immigrant inviability (local adaptation). In spite of high rates of intra- and interspecific gene flow (and concomitantly high genetic connectivity among populations), local adaptation, particularly with respect to climate-related traits, is very high in trees (see Fetter, Gugger & Keller, this volume). While most studies of local adaptation in trees involve intraspecific common gardens or reciprocal transplants, they clearly demonstrate a likely role for immigrant inviability in the maintenance of species boundaries. On the other hand, high gene flow, coupled with continuous species distributions, makes it more difficult for ecological speciation to occur in trees. Multiple reproductive barriers, acting simultaneously, are therefore likely required to establish RI. Where a single reproductive barrier can generate some level of isolation in presence of gene flow, multiple reproductive barriers can cause complete or nearly complete RI (Sobel and Chen 2014). Adaptive divergence may be the most common initial barriers to gene flow in angiosperm trees, and intrinsic post-zygotic isolation may lead to reinforcement. The presence of chromosomal rearrangements and gene-duplications in the *Populus trichocarpa* genome suggests some role of intrinsic post-zygotic RI, but little is known about structural rearrangements in other species that may facilitate recombinational speciation. Recent advances in sequencing technologies (e.g., the Pacific Biosciences RSII platform) bring studies of structural variation within reach, and such data should shed additional light on the contribution of genome lability to speciation in trees.

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Perennial Growth, Form and Architecture of Angiosperm Trees

David J. Hearn

Abstract The architecture of angiosperm trees reflects multiple developmental processes that interact to produce complex forms. In this review, I summarize our understanding of major developmental processes determining angiosperm tree architecture, as well as what is known about underlying molecular mechanisms and dynamical models that recreate developmental patterns. Meristem maintenance, internode elongation, branching, and vascular development all contribute to the modular architecture of trees. The regulation of these processes involves activator-inhibitor dynamics, and controlled transport of key hormone regulators in spatial scales ranging from cell-to-cell to long-distance are central to most of these patterning processes. A model of angiosperm tree architectural dynamics that is grounded in molecular details is on the horizon for at least some model tree species, but additional comparative genomics approaches will be required to determine how the stunning diversity of angiosperm tree architectures arose from evolutionarily conserved building blocks.

Keywords Activator-inhibitor dynamics • Biological pattern formation • Genotype to phenotype mapping • Meristem function • Plant form • Structural modularity

Introduction

The study of perennial plant form and architecture involves spatial and temporal scales of organization from molecules to some of the largest and oldest organisms on Earth. Within the past decade, a merging of genomics, molecular-genetics, and systems approaches has yielded remarkable insights about the molecular mechanisms that ultimately determine spatial and temporal patterning during development. This chapter describes some of the primary developmental processes that determine the architecture of angiosperm trees, what is known about underlying molecular mechanisms and their representation in dynamical mathematical models,

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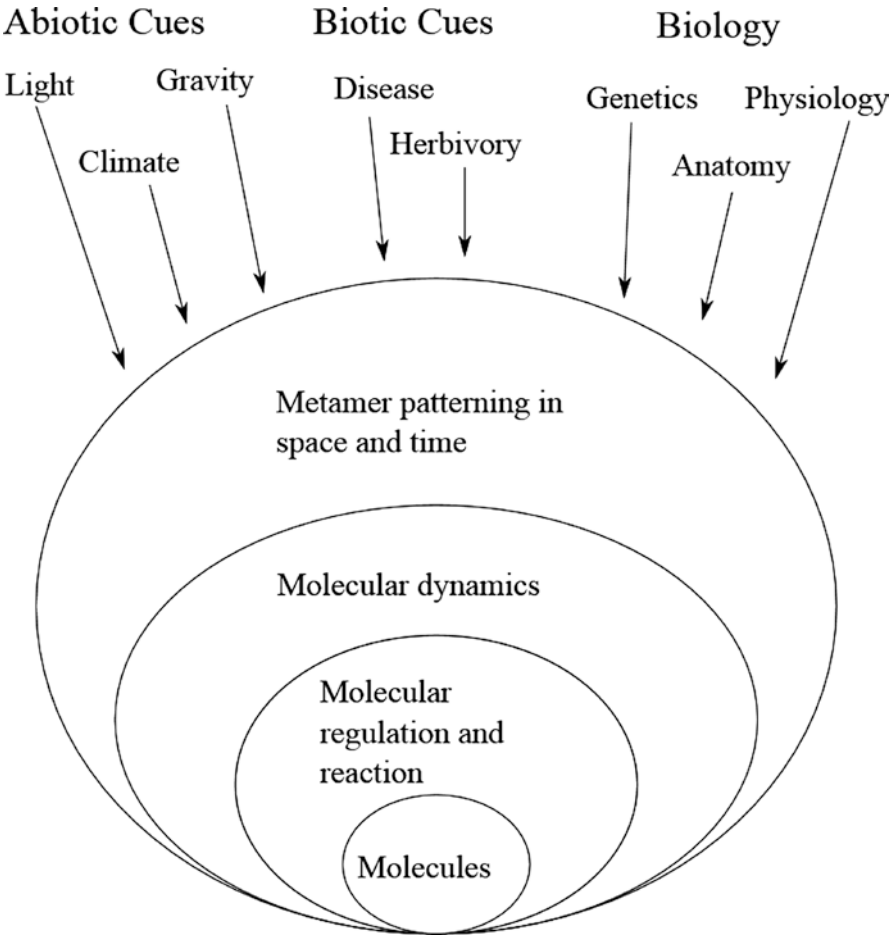

Fig. 1 Hierarchical scales of description used in plant architectural research. Each oval depends on the components of the ovals within it. *Top-down* influences (*light*, *disease*, etc.) can impact any of the other levels. See Introduction for more details

and how future comparative genomic studies could reveal the mechanistic variation associated with the amazing diversity of angiosperm tree architecture.

The study of tree form is, at core, a study of biological pattern formation across multiple scales of organization (Fig. 1), and many features of tree architecture are the same fundamental features that pervade biological systems more generally. At the molecular scale are the networks of chemical reactions and genetic regulation that interact and integrate environmental cues to affect developmental outcomes. These molecular mechanisms are embedded in a physical system of cells and tissues where they interact spatially (e.g. cell-to-cell signaling) over time. Mathematical models of these phenomena explain how pattern and form emerge from the molecular interactions and provide testable, quantitative predictions about the morphological impacts of changes to the molecular components as found in mutant lines.

Ultimately, tree architecture reflects the integration of environmental cues into developmental pathways to produce an overall form that balances energetic gains and mechanical soundness with costs from growth, metabolism, and organ loss to weather, disease, and herbivory. Environmental factors influence almost all aspects of tree architecture. Light, gravity, atmospheric conditions, and processes of herbivory, disease, and rot can all contribute to form directly or serve as cues affecting developmental pathways. As possible, this review will attempt to integrate across these different scales in consideration of the development of form in angiosperm trees.

Like many complex systems, the diversity and complexity of tree architecture stems from modular components of development in which spatial and temporal combinations of a few building blocks can produce complex form. What are these building blocks? At the level of morphology, with few exceptions (e.g. Podostemmonaceae), the primary aerial body of all land plants is constructed from the same basic building block (Barthélémy and Caraglio 2007). Asa Gray (1874) called this a phyton and more recent treatments refer to it as a phytomer (e.g., Hollender and Dardick 2015). It consists of a shoot apical meristem (SAM), a node with one or more lateral buds, and an internode in which stem vascular development occurs. In angiosperm trees, architecture reflects how the phytomer is produced and modified over time, including apical growth, elongation, phytomer production from axillary buds, and abscission. Secondary, radial growth in trees can dramatically alter phytomers and ultimately integrates them as part of woody stems. Due to length considerations, this chapter focuses on stems and ignores the often buried but important roots, flowers, inflorescence structures, and fruits, all of which influence the outward appearance of plants (reviewed by Hollender and Dardick 2015).

Key processes that give rise to, modify, and integrate phytomers and their recursive patterning in trees (Fig. 2) include (1) patterning at the shoot apex, (2) internode elongation, (3) release of apical dominance, apical control, and lateral shoot development, (4) branching angle, (5) senescence and self-pruning, (6) establishment of the primary and secondary vascular systems, and (7) determination of plant height. These processes provide an outline of sections in this chapter; in each, I introduce the component of tree architecture, describe what is known of the biophysical processes and molecular mechanisms responsible for their patterning, and highlight emerging areas of research. Since this chapter is part of a book on comparative genomics, I highlight how genomics studies yielded insights in these areas, and when such studies are missing, I illustrate how comparative genomics approaches may contribute.

Patterning at the Shoot Apex

It may seem strange to start a chapter on tree architecture with a scale of organization that requires a microscope to observe adequately. Yet, the patterns that are established at the shoot apex set the stage for the form of a tree. At the shoot apex, the patterning of leaf primordia is established, the arrangement of lateral buds follows in suit, and below the apex, vascular development initiates. Although the

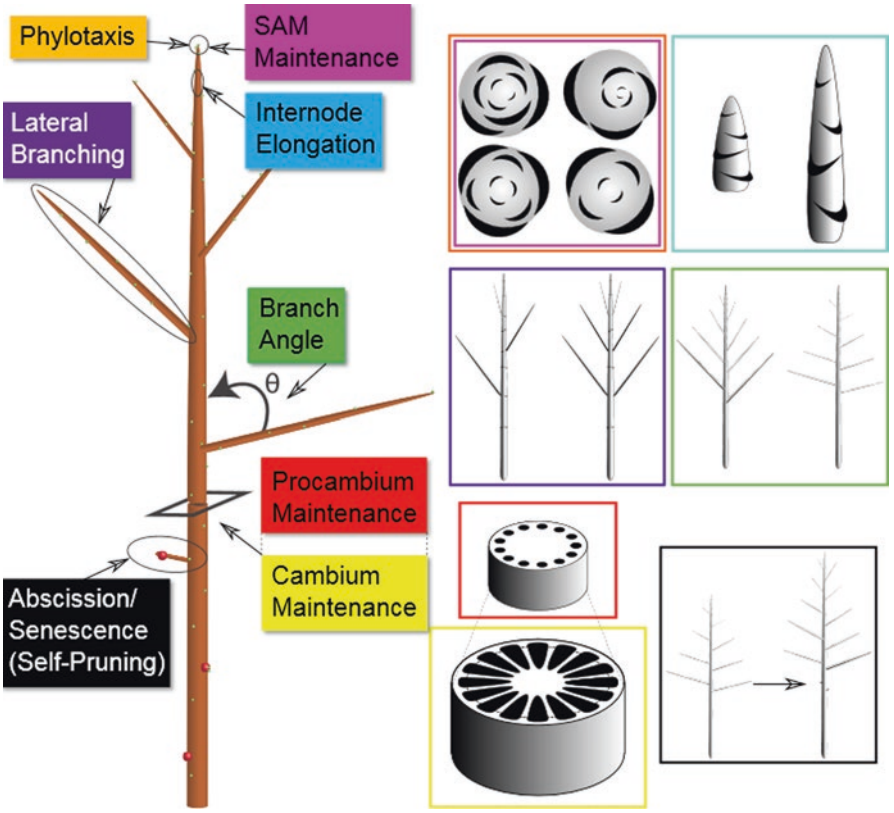

Fig. 2 Illustrations of developmental processes. The overview indicates where the process takes place (*black ovals*). *Green* spheres are nodes, red spheres are self-pruned branches. Processes are color coded: SAM maintenance (*pink*), phyllotaxis (*orange*; four phyllotaxis patterns are presented: monistichous, Fibonacci spiral, tricussate, opposite decussate), internode elongation (*light blue*), cladotaxis (*purple*), branch angle (*green*), procambium maintenance (*red*), vascular cambium maintenance (*yellow*), self-pruning (*black*)

patterning of primordia is typically viewed in terms of the arrangement of primordia and leaves (phyllotaxis), it is also where the branching pattern of the tree is established (with the exception of adventitious buds, discussed below in the section "Apical dominance, apical control and lateral shoot development").

As Goethe defined it (von Goethe 2009 reprint of the 1790 work), a leaf is that enation from the stem characterized by a bud located in the axil above the point of leaf attachment. Phyllotactic patterning therefore determines the initial set of possible branching points and branch arrangement.

The study of phyllotaxis provides a nice example of the marriage between molecular developmental genetics and quantitative mathematical modeling. Like many questions in biological development, the question of phyllotaxis (and branch arrangement) is a question of polarization and breaking of symmetry: going from

uniformity to patterned heterogeneity. The production of spatial regions that are locally enriched or locally depleted in a key regulator of cell division or differentiation often precedes the initiation of organ (e.g., leaf) development. In the shoot apex, rapidly proliferating stem cells produce a morphologically uniform dome (Fig. 2a). An understanding of phyllotaxy, then, is an understanding of when and where this uniformity is broken into primordia, which are the sites of leaf development.

The controlled movement of molecules, and not just their genetic regulation, is essential to the patterning process during phyllotaxis. In organisms such as *Acetabularia* and *Caulerpa*, the plant body consists of a syncytium with no delineation of cellular spaces through cytokinesis and, in the case of *Acetabularia*, it can be regulated by a single nucleus (Coneva and Chitwood 2015; Goodwin 2001). Yet radial arrangements of cell wall enations develop; cellular differentiation is not the explanation, and the biophysics of membranes and molecular movement must be responsible, at least in part, for the enation patterns, hence an early interest in biophysical models of phyllotaxis that considered membrane deformation (Green 1992) and repulsion or "inhibition fields" (Douady and Couder 1992; Smith et al. 2006b).

More recent models of phyllotaxis in *Arabidopsis* rely on controlled movement of auxin via PINFORMED (PIN) auxin efflux proteins. Reinhardt et al. (2003) suggested that auxin accumulates at incipient primordia and is depleted in areas surrounding primordia. Sites of auxin accumulation undergo cell division to produce leaf primordia and, ultimately, leaves. Mathematical models based on Reinhardt et al. (2003) of auxin and PIN1 dynamics recreate primordial patterning at the SAM and involve feedback between auxin concentration, polar auxin transport via PIN1 activity, and localization and expression of PIN1 (Jönsson et al. 2006; Smith et al. 2006a). The model of Smith et al. (2006a) also predicted altered phyllotactic patterns of *pin1* mutant lines, highlighting the predictive capabilities of the mathematical formulations. Additional feedback between PIN1 and auxin transport influence organ boundary specification by fostering the accumulation of CUP-SHAPED COTYLEDON 2 (CUC2) and SHOOTMERISTEMLESS (STM) proteins at the periphery of incipient primordia (Jönsson et al. 2006).

Genomics studies are identifying additional ingredients involved in leaf and bud patterning. For example, Vernoux et al. (Vernoux et al. 2014) used high-throughput yeast two-hybrid assays, in situ hybridization, and GUS translational lines to identify at least 50 transcriptional regulators involved in phyllotaxis including 23 auxin response factors and 29 Aux/IAAs.

Internode Elongation

Stem length is in part determined by the number of nodes and their timing of production at the shoot apex, but its primary determinants are the extent of internode elongation and the duration of growth in the shoot apex. Stem elongation occurs at the growing apex and from cell elongation and division in subapical rib meristems

before secondary growth and significant lignification (coincident with the onset of wood formation) preclude additional elongation. Internode length has a significant effect on overall tree form and dimensions. Cosgrove (2016) reported that the tallest tree species would be no taller than human size without the cell elongation processes that lengthen internodes. However, coupled with cell elongation is cell division in the rib meristems, which is an underappreciated contributor to internode elongation (Zimmernamm and Brown 1971). Indeed, early studies (Harting 1845; Moll 1876; reviewed by Sachs 1965) reported that fewer – rather than shorter – cells are found in short internodes. Cell division in rib meristems can continue, even 10 cm away from the SAM in some tree species (Zimmernamm and Brown 1971).

Additionally, internode length determines the spacing between leaves and, together with phyllotaxis and leaf orientation, plays a major role in determining light interception. Leaves that are too close along the stem and not angled appropriately shade one another and may diminish the net photosynthetic input (Niklas 1992, 1997). Presumably, natural selection favors internode lengths that optimize the balance between energetic inputs due to photosynthesis and costs due to materials used in the synthesis of internode tissue.

Internode lengths can vary substantially among species and within individuals. In many tree species, branches with markedly different internode lengths occur. Those branches with short internodes, "short shoots", can occur with high predictability and regularity, particularly in gymnosperms such as *Pinus* and *Larix*. In angiosperms such as the rosaceous *Malus*, *Prunus*, *Pyrus*, and *Crataegus*, short shoots regularly sprout from lengthened lateral "long shoots". In, e.g., *Fagus*, *Betula*, and *Acer*, short shoots are less predictable, but they increase in frequency with age (Zimmernamm and Brown 1971). In some species (e.g., *Liquidambar styraciflua*, Zimmernamm and Brown 1971), "reversion shoots" alternate between short and long internode growth among years.

Studies of internode length at the molecular level are surprisingly few and largely limited to model organisms like pea (Behringer et al. 1990; Reid et al. 1983; Reid and Potts 1986; Swain and Reid 1992). Cell elongation is largely determined by the extensibility of the cell wall and the threshold turgor when the cell wall yields (Cosgrove 1985). Remodeling of the typically rigid cell wall to accommodate expansion occurs mainly through the activities of expansins, but it also includes activities of endoglucanases, xyloglucan endotransglycosylase/hydrolases, and pectin methylesterases (reviewed by Cosgrove 2016).

The role of gibberellic acid (GA) has been known for some time in the elongation of internodes in plants via both cell division and elongation (Ingram et al. 1986). Auxin (Cleland 2010) and brassinosteroids (Clouse and Sasse 1998) also contribute to internode elongation, whereas abscisic acid (ABA), ethylene (ETH), and jasmonic acid (JA; Heinrich et al. 2013) inhibit elongation (Davies 2010). In GA deficient pea lines, osmotic and turgor pressures were not correlated with growth rate (Behringer et al. 1990). Instead, such lines varied in internode elongation rates either through altered levels of GA or changes to GA sensitivity (Reid and Davies 1992) that impact changes in cell wall extensibility and threshold turgor (Behringer et al. 1990). Additionally, strigolactones (SLs) increase internode cell

number, but not length, and their activities are not correlated with GA content in peas (de Saint Germain et al. 2013). The apparently independent activities of these hormones suggest multiple interacting and coordinated pathways in the regulation of internode elongation.

Not surprisingly, multiple genes and their products such as DELLA and GA oxidases (GAox) involved in the regulation of the above-mentioned hormones are central to regulation of internode length. DELLA proteins repress GA (Zawaski and Busov 2014) and mediate cross-regulation with auxin and ethylene pathways (Gallego-Bartolomé et al. 2011). GA exerts an inhibitory effect on DELLA proteins that, in turn, appear to inhibit GA synthesis in a feedback loop (de Lucas et al. 2008; Zentella et al. 2007). GA biosynthesis genes can also be regulated by auxin genes, as observed in pea (Ross et al. 2011).

Multiple environmental signals influence internode elongation through regulation of GA bioactivity. In poplar, trees responding to either reduced watering or short day length (SD) showed increased expression of *GAox* and *DELLA* encoding genes (Zawaski and Busov 2014). In a transcriptomics study (Busov 2014), GA-insensitive mutants, GA-deficient mutants, SD-dormant plants, and plants experiencing drought had a shared "regulon" of 684 differentially expressed genes and exhibited dramatic changes in height, crown architecture, leaf shape, and phenology (Zawaski et al. 2011). Responses to photoperiod involve DELLA proteins that bind to phytochrome-interacting transcription factors (PIFs; de Lucas and Prat 2014; Richter et al. 2010) and prevent PIFs from activating target genes that enhance elongation during etiolation responses (specifically skotomorphogenesis). Collectively, these studies suggest that GA signaling influences both physiological responses to environmental stimuli as well as acclimation in growth response to environmental stressors that result in decreased growth.

Apical Dominance, Apical Control, and Lateral Shoot Development

Lateral branch patterning is central to a tree's fitness and determines, in large part, the overall architecture of a tree. Branching patterns impact access to light, and they influence the presentation of reproductive organs, accessibility to herbivores and other animals, and tolerance to mechanical wear due to wind and precipitation. Plants that have no suppression of lateral growth and branch at each node are likely to suffer from self-shading and the expense incurred by the extraneous material in plant tissue, whereas plants that are incapable of responding to apical damage are doomed to remain as stunted twigs with no branches and limited reproductive capacity. Evolution presumably fine-tunes when and how often to initiate lateral growth to balance construction costs, photosynthetic gains, and reproductive output (Dun et al. 2006; Niklas 1992).

The quiescent lateral buds that are established in leaf axils form a repository of potential branching points. Within individual stems, the phenomenon of apical

dominance describes the suppression of outgrowth of lateral buds by the apex. As discussed below, apical dominance is increasingly well understood at the molecular level, mainly based on studies in *Arabidopsis* and other herbaceous annuals. But trees have at least three additional levels of regulation to consider in the development of overall crown form: (1) Apical control, which refers to the influence of the "leader" on the outgrowth and angle of subtending branches; (2) Growth from buds formed in the same year as flushing (forming sylleptic branches) versus growth from overwintered buds formed in the previous growing season (proleptic branches); and (3) determinant versus indeterminate growth (discussed more fully in the section "Tree height").

As with internode elongation, both endogenous and exogenous processes influence the development of axillary buds (Dun et al. 2006; Hayward et al. 2009; Ongaro and Leyser 2008; Schmitz and Theres 2005; Yang and Jiao 2016). Hormone ratios, sugar levels (Mason et al. 2014), and other factors (Wilson 2000) all contribution to the release of bud dormancy. Apical dominance is removed when the apex senesces or when external processes such as herbivory, damage, or disease halt apical growth. Exogenous changes in light level influence rates of lateral branching. Higher light levels generally contribute to increased branching, whereas absence of light can suppress branching altogether (Leduc et al. 2014).

Although it is becoming clear that other signals from roots and shoots can also modulate timing of lateral branch initiation (Beveridge et al. 1996; Mason et al. 2014; Napoli 1996), focus has traditionally been on the inhibitory role of auxin (Dun et al. 2006; Sachs and Thimann 1964; Thimann and Skoog 1934). However, strigolactones (SL) inhibit lateral shoot development more directly than auxin. Auxin polar transport promotes SL synthesis via *MORE AXILLARY SHOOTS* (*MAX*) genes (Gomez-Roldan et al. 2008; Umehara et al. 2008).

Although the arrangement of lateral branches on the main stem – i.e., cladotaxis (Shushan and Johnson 1955) – can resemble the phyllotactic pattern (Kawasaki and Bell 1991), the secondary inhibition by apices of lateral branches can result in cladotaxis that differs from phyllotaxis. In Myristicaceae a distinctive field character of this family is that many branches are produced close together, producing a whorl around the trunk and giving the tree a pagoda-like architecture even though the leaves are alternately arranged. This is also apparent in some pines such as *Pinus strobus* (white pine), which, like other pines, have leaves in tight fascicles, but have whorled branches on the main trunk. Not only can cladotaxis differ from phyllotaxis, but cladotaxis on the leading (orthotropic) stem can differ from cladotaxis on lateral (plagiotropic) stems, as witnessed in *Laetia procera* (Kawasaki and Bell 1991).

It may seem counterintuitive that trunks readily produce lateral branches when trunks are released from shade, as the axillary lateral buds that form during primary growth would seem to be engulfed by the developing cambial tissues. However, two additional mechanisms can produce lateral branches from the central bole of a tree: epicormic growth and adventitious buds (Zimmernamm and Brown 1971). Epicormic growth occurs when preexisting buds are activated. Epicormic buds grow in pace with the cambium, but inhibition of the rib meristem by basipetally trans-

ported auxin limits further growth, as evidenced by the release of inhibition of epicormic buds below trunk girdles (Zimmernamm and Brown 1971). In contrast, adventitious buds form de novo and are not constrained by existing cladotactic patterns. Adventitious buds can arise in response to injury that produces callus cells (e.g., in *Quercus*) or in cortical or phellogen cells associated with lenticel formation (Zimmernamm and Brown 1971). Virtually nothing is known of the molecular mechanisms of epicormic and adventitious bud formation in trees.

Although interest in mathematical modelling of branching processes has a long history (including L-systems; Lindenmayer 1968; Lindenmeyer and Prusinkiewicz 1990) and such models give rise to realistic plant architectures, they have traditionally been divorced from the molecular details and so offered few insights about how changes at the molecular level translate to architectural changes. A merging, now underway (e.g., Prusinkiewicz and Runions 2012), of top down modelling with bottom-up molecular mechanisms promises to greatly increase our understanding of how molecular events lead to larger-scale branching patterns.

Branching Angle

After apical control is released, not only does outgrowth of lateral buds commence, but girth of lateral branches increases, and lateral branches may bend upwards (Wilson 2000). Hollender and Dardick (2015) distinguish among three lateral branching angles. The first, the crotch angle, is the angle between the bud and the stem. A second is the geotropic angle, the angle at the growing tip of a lateral branch. A third, variously called the equilibrium angle (Wilson 2000), the angle of inclination (Brown 1971), or the gravitropic setpoint angle (Roychoudhry et al. 2013) is the angle between a lateral branch and the main axis. This branching angle represents a dynamically stable equilibrium. When the branching angle is experimentally changed, the branch returns to the initial angle through a combination of bending and growth, at least in some herbaceous stems (Roychoudhry et al. 2013).

The gravitropic setpoint angle emerges as a compromise between competing constraints (Niklas 1992). Lateral branches that are at a 90° angle with the main axis reduce self-shading along the lateral branch, but torque due to gravity is maximized. Orthogonal lateral branches are therefore at the greatest risk of breaking, and they either need to be short when soft-wooded (in many *Pinus*) or have strong, but energetically expensive, wood (in many oaks) to resist breakage. So there is a tradeoff between light capture efficiency, cost to wood production, and probability of breakage. Presumably, branch angle is optimized when the net energy gain (energy gain through photosynthesis minus energy invested in wood strength and energy lost to breakage) is maximized through natural selection (Niklas 1992). These predictions appear to match observed branching angles in some taxa. Soft-wooded trees, such as those of columnar cacti, either are unbranched, or they branch only when trunks are old and strengthened (often at the woody base of the main axis), or the branches are vertically oriented to minimize torque.

Even within an individual plant, branch angle varies. Lower branches tend to be more horizontal whereas upper branches are closer to vertical (Hollender and Dardick 2015). Presumably, this variation in branching angle optimizes light capture. After decapitation of the leader, one or more subtending branches can reorient upwards through production of reaction wood (reviewed by Hollender and Dardick 2015).

The roles of gravity and light tropisms in branching angle have been recognized for over a century. Sachs (1882) recognized variation in the angling of branches from response to gravity, and Darwin (1880) recognized a positive phototactic response to light. In both cases, auxin (IAA) accumulates on the side of the stem away from the direction of movement and at that site, IAA promotes cell growth leading to asymmetrical growth. D'Arcy Thompson (Thompson 1942) recognized, more generally, that different rates of growth on opposing sides of an organ or different rates of secretion lead to bending, and when the asymmetries in rates are maintained at a fixed site of growth or secretion, spiraling – such as that of the ram's horn – results. Although spiraling in stems can occur (e.g., spiral grain in many gymnosperms, spiral stems in *Costus* and some *Salix* cultivars), the specific feedback mechanisms that maintain a constant, rather than spiraling, equilibrium angle in most taxa are unclear. In herbaceous stems, stem angles adjust to an equilibrium value when stems are experimentally reoriented. Explanations for this response rely on opposing tropisms (phototropism, gravitropism, proprioception, epinasty) in equilibrium (Bastien et al. 2013, 2015; Mattheck 1991; Roychoudhry et al. 2013; Roychoudhry and Kepinski 2015). In herbaceous taxa, gravity sensing cells have amyloplasts that sink in response to gravity and alter the distributions of PIN proteins that assist in polar auxin transport (Roychoudhry and Kepinski 2015). This polarization of PINs presumably facilitates auxin transport in the direction of gravity and along the main axis of the stem (Kleine-Vehn et al. 2010).

It is an open question whether similar mechanisms are at work in woody plants. In trees, changes to the equilibrium angle usually come about with the production of reaction wood (reviewed by Groover 2016) – tension wood in angiosperms (Wilson 2000) or compression wood in gymnosperms (Timell 1986). Tension wood forms on the upper portion of limbs or leaning stems, and pulls the stem upwards through cellular contraction (Guerriero et al. 2014). In contrast, compression wood forms at the lower portion of limbs and pushes stems upwards through wood expansion (Mattheck 1991). Transcriptomics studies have begun to identify molecular mechanisms involved in reaction wood formation in angiosperm trees (Chen et al. 2015; Gerttula et al. 2015). Gerttula et al. (2015) concluded that the gravity sensing mechanism that shifts branch angle upwards in herbaceous plants is "fundamentally different" from that in woody angiosperm stems. In both woody and herbaceous stems, sedimentation of amyloplasts and polarization of PIN localization alter the distribution of auxin, but the functional targets of auxin differ. In herbaceous stems, accumulation of auxin in cells in the underside of stems increases their growth thereby reorienting stems upwards. In poplar stems, Gerttula et al. (2015) suggest that poplar PIN3-expressing cells transport auxin towards the cambium in the top of the stem, triggering tension wood development, whereas transport away from the cambium in the bottom of the stem fosters opposite wood formation.

Senescence and Self-Pruning

In many angiosperm trees, the trunk produces multiple lateral branches that are self-pruned as the tree grows. The frequency and distribution of lateral stems on trunks is a complex function of both endogenous developmental and physiological processes, and environmental factors. Lateral branches provide a platform to display leaves that acquire resources through photosynthesis. When those leaves become shaded by branches that are higher up on the trunk or by neighboring plants, the compensation point is passed, and the energy required to maintain the stem and its leaves surpasses the energy acquired through photosynthesis. When this occurs, natural shedding of the branch can reduce loss of energy. A tree when grown in an open environment will typically have more lateral branches that occur lower on trunks than when grown in a forest environment where lower lateral branches are lacking. And yet, the pattern of lateral branching is often characteristic of the tree species regardless of which environment the tree occurs, so there are endogenous factors that give rise to the species-specific patterns of branching and their shedding as well. A full understanding of self-pruning requires linking endogenous and exogenous signals to senescence processes.

Although self-pruning is a key aspect of plant development, the molecular mechanisms are poorly understood. There are likely to be multiple different senescence processes involved. One of them called cladoptosis is found in a relatively limited set of taxa that include many conifers, some oaks, and some tropical trees (reviewed by Millington and Chaney 1973). During cladoptosis, an abscission zone forms at the site of branch shedding that is much like abscission zones associated with fruit and leaf shedding. These abscission zones occur through cell wall disassembly, cell separation, and the hardening of a callous zone. The current hypothesis for cladoptosis is that the same molecular mechanisms for leaf and fruit abscission occur during cladoptosis, as discussed below.

Zhang et al. (2014) used microarray analysis to survey gene expression during three phases of cladoptosis in *Citrus sinensis*. They identified 1378 differentially expressed genes. Some of these function in programmed cell death through reactive oxygen species, and others were associated with cell wall biosynthesis and metabolism; multiple hormone biosynthesis genes were also differentially expressed. They concluded that cell wall modification pathways with some similarities to abscission of other organs are activated during self-pruning.

Not all shedding of lateral branches is associated with abscission zones and cladoptosis. In many trees, lateral branches die before they are shed, and in many cases dead branches persist on the trunk for multiple years until exogenous processes remove the branch. Rot, herbivory, gravity, and force loadings (that include wind and snow) contribute to the ultimate shedding. Again, it is an open question whether the same mechanisms of plant senescence more generally are responsible for the localized senescence of these lateral branches.

Our understanding of self-pruning is at its infancy. These studies are challenging because many plant models such as *Arabidopsis* do not exhibit lateral branch shed-

ding that is witnessed in trees. The work on *Citrus* (Zhang et al. 2014) provides multiple candidates that can form the basis for functional molecular genetic studies in tree taxa.

Establishment of the Vascular System and Regulation of Plant Girth

Secondary vascular growth increases the girth of stems in trees and other woody plants. Activities at the vascular cambium (VC) regulate the girth of plant stems, and variant cambial activities influence the sculpturing of stems (e.g., fissures, pleats, and buttresses) and the overall form of the tree. Leonardo da Vinci was among the first to recognize that tree branch diameter is influenced by apical control, and that cross sectional areas of lateral branches often sum to the cross sectional area of the main trunk Minamino and Tateno (2014). In addition to Minamino and Tateno (2014) biophysical models, which recreate da Vinci's rules under light loadings, more recent formalizations of da Vinci's relationships include modeling of resource distribution networks that result in the pipe model (Shinozaki et al. 1964) and power law scaling relationships (reviewed by Dahle and Grabosky 2009).

The vascular system in trees functions as a resource distribution network and as a support system countering gravity. In specialized woods such as those of pachycaul trees, storage is the predominant function, and trunks can become disproportionately inflated due to the proliferation of storage parenchyma in the axial system (Hearn et al. 2013). Suits of traits relating to conduction, support, and storage can evolve in a modular fashion that putatively maintains the functional coherence of these traits yet permits evolutionary processes to explore different allocation strategies to these functions (Hearn 2013). Tradeoffs between conductive efficiency, mechanical support, and storage influence the stature and architecture of plants (Baas et al. 2004; Niklas 1992) and also influence the adaptive responses to varying climates (Carlquist 2001). Dense, narrow vessels increase mechanical support but limit conduction, and soft storage woods decrease mechanical support. The vascular system also integrates and unifies the modular phytomers, often blurring lines between phytomers in older stems and trunks.

Insights are being made into the molecular regulation of secondary growth and wood formation. For example, members of the HD-ZIP III family are essential for procambial specification and xylem differentiation (Baima et al. 2001; Benítez and Hejátko 2013; Byrne 2006; Emery et al. 2003; Ilegems et al. 2010; Prigge et al. 2005; Zhong and Ye 2004) in vascular bundles of leaves, roots, and shoots through coordinated activities with auxin, KANADI genes (Ilegems et al. 2010), and brassinosteroid (Caño-Delgado et al. 2004; Ibañes et al. 2009). Members of the HD-ZIP III family are themselves post-transcriptionally repressed by miRNA165/166 (Sakaguchi and Watanabe 2012). It therefore seems likely that members of the HD-ZIP III family act as activators, miRNAs act as inhibitors, and coordinated activities with auxin and PIN proteins promote polar transport. Systems biological

modeling and associated experimental studies in *Arabidopsis* roots support this hypothesis (Muraro et al. 2014).

In tree taxa studied thus far, HD-ZIP III family genes play central roles in the initiation of the cambium (Robischon et al. 2011), regulation of patterning of secondary vascular tissues, and regulation of cell differentiation during secondary growth (Du et al. 2011; Zhu et al. 2013). In both herbaceous stems of *Arabidopsis* (Zhong and Ye 2004) and woody stems of poplar (Robischon et al. 2011), misexpression of HD-ZIP III family genes orthologous to *REVOLUTA* results in polarity patterning changes: primary amphivasal vascular bundles with xylem surrounding phloem form in *Arabidopsis REVOLUTA* mutants, while ectopic cambia with polarity reversals can form when REVOLUTA is overexpressed during secondary growth in poplar.

Molecular insights into the maintenance of the vascular cambium and differentiation of vascular cambium daughter cells are also forthcoming. The receptor-like kinase encoding *PHLOEM INTERCALATED WITH XYLEM* (*PXY*) (Fisher and Turner 2007) and the *WUSCHEL*-related homeobox gene *WOX4* (Ji et al. 2010) are fundamental cell autonomous regulators of the cambium. The *CLAVATA3/ESR-RELATED* (*CLE*)-encoded TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF) peptide expressed in phloem moves into the cambium and activates the PXY receptor Hirakawa et al. (Hirakawa et al. 2008) which, in turn, enhances WOX4 production. WOX4 promotes cambium proliferation and does not inhibit commitment to xylem differentiation (Hirakawa et al. 2010). In trees, the *PXY – CLE* pathway influences woody tissue organization and regulates cambial cell division rate, with tissue-specific over-expression lines showing two-fold increases in wood production over the wildtype (Etchells et al. 2015). Etchells et al. (2015) concluded that the *PXY – CLE* pathway was likely to be central to how trees evolved woody growth.

In trees studied thus far, rates of woody growth, and hence trunk and stem dimensions, are also directly affected by the activities of GA and Class I KNOX homeodomain transcription factor-encoding genes (*ARBORKNOX1* and *2* – orthologs of *Arabidopsis STM* and *BREVIPEDICELLUS*, respectively), which regulate cell differentiation during secondary growth (Du et al. 2009; Groover et al. 2006). GA overexpression in aspen increases growth rate and biomass, and antagonizes root initiation (Eriksson et al. 2015), whereas GA-insensitive or –deficient transgenic plants or mutants showed increased root growth and reduced shoot growth (Zawaski and Busov 2014; Zimmermann and Brown 1971). GA mutant lines show altered expression of photoperiod response genes, thereby linking cambial growth to photoperiod (Zawaski et al. 2012). GA stimulation of wood formation (tension wood, specifically) is sensitive to abundance of *ARK2* whose overexpression appears to limit woody growth (Gerttula et al. 2015). In a CHiP-Seq experiment, Liu et al. (2015) discovered that HD-ZIP III family members (*ptHB1*, 2, 3, and 5) are targeted by *ARK1*, but *ARK1* overexpression lines show no significant changes in HD-ZIP III family members. They proposed that regulation of HD-ZIP III genes requires the activities of other transcription factors in combination with *ARK* transcription factors at promoters of HD-ZIP III genes.

Tree Height

Tree height is a central ingredient of architecture, and it is a defining feature of the tree habit. It is central to the success of land plants by influencing access to light and dispersal ability (Niklas 1997). Plant size is correlated with metabolic rate (Enquist et al. 1998), lifespan, leaf metrics, reproductive output, precipitation levels, and a suite of other traits (reviewed by Moles et al. 2009).

Like other features of plant architecture, both internal and external factors influence tree height. When mechanical constraints due to the loading of gravity are considered, the critical (buckling) height of trees is predicted to be proportional to the diameter raised to the 2/3rds power according to the Euler buckling formula (Greenhill 1881). The realized height of trees does indeed follow a slope of 2/3 on a log-log plot of height vs. diameter, but the height of trees is considerably shorter than the predicted maximum (Niklas 1992). Traditionally, heights below the theoretical maximum were attributed to "mechanical overbuilding" of trees to accommodate both static loadings due to stem weight and additional dynamic loadings due to wind and precipitation (e.g., snow and ice; Niklas 1992) that effectively increase the trunk weight. More recently, a model of hydraulics better explained the observed relationship between height and diameter, suggesting that limits to water conduction, and not loading, are more critical biophysical determinants of height (Niklas and Spatz 2004, discussed further by Niklas 2007).

Developmental determinants of plant height are once again strongly influenced by activities at the SAM. In plants with a single main trunk (columnar and excurrent growth of Zimmernamm and Brown 1971), such as many conifers, the SAM of a single leading stem is maintained throughout the life of a plant, whereas in sympodial plants (decurrent growth of Zimmernamm and Brown 1971), lateral branches successively supplant the former leading stem (seen in many angiosperm trees and pachycauls such as *Adenia venenata* and *Dendrosicyos socotrana*; Hearn 2006; Olson 2003). In decapitated plants, or in plants in which the leading stem droops due to gravity or other influences (e.g., a lianous ancestry; Olson 2003), a lateral branch assumes the role of the leading stem by reorienting itself vertically through the development of tension and compression woods (Mattheck 1991; Wilson 1998).

The orchestration of the succession of leading stems revolves around the interplay between apical control and the maintenance of the SAM. In SAMs where maintenance activities cease, growth is determinate and stems tend to be short, whereas SAMs with lasting maintenance exhibit indeterminate growth. An activator-inhibitor loop between *CLAVATA* (*CLV*) and *WUSCHEL* (*WUS*) homeodomain transcription factor maintains the set of the central stem cells in the SAM (reviewed by Aichinger et al. 2012). In *Arabidopsis*, *CLV3* represses *WUS* outside of the central zone of stem cells where *WUS* is active, and *CLV3* appears to promote organ initiation (Schoof et al. 2000), whereas *WUS* enhances *CLV* expression and contributes to stem cell identity and maintenance and cytokinin production (Laux et al. 1996; Mayer et al. 1998; Schoof et al. 2000; reviewed by Carles and Fletcher 2003). Additionally, *KNOX* homeobox genes (e.g. *ARK2*, *STM*; Endrizzi et al. 1996) down-

Table 1 Dynamical processes responsible for patterning of architectural components

Architectural component	Dynamical process
Phyllotaxis	Molecular movement; redistribution of auxin resulting in auxin peaks at sites of primordia
Internode length	Constitutive (?) and environmentally determined rates of cell expansion and division in internode tissue
Cladotaxis	Molecular movement; transport of auxin basipetally; suppression by strigolactones (above threshold level?)
Branching angle	Uneven rates of growth on opposing sides of organ; equilibrium between upward and downward growth forces
Self-pruning	Apoptosis and/or carbohydrate starvation (?)
Procambium development	Activator-inhibitor dynamics; threshold activator initiates procambial development (?)
Vascular cambium maintenance	Activator-inhibitor dynamics
Shoot apical meristem maintenance	Activator-inhibitory dynamics

Question marks indicate areas requiring further clarification. See text for references and further descriptions of each component

regulate GA and promote cytokinin production in the SAM (Jasinski et al. 2005), and they maintain pluripotency of stem cells (reviewed by Hay and Tsiantis 2010).

Themes

Several general themes emerge from the above analysis of tree form and architecture.

1. Relatively few, dynamic mechanisms account for much of a plant's metameric patterning (Table 1). In particular, (i) activator-inhibitor dynamics influence multiple patterning events: at the SAM in a negative feedback loop between *CLV* and *WUS* (Fujita et al. 2011; Schoof et al. 2000), among *STM*, *CUC1*, and miRNA164 (Spinelli et al. 2011), in vascular patterning by interaction of HD-ZIP III genes with miRNA165/166, and between *MOL1* and *RUL1* as opposing regulators of secondary growth. (ii) Transport dynamics of key regulators, and not just changes in expression, are essential for spatial pattern formation. In particular, polar transport of auxin via asymmetrical cellular localization of PIN efflux carrier proteins is necessary for transducing environmental signals (Kleine-Vehn et al. 2010; Friml et al. 2002) and for establishing phyllotaxy (Reinhardt et al. 2003; Smith et al. 2006a), cladotaxy, and vascular patterning (Ibañes et al. 2009). (iii) Constitutive or, especially, environmentally determined expression rates contribute to spacing of metameric units. This is witnessed in internode length distributions and patterning of secondary growth. There are likely to be other dynamical themes yet to emerge. Among these, threshold dynamics vs. dose-dependent effects are worth exploring more. For example, Müller et al. (2016) state that

increased HD-ZIP III expression promotes metaxylem and lower levels promote protoxylem in a dosage-dependent fashion.

2. Different members of the same gene family often regulate different meristematic regions within a plant where they are vital to stem cell homeostasis (Agusti et al. 2011; Groover 2005; Groover et al. 2006). In root apical meristems (RAMs), the HD-ZIP III *PHABULOSA* (*PHB*) regulates vascular patterning by coordinating an auxin signaling loop (Müller et al. 2016; Muraro et al. 2014), whereas the HD-ZIP III *HB8* carries out a similar role in in the stem. The WUSCHEL-like genes *WUS*, *WOX5*, and *WOX4* maintain meristems in the SAM, RAM, and cambium, respectively (Agusti et al. 2011). *CLE* (TDIF) and *TDR/PXY* that promote *WOX4* expression in the cambium share a high similarity with *CLV3* and *CLV1*, respectively, which help to maintain the SAM (Hirakawa et al. 2010; Ogawa et al. 2008).
3. Different pathways appear to be connected through relatively few key regulators (Fig. 3). Auxin, in particular, influences most processes reviewed above. The involvement of shared regulators among different developmental processes may help to coordinate tradeoffs during evolutionary changes among developmental processes. For example, strigolactones coordinate apical control and lateral branching growth and angle. Coordination between loss of apical control and re-orientation of subtending lateral branches to vertical can optimize light capture by reducing self-shading and maximizing light exposure to the leading branch.
4. Conserved pathways and ortholog functions in distant relatives suggest that many fundamental plant patterning processes were inherited from an ancient common ancestor and conserved during evolutionary diversification. Examples are numerous. HD-ZIP III genes influence vascular patterning in poplar, *Petunia*, *Arabidopsis*, and others; *MAX* genes involved in strigolactone biosynthesis share similar roles in apical control and lateral branching in rice, *Arabidopsis*, and petunia (Arite et al. 2007; Johnson et al. 2006).

Role of Comparative Genomics

With the emerging theme that conserved genetic regulatory pathways contribute to similar developmental processes across multiple lineages, a somewhat paradoxical situation results. One the one hand, similar genes and functions are conserved, yet the diversity of plant architecture is vast. Transitions between woody tree forms and herbaceous forms evolved repeatedly in multiple plant lineages demonstrating the flexibility of evolutionary-developmental processes that shape plant architectural variation (Carlquist 2013; Groover 2005).

Carroll and others (Carroll 2008, and references therein), suggested this biological diversity that is underpinned by conservatism is due to shifts in expression of key genetic regulators caused by evolutionary changes to the DNA sequences of their promoter regions. This explanation is one component of a panoply of possible

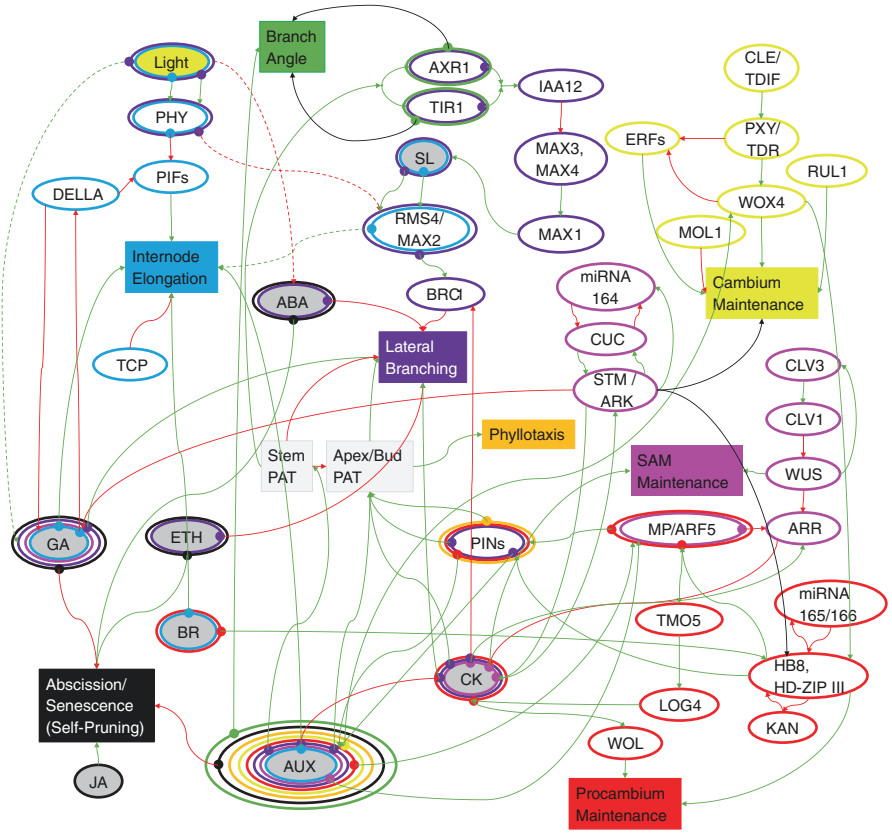

Fig. 3 Molecular interaction network organized by developmental process. Molecular components (*black letters*) and light signals are encircled by the color-coded process in which they are involved. Color codes follow those of Fig. 2. Molecules involved in multiple processes have multiple concentric ovals of different colors. *Green* links indicate pre- or post-transcriptional activation, red links indicate suppression, and *black* links indicate a general, unspecified interaction. *Arrows* indicate the direction of regulation, with a dot indicating the start of the interaction link for molecules participating in multiple processes. Links are not necessarily direct, and molecular intermediaries are missing for several of the links. Dotted lines represent especially indirect or uncertain interactions. Abbreviations for hormones (*gray background*) and gene names are standard. References that provided regulatory information are cited in relevant sections of the text and also include Aguilar-Martínez et al. (2007), Azizi et al. (2015), Kieffer et al. (2011), Laufs (2004), and Traas and Vernoux (2002)

explanations that also include changes to gene function and changes to patterns of molecular movement. Comparative genomics, and in particular transcriptomics, approaches provide a means to address Carroll's insight by identifying genes with altered expression between different species, or from exposure to differing environmental stimuli, or in tissues undergoing different stages of development.

Although these comparative genomics studies are making inroads towards identifying the key molecular building blocks, several largely unanswered questions can

be further aided by additional transcriptomic analysis. Many of these questions concern the nature of evolutionary parallelism in different clades. Multiple different signals can feed into shared developmental pathways through different "side pathways". For example, multiple regulatory pathways feed into processes of internode elongation (de Saint Germain et al. 2013; Wang and Li 2008), SAM maintenance (through the feedback loops of *CLV* and *WUS* or *STM*, *CUC* genes, and miRNA164), senescence (reviewed by Morkunas et al. 2012; Reape et al. 2008), and lateral branching (Arite et al. 2007). Are the multiple regulatory pathways the same across species, or are there additional side pathways that link to core developmental processes in novel ways, or are some of the links to these side pathways lost in some clades? Are different or unique genetic regulators responsible for specialized architectures in particular clades?

Conclusions

The approach here has been one of generalities focused around growth processes that shape major components of perennial plant architecture. Prior surveys of plant architecture focused on classifications of architectures (Halle et al. 1978; Tomlinson 1978) and more recently on the molecular basis of tree architecture (e.g., Groover 2005; Hollender and Dardick 2015; Wang and Li 2008) or on systems perspectives (e.g., Barthélémy and Caraglio 2007). Here, I have tried to integrate these studies by highlighting connections between the genes and their dynamical processes that generate spatio-temporal pattern. Increasingly, studies are integrating the molecular regulatory processes with the dynamical pattern formation processes through a combination of genomics analyses, wet-lab bench work and computational modeling (e.g., Smith et al. 2006a; Fàbregas et al. 2015; Fujita et al. 2011; Ibañes et al. 2009; Muraro et al. 2014). Such systems-level studies are paving the way to a more thorough understanding of plant architecture and the processes that translate genetic information into morphological form.

Our understanding of multiple processes of fundamental importance to plant architecture is still in its infancy. Specifically, little is known about the molecular processes that (1) define cladotaxis differently from phyllotaxis, (2) determine shifts in phyllotaxis or cladotaxis within individuals, (3) contribute to the equilibrium angles of lateral branches (e.g., Carvalho and Paiva 2013; Li et al. 2013; Sato et al. 2014), (4) initiate branch senescence and self-pruning (e.g., Zhang et al. 2014), (5) model how transitions between primary and secondary vascular development come about (e.g. Jouannet et al. 2015), and (6) determine spatio-temporal patterning of the products of the vascular cambium.

Transformational studies of relatively well-studied topics, such as apical dominance, are still uncovering previously unrecognized regulatory components, such as strigolactones that play a pivotal role in regulating apical dominance and lateral branch angle and growth. It is likely that additional surprises are in store as next-generation approaches facilitate the exploration of unique and variant plant architectures in woody non-model and model organisms, alike.

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The Genomics of Wood Formation in Angiosperm Trees

Xinqiang He and Andrew T. Groover

Abstract Advances in genomic science have enabled comparative approaches that can evaluate the evolution of genes and mechanisms underlying phenotypic traits relevant to angiosperm forest trees. Wood formation is an excellent subject for comparative genomics, as it is an ancestral trait for angiosperms and has undergone significant modification in different angiosperm lineages. This chapter discusses some of the traits associated with wood formation, what is currently known about the genes and mechanisms regulating these traits, and how comparative evolutionary genomic studies can be undertaken to provide more comprehensive views of the evolution and development of wood formation in angiosperms.

Keywords Wood formation • Wood development • Wood evolution • Transcriptional regulation • Epigenetics • Comparative genomics

Genomic Perspectives on the Evolutionary Origins and Variation in Angiosperm Wood

Introduction

The evolutionary and developmental biology of wood are fundamental to understanding the amazing diversification of angiosperms. While flower morphology and reproductive characters associated with angiosperms have been the focus of numerous genetic and genomic evo-devo studies, wood development is a relatively neglected trait of angiosperm evolution and development that is now tractable for comparative and evolutionary genomic studies. The ability to produce wood from a

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vascular cambium is an ancestral trait of the angiosperms, but has been extensively modified in both basal lineages as well as in more recent, derived taxa. Interestingly, some wood development traits have arisen independently in unrelated taxa, presenting the question of whether common or multiple mechanisms can produce similar developmental changes.

Wood is the product of the vascular cambium. The vascular cambium is a lateral meristem whose initials divide to provide daughter cells that differentiate into secondary xylem (wood) towards the center of the stem, or secondary phloem (inner bark) towards the outside of the stem (Larson 1994). This radial growth from a vascular cambium in woody stems is collectively referred to as secondary growth. For most angiosperms, there are two types of cambial initials. Fusiform initials produce the axial tissues and cell types that can include water conducting tracheary elements, fibers, and xylem parenchyma. Ray initials produce the radial tissues, the rays. Rays are believed to serve various functions including radial transport across the stem, storage of water and nutrients, and biochemical functions. The axial tissues function primarily in water and nutrient conduction, and mechanical support. Significant quantitative, biochemical, and morphological variation exists among angiosperms for many aspects of secondary growth. For example, striking examples of morphological variation include gain and loss of the cambium, successive cambia, xylem furrowed by phloem, and interxylary phloem (Spicer and Groover 2010). The mechanisms underlying this variation are only recently being revealed.

Wood serves multiple functions, including water conduction, water and nutrient storage, and mechanical support. Wood development thus reflects developmental mechanisms that integrate environmental cues to produce highly complex tissues (Arnold and Mauseth 1999). A familiar example of this integration is the annual rings of many temperate tree species, which reflect modification of wood development in response to the changing environmental conditions within and between growing seasons. How this integration occurs is still opaque, in part because of traditional divisions among the different disciplines associated with wood development. Indeed, wood development has been studied since the earliest days of botany and has provided important insights into the evolution and development of plants in general, and of trees in particular (Baas 1982; Groover and Cronk 2013). More recently, advanced genomic approaches have been applied to wood development, including studies in a limited number of angiosperm model tree species with fully sequenced genomes. Integrating genomic studies with classical literature and traditional disciplines including anatomy and physiology could be extremely effective for comparative studies. An ultimate goal is to describe the molecular mechanisms regulating wood formation, structure and physiological functions, including how these biological processes have been modified in different angiosperm lineages and how environmental cues are integrated into developmental processes.

Arguably, wood formation is an excellent subject for comparative and evolutionary genomic studies because (1) wood development is experimentally tractable; (2) wood is of extreme economic and ecologic importance; (3) wood shows incredible phenotypic variation across angiosperm taxa; (4) there is an extensive literature detailing angiosperm wood anatomy; and (5) insights are being made into at least some of the regulatory mechanisms regulating wood development.

Additionally, detailed angiosperm phylogenies combined with new sequencing technologies make possible the comparison of gene function across diverse angiosperm taxa, even for non-model species that display unique anatomical or developmental attributes. This chapter presents some of the fundamental information and concepts underlying current and future comparative and evolutionary genomic approaches for wood development in angiosperms.

Expectations for Evolution of Regulatory Mechanisms Regulating Wood Development

Wood development can be subdivided into interrelated processes including the regulation of cell division in the cambial zone, cell expansion and differentiation in the developing xylem, tissue patterning (e.g. the location of vessels within the wood), and numerous other traits. Wood development is also highly plastic, and varies based on stage of development, time of year, and in response to myriad environmental and physiological cues. Anatomical variation has been well-described for large numbers of angiosperms for many wood-related traits in the classical wood anatomy and wood paleobotanical literature (Carlquist 2001). At the same time, increasingly robust phylogenies for angiosperms are being produced using DNA sequence (The Angiosperm Phylogeny 2009). Designing comparative and evolutionary genomic studies of wood formation can take advantage of this information. As discussed later in this review, different phylogenetic scales can be considered for such studies, ranging from ancestral traits that have been modified in different angiosperm lineages, to traits that have arisen independently through convergent evolution in different taxa.

There are a number of wood-related traits that were present in ancestral angiosperms that could be excellent case studies for comparative genomic studies – for example, the tracheary element. Tracheary elements are water-conducting cells that undergo an elaborate differentiation process that supports biosynthesis of a lignified secondary cell wall, and ultimately ends in programmed cell death and lysis to produce a hollow cell corpse (Groover and Jones 1999; Escamez and Tuominen 2014). There are two major forms of water-conducting tracheary elements: tracheids, which are elongated cells with pits that facilitate water transport, and vessel elements, which tend to have larger diameters than tracheids and have openings at each end of the cell, termed perforation plates, that vary from ladder-like scalariform perforations to a simple opening (Esau 1977; Bliss 1921). Within the angiosperms, extensive variation can be seen for tracheary element morphology at different taxonomic levels, among organs within a species, among individuals within a species in response to different environmental conditions, and within individuals at different stages of development (Carlquist and Schneider 2002). This variation in tracheary element morphology and associated functional traits has been the basis of major hypotheses of early angiosperm evolution and diversification (Carlquist 2001, 2009; Bailey 1944). Early angiosperms are characterized by specific wood traits including tracheids, long cambial initials, and upright ray cells. The wood of more derived angiosperms possess vessel elements (or vessel

elements and tracheids, or tracheary elements with features of both vessel elements and tracheids), contains parenchyma that can serve to refill embolized vessels, and have procumbent ray cells (Carlquist 2009). Modification of wood anatomies and associated physiological conditions in different lineages likely played a primary role in angiosperm diversification and expansion into extremely diverse habitats. Indeed, xylem heterochrony is a primary factor in understanding the evolution and diversification of early angiosperms (Carlquist 2009).

There are striking innovations in wood anatomy among angiosperms. For example, for at least for some if not all species with successive cambia, a "master cambium" produces conjunctive tissue along with new cambia that then produce secondary xylem and phloem (Carlquist 2007). The result is a stem with repeating layers of secondary xylem and phloem embedded in a matrix of conjuctive tissue. Successive cambia have evolved within 14 orders scattered through the eudicots, and may enable flexibility in woody stems and lianas (Carlquist 2007; Spicer and Groover 2010).

Wood properties can also be examined from an ecological evolutionary perspectives. Since wood is the water conducting tissue of stems, wood properties ultimately determine the ability of a species or individual to grow in different habitats or respond to environmental stresses such as drought. For example, changes in wood properties (specifically, smaller tracheary elements less prone to cavitation) was a major factor in the successful movement of woody angiosperms into freezing environments (Zanne et al. 2014). Interestingly, woody perennial growth has also been found to be associated with reduced exploration of climate space (Smith and Beaulieu 2009) and slower rates of molecular evolution (Smith and Donoghue 2008). Parenchymatous woods illustrate how anatomy affects physiology and adaptive traits, for example in cacti (Mauseth and Plemons-Rodriguez 1998). The number and diameter of tracheary elements produced can vary dramatically in response to water availability, although the mechanisms controlling these responses are poorly understood. These and related topics regarding wood evolution and development have direct relevance to understanding how different species will respond to ongoing climate change.

As discussed below, genes and mechanisms regulating wood formation have been identified that can now be examined in an evolutionary context. While this review cannot cover all of these subjects comprehensively, some examples of genes and mechanisms regulating wood formation are presented in the next section.

Transcriptional Regulation of Wood Formation

Transcription appears to be one of the primary points of regulation for wood formation. This can be inferred from studies in which gene expression was measured using microarrays for successive tissue samples were taken across a developing *Populus* stems, from the phloem across the cambium and into different stages of secondary xylem maturation (Schrader et al. 2004). In general, changes in gene expression across the tissue profiles are highly correlated to developmental events,

with obvious correlations seen between the function of genes associated with xylem development and processes occurring during progressive stages of tissue development (e.g. cell division, cell elongation, and cell wall biosynthesis). Additionally, as discussed below, key transcription factors have been identified that regulate specific aspects of secondary growth and wood formation.

A comprehensive view of the developmental mechanisms underlying wood formation and secondary growth is still emerging. As in other plants and animals, the transcriptional networks regulating gene expression are highly complex, but are beginning to be modeled through integration of different genomic data types using computational approaches. The complexity of transcriptional regulation is shown by ChIP-seq results indicating that individual transcription factors can bind many hundreds or thousands of loci throughout the *Populus* genome (Liu et al. 2015a). Additionally, there is relatively low correlation between the binding of the individual transcription factors surveyed to date and the expression of bound genes (Liu et al. 2015b), precluding simple models relying on strong correlation of transcription factor binding and transcriptional outcomes for target genes. The lack of strong correlation between transcription factor binding and transcription of target genes may reflect that most genes require the cooperative binding of multiple factors and permissive chromatin states to result in expression change. It may also reflect the type of transcription factors surveyed to date, and "master regulator" transcription factors such as NACs may show more direct correlations between binding and gene expression. Nonetheless, it is obvious that, as has been found by the humane ENCODE project (Consortium 2012), transcription factors tend to bind to large numbers of targets and predicting gene expression requires data describing the binding of multiple transcription factors and chromatin states to be robust.

Several genomic-based approaches have been taken to uncover genes and mechanisms regulating wood formation in angiosperms. Each approach has different strengths and caveats, and ultimately surveys genes affecting wood formation at different levels. For example, association mapping identified single nucleotide polymorphisms in *Populus* that were correlated with a number of wood chemistry and ultrastructure phenotypes for a large population-based survey (Porth et al. 2013b). Candidate genes identified with strongest effects on phenotypes were primarily structural genes (23 genes), but at least three transcription factors were identified. Limitations to association mapping include that only genes with allelic variation having significant effects on phenotypes can be identified, and the large majority of genes involved in wood development are transparent to this approach. However, the approach does provide insight into the mechanisms and specific genes that are being acted upon by selection. More comprehensively, gene expression during wood formation has been profiled using microarrays and sequencing-based approaches to provide complete catalogues of wood-related genes in species including poplar (Schrader et al. 2004), Eucalyptus (Etienne Paux 2005) and Arabidopsis (Zhao et al. 2005). These approaches do not directly provide functional insights into gene networks or natural genetic variation, however.

Characterization of individual transcription factors has provided important insights into the regulation of wood formation. Transcription factors regulating wood development have been most extensively characterized in Arabidopsis and

Populus. While the rosette-form of Arabidopsis lacks fundamental features of arborescent angiosperms such as rays, it does possess a vascular cambium and makes limited secondary xylem in the vegetative rosette. Arabidopsis can also produce limited secondary vascular tissue and interfascicular fibers in the inflorescence stem. Nonetheless, it should be noted that some aspects of wood formation may not be well represented in Arabidopsis.

Developmental genetic, yeast one hybrid and other approaches have defined a hierarchy of NAC-domain and MYB-related transcription factors that are master regulators of tracheary element and fiber differentiation (Brady et al. 2011; Taylor-Teeples et al. 2015; Zhong et al. 2008; Zhong and Ye 2015). Generally, NAC-domain transcription factors occupy the top levels of this hierarchy, and regulate MYB-related transcription factors that in turn regulate genes encoding enzymes participating in secondary cell wall synthesis. Interestingly, misexpression of *VASCULAR-RELATED NAC-DOMAIN6* (*VND6*) results in the differentiation of parenchyma into metaxylem-like vessel elements, while misexpression of *VND7* results in differentiation of parenchyma into protoxylem-like vessel elements (Kubo et al. 2005). Furthermore, dominant repression of *VND6* and *VND7* specifically inhibits metaxylem and protoxylem vessel formation in roots, respectively. Other NAC family members including *NSD1*, *NST1* and *NST2* regulate the differentiation of fibers, and *nsd1*/*nst1*/*nst2* mutants fail to produce secondary cell walls in fibers (Zhong and Ye 2015; Zhong et al. 2006). Thus, NAC-domain transcription factors define mechanisms fundamental to the diversification of the ancestral tracheid cell type into vessels with different morphologies as well as fibers.

New insights into the regulation of the cambium and secondary vascular tissue differentiation was initially identified using the *in vitro* system for tracheary element differentiation in Zinnia (Ito et al. 2006). In this system, isolated mesophyll cells are induced to differentiate into tracheary elements Differentiation is dependent on exogenous nutritive factors, hormones and a secreted peptide factor, TDIF (Ito et al. 2006). In the plant, TDIF is expressed in the secondary phloem and is perceived by the LRR-like receptor, TDIF RECEPTOR/PHLOEM INTERCALATED WITH XYLEM (TDR/PXY). TDR/PXY in turn modulates expression of a WOX-like transcription factor, WOX4, that regulates the rate of cambial cell division (Hirakawa et al. 2008 2010; Etchells and Turner 2010). Interestingly, this signaling circuit appears to be evolutionarily ancient, with TDIF activity conserved among extant euphyllophytes (Hirakawa and Bowman 2015). Variation in TDIF signaling is thus an attractive mechanism to survey in angiosperm tree species.

More obscure are the mechanisms that regulate the patterning of secondary vascular tissues and the rate of differentiation of cambial derivatives. Class I KNOX transcription factors have been identified that are expressed both in the shoot meristem and cambial zone of *Populus* (Du et al. 2009; Groover et al. 2006). Both *ARBORKNOX1* (*ARK1*) and *ARK2* alter the differentiation of lignified cells within secondary xylem when misregulated. This function is similar to better characterized orthologs in Arabidopsis (*SHOOTMERISTEMLESS* and *BREVIPEDECELLUS*) which generally act to repress differentiation within the shoot apical meristem (Long et al. 1996). The expression of these transcriptional regulators in both the

apical and cambial meristems presents an interesting opportunity to better understand the cooption of genes and mechanisms from the shoot apical meristem during the evolution of the cambium.

Another potential example of cooption of mechanisms to the cambium from the shoot apical meristem is given by Class III HD ZIP transcription factors. This small family of transcription factors includes members that function in the patterning and polarity of lateral organs and vascular tissues. For example, mutations in the Arabidopsis Class III HD ZIP, REVOLUTA, conditions phenotypes that include adaxialization of both lateral organs and vascular tissues. Mechanistically, REVOLUTA acts antagonistically with another small family of transcriptional regulators, YABBYs. In *Populus*, misexpression of the *REVOLUTA* ortholog results in mispatterning of secondary vascular tissues, including reversal of polarity of xylem and phloem and formation of ectopic cambium in the cortex (Robischon et al. 2011). The Class III HD ZIPs are evolutionarily ancient, but functions such as polarity regulation in secondary vascular tissues are derived (Floyd et al. 2006).

Promising advances are being made towards comprehensively modeling transcriptional networks, and identifying correlations between network features and phenotypes. For example, integration of genetic, genomic and phenotypic data was used to identify small, directed networks of genes affecting wood biochemistry (Porth et al. 2013a). Gene co-expression studies can be used to cluster genes into modules that show similar expression pattern across different genotypes, mutants, or in response to experimental treatments. Correlations can then be tested between phenotypic traits and the module eigengene (conceptually, the average) expression values. This approach has the advantage of reducing the dimensionality of the data and minimizes the problems associated with multiple testing commonly encountered in single-gene approaches. In one example, microarray data were used to identify gene modules associated with leaf development in *Populus*, including putative mechanisms conserved between *Populus* and Arabidopsis (Street et al. 2011).

Network-based approaches were used for wood development in a recent study of tension wood development. Different *Populus ARK2* genotypes were subjected to gibberellic acid (GA) or control treatments, and then either placed horizontally to induced tension wood formation or left upright (Gerttula et al. 2015). Differentiating tension wood, opposite wood and normal wood from the trees and used for mRNA-sequencing. The resulting transcript abundance data were subjected to a co-expression analysis to place genes into modules, which were then correlated to wood properties, wood types and treatments. Genes within modules were then further analyzed to identify candidate transcription factors responsible for regulating the expression of other genes within each module. This proof of concept study could be expanded by including additional genetic or experimental perturbation, which would allow more precise assignment of genes to smaller gene modules. Importantly, these same approaches and concepts can be used to calculate and compare co-expression networks across species, supporting analyses capable of identifying ancestral mechanisms such as regulation of cambium division as well as taxa-specific derived traits.

Post-transcriptional Regulation of Wood Formation

Wood formation is regulated and modified post-transcriptionally by microRNA, by the regulation of rptoein abundance, and by a host of post-transcriptional protein modifications. The relative abundance of specific gene transcripts can be negatively regulated by non-coding microRNAs (miRNAs). At the protein level, transcript stability, rate of translation, protein stability and other factors can result in significant differences between relative rates of transcription and actual protein levels for a given gene. Additionally, phosphorylation, glycosylation, lipidation, ubiquitination and other post-translational modifications can have major effects on the abundance, localization, and activity of proteins. As discussed in this section, 'omics' approaches are defining the roles of these various types of post-transcriptional modifications during wood formation.

miRNA Regulation of Transcript Abundance During Wood Formation

miRNAs are short, nuclear-encoded non-coding RNAs involved post-transcriptional regulation of gene expression. In plants, the post-transcriptional regulation by miRNAs is achieved by first processing a miRNA precursor by Dicer-Like1 (Dcl1) into a 21 nucleotide miRNA/miRNA* duplex. The processed miRNA is then incorporated into an Argonaute-associated miRNA-induced silencing complex (miRISC). Specific complementary base pairing between a given miRNA and transcripts from target genes results in cleavage of the target transcript by the miRISC (Meng et al. 2011). miRNAs have been recognized to play crucial roles in diverse biological processes in plants and animals, including cambium differentiation and wood formation in Arabidopsis, as well as *Populus* and other tree species (Sun et al. 2012).

In a pioneering study of miRNAs in wood formation (Lu et al. 2005), miRNAs families were identified by DNA sequencing of developing secondary xylem tissues of *Populus trichocarpa*. In this study, a comparisons were made of tension wood and opposite wood from leaning stems, and normal wood from upright trees. Among the miRNAs identified, 12 families were either identical or very similar to Arabidopsis miRNAs, suggesting that these represent evolutionarily-conserved mechanisms. Interestingly, 10 *Populus* miRNA families were not conserved with Arabidopsis, and the majority of these non-conserved miRNAs were associated with tension wood and/or opposite wood formation. These results indicate that there are species-specific miRNAs, and that miRNAs may regulate tree-related traits such as tension wood formation (Lu et al. 2005).

Evolutionarily conserved miRNAs have been implicated in regulating specific processes underlying wood formation. miR165/166 directly targets transcripts encoding Class III HD-ZIP transcription factors that have been shown to play central roles in organ and vascular tissue polarity, and regulate xylem differentiation in

Arabidopsis roots (Carlsbecker et al. 2010) and shoots (Emery et al. 2003). The function and conserved role of Class III HD-ZIP genes has also been studied in *Populus*. Overexpression of miRNA-resistant Class III HD-ZIP *POPCORONA* resulted in delayed lignification of xylem and phloem fibers (Du et al. 2011), while overexpression of a miRNA-resistant form of *popREVOLUTA* resulted in the formation of ectopic cambial layers with reversed polarity within cortical parenchyma (Robischon et al. 2011). miRNAs also regulate structural genes involved in wood formation including laccase-encoding genes, whose protein products mediate the polymerization of monolignols during lignification. The *Populus* miRNA, Ptr-miR397, was found to directly target 29 of 49 predicted *Populus* laccase gene transcripts (Lu et al. 2013).

miRNAs also appear to be involved in perennial regulation of secondary growth and wood formation. Deep sequencing of short RNAs in the cambium zone of *Populus* stems identified more than 100 miRNAs with significant expression changes between active growth and dormancy, including developmental-, phytohormone- and stress-related miRNAs. Most of the development-related miRNAs were enriched in the active growth stage, such as miR164, miR396, miR168, miR319, miR171 (Ding et al. 2014). In contrast, miR166, which targets Class III HD-ZIP transcripts, was more abundant in dormancy and had low expression level during active growth (Ding et al. 2014; Ko et al. 2006), indicating that miR166 plays a role in regulating the annual growth cycle.

Profiling miRNAs expressed during wood formation has also been undertaken in a handful of other tree species. In *Eucalyptus*, the investigation of miRNAs from xylem, phloem and leaves identified 20 miRNAs of 5 families known in other plants and 28 novel miRNAs of 8 additional families (Victor 2006). In *Acacia*, 6 highly conserved miRNA families were identified as the potential regulators of secondary wall formation in xylem development, including miR166, miR172, miR168, miR159, miR394 and miR156.

Regulation of Protein Abundance and Post-translational Modification During Wood Formation

The term proteome refers to the total set of proteins and post-translational modifications found in a biological sample. A primary goal of proteomics for wood formation is to determine where and when thousands of individual proteins are produced in secondary vascular tissues, and how they are modified and interact.

Different approaches have been used to catalogue the proteins involved in secondary growth and wood formation. In an early wood-related proteomics study, 15 proteins were identified from differentiating xylem, mature xylem and bark of *Populus* by 2-Dimensional Electrophoreses (2-DE) coupled with mass spectrometry (MS), including key participants of the phenylpropanoid pathway and lignification (Mijnsbrugge et al. 2000). In addition, highly abundant proteins with unknown function were identified, underscoring that many genes and proteins vital to wood

formation remain anonymous. In *E. grandis*, the cambial region proteome was compared for three ages of growth, and 240 proteins of various putative functions were identified using a 2-DE-LC-MS/MS strategy (Fiorani et al. 2007). From xylem sap of *P. trichocarpa* x *P. deltoides*, 97 proteins were identified, including metabolic and glycolytic enzymes and defense proteins (Dafoe and Constabel 2009). The regeneration of secondary vascular tissues after removal of bark provides another useful experimental system for the study of secondary growth. Using this system, 2-DE was used in combination with MALDITOF-MS to identify 244 differentially expressed proteins during the secondary vascular tissue regeneration in *Populus*, 199 of which were assigned and classified under different functional classes including metabolism, signaling, cytoskeleton functions, cell cycle and secondary cell wall formation (Du et al. 2006). In another study performed by shotgun proteomics on xylem and phloem tissues from two *Populus* species: *P. deltoides* and *P. tremula*×*alba*, 7505 proteins were identified, 2627 were confidently identified in both xylem and phloem, 606 unique in xylem and 461 in phloem (Abraham et al. 2012). As can be seen from these studies, technical advances in proteomics are quickly providing a more accurate and informative characterizations of the proteins involved in wood formation.

Proteomic approaches have also been applied to more specific aspects of wood formation in angiosperm trees, including reaction wood formation. Using 2-DE, 140 protein species were identified in the upper side of a leaning stems of *E. gunnii*, with 12 proteins significantly associated with tension wood formation (Plomion et al. 2003). A proteomic approach of tension wood formation in *Populus* revealed 39 proteins, primarily cell wall-related, in the G-layer in mature xylem by 2-DE and gel-free MS methods (Kaku et al. 2009). A quantitative proteomic and phosphoproteomic analysis of tension wood formation in *Populus* revealed remarkable developmental plasticity, identifying 1155 proteins and phosphorylation events in comparison of normal wood and tension wood (Mauriat et al. 2015). These results underscores the importance of rapid and reversible post-translational modifications through phosphorylation during wood formation.

Other studies have sought to identify proteins that are uniquely expressed during secondary vascular development. An investigation of plasma membrane proteins from leaves, xylem, and cambium/phloem in *Populus* found that proteins involved in cell wall and carbohydrate metabolism, membrane trafficking were most abundant in the xylem plasma membranes, in agreement with the large role of cell wall biosynthesis in wood formation. Interestingly, the proteins uniquely found in xylem plasma membranes included enzymes involved in lignin biosynthesis, suggesting that they may exist as a complex linked to the plasma membrane, possibly in close proximity to a transporter translocating lignin monomers across the plasma membrane (Nilsson et al. 2010). Another proteomic analysis of the membrane proteins in differentiating secondary vascular tissues of *Populus* (Song et al. 2011) found a total of 226 proteins identified as integral plasma membrane proteins, including receptors, transporters, cell wall formation related or intracellular trafficking proteins. In particular, a group of RLKs were identified in the differentiating xylem and phloem, suggesting their involvement in secondary vascular development. An

endo-1,4-β-mannanase protein identified in the plasma membrane of differentiating xylem tissue potentially produces oligosaccharides that could serve as signaling molecules to suppress cell wall thickening (Zhao et al. 2013).

Culture-based systems for tracheary element differentiation can provide populations of synchronously differentiating cells for proteomics. The secondary cell wall patterning of tracheary elements is guided by underlying microtubules. Using an in vitro system for tracheary element differentiation, microtubule pulldown experiments paired with quantitative proteomic analysis of labeled microtubule interacting proteins identified 605 microtubule interacting proteins associated with specific stages of differentiation. The proteins associated with membrane trafficking, protein synthesis, DNA/RNA binding, and signal transduction peaked during secondary cell wall formation, while proteins associated with stress peaked when approaching tracheary element cell death (Derbyshire et al. 2015). This study thus provided an entire functional microtubule interactome during tracheary element formation, and expanded our understanding of the complexity of microtubule function in xylem development.

Ultimately the integration of proteomics data with other genomic and phenotypic data is required to comprehensively understand a given biological process, requiring the use of systems biology approaches. Bylesho et al (2009) devised a strategy for data generation and integration to model systematic changes in transcript, protein and metabolite profiles associated with lignin biosynthesis in hybrid aspen (Bylesjo et al. 2009). The joint covariation for all profiling platforms was calculated using multiple O2PLS models, and the results quantified genotype-specific perturbations affecting lignin biosynthesis and growth (Bylesjo et al. 2009). A systems biology approach was also applied to analyze effects of oxidative stress in *Populus* after acquiring transcriptomic, proteomic and metabolomic profiles of the cambial region of hybrid aspen plants, and then a multivariate analysis method OnPLS was used to integrate the three types of 'omics' data. The results provided a first comprehensive model of multi-level responses to oxidative stress in the vascular cambium (Srivastava et al. 2013).

Comparative Evolutionary Genomics Approaches for Wood Formation

There are a number of established approaches for comparative and evolutionary genomic studies that have been applied in both plants and animals, which could now be applied to wood formation in angiosperm trees. At one end of the spectrum, one common approach is to make detailed comparisons (e.g. of expression pattern) for one or a few genes of interest across species with interesting variation for a trait. At the other extreme, comparative genomic approaches can be used to compare DNA sequence, gene expression, or other comprehensive "omics" data within and across species of interest. Comparative genomic approaches are both more comprehensive as well as more powerful than one-gene-at-a-time approaches, and allow integration

of quantitative genomic, morphological, biochemical, and other wood-related data. This approach also has the advantage of discovering novel genes and mechanisms that have not been previously described.

A critical challenge for most comparative genomics projects is the determination of orthologous relationships of genes across species. For angiosperms, this is especially challenging given the complex evolutionary histories of angiosperm genomes, with many lineages characterized by whole genome duplication events, ploidy variation, hybridization and structural variation (Soltis et al. 2009). Conceptually and practically, the determination of orthologous genes (formally, genes that are homologous or of common decent in the species being compared) varies depending on the phylogenetic distance and evolutionary history of the genomes of the species being compared. At one extreme, closely related species that have not undergone any genome duplications or rearrangements since diverging from a common ancestor have relatively simple and direct orthology (e.g. orthologous genes having one-to-one orthologous relationships). In cases where one species lineage has undergone whole genome duplication since divergence from a common ancestor, homology may be characterized by one-to-two or one-to-many orthologous relationships. Several different computational approaches have been used to define orthologous relationships, and software is available for performing reciprocal BLAST-based approaches, tree-based approaches, and graph-based clustering approaches for estimating orthologous groups, for example see (Huerta-Cepas et al. 2016; Nakaya et al. 2013; Afrasiabi et al. 2013; Ye et al. 2013; Fischer et al. 2011; Li et al. 2003). In the case of related species with fully sequenced genome, syntenic relationships can be used to further infer orthologous relationships in addition to simple sequence data (Lechner et al. 2014). Morphological or experimental data can ultimately be integrated with orthology data to ask questions regarding the genetic evolution of traits, including wood formation.

For the study of the evolution of wood formation in angiosperms, one approach would be to identify genes and pathways regulating wood formation that have been described in model systems (e.g. *Populus* or Arabidopsis) and survey their diversification among different lineages. This general approach has been successful in providing fundamental insights into the variation in flower (Becker et al. 2011; Irish and Litt 2005) and leaf (Tsiantis and Hay 2003; Dkhar and Pareek 2014; Tsukaya 2014; Champagne et al. 2007) morphology within the angiosperms. Examples of genes and mechanisms associated with wood formation that could be surveyed include those previously discussed, such as NAC-domain, Class I KNOX and Class III HD ZIP transcription factors, or the mechanisms described by the TDIF/CLE TDR/PXY WOX signaling pathway. However, the selection of candidate genes for study is still relatively subjective, and differences in sequence resources (e.g. genome-level sequence) and experimental tractability (e.g. ability to transform) among species makes this candidate gene approach challenging and non-comprehensive. Indeed, because efficient transformation is difficult for many woody species, performing functional assessment of individual genes in multiple species

would be difficult. However, techniques for visualizing gene transcripts using in situ hybridization or protein epitopes using immunolocalizations within tissue sections have been extended to woody models including *Populus* (Du et al. 2009; Gerttula et al. 2015), and are relatively transferable across species. Examining differences in expression patterns in wood forming tissues during secondary growth could potentially reveal informative differences in the timing or spatial expression of candidate genes among species with unique anatomical, biochemical or other differences in wood development.

A more comprehensive and widely applicable approach would be to us gene expression-based comparative genomics approaches to discover and describe genes and mechanisms underlying phenotypic variation across species. Next generation sequencing (e.g. mRNAsequencing) can now be used to develop the datasets required for such studies from practically any species. This approach has the advantages of discovering genes and mechanisms that may be invariable in population-based approaches or transparent to mutational approaches in model systems. Importantly, this strategy can simultaneously discover mechanisms regulating development and relate them to variation within specific lineages. An example to this approach is given by co-expression gene networks. In this approach, genes that display similar expression across a range of biological conditions (e.g. tissue types or developmental time points) are assigned to coexpression "modules" that often represent genes that work together on common biological processes. For example, a comparative gene regulatory network approach was used to comprehensively describe the transcriptional differences underlying variation in leaf shape for survey of tomato species (Ichihashi et al. 2014). Gene modules were defined based on co-expression relationships, and were then annotated with features including enrichment for genes in specific Gene Ontology classes or correlation with phenotypic traits. To apply such an approach to wood formation, trees from a taxonomic survey spanning some relevant taxonomic range would ideally be grown in a common environment, and then subjected to a variety of experimental perturbations that alter wood development (e.g. hormone treatment). Wood forming tissues would then be harvested and subjected to mRNA sequencing to provide data for comparative co-expression analyses within and among species. Challenges to this approach include collecting together and propagating the required plant material from arboreta or other sources (Groover and Dosmann 2012), and reliably determining orthologous relationships among genes among the different species. However, this approach is currently technically tractable and can utilize analysis approaches and tools developed in other systems. Ideally, such a comparative approach could identify gene modules commonly involved in wood formation across species that might represent ancestral mechanisms, as well as lineage or species-specific modules that could underlie phenotypic differences among species.

New approaches could also be developed for functional genomics within at least some model tree systems that could be scaled to survey larger numbers of

genes. An example of such an approach is given by an irradiation hybrid mutagenesis screen in *Populus*. In this approach, a controlled cross was made between two *Populus* species in which the pollen from the male parent was irradiated to create chromosomal breaks. In a cross between *P. nigra* and *P. deltoides*, over 55 % of the 500 F1 progeny produced contained deletions or insertions of chromosomal segments (Henry et al. 2015). The insertions and deletions were mapped with precision in each F1 progeny using low-coverage, whole-genome sequence data. The genomic data can now be used to assist in reverse or forward genetic screens, including association between altered gene dosage in specific regions of the genome and phenotypes of interest. This population also represents a rich source of genetic perturbation useful in ultimately modeling gene regulatory networks (Filkov 2005). A primary aim of this approach is to create novel genetic changes that alter the complex gene dosage relationships that are believed central to the regulation of heterosis and complex quantitative traits (Birchler et al. 2006) including wood development.

Lastly, imaging-based techniques and data are especially useful for all comparative studies of wood formation. Secondary vascular tissues and wood are characterized by complex three dimensional tissues comprised of multiple cell types, and have both radial and longitudinal developmental gradients to consider. An increasing number of options are available to visualize and quantify molecular and anatomical features that can then be integrated with genomics level information. An example of the power of imaging-based techniques is shown in Fig. 1. To determine how woody stems perceived and responded to gravity, an antibody was raised against a peptide from a *Populus* PIN-like auxin transport protein and used in immunolocalizations to reveal the gravity-sensing cells within the stem (Fig. 1a, b). Using an auxin-responsive DR5:GUS reporter, the consequence of radial auxin transport by these PIN-expressing cells was revealed in stems placed horizontally to induce tension wood on the upper side of the stem (Fig. 1c) or opposite wood on the lower side of the stem (Fig. 1d). Differential auxin response in the cambial zone versus the cortex in tension wood and opposite wood, respectively, provides insights into the mechanisms regulating these distinct wood development programs that can now be surveyed in angiosperm trees with differing gravitropic stem responses. Molecular phenotypes including cell wall components can be surveyed using an extensive battery of publically available antibodies developed against cell wall epitopes (http://www.ccrc.uga.edu/~mao/wallmab/Home/Home.php). An example is shown in Fig. 2 for an arabinoglactan-recognizing antibody that labels mature gelatinous layers (G-layers) in tension wood fibers in *Populus*. A more generalized technique for high resolution, three dimensional imaging of wood tissues is laser ablation tomography (Fig. 3). In this technique, a high powered laser is used to progressively and precisely remove successive layers of tissue, with each layer imaged at high resolution. The images can then be reconstructed in three dimensions and features quantified, such as number, patterning, length, and interconnectivity among vessels. This technique could presumably be applied to any woody species or sample.

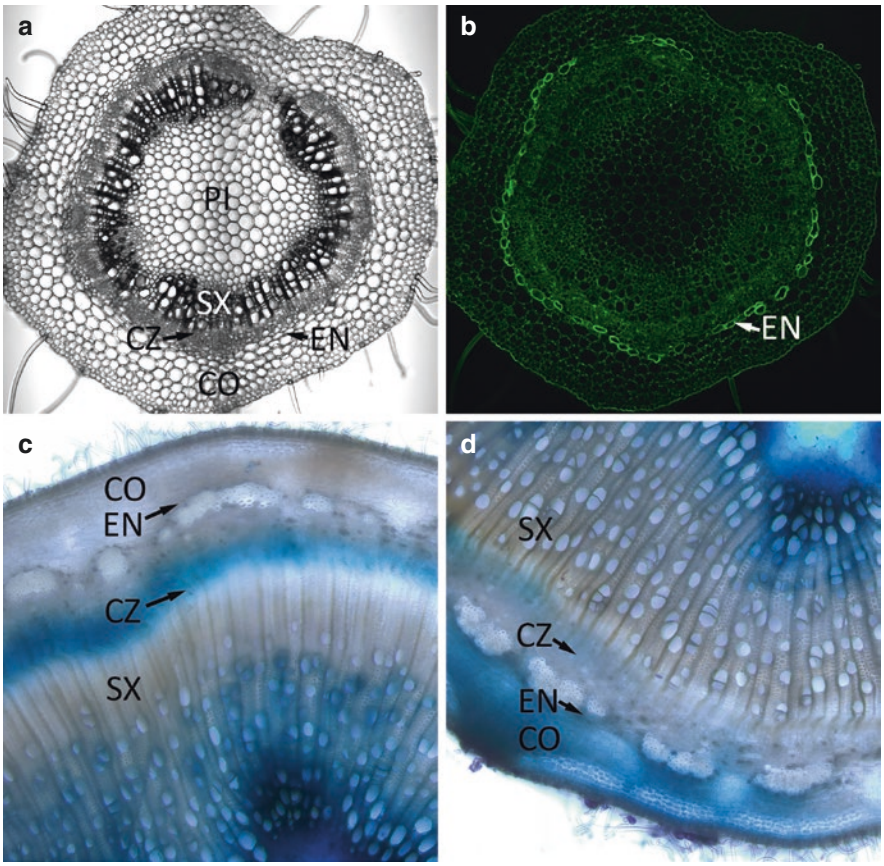

Fig. 1 Imaging of molecular events associated with tension wood formation in *Populus*. (**a**) Bright field image of stem cross section. (**b**) Confocal image of immunolocalization of a Populus PIN3-like protein in the same tissue section. The strong green signal corresponds to the endodermis, which is the innermost layer of the cortex. (**c**) Tension wood from DR5:GUS*Populus* stem of a GA treated tree. (**d**) Opposite wood from DR5:GUS*Populus* stem of a GA treated tree. Blue signal corresponds to auxin response (Note response is strong in the cambial zone of tension wood, versus the cortex of opposite wood. *CO* cortex, *CZ* cambial zone, *EN* endodermis, *SX* secondary xylem, *PI* pith)

Conclusions and Future Perspectives

The quickly advancing fields of genomics and computational biology make comparative and evolutionary genomic studies increasingly attractive. The large number of diverse angiosperm tree species of ecological or economic interest makes it infeasible to produce the range of resources for each to become a model species. However, sequencing-based experimental approaches such as mRNAsequencing paired with computational approaches such as comparative gene co-expression

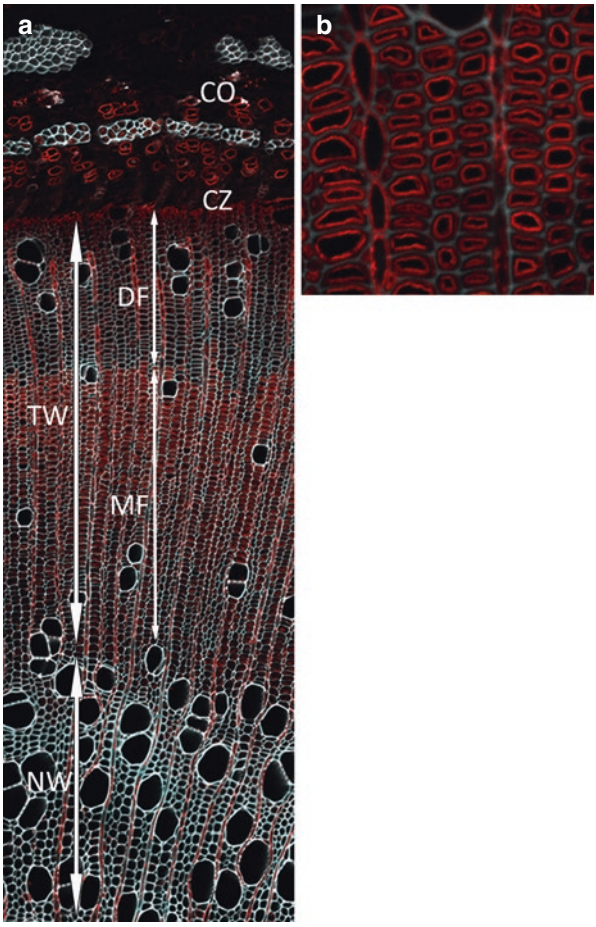

Fig. 2 Imaging of arabinogalactan protein epitopes in Populus tension wood. (**a**) A cross section of a Populus stem that has transitioned to tension wood formation. Strong labeling by the antibody JIM14 (*red signals*) of the gelatinous cell wall layer in the lumen of fibers within tension wood takes time to mature and be labeled by the antibody, providing a molecular marker of fiber type and differentiation. (**b**) Higher magnification of tension wood fibers with labeled gelatinous layers. *Blue signal* is UV autofluorescence. *CO* cortex, *CZ* cambial zone, *DF* differentiating fibers, *MF* mature fibers, *NW* normal wood, *TW* tension wood

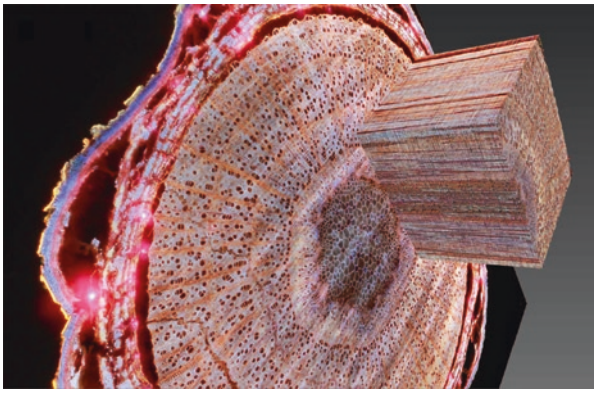

Fig. 3 Three dimensional rendering of wood sample using laser ablation tomography. Wood block rendered in three dimensions by integration of multiple z-stacked images. For information about this technology see http://l4is.com/Image courtesy of Lasers for Innovative Solutions, LLC

network analysis could be extended to most species. Importantly, comparative approaches can actually be much more powerful and address questions that one-species-a-time approaches cannot. The power of looking beyond a handful of model tree species would enable answers to such questions as, "what are the core set of genes and mechanisms that make a tree a tree."

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Phase Change and Phenology in Trees

Amy M. Brunner, Erika Varkonyi-Gasic, and Rebecca C. Jones

Abstract A long life span and large size are central characteristics of the tree growth habit. This growth habit requires a prolonged management of meristems as well as the long-term maintenance of above-ground tissues that are exposed to a variety of abiotic and biotic conditions, both seasonally recurring as well as episodic. Phase change and phenology, the timing of life cycle events, are key adaptive traits that alter meristem activity and identity as well as other aspects of growth and physiology. We review these processes and illustrate some of the diversity among taxa. The increasing genomic resources for trees and technological innovations are enabling the elucidation of the complex regulatory networks underpinning these processes as well as the variation within and between tree taxa. We address the current state of knowledge of environmental signals, genes and pathways regulating the multiple component processes of vegetative and reproductive phase change and phenology in trees.

Keywords Flowering time • Dormancy • Maturation • Phase change • Phenology

Introduction

Complex networks of endogenous and environmental signaling determine when life cycle events occur in plants. Phase change or maturation refers to changes that are usually stable. These include various alterations in vegetative morphology and physiology, such as leaf shape, which change gradually or abruptly over the tree's life. Phenology typically refers to the timing of recurring (except in annuals) events

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that are usually associated with seasonal climatic changes. Reproductive maturation is the competency to respond to signals promoting flowering, while reproductive phenology is the seasonal timing of the floral transition, anthesis and fruiting. Although phase change and phenology are somewhat distinct, they are also interconnected (Poethig 2013). In monocarpic Arabidopsis, vegetative phase change and reproductive transitions are clearly evident along the length of a shoot, however, the spatial and temporal aspects of phase change and phenology are much more complex in trees. Trees are characterized by a long non-flowering period that can last years to decades: e.g. 1–5 years in *Eucalyptus globulus* (Jordan et al. 1999), up to 40 years in *Fagus sylvatica* (Wareing 1959), which is thought to be a strategy to cope with different selection pressures that vary over the long life span of the tree (Day et al. 2002).This long juvenile period, however, is a major impediment to tree breeding and presents an economic hurdle to fruit growers, who face years of unproductive orchards. On the other hand, an extended non-flowering period may be desirable in biomass production plantations, especially in cases of deployment of exotic or genetically modified trees (Brunner et al. 2007).

As long-lived organisms, and in nearly all cases polycarpic, only a subset of an adult tree's shoot meristems transition to flowering during a given year. Moreover, reproductive phenology is integrated with vegetative phenology. Although usually discussed in terms of dormancy that enables survival during winter, the ability to alter vegetative meristem activity is central to the tree life style in any climate. Tree populations have adapted their phenology to local climates and accordingly, phenology is a major determinate of species distribution (Chuine 2010). The vegetative and reproductive phenology of trees are strong indicators of climate change (Polgar and Primack 2011). Correspondingly, the effects of climate change on the health and productivity of native forests and woody crops are a major concern and facilitated movement of tree populations is being considered (Aitken and Bemmels 2016). In this chapter, we describe tree maturation and phenology, how these vary among tree taxa and the regulation of these processes. We also include woody vines and shrubs in some aspects of this review, especially in regards to studies of phenology. The long generation times of trees and the difficulty in producing mutants that allow functional studies of specific genes pose challenges. Nevertheless, there are increasing options for deciphering the regulation of maturation and phenology in trees. We conclude this chapter with suggested approaches to advance our understanding of the variation in molecular regulation within and among taxa, as well as for more effectively leveraging the most experimentally tractable taxa for depth of understanding of these complex processes.

Phase Change

The differences between juvenile and adult vegetative phases of annual plants such as Arabidopsis are subtle, and displayed at the level of a single shoot (Telfer et al. 1997). In contrast, many forest trees are heteroblastic, with dramatic and abrupt

differences in morphology between the juvenile and adult form. Moreover, these changes occur at the whole-tree level such that there can be multiple juvenile branches nearer the base of a tree (Fig. 1). The most obvious phase change is the transition to reproductive development, and once reproductively mature, a subset of a tree's meristems transition to flower development usually at specific seasonal times (see section "Reproductive Phenology"). The timing of transition to first flowering is generally under strong genetic control in trees (e.g. Jordan et al. 1999) but with a significant environmental component. The juvenile vegetative phase is usually characterised by the inability to initiate floral development. However there is

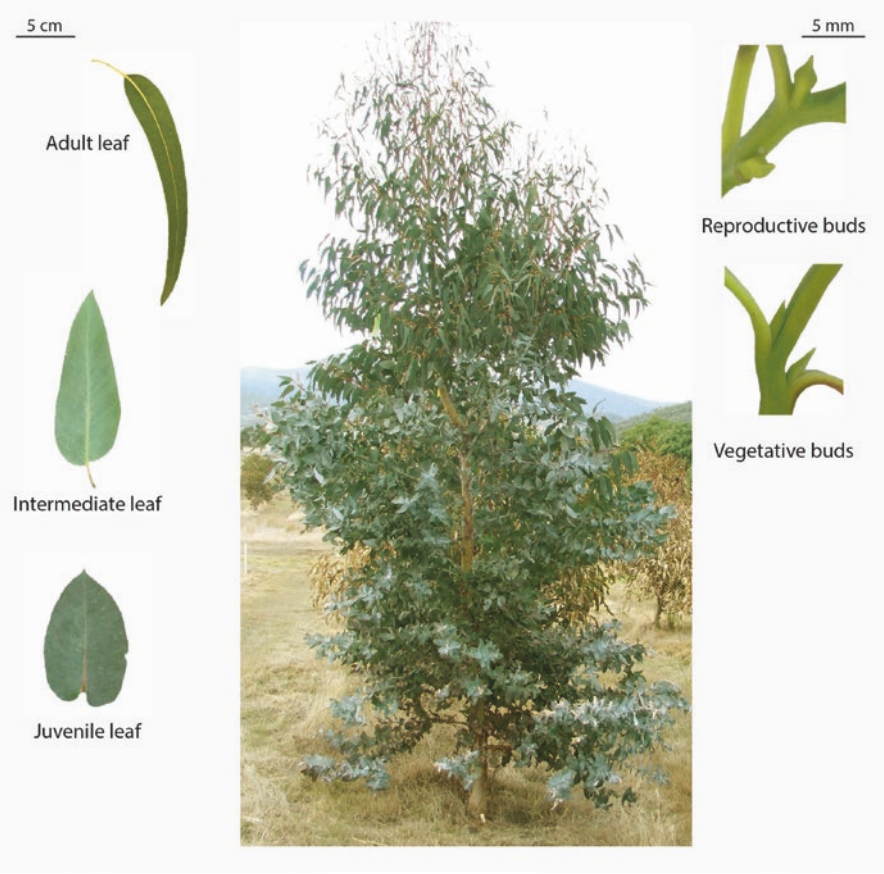

Fig. 1 Vegetative and reproductive phase change in *Eucalyptus globulus*. There is a dramatic morphological difference between leaves collected from the upper (adult), middle (intermediate) and lower (juvenile) canopies: juvenile leaves are horizontally oriented, sessile, opposite and glaucous, and are carried on square stems, while adult leaves are vertically oriented, petiolate, alternate and shiny green, on cylindrical stems. Vegetative buds are terminal as well as axillary, while reproductive buds are only borne in the leaf axils. Most eucalypts are only reproductive after transitioning to the adult vegetative form, but these processes are independently regulated and some eucalypts are reproductive in the juvenile leaf phase (Tree and leaf photos: Robert Wiltshire, Corey Hudson)

evidence that the vegetative and reproductive transitions are independently regulated, including in forest trees such as eucalypts (Jaya et al. 2010; Jordan et al. 1999; Wiltshire et al. 1998). Several species from diverse eucalypt lineages reach the adult reproductive phase while bearing only juvenile foliage in the canopy (Potts and Wiltshire 1997) including *Eucalyptus risdonii* which reproduces in the juvenile vegetative phase, and may be the product of relatively recent changes in developmental timing (heterochrony) from the closely related *E. tenuiramis* which reproduces in the adult leaf phase (Turner et al. 2000; Wiltshire et al. 1991). Conversely, there can be a considerable delay between vegetative and reproductive phase change in some trees: most *Acacia* species progress from the juvenile to adult vegetative stage early in seedling development (Gardner et al. 2008), years before reaching reproductive competency. The uncoupling of these processes in many forest trees provides a unique opportunity to study these transitions independently (Jameson and Clemens 2015). Since the vegetative and reproductive phase changes coincide in many plants, some authors conflate these two transitions, but in this review the two processes will be treated separately.

Vegetative Phase Change

Vegetative phase change in trees can involve dramatic differences in leaf morphology. For example, juvenile and adult leaves are well differentiated in many *Acacia* species: juvenile leaves are horizontally oriented, bipinnate and compound, while adult "leaves" are vertically oriented phyllodes (Kaplan 1980). The differences between juvenile and adult leaf forms are also striking in the Australian eucalypts, with juvenile and adult plants of *Eucalyptus globulus* contrasting so markedly (James and Bell 2001) that they have even been mistaken for different species (Maiden 1913). In *E. globulus*, juvenile leaves are horizontally oriented, sessile, opposite and glaucous, and are carried on square stems, while adult leaves are vertically oriented, petiolate, alternate and shiny green, on cylindrical stems (Brooker and Kleinig 2006) (Fig. 1). New Zealand also has a high proportion of distinctly heteroblastic trees, particularly those with a divaricate form in the juvenile state (Cockayne 1911; Jameson and Clemens 2015). Northern hemisphere forest trees such as poplar and oak also have differences in juvenile and adult leaf morphology (Wang et al. 2011) as do trees of horticultural interest such as olive (Moreno-Alias et al. 2009) and apple (Xing et al. 2014), though differences are in many cases more subtle and less abrupt than the southern hemisphere examples. For example, cottonwood leaves become gradually wider with longer petioles through development (Kearsley and Whitham 1998). The clear differences between juvenile and adult forms of many forest trees make them ideal candidates for studying the genetic control of vegetative phase transitions. However, reviews on the molecular control of developmental phase transitions in plants (Huijser and Schmid 2011) regularly highlight the lack of studies in woody plants, where vegetative phase change is most obvious and has the most practical significance (Wendling et al. 2014).

While the distinction between juvenile and adult vegetative stages is most obvious in the case of leaf forms, there are also important changes in many other aspects of morphology, biochemistry and physiology (reviewed in Wendling et al. 2014; Hackett 1985). For example, there is a general trend towards lower rates of photosynthesis as trees age (Bond 2000) in trees such as *Eucalyptus occidentalis* (Jaya et al. 2010), *Metrosideros excelsa* (Kubien et al. 2007) and *Acacia melanoxylon* (Brodribb and Hill 1993), though there are exceptions to this trend (Zotz et al. 2011). The reduced rooting ability in mature vegetative stages is well documented (Wendling et al. 2014) as are changes in wood properties of economic interest, such as fibre/tracheid length, microfibril angle and lignin content which result in higher density, stiffness and strength in mature wood compared with juvenile wood (Kumar et al. 2009). Differences in leaf defensive chemistry between developmental stages have been documented in eucalypts (Goodger et al. 2007; O'Reilly-Wapstra et al. 2007) and poplar (Rehill et al. 2006) and given that leaf chemistry can influence mammal browsing, decomposition rates and nitrogen cycling, these differences can have broad ecological implications.

Environmental and Hormonal Regulation

The juvenile-to-adult vegetative transition in forest trees has a genetic basis (Hudson et al. 2014; James and Bell 2001; Jaya et al. 2010; Jordan et al. 2000; Wiltshire et al. 1998; Hackett 1985) although there are interactions with a variety of environmental conditions including hormonal and nutritional factors (Hackett 1985). However, these environmental factors mainly appear to act by stimulating growth, as the size (i.e. node number), rather than the age of the shoot is more important in determining timing of phase change (Wiltshire and Reid 1992; Hackett 1985). Gibberellins (GAs) promote vegetative phase change in Arabidopsis and maize (Telfer et al. 1997; Evans and Poethig 1995) but their effects are variable in other species, including trees (Hackett 1985). Recent research in Arabidopsis and other model species, however, provide strong evidence that sugars produced by photosynthesis have a regulatory role that ensures that vegetative phase change occurs in favourable conditions (Yang et al. 2013; Yu et al. 2013, 2015). Though there is no direct evidence for this yet in trees, observations that pruning and defoliation increase the production of juvenile leaves in some trees (Libby and Hood 1976; Schaffalitzky De Muckadell 1954; Jaya et al. 2010) and that phase change is correlated with the cumulative amount of light received by the shoot (Wiltshire and Reid 1992) are consistent with a role for photosynthates.

Insights from Population, Functional and Comparative Genomics

Recent breakthroughs in the understanding of vegetative phase change in plants have defined a pathway with a central role for the microRNA (miRNA) miR156 (Poethig 2013). Expression of this plant-specific miRNA is high in seedlings and

decreases during development. It controls vegetative phase change in Arabidopsis and maize by downregulating a family of plant-specific transcription factors: the *SQUAMOSA PROMOTER BINDING-LIKE (SPL)* gene family (Poethig 2009; Wu et al. 2009), which go on to affect various aspects of plant development (Preston and Hileman 2013). SPL transcription factors also regulate flowering indirectly, by promoting the expression of miR172, which promotes flowering by supressing several AP2-like transcription factors that inhibit expression of the floral inducer *FLOWERING LOCUS T* (*FT)* (Zhu and Helliwell 2011). In this way, vegetative phase change and reproductive phase change are linked, but flowering is controlled by a complex pathway (see below) that can be altered independently of miR156 (Poethig 2013; Wang 2014), which perhaps explains the uncoupling of these processes in some forest trees described above.

MiR156 is present in all land plant groups (Axtell and Bowman 2008), and its function in vegetative phase change has been demonstrated in a number of taxa (Poethig 2013). Evidence suggests that miR156 and *SPL* genes are involved in vegetative phase change in angiosperm trees such as *Acacia*, *Quercus* and *Populus* (Wang et al.), *Eucalyptus* (Hudson et al. 2014; Wang et al. 2011), *Olea* (Garcia-Lopez et al. 2014) and *Malus* (Xing et al. 2014; Sun et al. 2013) and also gymnosperms such as *Sequoia* (Chen et al. 2013). Though most of the evidence in trees is correlative rather than functional, research using the model trees *Populus* and *Eucalyptus* in particular implicate miR156 in the genetic control of vegetative phase change. In both taxa, miR156 and *SPL* expression was correlated with vegetative phase: miR156 was highly abundant in juvenile leaves and was expressed at much lower levels in adult leaves, while two miR156 targets, poplar and eucalypt homologs of *SPL3* and *SPL9* were upregulated in adult plants indicating that changes in miR156 expression have functional significance (Wang et al. 2011). In addition, overexpression of miR156 in poplar drastically prolonged the juvenile stage, and reduced the expression of miR172 and homologs of *SPL3* and *SPL9* (Wang et al. 2011). In *Eucalyptus*, Hudson et al. (2014) identified a miR156 precursor gene near a major quantitative trait locus (QTL) for the large difference in the timing of the transition between early phase change and normal tree forms of *E. globulus*, and expression analyses indicated that this precursor gene was differentially expressed in trees with normal and precocious phenotypes. This QTL also co-located with a QTL for node of first flowering (Hudson et al. 2014), further implicating miR156 as it is involved in both vegetative and reproductive phase transitions in Arabidopsis and other plants (Wang 2014). This suggests that changes in this gene could have played a role in differentiating the precocious and normal ecotypes of this species, and may be a means of rapid heterochronic evolution in this genus.

Early hypotheses suggested that the long juvenile phase of trees could be explained by epigenetic processes such as DNA methylation, with a gradual increase in methylation explaining the corresponding changes in gene expression as trees age (Poethig 1990). However, patterns of methylation between juvenile and adult material vary among studies, with some reporting higher methylation in the adult stage compared with the juvenile stage and others reporting the opposite (Wendling et al. 2014). These discrepancies might reflect that many of the studies were conducted

on *in vitro* material, and thus confounded by stress conditions. However, the overall pattern of increased DNA methylation from juvenile to mature plants does appear to be correlated with phase change in *Eucalyptus* (Mankessi et al. 2011) and some conifers (Fraga et al. 2002; Valledor et al. 2010; Monteuuis et al. 2008). Moreover, increased levels of histone H3 lysine 27 trimethylation, a repressive chromatin modification, in *MIR156* genes is associated with their downregulation during vegetative phase change in Arabidopsis (Xu et al. 2016). Thus, the role of such epigenetic mechanisms in regulating the expression of *MIR156* genes during maturation in trees is worthy of further investigation.

Reproductive Phase Change

Most seedlings enter a juvenile phase during which they are incompetent to flower (Amasino 2010). In trees, reproductive maturation is considered to be the onset of first flower bud initiation (measured in terms of node number or age) and this has been shown to be under strong genetic control in *Eucalyptus* (Jordan et al. 1999; Wiltshire et al. 1998) and in fruit trees such as apple (Visser 1965), almond (Dicenta et al. 1993) and peach (Hansche 1986). However, in adult trees, meristems usually only commit to flowering during a limited seasonal time, suggesting that there is a recurring seasonal acquisition of competency to flower. As discussed in more detail below, studies suggest that maturation and the seasonal timing of the floral transition in adult trees might not be regulated by completely independent mechanisms.

Environmental and Hormonal Regulation

As in annual species, environmental variables such as photoperiod, vernalization (a prolonged period of chilling temperatures) and nutrition affect flowering onset though these may result in contrasting flowering responses to those observed in annual plants. While flowering onset is often photoperiod-dependent in annual plants, this has rarely been demonstrated in trees (Wilkie et al. 2008); however, 16 h photoperiods induced precocious flowering in *Eucalyptus occidentalis* seedlings (Bolotin 1975). Extended photoperiod also reduced juvenility in apple (Aldwinckle 1975). In a number of tree species such as eucalypts, vernalization promotes precocious flowering (Meilan 1997; Moncur 1992; Moncur and Hasan 1994; Wilkie et al. 2008). There is also evidence that improved mineral nutrition induces tree flowering, though this may occur mainly via stimulating faster growth as size is often correlated with first onset of flowering (Meilan 1997). Dwarfing rootstocks are widely used in fruit tree breeding and commercial cultivation to shorten the juvenile period, reduce vegetative growth and increase flowering of the scion (Warschefsky et al. 2016). Grafting affects transport of water nutrients and hormones, responses to hormones and movement of proteins and RNA.

In contrast to many annual plants, GA inhibits or has little effect on flowering onset in many woody angiosperms and the GA-inhibitor paclobutrazol is often used to stimulate flowering in trees of commercial importance (Meilan 1997). In *Eucalyptus globulus*, the application of paclobutrazol stimulated flowering 3 years earlier than would be expected under natural conditions, on seedlings that were still bearing juvenile foliage (Hasan and Reid 1995). In contrast, paclobutrazol application did not shorten the juvenile phase in *Populus deltoides* (Yuceer et al. 2003a). Floral bud-bearing dormant branches from adult trees are rooted in soil for *Populus* breeding, but phytomers initiated after rooting revert to juvenility. However, a combination of paclobutrazol, root pruning and water stress induced newly formed inflorescences on rooted cuttings from mature *P. deltoides*, suggesting that rejuvenation was at least partially prevented (Yuceer et al. 2003a). Genetic manipulation also suggests that hormones play a role in reproductive maturation. Disruption of GA or ethylene signaling by introduction of a dominant mutant allele induced early flowering in *Populus* and birch, respectively (Table 1) (Ruonala et al. 2006; Zawaski et al. 2011).

Insights from Population, Functional and Comparative Genomics

In Arabidopsis, competency to respond to conditions promoting flowering is acquired through the age-related decline in miR156 and many ecotypes also require vernalization. Vernalization reduces the level of *FLOWERING LOCUS C (FLC),* a repressor of flowering. Both miR156 and FLC act directly and indirectly on genes promoting flowering. Thus, decreasing levels of miR156 and FLC increase the plant's ability to respond to flowering promoting signals. *Antirrhinum CENTRORADIALS (CEN*) and its Arabidopsis homolog *TERMINAL FLOWER1 (TFL1*) promote indeterminate growth and differences in *CEN/TFL1* expression were proposed to contribute to the diversity in plant growth habits (Bradley et al. 1997; Ratcliffe et al. 1998). Experimental work and modelling support that the ratio of TFL1 and the floral promoter FT is a major determinate of vegetative versus flowering fate (Jaeger et al. 2013; Lifschitz et al. 2014). Downregulation of *TFL1* homologs in *Populus* and Rosaceae species induced earlier first onset of flowering (Freiman et al. 2012; Kotoda et al. 2006; Mohamed et al. 2010). Ample evidence confirms the role of *FT* genes as activators of flowering in woody perennial plants; overexpression of either endogenous or heterologous *FT* genes resulted in extremely early flowering in diverse taxa (Table 1). *FT* delivery by plant viral vectors resulted in extreme precocity in apple and pear (Yamagishi et al. 2016) and the *FT*-mediated precocity has been harnessed for a rapid breeding scheme (Wenzel et al. 2013; Srinivasan et al. 2012). A transcriptomic study detected significant increases in *FT* and other flowering time gene homologs in *Malus* dwarfing rootstock accessions relative to more vigorous genotypes (Foster et al. 2014). An interstock is often sufficient to induce precocious flowering, suggesting that the signal may be vascular derived. Two quantitative trait loci (QTL), Dw1 and Dw2, are primarily responsible for rootstock-induced dwarfing and precocity in apple (Foster et al. 2015) and a

Table 1 Genes affecting phenology and maturation in woody plants

Gene family	Gene[a]	Genus[b]	Loss-of-function		Gain-of-function phenotype	References
			Type	Phenotype		
	MIR156	*Populus*			Prolonged juvenile vegetative phase	Wang et al. (2011)
PHY	*AsPHYA*	*Populus*	Antisense	Altered circadian rythyms; earlier SDI bud set	Delayed SDI bud set	Olsen et al. (1997); Kozarewa et al. (2010)
SANT/MYB	*LHY1/LYH2*	*Populus*	RNAi	CDL & freezing tolerance reduced; delayed bud flush		Ibanez et al. (2010)
PRR	*TOC1*			CDL for growth cessation reduced		
PEBP	*FT1*	*Populus*	RNAi	SDI budset accelerated (*FT1/FT2* knockdown)	Precocious flowering (WT-like inflorescences); delayed SDI budset	Bohlenius et al. (2006); Hsu et al. (2006, 2011)
PEBP	*FT2*	*Populus*			Precocious single flowers; delayed SDI bud set	
PEBP	*FT*	*Actinidia*			Reduced vigor and secondary growth; shoot-tip abortion	Varkonyi-Gasic et al. (2013)
PEBP	*FT*	*Jatropha*			Flowering in WT scion grafted on precocious flowering transgenic rootstock	Ye et al. (2014)
PEBP	*FT*	*Poncirus*			Precocious flowering	Endo et al. (2005)
PEBP	*FT*	*Vaccinium*			*In vitro* flowering	Song et al. (2013)
PEBP	*FT*	*Malus*			*In vitro* flowering	Kotoda et al. (2010)

(continued)

Table 1 (continued)

Gene family	Gene[a]	Genus[b]	Loss-of-function		Gain-of-function phenotype	References
			Type	Phenotype		
PEBP	*AtFT, PtFT1*	*Malus*			Precocious flowering	Yamagishi et al. (2011); Wenzel et al. (2013)
PEBP	*PcoFT2*	*Malus*			Delayed dormancy and leaf senescence in SD/LT conditions	Freiman et al. (2015)
PEBP	*AtFT, PtFT1*	*Eucalyptus*			Precocious flowering	Klocko et al. (2016a)
PEBP	*CEN1*	*Populus*	RNAi	Earlier, more prolific flowering; reduced depth of dormancy	Delayed dormancy release/ bud flush	Mohamed et al. (2010)
PEBP	*TFL1*	*Malus*	Antisense VIGS	Precocious flowering		Kotoda et al. (2006); Sasaki et al. (2011a)
PEBP	*TFL1*	*Pyrus*	RNAi	Precocious flowering		Freiman et al. (2012)
PEBP	*TFL1*	*Pyrus*	VIGS[c]	Precocious flowering		Yamagishi et al. (2016)
bZIP	*FDL1*	*Populus*	RNAi	Slightly earlier SDI bud set	Delayed SDI budset	Tylewicz et al. (2015)
bZIP	*FD2*[d]	*Populus*			Precocious flowering; small leaves, many branches; SDI bud set delayed	Parmentier-Line and Coleman (2016)
AP1/FUL (MADS-box)	*MADS4, AP1*	*Betula*			Precocious flowering	Elo et al. (2007); Huang et al. (2014)

Gene family	Gene[a]	Genus[b]	Loss-of-function		Gain-of-function phenotype	References
			Type	Phenotype		
AP1/FUL	*BpMADS4*	*Malus*			Precocious flowering	Flachowsky et al. (2007); Weigl et al. (2015)
AP1/FUL	*BpMADS4*	*Populus*			Delayed budset under SD/LT regime	Hoenicka et al. (2008)
AP1/FUL	*LAP1*	*Populus*	RNAi	SDI growth cessation slightly accelerated	Delayed SDI bud set	Azeez et al. 2014
AP1/FUL	*AtAP1*	*Citrus*			Precocious flowering	Pena et al. (2001)
LFY	*AtLFY*					
LFY	*PtFL*	*Populus*	RNAi		Small inflorescences; sterile flowers	Klocko et al. (2016b)
AP2/ERF	*AIL1, AIL3*	*Populus*			Delayed SDI bud set	Karlberg et al. (2011)
AP2/ERF	*PpCBF1*	*Malus*			Increased freezing tolerance; SDI dormancy & leaf senescence in daylength insensitive *Malus*	Wisniewski et al. (2011)
AP2/ERF	*EBB*	*Populus*	ami	Delayed bud flush	Early bud flush; increased SAM cell division	Yordanov et al. (2014)
SVP/StMADS11	*DAM1–6*	*Prunus*	Deletion	Continuous growth under SDs		Bielenberg et al. (2008)
SVP/ StMADS11	*PpDAM6*	*Populus*			Bud set under LDs	Sasaki et al. (2011b)

(continued)

Table 1 (continued)

Gene family	Gene[a]	Genus[b]	Loss-of-function		Gain-of-function phenotype	References
			Type	Phenotype		
SVP/ StMADS11	*SVP3*	*Actinidia*			Floral development period extended	Wu et al. (2014)
GA20ox	*AtGA20ox*	*Populus*	Antisense	Earlier SDI bud set	Delayed SDI bud set	Eriksson et al. (2015)
GA2ox	*PcGA2ox*	*Populus*			Greater rate of growth reduction in SDs, delayed leaf senscence; delayed spring bud flush	Zawaski et al. (2011); Zawaski and Busov (2014)
DELLA	*At-gai*[e]				Greater rate of growth reduction in SDs & early flowering	
B3	*ABI3*	*Populus*	Antisense	SDI buds have more and larger bud scales	SDI buds remain open with enlarged embryonic leaves	Rohde et al. (2002)
Ethylene receptor	*At-etr1–1*	*Betula*	DN allele	SDI bud development disrupted, leaf senescence, dormancy delayed; early flowering		Ruonala et al. (2006)

SDI short day-induced, *LT* low temperature, *CDL* critical daylength, *DN* dominant negative, *ami* artificial miRNA, *WT* wild-type

[a]Unless indicated, gain-of-function transgenes use gene sequences from the recipient taxa for transformation. Prefixes used for genes from taxa that are different from the transgenic taxa: *As-Avena sativa ; Bp-Betula pendula; At-Arabidopsis thaliana; Pc-Phaseolus coccineus; Pco-Pyrus communis; Pp-Prunus persica; Pt-Populus trichocarpa*

[b]Taxa with identified mutation or used for genetic transformation

[c]Concurrent expression of *AtFT* and silencing of *TFL1* using a viral vector

[d]Tylewicz et al. (2015) and Parmentier-Line and Coleman (2016) named different genes *FDL1* and *FD1*, respectively. Thus, to avoid confusion, we have renamed "*FD1*" to "*FD2*"

[e]GA insensitive allele

locus syntenic to apple Dw1 controls dwarfing in pear (Knabel et al. 2015), but the molecular nature of Dw1 and Dw2 has not yet been identified.

Early onset of flowering has been induced in trees by other flowering time gene homologs (Table 1). Overexpression of *Populus FD2*[1] resulted in precocious flowering in *Populus* (Parmentier-Line and Coleman 2016), consistent with the conserved floral-promoting role of the FT/FD-containing complex in diverse annual plants (Taoka et al. 2013). This complex directly or indirectly activates additional flowering time and floral meristem identity genes, including *LEAFY (LFY)* and the related MADS-box genes *APETALA1 (AP1)* and *FRUITFUL (FUL)*. However, unlike *FT*, their sufficiency for inducing early flowering onset is more variable among species. For example, *AP1* and *LFY* homologs induced early flowering in citrus and birch (Elo et al. 2007; Huang et al. 2014; Pena et al. 2001). In contrast, *LFY* only induced individual flowers rather than inflorescences in certain *Populus* genotypes and *Populus LFY (PtFL)* did not induce flowering in any of the clones tested nor did *AP1/FUL* homologs (Azeez et al. 2014; Hoenicka et al. 2008; Rottmann et al. 2000; Weigel and Nilsson 1995).

Induction of early flowering by overexpressing a floral promoter does not necessarily indicate that these genes regulate phase change. It might be that the abnormally high level of a flowering promoter overcomes the threshold set by repressive mechanisms. However, there are some indications that flowering promoters, such as *Populus FT* homologs, show both seasonal (see section "Phenology") and age/size-related expression changes, suggesting they could have roles in both phase change and seasonal flowering time (Bohlenius et al. 2006; Hsu et al. 2006, 2011). In addition, experiments with a heat-shock promoter driving *FT* expression in *Populus*, revealed a positive correlation between plant height and flowering (Zhang et al. 2010). A recent study reported efficient induction of precocious fertile flowers in *Populus* by combining inducible *FT* expression with a subsequent vernalization treatment (Hoenicka et al. 2016). Considered together these studies perhaps reflect that reproductive maturation and the seasonal induction of flowering share some regulatory components or that seasonal regulation imposes limits on age-related reproductive competency. Moreover, interconnections between reproductive phase change and phenology appear to vary among taxa.

There has also been some success in inducing flowering through DNA demethylation of flowering genes in citrus (Zhang et al. 2014). The earliest flowering Arabidopsis mutants are *embryonic flower1* (*emf1*) and *emf2* (Bratzel and Turck 2015). These genes encode components of POLYCOMB REPRESSIVE COMPLEXES (PRCs) that mediate gene repression via histone modification and EMF1 is needed to maintain repression of *SPLs* and *miR172* genes during the juvenile phase. As discussed above for vegetative phase change, studying the epigenetic changes associated with specific genes could provide insight into the mechanisms controlling reproductive maturation in trees.

[1] Tylewicz et al. (2015) and Parmentier-Line and Coleman (2016) named different *Populus* genes *FDL1* and *FD1*, respectively; to avoid confusion we have renamed *FD1* to *FD2*.

Phenology

The ability to control the timing of critical developmental transitions provides immobile plants with a mechanism to cope with intra-annual variation in abiotic and biotic conditions. Phenology is a crucial dimension of plant survival and reproductive strategies. Longevity and the large, persistent shoot systems of trees pose challenges to vegetative and reproductive growth and development not experienced by herbaceous plants. Vegetative phenology has been most extensively studied in temperate and boreal trees, where dormancy enables survival through the winter. In these climates, dormancy is also part of reproductive phenology. The seasonal timing of growth and dormancy transitions are closely matched to local climates to effectively balance the trade-offs of optimizing growth and avoiding frost injury. The timing of flowering affects fruit and seed number, quality, exposure to herbivory and success of pollination. The timing of fruit development and ripening determines the efficiency of seed dispersal. Thus, reproductive phenologies are key determinants of plant fitness (Kozlowski 1992).

Locally adapted phenology involves selection of alleles engaged in the perception and transduction of environmental signals that serve as reliable markers of seasonally-associated changes. The environmental signals regulating vegetative and flowering phenology in temperate zones are generally the same. In their review on flowering time, Bernier and Perilleux (2005) identified photoperiod and vernalization as the most reliable signals for monitoring seasonal time in temperate climates and thus, usually the primary signals regulating phenology. Less predictable factors modulate the effects of the primary signals and in some cases, these can substitute for the primary signal. In addition, the relative contribution of a given environmental cue varies between and within species. As discussed in more detail below, this is also the case for vegetative phenology. Although the ecological drivers for phenological patterns are different, there is also evidence suggesting that some of the same signals are used by some tropical trees.

Short-day (SD)-induced growth cessation and dormancy induction have been studied more than other seasonal transitions from a molecular perspective, particularly in *Populus* (Table 1) (Ding and Nilsson 2016; Petterle et al. 2013). These studies have revealed that homologous genes control photoperiodic regulation of growth and dormancy transitions in trees and flowering time in plants. The similar regulatory modules controlling vegetative and flowering phenology reflect their shared environmental cues. However, in an adult tree, vegetative and floral seasonal transitions are not always coincident or controlled by the same environmental cue. Although we are only beginning to decipher the genetic regulation of tree phenology, results to date hint that gene duplication and divergence could contribute to the separation of environmental signaling controlling vegetative versus flowering phenology. It has been nearly impossible to definitively differentiate the endogenous function of related genes in trees. Paralogous genes often encode proteins with some degree of functional equivalency and a transgene needs to have a dominant effect in trees where breeding to homozygosity is not feasible. It may not be possible to select a distinct gene fragment for RNA-mediated downregulaton (RNAi or

antisense transgenes), and even if the fragment is highly distinct, transitive effects can lead to downregulation of paralogs (Van Houdt et al. 2003). Fortunately, genome-editing approaches can now circumvent this limitation and efficiently induce gene-specific biallelic or homozygous mutations in trees (Zhou et al. 2015).

Vegetative Phenology

Woody plants recurrently suspend and resume apical and lateral growth in response to environmental and endogenous signals. Apical bud and cambial growth cessation can be induced by sporadic events of stress, but are typically associated with seasonal climatic patterns. As discussed in recent reviews (Cooke et al. 2012; Olsen 2010; Rohde and Bhalerao 2007), the terminology used to describe 'lack of visible growth' varies and the widely adopted definitions of Lang (1987) are problematic in light of advances in our understanding of dormancy. In this review, we use 'dormancy' to refer to a meristem that is insensitive to growth promoting conditions until it is released from dormancy by an environmental cue, and the quantitative nature of this phase is described by the term 'depth of dormancy'.

In trees of temperate and boreal climates, growth cessation and apical bud set precede entry into dormancy. Some species, however, do not form apical buds and the shoot apex is abscised, which in some cases is an age-related phenomenon (Junttila 1976). The pattern of growth and rest during a growing season varies among taxa (Fig. 2) and among shoots within a tree. A dormant bud contains a preformed shoot with a number of leaves and leaf primordia and axillary meristems are often already formed in the older leaves (Cooke et al. 2012; van der Schoot et al. 2014). An annual shoot can consist of only preformed phytomers or the shoot can continue to elongate by producing neoformed phytomers (i.e., leaves initiate and expand within a growing season). Free-growing species such as *Populus* spp., and *Betula* spp. can continue neoformed growth until their critical daylength (CDL) for growth cessation and dormancy is perceived. However, as these trees increase in size, the crowns contain an increasing proportion of short shoots consisting of only preformed phytomers as well as shoots that have ceased neoformed growth before the CDL (Critchfield 1960). A fixed growth pattern, where annual shoots consist of only preformed phytomers has been reported for some angiosperm trees; however, there is uncertainty as to whether this is strictly accurate or alternatively, that they lose the capacity for neoformed growth with age/size or other factors (Pallardy and Kozlowski 2008). Several species of oak, including *Quercus rubra* and *Q. robur*, display rhythmic growth with successive phases of shoot apical meristem (SAM) rest and activity during the growing season (Farmer 1975; Kuster et al. 2014). Trees grown in controlled environments, including both with and without the nutritional benefits provided by association with mycorrhizal fungi, display this rhythmic pattern (Herrmann et al. 2015). Thus, oak's rhythmic growth is endogenously controlled. However, the number, amplitude and frequency of growth and rest oscillations are influenced by environmental factors and age/size (Bobinac et al. 2012; Farmer 1975; Kuster et al. 2014).

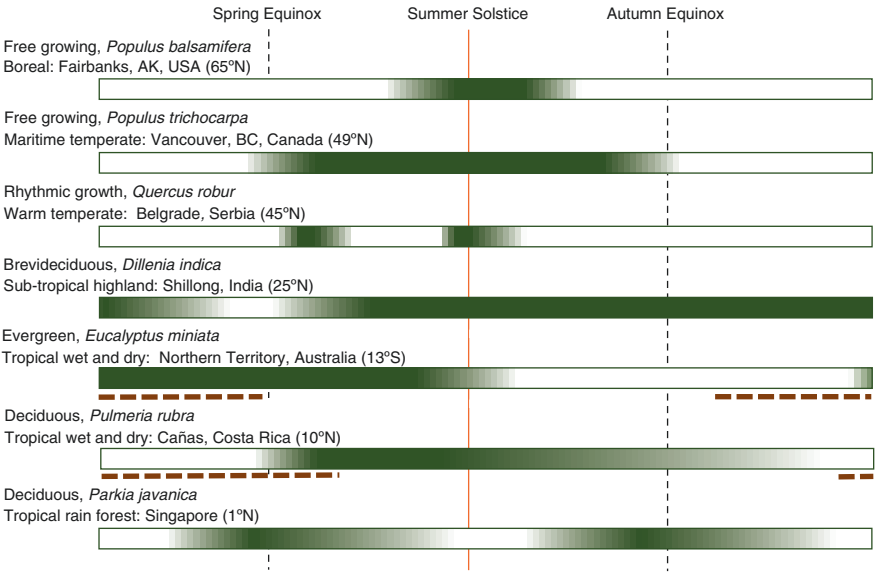

Fig. 2 Examples of annual growth patterns in tropical to boreal trees. *Shaded areas* indicate growth and *white areas* represent periods of rest or dormancy. The bars start with January 1 for northern latitudes and July 4 for southern latitudes. Climate descriptions are based on Köppen classifications. *Horizontal dotted lines* indicate a dry season. The patterns are derived from the reports of Olson et al. (2013) (*P. balsamifera*), McKown et al. (2014) (*P. trichocarpa*), Bobinac et al. (2012) (*Q. robur*), Venugopal and Liangkuwang (2007) (*D. indica*), Williams et al. 1999 (*E. miniata*); Borchert and Rivera (2001) (*P. rubra*), and Calle et al. (2010) (*P. javanica*)

The seasonal development of tropical trees is often characterized by distinct periods of rest and shoot growth (flushing). Patterns range from fully deciduous species that have an extended leafless period to evergreen taxa that have periods of leaf exchange (Borchert and Rivera 2001). Similar to winter dormancy in temperate trees, cambial inactivity has been correlated with leaf shedding and leafless periods (Morel et al. 2015). For some tropical species, shoot phenology is irregular or does not show seasonal variation (e.g., evergreens that lose a small proportion of their leaves each month), but many species show predictable annual patterns. Survey of a large number of tropical species at diverse sites found two main patterns of leaf flushing (Borchert et al. 2015; Calle et al. 2010). At equatorial sites there were two distinct flushes per year, whereas at and above 10° latitude, there was only one leaf flush (Fig. 2). Some tropical species, such as *Theobroma cacao*, appear to show endogenously controlled rhythmic growth similar to oak (Greathouse et al. 1971). In a 100-day-long constant environment study, *T. cacao* exhibited nearly four growth-rest cycles.

Trees exhibit changes in morphology, physiology and biochemistry that are adaptations to seasonal conditions. Some changes occur during each growing season. One of the most common growing season variations is in wood development. In temperate climates, earlywood develops after growth resumes in spring and is characterized by vessels with large lumens. Subsequent latewood is denser and has

smaller diameter vessels; thus, it is less vulnerable to embolism induced by water stress, which is more likely to occur in summer (Plomion et al. 2001). This annual pattern is generally consistent year to year, but xylem anatomy also shows plasticity and variation in characteristics such as maximum latewood density is used to reconstruct climatic variation (Fonti et al. 2010). Some tropical species also produce earlywood and latewood; for example, *Tectona grandis* (teak) growing in the tropical wet and dry climate of East Timor (Cardoso et al. 2015). Secondary metabolites that are associated with plant defense have been shown to change with leaf development or seasonal time in *Populus*, *Juglans* and *Eucalyptus* (Holeski et al. 2012; Simmons and Parsons 1987; Solar et al. 2006).

In temperate and boreal trees, the transitions of shoot apical and cambial meristems into and out of dormancy take place over a number of weeks and are integrated with a suite of processes with overlapping phenologies (Fig. 3). Seasonal changes in development, cell structure and physiology enable survival during the winter and resumption of growth in spring. A number of cellular changes enable meristems and other tissues to survive the prolonged freezing and dehydration stress of winter (Strimbeck et al. 2015; Welling and Palva 2006; Wisniewski et al. 2014). These include osmotic adjustment, desaturation of membranes and changes in the cell's ultrastructure. The vast majority of temperate and boreal angiosperm trees are deciduous. Autumn leaf senescence is a form of programmed cell death during which proteins are degraded and nitrogen (N) recycled into vegetative storage proteins (VSPs) that are often localized in stems and roots (Keskitalo et al. 2005; Millard and Grelet 2010). Evergreen species also store N in leaves. The stored N is remobilized to actively growing tissues in spring. VSPs also show seasonal patterns in some tropical trees. In *Swietenia macrophylla* (mahogany), VSPs accumulated as new shoots matured and rapidly decreased when the next growth cycle began (Tian et al. 2003). In the wet/dry tropics of northern Australia, bark proteins accumulated during the dry season in a number of species, such as deciduous *Acacia latescens* (Schmidt and Stewart 1998).

Environmental Regulation of Vegetative Phenology

Temperate and Boreal Trees

Photoperiod and temperature are the primary environmental signals regulating most temperate and boreal tree dormancy transitions and associated processes (Fig. 3). Variation in these two parameters along latitudinal and elevational clines are reflected in the strong genetic differentiation revealed by common garden studies (Alberto et al. 2011; Keller et al. 2011). Controlled environment studies of ecotypes have shown that the CDL for bud set and dormancy induction in various angiosperm and gymnosperm species increases with latitude of origin (Bohlenius et al. 2006; Heide 1974; Junttila 1980, 1982). Similar to SD flowering plants, night breaks of white or red light (R), but not R followed by far-red light (FR), disrupt SD-induced growth cessation, cold acclimation and dormancy in a number of woody plants

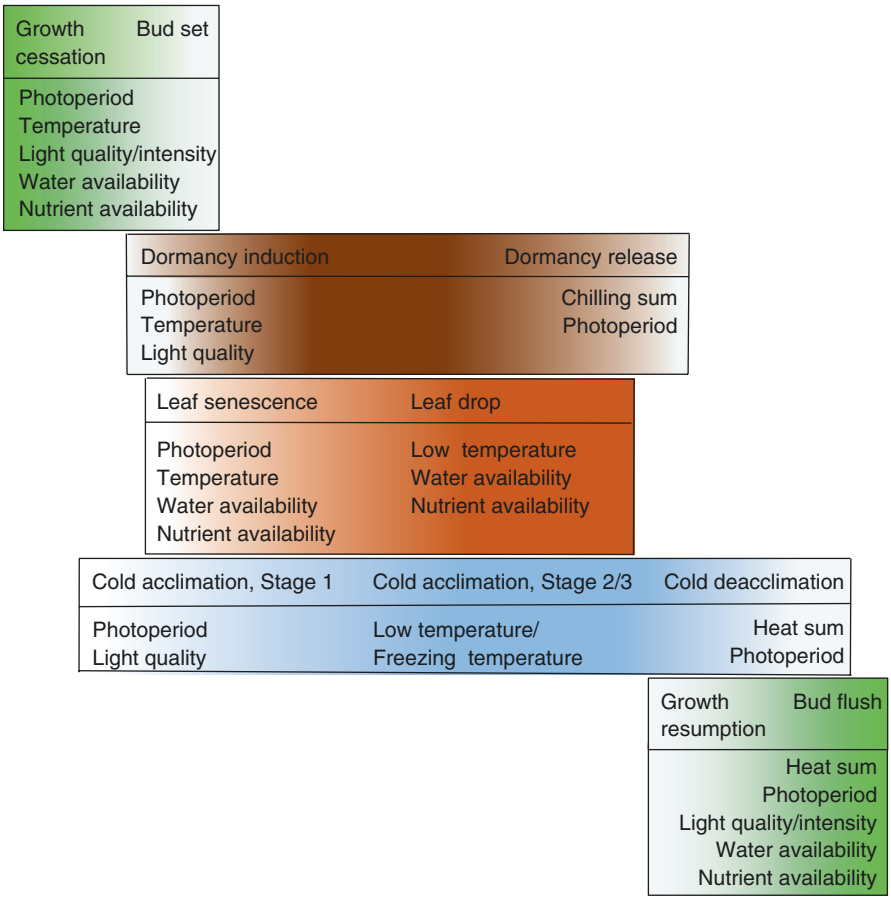

Fig. 3 Environmental signals regulating vegetative phenology. Processes are arranged in a seasonal sequence typical of a free-growing temperate zone species. The primary signals regulating these transitions in most temperate trees are listed first. *Gradient shading* indicates that transitions are gradual and quantitative. See text for further details

(Howe et al. 1996; Nitsch 1957; Williams et al. 1972). R converts PHYTOCHROME (PHY) to a biologically active form (Pfr), and FR reverts it to an inactive form (Demotes-Mainard et al. 2016); hence, these night break experiments implicated PHY in SD-induced bud set. However, studies of high latitude ecotypes suggest that night length monitoring is not always the major mechanism controlling these seasonal development transitions (Luttge and Hertel 2009; Olsen 2010). Trees in the arctic need to set bud at times without a distinct night period. In many plants, particularly shade-intolerant species, stem elongation is enhanced by a low R:FR ratio (Demotes-Mainard et al. 2016). The angle of incidence of solar radiation, and thus light quality and intensity, vary with latitude, time of day and time of year (Linkosalo and Lechowicz 2006). Diurnally, a lower R:FR ratio occurs at twilight, and length

of the twilight period increases with latitude. Moreover, the seasonal changes in twilight length are more pronounced at high latitudes, where twilight lasts past local midnight near the summer solstice. Studies in *Salix pentandra*, and *Picea abies* indicate that the need for FR light to maintain growth increases with latitude of origin (Clapham et al. 1998; Junttila and Kaurin 1985; Molmann et al. 2006). Daylength extension with FR-depleted lamps or monochromatic R LEDs was sufficient to sustain growth in ecotypes from below 60–64°N, but not in ecotypes from more northern latitudes. End of day treatments using different intensities of broad-spectrum light also indicated differences among latitudinal ecotypes of *S. pentandra* (Junttila 1982). Light sensitivity showed an inverse relationship with latitude of origin; the lowest light intensity prevented bud set in only the southernmost ecotype (59°40′N). Thus, light quality or light intensity rather than photoperiod might be a primary signal for trees at high latitudes.

In photoperiodic responsive species, temperature has an important modulating role in growth cessation, dormancy induction and depth of dormancy. Moreover, temperature can substitute for photoperiod in certain cases and it is the primary signal in some species. The temperature responses of high latitude (65–70°N) ecotypes of *Salix pentandra* and *Cornus sericea* differed from more southern ecotypes (Junttila 1980; Tanino et al. 2010). Low night temperature (15/6 °C day/night for *S. pentandra* and 20/5 °C for *C. sericea*) induced growth cessation and dormancy under long daylengths (LDs) only in the northern ecotypes. SD-induction of growth cessation and dormancy in three *Prunus* species was highly dependent on temperature; all trees were unresponsive at 21 °C (Heide 2008). At lower temperatures, responses varied among species. In a wild *P. avium* genotype (59°30′N), growth ceased in response to SDs only under the lowest temperature tested (9 °C). Both *P. cerasus* cultivars responded to SDs at 15 °C, whereas at 9 °C, both *P. insititia* cultivars ceased growth under LDs. In other Rosaceae species, including *Malus pumila, Pyrus communis* and *Sorbus aucuparia*, SDs did not induce growth cessation and dormancy, but temperatures below 12 °C consistently induced dormancy under both LDs and SDs (Heide 2011; Heide and Prestrud 2005). Conversely, relatively warm night temperatures (18.5/13.5 °C day/night versus 18.5/3.5 °C) accelerated SD-induced growth cessation, dormancy and cold hardiness in hybrid poplar clones (Kalcsits et al. 2009). A nuanced study of growth cessation and bud set stages in hybrid poplar families replicated at different temperate latitudes suggested that warmer temperatures delayed growth cessation but accelerated the rate of bud formation (Rohde et al. 2011). In *Betula spp.* and *Alnus glutinosa* seedlings originating from 60–61°N latitudes, depth of dormancy increased with increasing temperatures during the SD treatment (Heide 2003). These studies show that temperature effects are complex and variable among species and ecotypes, in relation to other environmental factors and among phenology stages.

Following dormancy induction, prolonged exposure to low, non-freezing, temperatures (quantified as accumulated low temperature units or chill sum) is needed to release dormancy, although LDs can partially substitute for chilling in some species and ecotypes (Myking and Heide 1995; Zohner and Renner 2015). Release from dormancy often occurs several weeks before bud flush, which is primarily determined

by accumulated warm temperature units (heat sum) in many trees. Conditions during preceding phases influence subsequent phases (Heide 2003; Howe et al. 1999; Junttila and Hanninen 2012; Myking and Heide 1995). For example, temperatures during dormancy establishment affect depth of dormancy and hence the subsequent chilling requirement for dormancy release. As the length of the chilling period increases, days to bud burst under subsequent growth promoting conditions are reduced as is the minimum temperature for bud flush. Although shoot meristems have been studied more than cambial meristems, the dormancy-activity transitions of both meristems are coordinated and influenced by the same environmental signals (Baba et al. 2011; Begum et al. 2013; Espinosa-Ruiz et al. 2004). In daylength-sensitive trees, SDs induce cessation of cambial cell divisions and dormancy, which can be assessed by the ability of applied auxin to stimulate cell division. The cambium gradually regains responsiveness to auxin during chilling. Earlier cambial reactivation can be induced locally by heating a stem portion during winter (Begum et al. 2007).

Photoperiod and temperature also regulate autumn leaf senescence. The most detailed information comes from controlled and natural environment studies of *Populus tremula* (Fracheboud et al. 2009; Keskitalo et al. 2005). SDs trigger the onset of leaf senescence but the CDL for this is different from the CDL for bud set, and bud set appears to be prerequisite for senescence. Low temperature accelerates senescence and is necessary for leaf abscission. Similarly, *Populus* bark storage protein accumulates under SDs or at low temperatures under LDs (Coleman et al. 1992; van Cleve and Apel 1993). SDs induce an increase in cold-tolerance, low temperatures further increase cold-tolerance, and in some species, exposure to freezing temperatures provides an additional enhancement (Welling and Palva 2006). In spring, the level of cold deacclimation increases with duration of warm temperatures and LDs enhance this process (Kalberer et al. 2006; Welling and Palva 2006).

Whereas photoperiod and temperature are typically the primary environmental signals regulating dormancy transitions and associated changes, a number of other factors can induce at least some of these processes (Cooke et al. 2012; Luttge and Hertel 2009; Olsen 2010). For example, water and nutrient deficits can induce leaf senescence, growth cessation and bud set under LDs, reflecting a stress avoidance mechanism. However, how these factors modulate aspects of the primarily light- and temperature-regulated seasonal phenology is unclear. Water stress during a SD treatment reduced shoot elongation in *Betula pubescens*, but seemed to have little effect on dormancy induction and release (Rinne et al. 1994). In a study of potted *Quercus petraea* seedlings, repeated drought and re-watering during summer delayed autumn leaf senescence and spring bud flush (Vander Mijnsbrugge et al. 2016). After 3 years of successively more severe summer drought treatments, *Q. robur*, *Q. petraea* and *Q. pubescens* saplings showed earlier spring bud flush (Kuster et al. 2014). A number of species have longitudinal ranges that differ markedly in seasonal precipitation patterns. Thus, study of longitudinal ecotypes could provide insight into the effects and relative role of water availability on phenology. Freezing temperatures and drought induce similar stresses and plant responses (Cramer et al. 2011). Correspondingly, drought treatments under both LDs and SDs increased freezing tolerance in *Cornus sericea* (Chen and Li 1977).

Tropical Trees

The intra-annual shoot phenology of some tropical species is consistent among years, suggesting that specific changes in environmental variables are being perceived as indicators of seasonal changes. Major portions of the tropics and subtropics have a seasonally dry climate, and competition for resources or biotic interactions might also be drivers for locally adapted phenology. In wet/dry climates, phenology patterns vary among species, perhaps reflecting different adaptations to limited precipitation (e.g., deep roots, succulent stems) (Borchert and Rivera 2001; Vico et al. 2015). Observations that some species flush new growth during the dry season, suggest that water availability is not the primary signal in many cases (Fig. 2) (Borchert and Rivera 2001; Elliott et al. 2006). Moreover, altering natural daylength during different seasonal times indicated that photoperiods below 12 h arrested shoot growth in *Plumeria rubra.*

Recent studies have correlated daily insolation with the phenology of a number of tropical trees across a range of sites (Borchert et al. 2015; Calle et al. 2010; Yeang 2007). Daily insolation is a function of photoperiod and the intensity of irradiation. Thus, at the equator, insolation is only due to the total daily irradiation that depends on the angle of incoming solar radiation and other factors such as cloud cover. Insolation due to solar angle is maximal (irradiation perpendicular at noon) only at the equinoxes. This bimodal pattern of insolation maxima becomes unimodal above 10°N, and this was correlated with the shift from a bimodal to unimodal pattern of phenology in tree species distributed across these latitudes (Fig. 2; Calle et al. 2010). However, other studies have suggested that seasonal cloud cover is more important than solar angle for determining seasonal irradiation patterns and phenology in the tropics (Wright and Van Schaik 1994). In addition, more blue and UV light is received at the equinoxes when radiation passes through a thinner layer of air; in contrast, this effect is much smaller for longer wavelengths. Blue light has long been associated with inhibition of hypocotyl elongation, and alone or in combination with other wavelengths has been shown to increase growth rates, biomass and/or height in several species (Carvalho and Folta 2014). Considered together, these various studies suggest that trees closer to the equator or the arctic circle might primarily respond to the light intensity and light quality characteristics that exhibit the most reliable seasonal changes at their latitudes of origin. However, other factors such as changes in atmospheric humidity leading up to the wet season, are also possible signals.

Molecular Regulation of Vegetative Phenology

Growth Cessation, Dormancy Induction and Cold Acclimation

Perception of changes in daylength involves the circadian clock (Greenham and McClung 2015). Light entrains the clock and also acts on the clock's output pathways. Molecular components of daylength sensing were revealed by studies of

flowering time in Arabidopsis, a LD plant. The transcription factor LATE-ELONGATED HYPOCOTYL (LHY) and transcriptional repressor TIMING OF CAB EXPRESSION 1 (TOC1) are components of the circadian oscillator (Greenham and McClung 2015). Light and the circadian clock regulate a large portion of the transcriptome, including *CONSTANS (CO)*. Under SDs, *CO* expression peaks at night and the CO protein is degraded. Under LDs, *CO* transcription peaks late in the day and the CO protein is able to accumulate and activate *FT* expression. In addition to relaying input to the circadian oscillator, PHYB mediates the degradation of CO in the morning, whereas light-labile PHYA promotes the accumulation of CO late in the day under LDs. This photoperiod sensing occurs in the leaf, and the FT protein is translocated to the shoot apical meristem (SAM), where it promotes the floral transition.

For comparison, we highlight the genes that have also been characterized in trees (Table 1; also see Ding and Nilsson 2016). Constitutive expression of *PHYA* in *Populus* delays SD-induced growth cessation, whereas downregulation results in earlier bud set (Kozarewa et al. 2010; Olsen et al. 1997). *PHYB2* co-localized with QTLs for bud set and bud flush in a hybrid poplar mapping pedigree and two non-synonymous SNPs in *PHYB2* were associated with variation in bud set time in *P. tremula* (Frewen et al. 2000; Ingvarsson et al. 2008). RNA-mediated down regulation of *Populus* orthologs of *LHY* or *TOC* resulted in a shorter CDL for growth cessation (Ibanez et al. 2010). Downregulation of *FT* orthologs delayed and overexpression of *FT1* or *FT2* prevented SD-induced bud set in *Populus* (Bohlenius et al. 2006; Hsu et al. 2011). Study of latitudinal ecotypes under a 19 h daylength, which was below the CDL for northern but not southern ecotypes, showed that the phase of CO_2 expression varied among ecotypes. This resulted in *FT* being induced in southern but not northern ecotypes. However, constitutive expression of *CO* orthologs did not alter time of bud set in field environments, indicating other factors are needed for the SD-response (Hsu et al. 2012).

Hsu et al. (2011) showed that the two *Populus FT* co-orthologs have dramatically different seasonal expression patterns (Fig. 4). *FT2* is expressed in leaves when shoots are elongating and *FT1's* peak of expression occurs during winter. Extensive environmental manipulation of transgenics also supported that *FT2* is the paralog that promotes growth under LDs. Moreover, Evans et al. (2014) associated a SNP in *FT2* with variation in bud set time in *P. trichocarpa*. However, photoperiodic downregulation of *FT2* is not the only control point for time of growth cessation. Under 8 h daylengths, *FT2* was downregulated at the same time in different hybrid poplar clones, but these genotypes ceased growth at different times and displayed temporal differences in downstream transcriptional changes (Resman et al. 2010). *Populus* has three *FD* orthologs and overexpression of either *FDL1* or *FD2* delayed SD-induced budset and increased the expression of members of the *AP1/FUL* subfamily (Parmentier-Line and Coleman 2016; Tylewicz et al. 2015). Correspondingly, overexpression of a birch member of the *AP1/FUL* subfamily or a *Populus AP1* ortholog (*LAP1*) in *Populus* delayed bud set (Azeez et al. 2014; Hoenicka et al. 2008). Moreover, LAP1 binds to the *AINTEGUMENTA-like 1* (*AIL1*) promoter. AIL proteins promote cell proliferation (Horstman et al. 2014) and *Populus* contains 4

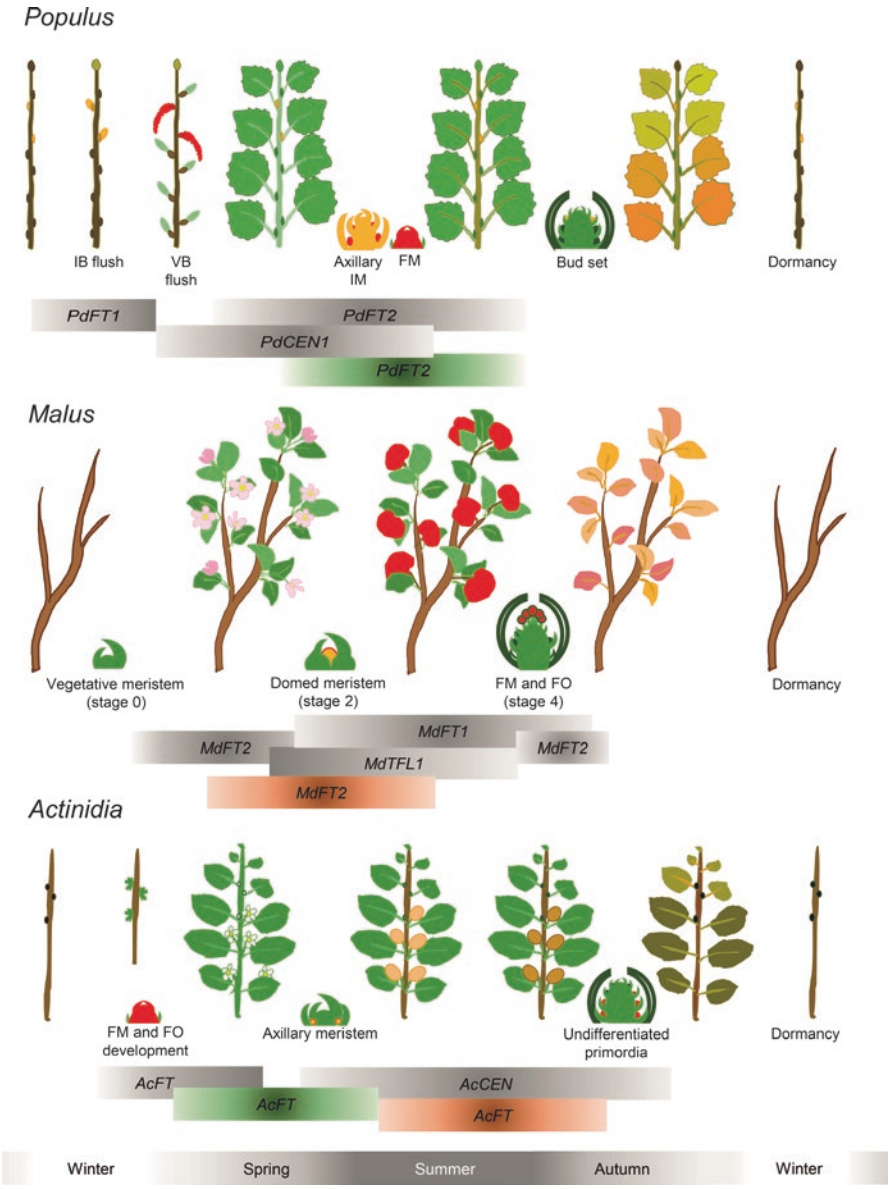

Fig. 4 Cycles of vegetative and reproductive growth correlate with expression of *FT* genes. Areas *shaded grey* indicate expression in terminal buds for *Malus* and *Populus* and axillary buds for *Actinidia*. Areas *shaded green* and *orange* indicate expression in leaves and fruit, respectively. Indicated times of bud and early flower development are approximate and adopted from Yuceer et al. (2003b), Foster et al. (2003) and Walton et al. (1997). Expression patterns are derived from Hsu et al. (2011), Kotoda et al. (2010) and Varkonyi-Gasic et al. (2013). *IB* inflorescence bud, *VB* vegetative bud, *IM* inflorescence meristem, *FM* floral meristem, *FO* floral organ

AIL genes (Karlberg et al. 2011). All are downregulated in the shoot apex by SDs and overexpression of *AIL1* or *AIL3* delayed SD-induced bud set.

A transcriptomic time series of the SD response in the *Populus* shoot apex showed that homologs of additional photoperiodic signaling genes were differentially expressed within the first 2 weeks of SD exposure (Ruttink et al. 2007). *PHY AND FLOWERING TIME 1 (PFT1)* promotes flowering in response to light quality via both *CO*-independent and –dependent mechanisms (Inigo et al. 2012). Genetic variation in a *Populus PFT1* homolog was linked to phenological variation (Evans et al. 2014). Moreover, light quality alters the ability of PHY to interact with clock components (Yeom et al. 2014) and a single amino acid change in Arabidopsis PHYA altered sensitivity to FR (Maloof et al. 2001). These examples illustrate that light aspects other than photoperiod provide input to the clock and also act directly on key regulators. As discussed above, light quality and light intensity might be primary signals regulating growth cessation in trees near the arctic and equator. Thus, it will be interesting to see if similar genes and variation within are involved in the differing responses of these ecotypes.

In addition to light, temperature and other environmental signals entrain the circadian clock (Greenham and McClung 2015). Studies in Arabidopsis have shown that light and temperature signaling are interconnected, but also involve distinct components (Franklin et al. 2014). Disruption of the circadian oscillator during dormancy under natural conditions or in response to chilling temperatures under LDs was demonstrated in chestnut (Ramos et al. 2005). Under LDs, the diurnally cycling expression patterns of *LHY* and *TOC1* resumed within 1 week following return to warm temperatures. The circadian clock also has a role in cold acclimation. Downregulation of *LHY* in transgenic *Populus* reduced freezing tolerance under SDs (Ibanez et al. 2010; Welling et al. 2002). *Populus* overexpressing *PHYA* did not cold acclimate in response to SDs, but responded to low temperature and acquired a level of freezing tolerance similar to WT trees (Welling et al. 2002). This in contrast to bud set, which was prevented under both SDs and low temperature/LDs by constitutive *PHYA* expression. Thus, connections between light and temperature signaling appear to differ between bud set and cold acclimation responses.

Environmental and endogenous signaling pathways have been shown to converge on integrators to control plant growth and development (Franklin et al. 2014). In Arabidopsis, photoperiod, light quality, temperature, hormone and sugar signaling pathways regulate *FT* to control flowering (Kaiserli et al. 2015; Pin and Nilsson 2012; Wahl et al. 2013). Similar to FT's role in integrating multiple flowering pathways, *Populus FT2* appears to integrate multiple signals to control growth. *FT2* expression is downregulated by SDs, low temperature and water stress, suggesting growth responses to different abiotic conditions share some regulatory components (Hsu et al. 2011). *FT* orthologs have a similar role in different tree species, but in some cases, it seems to be a repressor rather than a promoter of growth. For example, ectopic expression of pear *FT2* delayed dormancy and leaf senescence in apple (Freiman et al. 2015). In contrast, *Actinidia* (kiwifruit) *FT* was upregulated in axillary buds during winter and overexpression resulted in poor growth, including reduced secondary growth (Varkonyi-Gasic et al. 2013). Such functional variation

is not uncommon in the phosphatidylethanolamine-binding protein (PEBP) family. In angiosperms, PEBPs group into three major clades; two of these clades, named according to their founding members as FT and CEN/TFL1, have broadly conserved antagonistic roles in flowering (Klintenas et al. 2012). However, these roles can be flipped by a single amino acid change, and in beet and tomato, FT co-orthologs have antagonistic effects on flowering (Cao et al. 2016; Pin et al. 2010). Conifer *FT-like (FTL)* genes also have roles in seasonal growth patterns. In both *Picea abies* and *Pinus sylvestris*, *FTL2* expression is upregulated in needles before bud set and downregulated near the time of bud flush; *P. abies* transgenics support the notion that *FTL2* represses growth (Avia et al. 2014; Karlgren et al. 2013). Thus, it is plausible that PEBPs have generally conserved roles in controlling growth, dormancy and flowering in angiosperm trees, but their exact roles in these processes varies due to lineage-specific changes in gene number and regulatory and/or coding sequence evolution.

Whereas SDs rapidly downregulate *FT2* in *Populus*, *FDL1* expression increases after 3 weeks of SD exposure and promotes the expression of genes involved in freezing tolerance (Tylewicz et al. 2015). Thus, *FDL1* appears to act with *FT2* to control growth, but independently of *FT2* in cold acclimation. Many plants rapidly cold-acclimate to varying degrees in response to low temperatures and the C-repeat Binding Factors (CBFs) are key regulators in a broadly conserved cold-response pathway (Carvallo et al. 2011). Overexpression of *CBFs* in diverse woody plants increases cold-tolerance (Benedict et al. 2006; Walworth et al. 2012; Wisniewski et al. 2011). Studies also indicate that *CBFs* have diversified in trees to have roles in both the rapid cold acclimation that also occurs in many herbaceous plants as well as the dormancy-associated acquisition of a high level of freezing tolerance typical of woody plants. Induction of birch *CBFs* by low temperatures was rapid and transient during active growth under LDs, similar to Arabidopsis (Welling and Palva 2008). But in dormant trees under SDs, low temperature induction of *CBFs* was delayed. In *Populus*, different *CBFs* were induced by cold in leaves versus perennial stems and constitutive *CBF* expression induced different sets of genes in these two tissues (Benedict et al. 2006). There is also evidence that *CBFs* influence other responses to daylength. Constitutive expression of a peach *CBF* in day-neutral *Malus* conferred photoperiodic responsiveness; these transgenics set terminal buds and showed leaf senescence in response to SDs (Wisniewski et al. 2011).

As with other plant processes, multiple hormones regulate seasonal changes in trees and environmental signaling interacts with hormone metabolism and signaling. One of the first names for abscisic acid (ABA) was “dormin” due to its identification in sycamore leaves following exposure to the CDL for dormancy induction (Finkelstein 2013). The role of GA in promoting shoot elongation is broadly conserved and it acts antagonistically to ABA in a number of processes, including in control of seed dormancy (Sun 2011). Accordingly, genome-wide and gene-specific expression studies have supported that GA and ABA promote apical growth or dormancy in diverse tree species (e.g., Ruttink et al. 2007; Ueno et al. 2013). Moreover, *Populus* transgenics support a role for bioactive GA levels and DELLA protein-mediated repression, but not the level of the GA receptor in

the SD growth cessation response (Eriksson et al. 2015; Zawaski and Busov 2014) (Table 1). ABA synthesis and signaling genes were differentially expressed in the *Populus* shoot apex after 3–4 weeks of SD exposure, when a closed bud structure was forming (Ruttink et al. 2007). *ABA INSENSITIVE3 (ABI3)* is part of a conserved pathway promoting seed maturation (Suzuki and McCarty 2008). Overexpression of a *Populus ABI3* ortholog altered SD-induced bud development but not growth cessation, indicating that these processes are regulated separately to some degree (Rohde et al. 2002). Transcriptomic studies have also implicated changes in GA synthesis and signaling in the control of cambial growth and dormancy transitions (Druart et al. 2007). Moreover, regulation of auxin transport and signaling was linked to SD-induced cambial growth cessation and dormancy (Baba et al. 2011). In addition to being upregulated in *Populus* leaves during autumn, genes involved in ethylene signaling and perception were upregulated in shoot apices after 2 weeks of SD exposure (Andersson et al. 2004; Ruttink et al. 2007). Furthermore, disruption of ethylene signaling in *Betula* affected SD-induced apical bud formation but not growth cessation; dormancy was delayed (Ruonala et al. 2006).

The naturally occurring '*evergreen*' (*evg*) peach genotype does not cease growth in response to daylength or chilling temperatures (Rodriguez et al. 1994). QTL analysis implicated a deletion involving six tandemly arrayed *DORMANCY ASSOCIATED MADS-BOX (DAM)* genes as the causative mutation (Bielenberg et al. 2008). *DAM* genes are members of the *StMADS11/SHORT VEGETATIVE PHASE (SVP)* superclade of *MADS-box* genes that was present in the common ancestor of gymnosperms and angiosperms (Gramzow and Theissen 2015; Jimenez et al. 2009). Multiple peach and Japanese apricot *(Prunus mume*) *DAM* genes show expression correlated with growth cessation, dormancy induction and/or inversely correlated with chilling-mediated dormancy release (Jimenez et al. 2010; Li et al. 2009; Sasaki et al. 2011b). Moreover, constitutive expression of *PmDAM6* in *Populus* resulted in growth cessation under LDs (Sasaki et al. 2011b). Epigenetic changes have also been implicated in seasonal dormancy regulation in woody plants (Rios et al. 2014). In peach, the repression of *DAM6* during dormancy release was correlated with decreases in transcriptionally activating histone modifications and increases in repressive histone marks (Leida et al. 2012). The pattern of *DAM6* expression and its epigenetic regulation are similar to floral repressors that establish the vernalization requirement for flowering (Ream et al. 2012). In contrast to the highly conserved photoperiodic pathway, vernalization pathway genes have evolved independently multiple times. Thus, whether the role of *DAM* genes in dormancy is broadly conserved is yet to be determined.

Dormancy and Resumption of Growth

A central question to answer is, what regulates the dormant phase and explains its quantitative nature? The expression peak of *DAM6* suggests a specific role in maintaining dormancy, but the *evg* mutant does not cease growth in response to

SDs. However, multiple related *DAM* genes are deleted in the *evg* genotype and these show different expression patterns (Li et al. 2009). Seasonal upregulation of *DAM1/2/4* is correlated with growth cessation; thus, *DAM* paralogs might have diverged to have distinct roles in the seasonal cycle. Although peach is not amenable to transformation, transgenics are feasible for related species and gene-specific mutation via genome editing could test this hypothesis. Intercellular movement of proteins and other molecules is essential for meristem functioning (Benitez-Alfonso 2014). The regulation of plasmodesmata (PD) apertures is dynamic and important for control of plant development and environmental responses. The blocking of symplastic transport via the formation of 1,3-β-D-glucan-containing sphincters at PDs, appears to be a central control mechanism underlying dormancy of the SAM (Rinne et al. 2001, 2011). *Populus FT1* induces flowering (Table 1), but its chilling-induced upregulation and the association of SNPs in the *FT1* locus with bud flush time suggest an additional role in dormancy release (Evans et al. 2014; Rinne et al. 2011). Moreover, *Populus CEN1* is markedly upregulated in buds shortly before they flush, which is in between the seasonal peaks of *FT1* and *FT2* expression (Fig. 4). However, overexpression of *CEN1* in *Populus* greatly delayed spring bud flush (Mohamed et al. 2010). In controlled environments, *35S::CEN1* transgenics required a longer chilling period to release dormancy than wild-type, whereas transgenics with *CEN1/CEN2* downregulated required less chilling. Thus, low levels of *CEN* appear to be important for dormancy release. Both intercellular mobility and the relative amounts of *FT* and *TFL1/CEN* regulate flowering versus vegetative growth in Arabidopsis and it has been hypothesized that similar mechanisms could contribute to dormancy release in *Populus* (Brunner et al. 2014; Rinne et al. 2011). Putative 1,3-β-glucanase genes are upregulated by chilling, consistent with the role of these enzymes in removing callose plugs at PDs (Rinne et al. 2011). Moreover, constitutive and heat-inducible expression of *FT1* upregulated a 1,3-β-glucanase gene in *Populus* (Hsu et al. 2011). Chilling treatment to release dormancy also upregulates GA biosynthesis genes in the shoot apex (Rinne et al. 2011). Studies indicate GA4 is primarily responsible for shoot elongation in *Populus*, and application of GA4 induced bud flush of dormant axillary buds in a culture-based assay (Israelsson et al. 2004; Rinne et al. 2011).

Field-study of a *Populus* activation-tagged population identified an early bud flush genotype and led to the discovery of the transcription factor EARLY BUD BREAK1 (EBB1) (Yordanov et al. 2014). *EBB1* expression was very low during winter but upregulated in shoot apices shortly before bud break. *EBB1* is expressed in the L1/L2 layers of the shoot apex and adjacent leaf primordia and promotes cell division. Thus, *EBB1* appears to promote the reactivation of the SAM in spring. Study of seasonal expression patterns of *EBB1* homologs from *Picea*, and *Vitis* suggest that *EBB1*'s role in bud break is broadly conserved among woody plants (Busov et al. 2016). Resumption of shoot growth creates N sinks and studies support that that the remobilization of N from storage proteins is mediated by these sinks (Islam et al. 2015). Genes involved in N metabolism were the most enriched category among putative *EBB1* targets (Yordanov et al. 2014).

Reproductive Phenology

The flowering process in trees can be direct (Grainger 1939), with uninterrupted flower development in one season, but in nearly all temperate trees, flower development is initiated in one year but completed in the following season, after the winter dormancy period. However, phenological patterns of indirect flowering and the extent of floral development in the first versus the second season vary among taxa (Fig. 4). In trees such as apple, floral meristem and floral organ development progress prior to growth cessation and dormancy (Foster et al. 2003). However, in less well studied woody perennials (e.g. *Actinidia*), discrepancies in reported timing of floral commitment, ranging from the spring or summer of the first growing season to the spring of the second growing season (Snowball 1997; Walton et al. 1997), often reflect establishment of morphologically undifferentiated primordia, with potentially determined floral fate (Walton et al. 2001). In another woody perennial vine, grape, uncommitted primordia can give rise to inflorescences or tendrils (Vasconcelos et al. 2009), vegetative structures that are modified inflorescences (Boss and Thomas 2000). In a rare example of a temperate deciduous tree which resembles direct flowering, jujube (*Ziziphus jujuba*) flower bud differentiation occurs after dormancy release (Meir et al. 2016). In subtropical tree species such as olive and citrus, flower induction, floral organ development and blooming proceed in uninterrupted succession (Fabbri and Alerci 1999; Nishikawa et al. 2011), however in a deciduous trifoliate orange flower organ development stops during winter (Nishikawa et al. 2009).

Reproductive bud flush time, anthesis and anther dehiscence are often related to pollination mechanism (Fenner 1998). For example, in wind pollinated species, anthesis typically occurs before vegetative bud flush. Flower longevity and synchronicity of flowering within the plant populations determine the level of outcrossing versus selfing and competition for shared pollinators, while subsequent fruiting behavior ensures adequate seed development and dispersal. All these events include a genetic and environmental component and are increasingly subjected to omics studies.

Environmental and Hormonal Factors Controlling Flowering Time

The large size of many flowering trees precludes demonstration of specific environmental signals regulating the floral transition. However, controlled environment experiments have been performed using potted plants or defoliated shoots (Nishikawa et al. 2007; Yooyongwech et al. 2009; Guak and Neilsen 2013) and numerous field studies combined with modelling (Guo et al. 2014) provided the means to quantify the chilling and heat requirement of trees. These vary dramatically between species, cultivars and regions, but most trees from temperate climates require the accumulation of winter chill and subsequent heat during their dormant phase to resume growth and initiate flowering in the following spring. As an example, 69 Japanese apricot cultivars grown in Nanjing (altitude 10 m, lat. 32°03′N,

long. 118°46′E), China, were classified as low-, medium- and high-chilling, and their bloom time correlated with chilling requirement (Zhuang et al. 2016). Correlation between chilling requirement and bloom time has been demonstrated for other temperate taxa (Wall et al. 2008; Alburquerque et al. 2008; Egea et al. 2003), but the phenology of floral buds, especially bud flush time, is often not coincident with vegetative bud phenology. In pear (*Pyrus pyrifolia*), the chilling hours needed for 50 % vegetative bud break was 2 times greater than the chill sum needed for floral bud break (Hussain et al. 2015). This suggests that a difference in depth of dormancy and hence, the chilling requirement to release dormancy differs between vegetative and floral buds. This is a research area of significant interest and studies of inheritance of reproductive phenology traits and related QTL identification have been performed in apricot, almond and other *Prunus* species (Salazar et al. 2016; Fan et al. 2010; Sánchez-Pérez et al. 2014; Castède et al. 2015).

While chilling is usually seen as a requirement for dormancy break and flowering in temperate regions, some exceptions include *Ziziphus jujube* (Meir et al. 2016). Chilling of dormant plants was not necessary for flowering, but did accelerate anthesis. However, this appeared to be an indirect effect as chilling accelerated vegetative bud break, but days between bud break and blooming were constant following chilling treatments of different durations or in the absence of chilling. This suggests that in some cases the seasonal floral transition is primarily regulated by endogenous factors, perhaps related to energy and nutrient status. Similarly, in a deciduous woody perennial grapevine, environmental stimuli driving floral initiation are high temperature and high light intensity, corresponding to conditions the plant encounters at the forest canopy top and when the developmental and nutritional states are at the optimum (reviewed in Carmona et al. 2008). One possible mechanism may involve the sugar metabolite trehalose-6-phosphate, which appears to be involved in ensuring that flowering occurs when sufficient energy and nutrients are available in Arabidopsis (Wahl et al. 2013). In subtropical climates, floral induction usually occurs in response to cool temperatures (Chaikiattiyos et al. 1994; Nishikawa et al. 2007; Núñez-Elisea and Davenport 1995) and involves a mobile signal (Davenport et al. 2006; Nave et al. 2010). Floral induction in tropical conditions is often associated with water stress, which is predicted to have only an indirect effect (Chaikiattiyos et al. 1994; Núñez-Elisea and Davenport 1994).

Molecular Regulation of Seasonal Flowering

Despite differences in their life strategies and lifespan, it is clear that the underlying genetics of floral initiation and development between annuals and perennials share commonalities. *Eucalyptus* has only one *FT* gene and this also appears to be the case for *Ficus carica*, and in both species, *FT* expression was correlated with the floral transition (Vining et al. 2015; Ikegami et al. 2013; Jones et al. 2011). *FT* expression is correlated with cool temperature treatments and floral induction (Ikegami et al. 2013; Nishikawa et al. 2007, 2009, 2011), as well as irregular seasonal flowering in several tropical taxa (Nakagawa et al. 2012; Ziv et al. 2014). As discussed above, *FT* genes have diverged, acquiring roles unrelated to flowering

time (Pin and Nilsson 2012). This is commonly found to be the case in woody perennials that have multiple *FT* orthologs. For example, expression of only one of two *FT* genes in apple, and one of three *FT* genes in citrus appeared to be associated with seasonal transition to flowering (Kotoda et al. 2010; Nishikawa et al. 2007) (Fig. 4). *FT* homologs from kiwifruit and pear promote early flowering in model annuals but not when expressed in kiwifruit or apple (Varkonyi-Gasic et al. 2013; Freiman et al. 2015). Differential expression combined with phenotypes observed upon induced expression of *FT1* or *FT2*, suggested sub-functionalization in coordination of reproductive and vegetative growth (Hsu et al. 2011). Relatively low levels of *FT1* could induce wild-type catkins. However, there is a temporal gap between *FT1* upregulation and inflorescence development. This is perhaps due in part to the blocking of symplastic transport during dormancy (Rinne et al. 2001) that could delay accumulation of the *FT* protein in axillary meristems as well as to the warm temperatures needed to resume growth and development. These or other factors could also limit the competency of meristems to respond to *FT1* or other signals promoting flowering.

The main question remains: is FT acting as florigen in woody perennials? There has been little direct or indirect evidence that FT moves over long distances in trees. Unlike in annuals, where FT protein was detected in phloem sap (Lin et al. 2007; Giavalisco et al. 2006; Aki et al. 2008), FT protein or *FT* mRNA has not been detected in either phloem or xylem sap of trees. The viruses that were successfully used to deliver FT and dramatically promote flowering in apple were themselves moving into the shoot apical meristem (Yamagishi et al. 2011, 2014). Graft-transmissible early flowering in a small woody perennial shrub *Jatropha* provided evidence for FT florigen in woody perennials (Ye et al. 2014). Expression of *Jatropha FT* under the control of the relatively weak synthetic G10–90 promoter reduced flowering time and induced early flowering in non-FT scions. In contrast, grafting experiments in poplar and apple with rootstocks expressing Arabidopsis and poplar *FT* transgenes under the control of a heat-shock inducible promoter did not result in flowering in wild-type scions (Zhang et al. 2010; Wenzel et al. 2013). These contradictory findings may have a simple mechanistic explanation. The notable difference in plant size and physiology can impact on speed, range and effectiveness of the signal; the intensity and duration of heat-shock induced expression of *FT* combined with plant intrinsic sink and source relationships may have delivered FT amounts below the required threshold for floral induction.

One of the difficulties in FT florigen studies in perennials is the high probability that they are balanced out by competing flowering repressors, combined with functional diversification of proteins that interact with FT and their potential targets. As described above, downregulation of *CEN/TFL1* homologs induces earlier first onset of flowering in diverse trees. In *Populus*, the reduction in the juvenile phase was modest, but downregulation markedly increased the number of axillary meristems that seasonally transition to flowering (Mohamed et al. 2010). Thus, *Populus CEN1/2* appears to also have a recurring seasonal role in limiting the number of

meristems that commit to flowering. Little is known about the functions of tree homologs of other floral repressors identified in annual plants. Unlike *FT* and *TFL1*, the functions of these are less broadly conserved among herbaceous plants. In Arabidopsis, the MADS transcription factors SVP and FLC form a complex to repress genes promoting the floral transition (Mateos et al. 2015), but the *FLC* clade has been repeatedly lost during angiosperm evolution (Gramzow and Theissen 2015). Differences in expression pattern and heterologous function in Arabidopsis indicated that four kiwifruit *SVP* homologs have distinct roles in dormancy and flowering (Wu et al. 2012). Overexpression of kiwifruit *SVP3* did not alter growth, dormancy or flowering onset, but it prolonged the period from floral initiation to anthesis (Wu et al. 2014).

Orthologs of *LFY* and members of the *AP1/FUL* family are expressed in initiating floral meristems in diverse trees, consistent with a conserved function in promoting floral meristem identity (Elo et al. 2007; Fernando and Zhang 2006; Rottmann et al. 2000; Southerton et al. 1998). Strong downregulation of *PtFL* did not alter the age of first onset of flowering in *Populus*, but seasonal development of inflorescences was markedly altered, resulting in small catkins with poorly developed, sterile flowers (Klocko et al. 2016b). As described above, members of the *AP1/FUL* family alter SD-induced growth cessation in *Populus*; however, the predominantly floral expression of *Populus* and *Salix AP1* orthologs indicate a role in flowering (Chen et al. 2015; Fernando and Zhang 2006). There are 5 *Populus* members of the *AP1/FUL* clade; thus, CRISPR/Cas9-mediated gene-specific mutations are needed to clarify the endogenous functions of these paralogs.

As described in section "Reproductive Phase Change", the inhibition of GA promotes an earlier onset of flowering in some trees. Similarly, GA often inhibits the seasonal floral transition in adult woody plants. The grapevine green revolution mutant has tendrils converted into inflorescences due to a mutation that likely creates a constitutively active DELLA repressor of GA signalling (Boss and Thomas 2002). Many fruit tree crops show biennial bearing, where minimal floral induction occurs in the year following a high-yield fruit crop (Samach and Smith 2013). In addition, a high intensity of flowering can lead to fruits that are too small to be marketable. GA3 application during floral induction reduces the intensity of flowering in many trees, and thus, can partially overcome the problems associated with biennial bearing. Branch bending induces flowering in a number of fruit trees, and this was correlated with reduced levels of GA biosynthesis genes and bioactive GA in Japanese plum (Chutinanthakun et al. 2015) and apple (Xing et al. 2016). Genes affecting the amounts of auxin and GA co-located with the QTL intervals for biennial bearing in apple (Guitton et al. 2012) and the potential mechanism included differential accumulation of *FT* transcripts (Munoz-Fambuena et al. 2012; Nakagawa et al. 2012; Ziv et al. 2014) in response to different fruit loads. Differential responses of other flowering genes including age-related pathway SPL transcription factors were also detected (Guitton et al. 2016; Shalom et al. 2012; Zhou et al. 2015).

Perspectives

Insight into the genes and signaling pathways underpinning phase change and phenology has mainly come from a combination of comparative extrapolation of gene function (study of homologs in trees) and transcriptomic studies in a few taxa done before the advent of next-generation sequencing. Nonetheless, studies in trees have accentuated that environmental signaling controlling vegetative and reproductive phenology share commonalities, and that gene duplication and divergence has contributed to the distinctness of these phenologies. We know less about the regulation of phase change in trees. Studies hint that the acquisition of the competency to flower that occurs after years of growth and the recurring seasonal time of floral initiation involve both common and distinct regulatory components. Regulatory networks are modular, and this feature is thought to be a major contributor to the ability of populations to adapt to new environments (Clune et al. 2013). Angiosperm lineages have experienced different numbers of whole genome duplications and subsequent patterns of genome rearrangement and loss (Soltis and Soltis 2016). Moreover, the tree growth habit has been lost and gained throughout angiosperm evolution (Groover 2005). Thus, to what extent similar modules have been repeatedly duplicated, redeployed and/or rewired to control maturation and seasonal transitions central to the tree lifestyle is one of the many important questions yet to be answered. Studies based on comparison to work in annuals will continue to be fruitful, but we need less biased approaches to advance our understanding of these complex processes in trees beyond this "low hanging fruit". Fortunately, the increasing number of tree genome sequences and technical advances provide new opportunities.

Especially when a reference genome is available, NGS can be leveraged to give a more comprehensive view of genomic regulation by capturing non-coding transcripts and many post-transcriptional events (Mattick et al. 2010). NGS also enables unbiased genome-wide genetic association studies and selection scans to reveal SNPs associated with phenological adaptation and maturation. As shown by a genome-wide study of vegetative phenology in *P. trichocarpa*, most of the genes identified were not readily placed in a predicted pathway or not yet linked to adaptive traits (Evans et al. 2014). Moreover, most of the associated SNPs are located in non-coding regions, suggesting that the regulation of gene expression, the transcription of non-coding RNAs, and alternate splicing, have major roles in adaptation to different climates. Integration among approaches is needed to maximize advances in elucidating regulatory mechanisms. Moreover, NGS transcriptomic studies need to capture the temporal and spatial complexity of maturation and phenology and dissect the component transitions. Ruttink et al. (2007) showed the value of a multi-week time series under controlled conditions for providing insight into the overlapping events of growth cessation, bud formation, cold acclimation and dormancy. Similarly, nuanced dissection of growth cessation and bud set stage over multiple weeks in field environments provided insight into the role of temperature at different stages, potentially explaining some of the contradictory effects reported in other studies (Rohde et al. 2011).

Transcriptomic studies have focused on one tissue or organ, but simultaneous studies of multiple tissue types are needed for a fuller understanding these processes in trees. Perception of and response to environmental signals often involves different organs. Similarly, hormones are often transported long-distance and we also need to dissect the roles of nutrient and sugar signaling and transport between sources and sinks. Performing such detailed NGS transcriptomic studies in a subset of diverse taxa with reference genomes as well as a diverse ecotypes or mutants within these taxa, would allow construction of gene regulatory networks that reveal the conservation and divergence in regulation among trees. Although flowering is more challenging to study in trees, there are a diversity of taxa that flower at a relatively small size and young age, including a number of fruit trees as well as species or genotypes of *Eucalyptus, Betula* and *Salix.* Poethig (2010) emphasizes the need for a heteroblastic tree species with well-developed molecular tools for phase change research, and Zotz et al. (2011) suggest a eucalypt, the genome of which has now been sequenced (Myburg et al. 2014), as a possible model organism. The vegetative phenology of lower latitude temperate to tropical trees has received relatively little attention from both environmental signaling and molecular regulatory perspectives. As described earlier, the phenology of some tropical trees suggests they use specific seasonally changing environmental signals to adapt to wet/dry climates. Seasonal responses have been proposed to have evolved from primary responses to abiotic stress, particularly drought (Preston and Sandve 2013). Moreover, there is overlap between the transcriptomic changes induced by drought and SDs in *Populus* (Zawaski and Busov 2014). Thus, study of trees adapted to these climates would expand our understanding of the evolution of phenology regulatory networks.

Combined with genome-wide association studies, these regulatory networks can generate new hypothesis and hone in on genes for in-depth study, such as identification of transcription factor targets via ChIPseq, changes in histone modification or identifying protein-protein interactomes around select regulatory nodes. Moreover, they can improve selection of gene candidates likely to have a key role in phenology or maturation that can be validated by transgenic manipulation. Although the ability to efficiently regenerate transgenic trees remains a hurdle for most species, this can be done in a diversity of taxa that have reference genomes, thus, allowing the CRISPR/Cas9 system to be leveraged. *Populus* is the most efficient system for transgenesis and can be further advanced by using genome–editing to generate different types of mutations in different genes. For example, a mutation in a predicted downstream gene could be introduced in the SD-unresponsive *35S::FDL1* background. These various studies would provide genome-wide and in-depth insight into the regulation of these traits and the regulatory network context of genetic variation enabling adaption to different environments. Such knowledge is central to improving the productivity of plantations and managing native forests in a changing climate.

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Abiotic Stress

Héloïse Bastiaanse, Guillaume Théroux-Rancourt, and Aude Tixier

Abstract Abiotic stresses such as drought, are the main factors for forest declines globally. There is therefore an increasing interest in understanding the mechanisms underlying tree adaptations and survival to water deprivation. Angiosperm tree species demonstrate an amazing phenotypic plasticity. They sense and respond to adverse changing environmental conditions via a series of physiological, cellular, and molecular processes which are under a tight genetic control. In this book chapter, first we present some key morphological and anatomical features adopted by angiosperm tree species to survive drought. These traits are described at the roots, stem and shoots levels. Then, we provide insights into the dynamics of gene expression and components of regulatory networks associated with drought response, including comparisons among angiosperm tree species. Such comparative genomic approaches have the potential to provide a better understanding of the evolution and diversification process of drought response in angiosperm trees but also to the development of cultivars resilient to drought and other abiotic stresses.

Keywords Angiosperm • Tree • Abiotic stress • Drought Tolerance • Adaptation • Anatomy • Structure-function • Hydraulic conductance Water use efficiency • *Comparative genomics* • QTL • Candidate gene • Gene expression • Stress sensing • Stress signaling

Tree species, because of their long lifespans and their sessile nature, figure among the best biological examples of adaptation and acclimation to changing environmental conditions. Trees have to cope with seasonal changes, and can also be exposed to extreme climatic events involving extended periods of drought, flooding,

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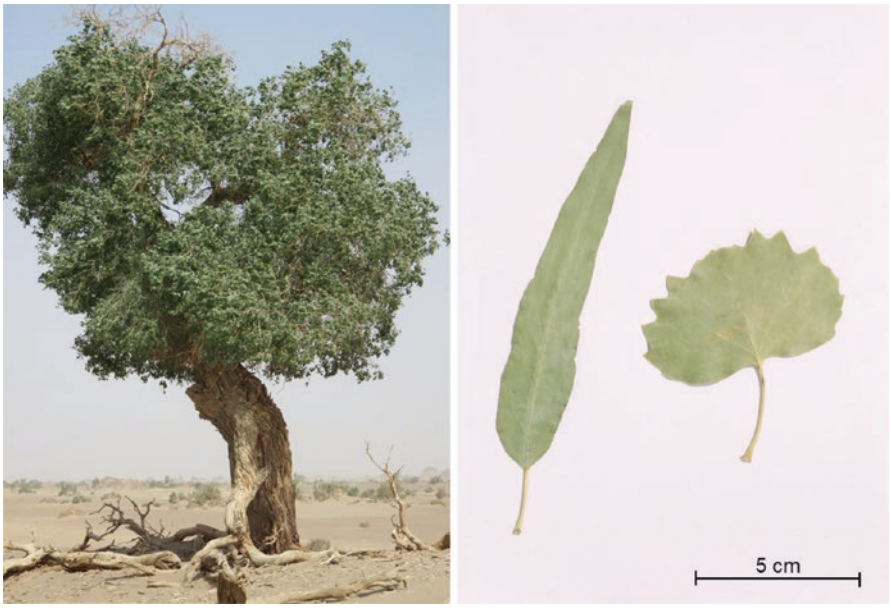


Fig. 1 The desert poplar *Populus euphratica* in China in the Inner Mongolia Autonomous Region, China (*left*) and examples of its heteroblastic dentate broad-ovate and lanceolate leaves associated with contrasting levels of drought tolerance (*right*) (Pictures from Andrew Groover and Suzanne Gerttula)

mechanical stresses, and temperature extremes. Trees can also inhabit marginal ecosystems, such as habitats of the desert poplar *Populus euphratica* characterized by limited water sources that contain high levels of salt (Wang et al. 1996) (Fig. 1). Such abiotic stress strongly influences tree distribution and survival (Sykes et al. 1996; Svenning and Skov 2004; Engelbrech et al. 2007; von Humboldt and Bonpland 2009). Since tree species often cover a broad range of geographic and environmental distribution, adaptation to abiotic stress has been shown to be plastic, with various responses being observed among population and within species (Sparks and Black 1999; Sánchez-Gómez et al. 2011).

To cope with adverse environmental conditions, angiosperm trees have evolved specific avoidance and tolerance mechanisms that allow them to maintain their reproductive fitness and survival. Morphological and physiological changes in response to abiotic stress are well documented at the whole plant level. These changes involve complex molecular processes ranging from stress sensing to the establishment of stress tolerance, which is under genetic and epigenetic control. Due to advances in sequencing technologies, tree responses and tolerance to abiotic stress are understood in increasing detail and have revealed enormous genetic diversity among species.

Here we outline the morphological and anatomical changes in angiosperm trees in response to abiotic stress, along with recent findings describing the genetic and molecular basis of abiotic stress tolerance. A special emphasis is given to drought,

as water limitation is one of the leading contributors to tree declines globally (van Mantgem et al. 2009; Allen et al. 2010; Choat et al. 2012).

Morphological and Anatomical Changes

As discussed below, morphological and anatomical responses to abiotic stress in angiosperm trees are observed in the roots, the stem and the canopy levels, with some changes being unique to angiosperm trees. The presence of a hydraulic system for long distance water transport and the need of maintaining functional tissues and organs for long periods of time are two important characteristics distinguishing woody species within the plant kingdom (Aranda et al. 2012). As compared to conifers, angiosperms are unique in some wood anatomy properties, notably the type of the tracheary elements (Fig. 2). Gymnosperm conducing system is composed of the ancestral tracheids which perform both the conductive and supportive functions. These elongated narrow tube like cell are unicellular, have thick lignified walls presenting bordered pits to facilitate the transfer of liquid between adjacent cells. Such pits present a heterogeneous structure, with an impermeable torus surrounded by a flexible margo. On the contrary, the evolutionary advanced angiosperm conduits are composed of larger vessel elements (and tracheids in some species). They are multicellular and are interconnected by means of plates with pores (perforation plates) through which the water moves up ward. Their wall are thickened in various ways, the pit membrane however, generally lacks the conspicuous specialization of the torus margo membrane and at least superficially has a relatively homogenous texture (Cochard 2006; Delzon et al. 2010). Some of these differences could be at the origin of contrasting levels of abiotic stress resistance observed between the two taxa.

Roots

Roots, as the primary sites of water and nutrient uptake by plants, represent important organs for plant growth and survival, and must respond to environmental cues including water availability and salinity levels. Roots evolved a remarkable capacity of sensing changes in environment conditions perceived from the physicochemical parameters of the soil, and relay that information to the shoot (Hamanishi and Campbell 2011). Roots also respond to external signals by modulating their architecture to find the supply of water and nutrients, which can be limited in abundance and localized (Malamy 2005). In woody plants, this architecture modulation and foraging for belowground resources mainly concerns the fine roots (<2 mm diameter) that are the most active portion of the root system in water uptake, as opposed to the more perennial coarse root system (>3 mm diameter) serving the functions of anchorage, carbohydrate storage, and the transport of nutrients and water (Comas et al. 2013).

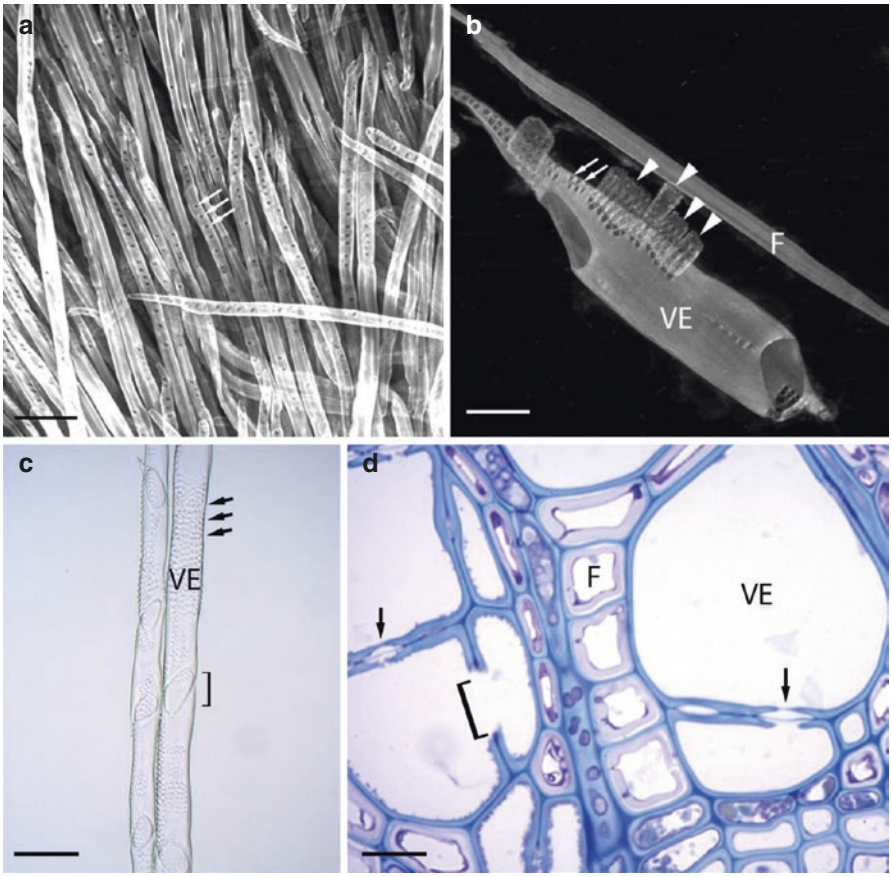


Fig. 2 Microscopic observations showing the anatomy of tracheary elements in gymnosperm and angiosperm tree species. (**a**, **b**) show confocal reflectance images of macerated wood cells, of respectively, *Podocarpus* spp and *Populus deltoides* × *P. nigra*, after staining with calcofluor. In (**c**) macerated vessel elements of *Populus tremula* × *alba* were observed under bright field microscope without any staining (image from Tixier 2013). The gymnosperm tracheids are narrow, elongated, unicellular and serve the double role of support and water transport. They have thick lignified walls presenting bordered pits (*arrows*) to facilitate the transfer of liquid between adjacent cells. The angiosperm vessel elements (*VE*) serve only the function of solute transport, while the fibers (*F*) provide support to the woody stem. VE are larger, multicellular and are interconnected by means of perforation plates (*squared bracket*) through which the water moves up ward. Their wall also present pits allowing liquid transfer radially between vessel elements, or in connection to ray parenchyma cells (*arrow heads*). (**d**) shows a cross section of *Populus tremula* × *alba* stem after toluidine blue staining (image from Tixier 2013). The homogeneous pit membrane of angiosperm can be observed. Hence they lack the conspicuous specialization of the torus margo membrane in gymnosperm tracheids. (not shown in this figure). Scale = 50 µm in (a, b), 100 µm in (c) and 10 µm in (d)

There is a great diversity of root architecture, in terms of root length, thickness, depth, partitioning and distribution that varies among forest trees, shrubs and horticultural trees species (Stone and Kalisz 1991). Such architecture can contribute to drought tolerance. For example, as a stress avoidance strategy, the deep and wide-

spreading roots systems of some drought-tolerant oak species seem to be important for optimizing the water intake in water-limited habitats, as opposed to the shallow root system of drought sensitive species such as dogwood and sugar maple (Hinckley et al. 1981). Other root traits associated with maintaining productivity of both herbaceous and woody plants under drought include smaller fine root diameters with greater specific root length. These features are conducive to higher hydraulic conductance by increasing the surface area in contact with soil water as well as the volume of soil that can be explored for water. In addition, this increases root hydraulic conductivity by decreasing the apoplastic barrier of water entering the xylem (Comas et al. 2013).

Water flows from the roots to the shoot in a continuum that is governed by a combination of the driving forces and the hydraulic properties of the pathway. During drought periods, water tension in the root xylem can reach critical values (Ψ_{crit}), leading to the interruption of the water column. Such embolized, air-filled xylem conduits become non functional (Domec et al. 2004). In many woody plants, hydraulic failure is more likely to take place in roots than in shoots. In fact, previous investigations have demonstrated that xylem in roots is not only more vulnerable to embolism than in stems, but also operates closer to the Ψ_{crit} than stems (Alder et al. 1996; Hacke and Sauter 1996; Martínez-Vilalta et al. 2002; Domec et al. 2004; Froux et al. 2005; Comas et al. 2013), so small reductions in Ψ_{soil} can significantly enhance root embolism rates. Further, fine (<2 mm diameter) and medium (2–3 mm diameter) roots are more vulnerable to embolism than coarse roots (>3 mm diameter) (Sperry and Ikeda 1997). Upon rewetting of the roots, root xylem functions can be restored by refilling the embolized conduits (Jaquish and Ewerts 2001; Domec et al. 2004), or by the growth of new roots (Domec et al. 2004). In the case of a persistent drought or for severely cavitated roots, embolisms may be irreversible (Seiler and Johnson 1988; Hacke et al. 2000) and a substantial reduction of the fine root biomass may be observed (Meier and Leuschner 2008; Zang et al. 2014). Because the roots and rhizosphere are likely to contain the most vulnerable components of the soil-to-leaf hydraulic pathway (Jackson et al. 2000), avoidance of hydraulic failure in drying soil may play a major role in the success of species growing under a wide range of precipitation regimes. It has been suggested that partial loss of root hydraulic conductivity through embolism could result in the generation of a hydraulic signal transmitted to the shoots that reduces stomatal conductance (g_s) (Colchard et al. 1996). This would in term reduce transpiration, and maintain shoot water potential at a nearly constant minimum value above Ψ_{crit}. This behavior, referred as an isohydric behavior, is opposed to anisohydric behavior where water potential decreases following the evaporative demand experienced during the day. (Cochard et al. 1996; Domec et al. 2004; Sade et al. 2012). A number of tree species exhibits at least partial isohydric behavior during gradual soil drying cycles (Gollan et al. 1985; Klein 2014), but the extent to which stomatal regulation of leaf water status is determined by shoot versus root and rhizosphere water status and hydraulic properties is largely unexplored (Domec et al. 2004).

In addition to some key architectural root traits, tree species adapted to dry climatic regimes also tend to have a reduced above ground biomass, resulting in a higher root-to-shoot ratio (Kozlowski and Pallardy 2002; Markesteijn and Poorter

2009). Such increase of the root-to-shoot ratio seems to largely depend on the level of drought stress, as well as on the tree species and even the population (Bongarten and Teskey 1987; Pallardy and Rhoads 1993; Yin et al. 2004; Aspelmeier and Leuschner 2006; Poorter et al. 2012; Kuster et al. 2013; Brunner et al. 2015).

Stem

In angiosperm trees, the evapotranspiration at the stomatal chambers of the leaves creates the negative pressure that supports water transport from the roots via the plant's conduit network of tracheary elements (Tyree and Zimmermann 2002). During drought events or freeze-thaw cycles in winter months, negative pressure increases in xylem vessels, leading to possible cavitation events (Lens et al. 2013). Resistance to cavitation seems to be an important factor in both angiosperm and gymnosperm evolution, since hydraulic failure has been found to drive tree mortality (McDowell et al. 2008). Hence, associations were found between increasing cavitation resistance and increasing aridity (Maherali et al. 2004; Choat et al. 2012). Different vulnerability to stem xylem embolism have been observed among tree species, and some phylogenic trends have been observed which might explain species distribution and survival (Sperry et al. 1994; Maherali et al. 2004; Choat et al. 2012; Meinzer and McCulloh 2013). For instance, angiosperm trees seem capable of surviving high levels of xylem embolism, with a threshold of 88 % loss of stem hydraulic conductivity for some angiosperm trees before leading to any irreversible drought damage, as compared to 55 % for conifers (Urli et al. 2013). But in the other hand, angiosperms were also found to be more prone to embolisms since they operate at a lower safety margin (defined as the difference between naturally occurring xylem pressures and pressures that would cause hydraulic dysfunction) than gymnosperms (Maherali et al. 2004; Choat et al. 2012). Additionally, species that show denser wood usually show higher cavitation resistance (Sperry et al. 2006).

Various attempts have been made to identify relevant wood anatomical parameters explaining the differences in cavitation vulnerability observed among tree species. The importance of the diameter of the conduits has been discussed, (Sperry and Tyree 1988; Sperry and Saliendra 1994; Hargrave et al. 1994; Lo Gullo et al. 1995; Hacke and Sauter 1996), with wider conduits generally being more vulnerable to xylem embolism as compared to narrower conduits. In that regard, the reduction of the size of the vessel elements observed in poplar, eucalyptus as well as in various oak species appears to be an adaptive response to drought stress (Villar-Salvador et al. 1997; Garcia-Gonzalez and Eckstein 2003; Corcuera et al. 2004; Eilmann et al. 2006; Arend and Fromm 2007). However, other studies suggest that vulnerability to cavitation would mainly be explained by the quantity as well as the quality of the interconduit pits present in the lignified cell walls of the tracheids and vessel elements (Zimmermann 1983; Choat et al. 2004; Cochard 2006; Jansen et al. 2011). Pits facilitate water exchange between neighboring cells but are also important sites for preventing air-seeding and spreading of embolism between

neighboring conduits (Lens et al. 2013). In term of quantity, the probability of a vessel to cavitate increases with the pit area (the rare pit area theory) (Christman et al. 2009; Lens et al. 2011), as well as the pit aperture diameter (Choat et al. 2003; Sano 2005; Jansen et al. 2009). Hence, the bigger the conduit, the larger its surface and the more pits are located in its wall, the greater probability of a defective, wide, or less efficient pit that could be an air-seeding starting point (Badel et al. 2015). In support of the rare pit hypothesis, there is a significant relationship between pit area per vessel and increasing vulnerability across species (Christman et al. 2012). In terms of quality, thicker and less porous pit membranes are associated with greater resistance to cavitation by air-seeding because more pressure is required to pull air across them (Lens et al. 2011). In this regard, the higher resistance to cavitation observed in gymnosperm might be due to the heterogeneous structure of the pit membranes of the ancestral gymnosperm tracheids, constituted by an impermeable torus surrounded by a flexible porous margo allowing for low-resistance water transfer between tracheary elements (Cochard 2006; Delzon et al. 2010). When a pit connects a functional tracheid with an embolized one, the margo stretches in response to the pressure difference, so that the torus seals the opening of the pit and prevents propagation of embolism. In the majority of angiosperm tree species, water flows through the homogenate membrane pit of the vessel elements, and air sealing of the pits depends entirely on the stretching of this membrane (Hacke et al. 2004). Mechanical modelling of pit membranes behavior in angiosperm tree species has provided better understanding of their resistance to cavitation (Sperry and Hacke 2004; Tixier et al. 2014).

If embolism occurs, studies have revealed that trees can potentially recover water conductivity by refilling the embolized xylem conduits or by developing new functional xylem tissue during the secondary growth (Zwieniecki and Holbrook 2009). Refilling of the embolized vessels is made possible by generation of a positive pressure on the xylem by the roots or by the stem itself (Tyree and Sperry 1989), by creating osmotic forces through the accumulation of soluble sugars in the vessels (Ewers et al. 2001; Secchi and Zwieniecki 2010). These sugars could notably be released from the starch stored in xylem parenchyma, and would create a low water potential leading to water movement from the parenchyma cells into the xylem vessels (Ewers et al. 2001; Ameglio et al. 2001; Nardini et al. 2011). This would occur mainly during winter and spring, when in absence of evaporative forces, xylem tensions are smaller than the positive osmotic pressure developed by the accumulation of solute (Ameglio et al. 2001; Ewers et al. 2001). The presence of a range of osmotic pressures within a tree allowing for refilling during the growing season is still debated (Brodersen et al. 2013; Cochard and Delzon 2013; Cochard et al. 2015; Choat et al. 2016; Bouche et al. 2016).

Xylem embolism resulting from freezing of water in the stem has been measured seasonally in angiosperm tree species of the Northern hemisphere (Sperry and Saliendra 1994; Zhu et al. 2000; Ameglio et al. 1995, 2001; Ewers et al. 2001; Sakr et al. 2003), including the sugar maple (*Acer saccharum*) (Sperry et al. 1988). Some phylogenic trends for tree capacity to refill embolized xylem have been noted, with a lower capacity in conifers as compared to angiosperms (Johnson et al. 2012). One

hypothesis is that such differences could reflect the lower amount of xylem parenchyma in conifers in comparison with angiosperm trees, as well as differences in non-structural carbohydrate concentrations in their stems which may be required for embolism repair (Johnson et al. 2012). However, there is some controversy over the possibility that the observation of xylem refilling in some of these studies was actually an artifact of the destructive sampling technique used (Cochard et al. 2000; Sperry 2013; Wheeler et al. 2013). Using non-destructive, high-resolution computed tomography, Choat et al. (2016) saw no evidence of refilling of coast redwood (*Sequoia sempervirens*) xylem after imposition of a severe drought stress. To address the gap in our understanding of embolism repair in woody plants, there is an urgent need of applying such direct methods in angiosperm tree species.

More generally, abiotic stress at the stem level has been shown to considerably impact tree growth with a significant reduction of stem diameter under drought (Spieß et al. 2012). In fact, the process of radial growth has been shown to be highly sensitive to water deprivation, with the demonstration of reduced tree rings size (Orwig and Abrams 1997; Kozlowski and Pallardy 1997; Breda et al. 2006). This impacts tree productivity for industry in term of wood quality and yield, but also increases tree susceptibility to insects, diseases as well as mechanical damages (Lundstrom et al. 2008).

Shoots and Leaves

Angiosperm trees manifest a great diversity of leaf shapes and structures that ranges from developmental sequences within a shoot in response to microenvironment, to variation among, and within species at the population level (Nicotra et al. 2011). Since leaves play a critical role by providing the necessary sugars needed for growth and survival through photosynthesis, it has been proposed that variation in the leaf shape would reflect the effects of natural selection. Leaf size, thickness, margin and venation characteristics have been found to follow a predictable pattern based on climate and growth habitat. For instance, leaves of desert or Mediterranean angiosperm species are more finely dissected than leaves of temperate-zone angiosperm trees (Hinckley et al. 1981), whereas mesic, evergreen tropical forests contain almost exclusively species with entire-margined leaves (Bailey and Sinnott 1916; Gentry 1969). Smaller, thick leaves are also commonly associated with more xeric sources of temperate-zone angiosperm trees (Ying and Bagley 1976; Kozlowski and Pallardy 1997; Abrams 1994), as well as harsh environmental conditions such as cold, hot, dry, saline environments or many of these factors in combination (Ying and Bagley 1976; Dancik and Barnes 1975; Pallardy 1981; Kozlowski and Pallardy 1997; Abrams 1994). In addition, leaves may also exhibit a remarkable phenotypic plasticity in response to abiotic stress (Kessler and Sinha 2004; Barkoulas et al. 2007). The leaf shape and structure are defined mainly in a brief period of primary morphogenesis based on the possible role of reaction–diffusion systems and can be altered by allometric expansion (Frank and Britton 2000; Dengler and Kang 2001). For instance,

in response to drought, modulation of total leaf area is important to regulate water use, and can be associated with the decline in cell and leaf size or leaf senescence, and hence tree productivity (Battaglia et al. 1998; Le Dantec et al. 2000). Drastic changes in leaf shape in response to environmental changes have been also observed in some tree species. In the *Eucalyptus* Wilsons Promontory seedlings, the rapid shift from the broad, thin, horizontally oriented, dorsiventral and hypostomatal juvenile leaves to the narrow, thick, vertically oriented, isobilateral and amphistomatal adult leaves may confer an adaptive advantage within its native habitat, which is exposed to strong winds and salt spray (Potts and Jordan 1994; James and Bell 2001). Heteroblasty is also observed in the desert poplar *P. euphratica* (Fig. 1), and seems to be correlated with drought and desertification, with changes in leaf shape from lanceolate to dentate broad-ovate, the latter being associated with increased drought tolerance (Li and Zheng 2005; Zheng et al. 2007).

Stomata, the pores found on leaf surfaces, play a key role in regulating water movement and retention in response to environmental conditions. As a short-term response enabling plants to contend with fluctuating water supply, the closure of stomatal aperture leads to a decrease in water loss from the plant, but also limits CO_2 entry into the intercellular airspace, thus potentially limiting photosynthesis (Cowan and Farqhar 1977; Chaves et al. 2003). Stomatal movements, and drought avoidance strategies to increase water use efficiency (WUE, defined as the amount of C assimilated, e.g. in term of biomass or μmol CO_2, per unit water loss) and maintain production were found to differ greatly among trees. For instance drought tolerant poplar clones were shown to exhibit lower gas exchange rates under well-watered conditions and a more gradual decline in photosynthesis and stomatal conductance as drought proceeds, while drought sensitive clones tend to achieve higher basal gas exchange when well-watered but then close their stomata more rapidly when subjected to drought, and hence show greatly reduced productivity (Silim et al. 2009; Theroux-Rancourt et al. 2015). In addition, stomatal closure is shown to prevent the development of substantial xylem embolism in aboveground woody tissue, if xylem reaches a critical tension during periods of high transpiration (Jones and Sutherland 1991). In this context, there is growing evidence of functional coordination between vulnerability to xylem cavitation and the regulation of stomatal conductance to maintain water potential above the range that would lead to cavitation (Maherali et al. 2006; Sparks and Black 1999). This mechanism has been demonstrated for several species of woody plants (Sperry et al. 1993; Nardini and Salleo 2000; Vilagrosa et al. 2003). However, relationships between stomatal conductance and xylem cavitation seem to be highly species-specific and were found to not hold true among certain species such as poplar hybrids, suggesting that other drought-resistant strategies may also play a key role in such genotypes exposed to drought stress (Fichot et al. 2010; 2011; Arango-Velez et al. 2011).

Finally, stomatal responses were found to be highly interactive to water availability in the soil, leaf, and atmosphere, but the response to these environmental and internal variables were shown to depend on the species (Running 1976; Sandford and Jarvis 1986; Bond and Kavanagh 1999). For instance, in the western United States, stomatal conductance of gymnosperms tends to be more sensitive to vapor

pressure deficit (VPD) than for angiosperms (Marshall and Waring 1984; Bond and Kavanagh 1999). The origin of such differences might be found in the leaf hydraulic design. The extent to which the time-varying flux of water through the leaf might affect the performance of the photosynthetic cells depends both on the hydraulic linkage between xylem and epidermis, and on the degree to which the mesophyll is hydraulically uncoupled from the transpiration stream. The former has implications for stomatal control of xylem tensions, while the latter bears on the effects of transient imbalances in supply and demand on the water status of the mesophyll (Zwieniecki et al. 2007). In that regard, the hydraulic isolation of the epidermis to the xylem in conifers could involve more negative water potential in the epidermis than in the xylem, e.g. following changes in VPD, and thus correspond to stomatal closure before the xylem has experience significant water potential drop (Zwieniecki et al. 2007). Inversely, the high hydraulic connectivity often observed between the xylem and the epidermis in angiosperms could allow for efficient water supply from the xylem and for fast stomatal control following variations in xylem water potential. Such fast stomatal closure does not necessarily impact photosynthesis, since the mesophyll of the majority of angiosperms is hydraulically uncoupled from the xylem. This allows those cells to buffer the propagation of transient changes in transpiration and minimize the degree to which short-term variation in transpiration affects the mesophyll (Zwieniecki et al. 2007). For instance, this lack of synchronicity between stomatal closure and the decline of photosynthetic traits such as mesophyll conductance (g_m), permit some poplar genotypes to maintain higher WUE during the early stages of drought stress (e.g. Flexas et al. 2002; Braatne et al. 1992; Theroux-Rancourt et al. 2014, 2015). Such genotypes even recover better from short term deficit in water supply by being able to restore high rates of carbon assimilation faster after a return to well-watered conditions (Theroux-Rancourt et al. 2015). Nonetheless, the longer the duration of the drought, the longer it takes for leaves to recover their pre-drought transpiration and leaf hydraulic conductivity rates. This often takes over a month and substantially limits leaf-level processes (Blackman et al. 2009). Other tree species, such as *Eucalyptus*, exhibit a high connectivity with the mesophyll, thus making the leaf highly responsive to the environment and, although not buffering the mesophyll cells, could allow for an increase in both photosynthesis and mesophyll conductance as stomatal conductance increases (e.g. Cano et al. 2014).

Finally, as mature leaves sense environmental conditions, plants can also develop longer lasting stomatal-based responses to persistent water deficit by adjusting stomatal density in developing leaves (Lake et al. 2001; Miyazawa et al. 2006; Casson and Hetherington 2010; Hamanishi et al. 2012). Lower stomatal density restricts the number of sites for water loss and decreases evapotranspiration. Modification of stomatal density in response to drought varies between tree species and is contingent on the severity of water deficit. For example, reduced stomatal densities after drought treatment have been observed in oak species (*Quercus series*, *Q. virgiana* and *Q. oleoides*; Cavender-Barres et al. 2007) as wells as in *Populus balsamifera* (Hamanishi et al. 2012) and *Betula pendula* (Pääkkönen et al. 1998), but no alteration in stomatal densities was found in *Prunus avium* (Centritto et al. 1999), *Q. petraea* and *Pinus pinaster* (Guehl et al. 1994).

Genetic Control of Abiotic Stress Tolerance of Angiosperm Trees

Tree response to abiotic stress is a complex biological process. The ability of trees to adapt and survive harsh environments is a consequence of a variety of biochemical and physiological processes at the whole plant level. Many of which are the result of stress signal perception leading to alterations in the transcriptome that result in an adaptive response (Kozlowski and Pallardy 2002; Lei et al. 2006). Numerous studies on woody plants show intra- and interspecific genetic variation in characteristics associated with drought resistance, such as vulnerability of xylem to cavitation (Kavanagh et al. 1999; Maherali et al. 2006), hydraulic conductance (Alder et al. 1996), WUE (Lauteri et al. 2004) and stomata size and density (Bruschi et al. 2003). Drought tolerance involves many genes and biochemical-molecular mechanisms, but also depends on large genome×environment interactions (Tardieu and Tuberosa 2010). In fact, in the case of forest tree species, such as *Castanea sativa*, *Populus przewalski*, and *Quercus suber*, differential responses to drought have been reported at the population level for different geographical origins (Lauteri et al. 1997; Lei et al. 2006; Ramirez-Valiente et al. 2009; Aranda et al. 2012). This suggests the maintenance of a large adaptive intra-specific genetic variability to acclimate to stressful environments. In recent years, in addition to our knowledge of phenotypic variations, much progress has been made in elucidating the molecular and genetic determinants of tree responses to abiotic stress. Such research particularly benefits from the completion of a draft sequence of various tree species such as *Populus trichocarpa* (Tuskan et al. 2006), *Malus×domestica* (Velasco et al. 2010), *Prunus persica* (Verde et al. 2013), *Picea glauca* (Birol et al. 2013), *Picea abies* (Nystedt et al. 2013), *Eucalyptus grandis* (Myburg et al. 2014), as well as the release of the loblolly pine *Pinus taeda* genome (Neale et al. 2014). This opens promising avenues for comparative genomics in woody plants to fully understand the evolutionary basis of gene function and molecular network underlying tree responses to abiotic stress.

Below we present and compare the main findings made across tree species in the understanding of the genetic basis of tolerance to drought. In particular, we discuss the degree of similarity or differences in drought response between species and how such information could be used to gain insight into tree adaptation to drought.

Quantitative Trait Loci Involved in Drought Stress Response

Using genomic and transcriptomic data, Street et al. (2006) identified 30 QTLs associated with drought tolerance in an F2 pedigree of *Populus trichocarpa×P. deltoides*. Some of these QTLs co-localized with genes that were found to be up or down-regulated in response to drought. These might represent candidate genes for the tree response to water depletion (e.g. *GIGANTEA* dehydration-stress protein, glutathione-peroxidase 1, universal stress protein). Interestingly, in many cases, candidate genes supported previous functional descriptions in other species. In F1 and F2 hybrid populations of *Salix* and *Eucalyptus*, from eight to 66 minor drought

QTLs were identified (Rönnberg-Wästljung et al. 2005; Teixera et al. 2011). Such studies highlight that many genes of modest effect control tree adaptation to drought, with each QTL explaining a low to moderate proportion of total phenotypic variance (from 5 to 30 %).

When similar experimental procedures and phenotypic parameters are used to evaluate drought response, the availability of orthologous markers among unrelated mapping families or species (Casasoli et al. 2006; Aranda et al. 2012) allows comparative QTL mapping. Such analyses have been conducted for QTLs controlling carbon isotope discrimination, an important measure of drought tolerance, in two closely related genus within the family Fagaceae. In comparison of the European white oak (*Quercus robur*) and the European chestnut (*Castanea sativa*), which are widely distributed across Europe, oak displayed seven QTLs controlling carbon isotope discrimination, which were then compared to the positions of eight QTLs identified in chestnut using orthologous microsatellite markers. Overall, the genetic architecture of this adaptive trait was similar between oak and chestnut in term of QTL number and contribution to the phenotypic variance, no QTL for carbon isotope discrimination was conserved between both species (Casasoli et al. 2006). This could reflect the complex and contrasting evolution strategies of tree responses to drought, even in two closely related genus. Additionally, there can be significant overlap or correlations between different traits associated with WUE or drought. For instance, a major QTL governing water-use efficiency in *Quercus robur* was found to co-localize with a major QTL governing stomatal density (Brendel et al. 2008; Gailing et al. 2008). Many QTLs for drought-specific traits also co-localized in a *Populus trichocarpa* × *P. deltoides* F2 population (Street et al. 2006). Such co-localization may occur by chance, due to clustering of genes, or may be a consequence of pleiotropic effects. The latter may indicate overlapping or similar molecular basis of both phenotypic traits.

Gene Expression

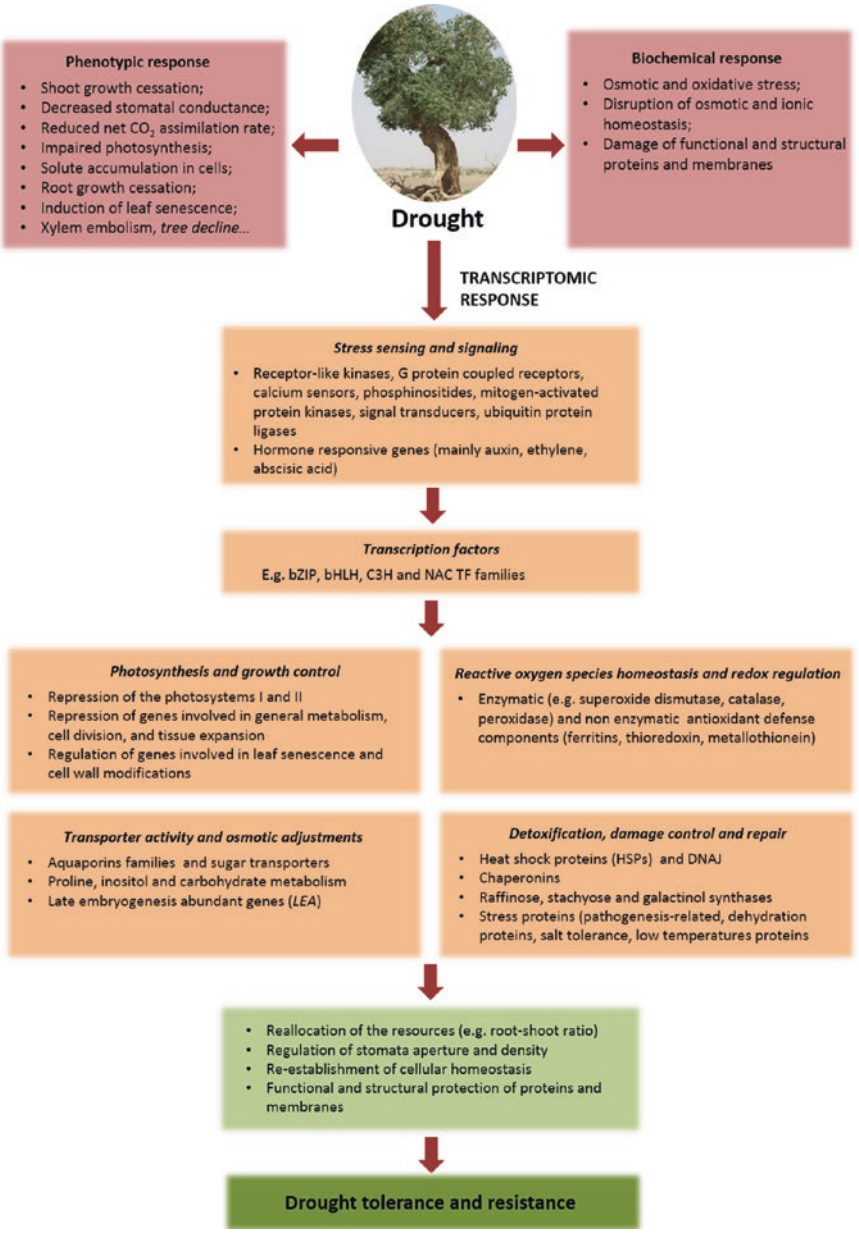
In the past years, numerous gene expression studies investigating water deprivation have been undertaken in various angiosperm tree species, with *Populus* being the most studied. Species included *Populus euphratica* (Brosché et al. 2005; Bogeat-Triboulot et al. 2007; Qiu et al. 2011; Yan et al. 2012; Tang et al. 2013), *P.* × *canadensis* (Caruso et al. 2008), *P. balsamifera* (Hamanishi et al. 2010), *P. simonii* (Chen et al. 2013), *P. trichocarpa* (Tang et al. 2015), *P. alba* (Berta et al. 2010), *P. yunnanensis* (Peng et al. 2012), *Quercus robur* (Spieβ et al. 2012), *Eucalyptus camaldulensis* (Thumma et al. 2012), *Malus* × *domestica* cultivars (Wisniewski et al. 2008), as well as various interspecific hybrids and genotypes: *Populus deltoides* × *P. nigra* (Wilkins et al. 2009; Cohen et al. 2010), *P. trichocarpa* × *P. deltoides* (Street et al. 2006), *P. nigra* × P. *maximowiczii* (Wilkins et al. 2009), *Salix viminalis* × (*S. viminalis* × *S. schwerinii*) (Pucholt et al. 2015).

Together, these studies revealed the regulation of hundreds or even thousands of differentially expressed genes involved in drought stress. These genes serve as

potential markers for tree breeding programs to develop genotypes resistant to the effects of drought. Such studies also provide a starting basis for comparative genomics initiatives, which have emerged as a powerful tool for deciphering the molecular basis of drought responses and reveal physiologically relevant processes (Wilkins et al. 2009; Cohen et al. 2010; Hamanishi et al. 2010). For example, comparative genomic studies conducted on poplar and willow have revealed some common, but also unique transcriptional pathways between and within tree species in response to drought (Cohen et al. 2010; Hamanishi et al. 2010; Pucholt et al. 2015; Tang et al. 2015). For example, Tang et al. (2015) highlighted differential expression of H^+-ATPase, rubisco activase, laccases and expansin genes in *Populus trichocarpa* but not in the desert poplar *P. euphratica*. In addition, when comparing pine and poplar drought transcriptomic responses to *Arabidiopsis thaliana*, strong species-dependent features were revealed, with none of the 27 genes reliably responsive to water stress in *Arabidiopsis* being regulated under drought in poplar nor pine (Bray 2004; Polle et al. 2006). This might reveal that angiosperm trees, regardless of species or hybrids, are likely to have different mechanisms in response to drought stress and they are highly variable among species and genotypes.

However, a major difficulty for comparing the outcomes of different tree transcriptomic studies is that the elicited responses not only differ among genotypes, but also differ depending on the organ and developmental stage of the plant sampled, as well as on the severity and duration of the stress applied (Cohen et al. 2010; Spieß et al. 2012; Pucholt et al. 2015). For example, when a severe stress might induce rapid responses such as stomatal closure, a gradual water depletion regime gives the plant the time to better react and adapt to the stress by implementing additional mechanisms of stress protection (e.g. adjustment of the leaf osmotic potential, which is a relatively slow process) (Arndt et al. 2001; Tesche 1992; Bruce et al. 2007; Blum 2009; Spieß et al. 2012). As a result, varying water depletion regimes seems to induce the transcriptional activation of different gene networks. For example, in oak, loblolly pine, and poplar, regulation of distinct sets of genes were observed across the time, and among the water deficit regimes applied (Watkinson et al. 2003; Spieß et al. 2012; Yan et al. 2012). In general, the higher the water deficit intensity was, the more wide-ranging the regulatory changes and the cellular responses observed (Bogeat-Triboulot et al. 2007; Hamanishi et al. 2010; Yan et al. 2012). To add to this complexity, transcriptional response to drought stress in trees, such as poplar, has been also found to vary depending on the time of the day (Wilkins et al. 2009; Hamanishi et al. 2010), on gender, as well as on the clone growth history. Vegetatively-propagated poplars from the same clone that were previously exposed to different environmental conditions developed differences in transcript abundance patterns and DNA methylation levels in response to the same drought treatment, indicating potential roles for epigenetic regulation of drought responses (Raj et al. 2011; Peng et al. 2012). These studies highlight the high complexity of gene expression studies in response to abiotic stress.

Among the various transcriptional studies investigating angiosperm trees response to drought, some general biological pathways can be identified. A description of such pathways along with putative genes involved is presented in Fig. 3. In the various studies, most genes displaying altered expression during water deficit


Phenotypic response
Shoot growth cessation;
Decreased stomatal conductance;
Reduced net CO_2 assimilation rate;
Impaired photosynthesis;
Solute accumulation in cells;
Root growth cessation;
Induction of leaf senescence;
Xylem embolism, tree decline...
Drought
Biochemical response
Osmotic and oxidative stress;
Disruption of osmotic and ionic homeostasis;
Damage of functional and structural proteins and membranes
TRANSCRIPTOMIC RESPONSE
Stress sensing and signaling
Receptor-like kinases, G protein coupled receptors, calcium sensors, phosphinositides, mitogen-activated protein kinases, signal transducers, ubiquitin protein ligases
Hormone responsive genes (mainly auxin, ethylene, abscisic acid)
Transcription factors
E.g. bZIP, bHLH, C3H and NAC TF families
Photosynthesis and growth control
Repression of the photosystems I and II
Repression of genes involved in general metabolism, cell division, and tissue expansion
Regulation of genes involved in leaf senescence and cell wall modifications
Reactive oxygen species homeostasis and redox regulation
Enzymatic (e.g. superoxide dismutase, catalase, peroxidase) and non enzymatic antioxidant defense components (ferritins, thioredoxin, metallothionein)
Transporter activity and osmotic adjustments
Aquaporins families and sugar transporters
Proline, inositol and carbohydrate metabolism
Late embryogenesis abundant genes (LEA)
Detoxification, damage control and repair
Heat shock proteins (HSPs) and DNAJ
Chaperonins
Raffinose, stachyose and galactinol synthases
Stress proteins (pathogenesis-related, dehydration proteins, salt tolerance, low temperatures proteins
Reallocation of the resources (e.g. root-shoot ratio)
Regulation of stomata aperture and density
Re-establishment of cellular homeostasis
Functional and structural protection of proteins and membranes
Drought tolerance and resistance

returned to the control levels after the plants were re-irrigated and allowed to recover, indicating that these are transient responses to drought (Bogeat-Triboulot et al. 2007). Besides these functionally annotated genes, many genes of unknown functions have been found to be differentially regulated in these studies and represent an additional source of candidate genes for drought tolerance (Wilkins et al. 2009; Thumma et al. 2012; Spieß et al. 2012; Yan et al. 2012).

Stress Sensing, Signaling and Transcription Control

Drought, cold and salt stress are different abiotic stresses yet share a cluster of signaling pathways linked to water stress. After sensing of water deficit at the cellular level (e.g. through changes in turgor pressure trough the roots), one of the first plant response is the activation of signal cascades triggering alterations in gene expression. This in turn leads to changes at the cellular, physiological and developmental level. In angiosperm trees, known drought response signaling genes encode for proteins including receptor-like kinases, G protein coupled receptors, calcium sensors, phosphinositides, mitogen-activated protein kinases, signal transducers (XLG2), ubiquitin protein ligases and calmodulin (Wisniewski et al. 2008; Berta et al. 2010; Spieβ et al. 2012; Tang et al. 2013). In various studies, a large number of genes encoding for transcription factors (TF) have also been found to be differentially expressed. Up to 25.1 % of genes encoding for TF genome-wide were regulated in the desert poplar *Populus euphratica* in response to water deprivation, indicating massive remodeling of gene expression (Tang et al. 2013). In this particular study, MYB and MYB-related TF family were the most abundant, followed by those matching to the bZIP, bHLH, C3H and NAC TF families. Plant hormones also seem to play central roles in the ability of plants to adapt to changing environments, and hence, response to abiotic stress, by mediating growth, development,

Fig. 3 Overall model of tree response to drought. At the whole plant scale, under a gradual soil water depletion, impact on the phenotype usually begins with shoot growth cessation, followed by decreased stomatal conductance, leading in turn to a reduced net CO_2 assimilation rate, impaired photosynthesis, solute accumulation in cells, root growth cessation and finally, when water availability is very low, induction of leaf senescence and plant decline (Passioura 1996). At the biochemical level, drought causes cellular damages and secondary stresses, such as osmotic and oxidative stress. In angiosperm trees tolerant to drought, such abiotic stress will activate a cascade of transcriptomic responses. First, stress perception at the cellular level (e.g. through changes in turgor pressure by the roots or through sensing of the secondary osmotic and oxidative stress) triggers downstream signaling cascades (hormone dependent or independent) and activation of transcriptomic factors. Such transcriptomic factors will in turn regulate genes acting in various biological processes such as photosynthesis and plant growth, reactive oxygen species (ROS) homeostasis and redox regulation, osmotic adjustments, detoxification, damage control and repair. Activation of such stress-responsive mechanisms participates in optimizing tree water use efficiency, re-establishing cellular homeostasis, and protecting and repairing damaged proteins and membranes

nutrient allocation and source/sink ratio (Peleg and Blumwald 2011). Differentially expressed genes associated with various hormones were identified in trees in response to drought (Street et al. 2006; Caruso et al. 2008; Berta et al. 2010; Cohen et al. 2010; Hamanishi et al. 2010; Qiu et al. 2011; Peng et al. 2012; Yan et al. 2012; Chen et al. 2013; Tang et al. 2013). Although auxin (IAA), ethylene (ET) and abscisic acid (ABA) figure among the most studied across different taxa, studies have also revealed the importance of brassinosteroid (BR), gibberellic acid (GA), jasmonate (JA), salicylic acid (SA), and cytokinin (CK). Recent evidence in plant physiology indicated that plant hormones are involved in multiple processes. For instance, among other important functions of ABA in seed maturation and germination, as well as in the regulation of gene expression, one of its most prominent roles is the adaptation to abiotic stress via the regulation of stomatal movements (Leung and Giraudat 1998; Wilkinson and Davies 2002). In addition, cross-talk between the different plant hormones often results in synergetic or antagonic interactions that play crucial roles for the adaptation of plants to abiotic stress (Peleg and Blumwald 2011).

Reactive Oxygen Species and Antioxidant Defense Machinery

Besides these signaling molecules, a broad repertoire of protective genes was found to be regulated in response to abiotic stress. Abiotic stress often leads to the production reactive oxygen species (ROS), which can be important in plant signaling but, if produced in excess, can also damage proteins, lipids, carbohydrates and DNA, and ultimately results in an oxidative stress (Gill and Tuteja 2010). In trees, enzymatic (superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, glutathione-S-transferase, oxidoreductase, aldehyde dehydrogenase) as well non-enzymatic antioxidant defense components (ferritins, thioredoxin, glutaredoxin, metallothionein) were found to be regulated in response to drought (Brosché et al. 2005; Street et al. 2006; Bogeat Triboulot et al. 2007; Cohen et al. 2010; Qiu et al. 2011; Peng et al. 2012; Spieβ et al. 2012; Yan et al. 2012; Chen et al. 2013; Tang et al. 2015). Such molecules might work in concert to maintain cellular redox homeostasis and protect plant cells from oxidative damage by scavenging of ROS.

Photosynthesis and Growth Control

Photosynthesis and cell growth are among the primary processes to be affected by drought. Hence, in addition to genes regulating the stomata aperture (e.g. *OPEN STOMATA* 1; genes related to the regulation of ABA; Chen et al. 2013) as well as stomata density and distribution (e.g. *STOMATOGEN*, *ERECTA*, *FAMA*; Hamanishi et al. 2012), down-regulation of many genes associated to photosynthesis was demonstrated in trees under drought stress. Such genes are mainly components of the electron-transport chains, the photosystems I and II (Wisniewski et al. 2008; Cohen et al. 2010; Peng et al. 2012; Yan et al. 2012; Spieβ et al. 2012; Thumma et al. 2012; Chen et al. 2013).

Alteration of photosynthetic metabolism can arise either from the direct decreased of CO_2 availability due to diffusion limitations through the stomata and the mesophyll, or can arise as a secondary effect of the oxidative stress (Chaves et al. 2009). In this latter case, one hypothesis would be that, in order to maintain ROS homeostasis under drought stress, trees would suppress photosynthetic capability by broadly reducing electron transport in photosystem I and photosystem II (Tang et al. 2015).

Together with the repression of tree growth observed in the various studies, transcriptome analysis revealed a strong down regulation of genes related to general metabolism, cell division, and tissue expansion (Cohen et al. 2010; Bogeat-Triboulot et al. 2007; Wisniewski et al. 2008; Thumma et al. 2012; Tang et al. 2015). Interestingly, premature leaf drop during drought was associated with the regulation of genes involved in senescence, such as *CLPR1*, *SENESCENCE-ASSOCIATED GENE 21*, as well as proteins including PUTATIVE SENESCENCE-ASSOCIATED PROTEIN, WRKY transcription factors as well as cysteine proteases (Brosché et al. 2005; Cohen et al. 2010; Spieβ et al. 2012; Tang et al. 2013; Chen et al. 2013). In addition, the observation in poplar of a pronounced induction of Asn Synthetase prior to leaf senescence might suggests a strong remobilization of nitrogen (Bogeat-Triboulot et al. 2007). Cell wall and cuticle properties have been also shown to change after drought treatment (Zwiazek 1991; Potters et al. 2007; Moore et al. 2008). Hence, various cell-wall related genes (e.g. pectin esterases, o-methyltransferases, expansins, cellulose synthases, laccases, cell wall-associated kinases, endoxyloglucan) and genes involved in epicuticular wax biosynthesis (e.g. *CER1*) have been found to be regulated in response to water deprivation (Street et al. 2006; Caruso et al. 2008; Berta et al. 2010; Cohen et al. 2010; Hamanishi et al. 2010; Spieβ et al. 2012; Thumma et al. 2012; Chen et al. 2013; Tang et al. 2015).

Transporter Activity and Osmotic Adjustments

Among other transporters, genes encoding for aquaporins (NIP, SIP, TIP and PIP) and sugar transporters (e.g., STP1; ATINT1; glucose-6-phosphate transport, GPT2) were the most represented (Wilkins et al. 2009; Brosché et al. 2005; Bogeat-Triboulot et al. 2007; Berta et al. 2010; Cohen et al. 2010; Qiu et al. 2011; Yan et al. 2012; Chen et al. 2013). Collectively, these products transport water and sugars through the plasma membranes and the tonoplast and can help cells adjust to changes in osmotic pressure. For instance, members of the PIP1 family of aquaporins have been shown to be important for recovery from xylem embolism, in poplar (Secchi and Zwieniecki 2010; Laur and Hacke 2013) and in spruce (Laur and Hacke 2014). Variation of transcript abundance of various aquaporins family members was also found in poplar species having contrasting drought response strategies at the whole plant level (Almeida-Rodriguez et al. 2010). Such variability may reflect the importance of aquaporins with respect to water transport and drought tolerance in trees (Hamanishi and Campbell 2011).

Osmotic adjustment was also evidenced by genes related to proline (e.g. delta-1-pyrroline-5-carboxylate synthase, *P5CS*; *P5C* dehydrogenase, *P5CDH*; ornithine aminotransferase, *OAT*), inositol (e.g. myo-inositol oxygenase; inositol-triphosphate

5- phosphatase 2 – like), carbohydrate metabolism (such as beta-amylase, endochitinase, malate dehydrogenase, sucrose synthase, starch synthase) as well as late embryogenesis abundant genes (*LEA* 4, 5- D, 14-A) that showed significantly up or down-regulation (Brosché et al. 2005; Street et al. 2006; Peng et al. 2012; Yan et al. 2012; Spieβ et al. 2012; Tang et al. 2013, 2015; Chen et al. 2013; Pucholt et al. 2015).

Detoxification, Damage Control and Repair

In the desert poplar *Populus euphratica*, a great diversity of heat shock proteins (HSPs) (including transcripts for DNAJ heat shock family proteins, members of small HSPs, HSP70, HSP90, HSP100), heat shock transcription factors (e.g. HSFA2, HSF4, HSFC1) and chaperonins were induced by all the different drought levels (Caruso et al. 2008; Wisniewski et al. 2008; Cohen et al. 2010; Peng et al. 2012; Thumma et al. 2012; Chen et al. 2013; Yan et al. 2012; Tang et al. 2013; Pucholt et al. 2015). Such molecules are involved in protein repair and protection against denaturation, which are normally synthesized in response to abiotic stress such as drought and high temperatures. That many more genes for heat-shock proteins and regulatory factors that were found in *P. euphratica* compared to other species (Spieβ et al. 2012) might suggest that this species has evolved a robust mechanism for preventing proteins from being denatured in harsh environments combining both water depletion and high temperatures. In accordance with ROS production and detoxification processes, up-regulation of raffinose, stachyose and galactinol synthases were detected in poplar leaves under drought. An increase in such compound could improve osmoprotection and ROS scavenging (Cohen et al. 2010; Hamanishi et al. 2010). Finally, various stress proteins were shown to be differentially regulated under drought stress: PR proteins (e.g. chitinase, osmotin, PR protein 1, peroxidase, thaumatin-like proteins), dehydration proteins (early-responsive to dehydration stress protein ERD4, ERD6, ERD7 and ERD15; dehydrin LEA; dehydration responsive factor RD19, RD21, RD22, RD26; drought-induced DI19, DI21; xerico), salt tolerance proteins (salt tolerance zinc finger proteins) as well as low temperatures induced genes (e.g. cold-regulated *LTCOR12*; rare-cold-inducible *RCI2A*, *RCI2B*; frostbite 1) (Bogeat-Triboulot et al. 2007; Caruso et al. 2008; Wisniewski et al. 2008; Cohen et al. 2010; Hamanishi et al. 2010; Thumma et al. 2012; Spieß et al. 2012; Yan et al. 2012; Chen et al. 2013; Pucholt et al. 2015).

Conclusion

Climate change, conducing notably to extreme drought events, affects forest tree survival worldwide. This emphasizes the need to better understand the mechanisms of tree adaptation to abiotic stress. Angiosperm tree species show a remarkable plasticity that shapes tree evolution and distribution. They respond to adverse changing environmental conditions via a series of physiological, cellular, and molecular processes culminating in stress tolerance. Such processes were shown to

be under a tight genetic control and a great variability of gene expression was found across species, but also within the same tree species.

Future studies making use of comparative genomic approaches have the potential to provide new insights into the evolution and diversification of drought response strategies in various angiosperm tree species and taxa. One critical aspect for future studies is to develop standardized experimental conditions for imposing drought treatments, and standardized methods for measuring drought response traits and sampling tissues for genomic analysis. Such efforts would greatly enhance the ability to make comparisons among species, and begin to systematically describe and quantify drought response mechanisms within and across species. These future studies have the potential to make significant contributions not only to the basic biology of drought response in angiosperm trees, but also to the development of cultivars resilient to drought and other abiotic stresses.

Glossary

Isohydric water-balance behavior involves the maintenance of a constant leaf water potential at midday, even under drought conditions. In contrast, plants exhibiting anisohydric behavior can markedly decrease water potentials following the evaporative demand experienced during the day. This permits lower leaf water potentials in the presence of drought stress.

Mesophyll conductance (g_m) estimates the restriction to the influx of carbon dioxide from the leaf internal airspace to the site of carboxylation. Together with stomatal conductance, it constitutes a crucial component of the diffusive limitation of photosynthesis.

Ψ_{crit} critical xylem water potential, beyond which hydraulic failure is likely to occur, expressed in megapascals (MPa)

Ψ_{soil} soil water potential (MPa)

Stomatal conductance usually measured in mmol m^{-2} s^{-1}, is the measure of the rate of water vapor exiting through the stomata of a leaf, from which one can estimate the rate of carbon dioxide (CO_2) entering.

Vapour Pressure Deficit (VPD) is the difference between the amount of moisture in the air and saturated vapour pressure, i.e. how much moisture the air can hold when it is saturated, at a certain temperature.

Water use Efficiency (WUE) represents the tradeoff between C assimilation and water loss, expressed as the ratio of C assimilated, e.g. in term of biomass or photosynthetic rate, to the rate of transpiration.

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Landscape Genomics of Angiosperm Trees: From Historic Roots to Discovering New Branches of Adaptive Evolution

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Abstract Landscape genomic studies analyze spatial patterns of genetic variation to test hypotheses about how demographic history, gene flow, and natural selection have shaped populations. For decades, angiosperm trees have served as outstanding model systems for landscape-scale genetic studies due to their extensive geographic ranges, large effective population sizes, abundant genetic diversity, and high gene flow. These characteristics were recognized early in the landscape genetics literature, and studies on angiosperm trees, particularly *Populus* and *Quercus*, tested hypotheses about how landscape features shaped neutral patterns of gene flow and population divergence. More recently, advances in sequencing and analysis methodologies have allowed for greater opportunities to directly test how natural selection acting locally across the landscape has shaped the genome-wide diversity of populations, often in the context of broad climatic gradients in growing season length. Despite, the methodological gains and successes of the last decade, landscape genomics studies face new challenges of study design, hypothesis testing, and validation. Here, we explore the development of landscape genetics and genomics in angiosperm trees and what we have learned from investigating the evolutionary consequences of life as a tree in heterogeneous landscapes. We outline the past, present, and potential future of landscape genomic studies in angiosperm trees, highlighting successes of the field, challenges to overcome, and ideas that scientists from all backgrounds engaged in landscape genomics should consider.

Keywords Genomics • Trees • Landscape Genetics • Association Genetics

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Introduction

The biological characteristics of some organisms lend themselves almost perfectly to the fields of study that have developed around them: *Drosophila* and population genetics; *Caenorhabditis elegans* and development; maize and plant architecture. Trees are, at first glance, unlikely model organisms, especially given their longevity and long generation time (Taylor 2002); however, as models for ecological and landscape genomics, they combine a unique set of features (Petit and Hampe 2006). Trees have large effective population sizes (Petit and Hampe 2006; Evans et al. 2015), disperse genes widely through pollen and seed over long distances (Nathan et al. 2002), produce millions of progeny that encounter strong selection (Augspurger 1984), can clonally propagate and persist for millennia (Ally et al. 2008), extensively hybridize with congeners (Moran et al. 2012), and have large lifetime exposures to pathogens and herbivores that can change annually (Floate et al. 2016). These varied characteristics result in complex evolutionary processes that play out in landscapes as small as a few hectares (Streiff et al. 1999), to entire biomes (Keller et al. 2010) and continents (Callahan et al. 2013), making trees excellent candidates for the study of how ecological selection and landscape features structure genetic diversity.

There is a rich history of ecological genetic studies in forest trees beginning with provenance trials of the eighteenth century (Langlet 1971). Foresters would collect range-wide samples of seeds or cuttings from diverse populations and propagate these into common gardens, often replicated in different climatic regions of the landscape, with the objective of assessing how genetics and environment influence growth and yield, especially patterns of vegetative bud phenology and the timing of adaptive seasonal dormancy (Savolainen et al. 2007). While these efforts began well before the age of genomics, researchers were already recognizing the importance of climate, disease, and other sources of selection in structuring forest tree adaptive diversity. With the rise of the genomic era, ecological geneticists working on trees have been increasingly in a position to evaluate the genetic architecture of local adaptation, identify regions of the genome associated with climate, and accelerate the selection and breeding process of tree domestication.

The expansion of genomic resources in poplars (*Populus*, Tuskan et al. 2006), *Eucalyptus* (Myburg et al. 2014), oaks (*Quercus*, Plomion et al. 2016), and other forest-trees (Liang et al. 2011; Fang et al. 2012) was critically important for opening new avenues of research in ecological and landscape genomics, and in many ways, studies on trees drove this field forward. *Populus* emerged as the model for tree biology in the 2000s due to the ease of clonal propagation, manageable genome size (~480Mb), ecological diversity, extensive hybridization, the availability of genomic resources (such as its transformation by *Agrobacterium*), and its candidacy as a source of cellulosic bioethanol production (Difazio et al. 2011). The accumulation of population genetic and genomic studies in *Populus* has resulted in large gains in our understanding of the genetic architecture of phenology (Keller et al. 2012; Olson et al. 2013; McKown et al. 2014a, b, c), wood and biomass growth (Porth et al. 2013), disease

resistance (Porth et al. 2015; Foster et al. 2015), and many other physiological processes. The *Populus* community is global and continues to grow in the fields of biomass production (Zhang et al. 2015), phytoremediation (Di Lonardo et al. 2011), the plant microbiome (Hacquard and Schadt 2015), and of course, among other fields, landscape genomics (Evans et al. 2014; Geraldes et al. 2014; Porth et al. 2015).

More recently, *Quercus* sp. are emerging as models for the study of ecological and landscape genomics (Petit et al. 2013). Consisting of over 400 species, oaks are dominant in many Northern Hemisphere forests, and represent a large carbon sink in terrestrial landscapes (Myneni et al. 2001). Many are foundational species that maintain biodiversity by providing habitat and food for numerous plants and animals (Block et al. 1990; Burns and Honkala 1990; Fralish 2004; McShea et al. 2007). They are also of high economic value for their lumber (Luppold and Bumgardner 2008; Espinoza et al. 2011) and ecosystem services that include carbon sequestration, riparian buffering and water quality enhancement (Dosskey et al. 1997; Kroeger et al. 2010), improvement of hunting and rangelands (Standiford and Howitt 1993; Kroeger et al. 2010), improved nutrient cycling (Dahlgren et al. 1997; Herman et al. 2003), and cultural significance (Pavlik et al. 1991; Anderson 2005).

In this chapter, we explore the development of landscape genetics and genomics in angiosperm trees and what we have learned from investigating the evolutionary consequences of life as a tree in a heterogeneous landscape. We focus especially on *Populus* and *Quercus*, which have made the greatest strides to date among landscape studies on angiosperm trees. We then highlight several areas where researchers in tree landscape genomics face challenges, or historically have been narrowly focused. Lastly, we offer recommendations for investigating overlooked aspects of tree biology and evolution in landscape genomics studies, and suggest new inquires for future research directions.

Historical Review

Landscape Genetics: Gene Flow and Population Structure

Before the mid-2000s and the onset of the next generation sequencing (NGS) revolution, landscape genetic studies focused primarily on how genetic diversity of anonymous markers were shaped by neutral demographic processes, including the effects of gene flow, clonality, isolation-by-distance, range expansion, and population structure (Manel et al. 2003). Polymorphic loci were rarely associated with adaptive signatures in allele frequency driven by environmental agents of selection (González-Martínez et al. 2006), a fact largely due to the type of molecular markers employed (e.g. amplified-length polymorphisms, AFLPs; random amplified polymorphic DNA, RAPDs; and simple sequence repeats, SSRs/microsatellites) and the typically unknown location of these markers in a genome. Nevertheless, these loci were sufficient to answer questions regarding demographic processes shaping genetic diversity.

Early landscape genetic studies in angiosperm trees focused on both contemporary gene dispersal (Sork and Smouse 2006) as well as larger-scale phylogeographic structure (Petit et al. 2002). Pollen and seed differ by the distances they are dispersed, their dispersal vectors, the genomes they carry, and their ploidy level; characteristics with pronounced effects on spatial patterns of diversity (McCauley 1995; Petit and Hampe 2006). Elegant early studies of landscape genetics in white oaks by Petit and colleagues laid the foundation for our understanding of how long-distance seed dispersal creates legacies of genetic structure during range expansion (Le Corre et al. 1997; Petit et al. 1997). In *Quercus robur* and *Q. petraea*, Petit et al. (1997) discovered homogenous patches of chloroplast (cpDNA) haplotypes on the landscape, suggesting that rare long-distance dispersal events followed by local population expansion, rather than a continuous wave of migration, structures landscape diversity in cpDNA. Finer-scale understanding of pollen and seed movement came from Sork and colleagues who demonstrated how landscape configuration and land use history shaped the effective number of pollen donors (N_{ep}). Fragmentation and habitat loss lowered N_{ep} in wind-pollinated California valley oaks (*Q. lobata*) (Sork et al. 2002), while fragmentation actually facilitated gene flow in the insect-pollinated flowering dogwood (*Cornus florida*) (Sork et al. 2005). Studies in *Q. robur* and *Q. petraea* demonstrated that pollen-mediated gene flow occurring over long distances (>200 m) is common, accounting for 67% of matings (Streiff et al. 1999). Studies in *Populus* have also informed our understanding of gene flow distances. Dispersal of exotic or transgenic alleles is a concern in hybrid *Populus* plantations. Carefully designed gene flow studies demonstrated that most pollination and seed establishment occurs near the source tree (within 450 m) (DiFazio et al. 2012), and pollination occurs less frequently when native tree populations are large. Establishment of exotic or transgenic genes in populations of native species is more likely when native population sizes are small, and removing native female trees near exotic or transgenic plantations may be an effective means of eliminating gene flow in managed landscapes (Meirmans et al. 2010).

Some angiosperm trees (especially in *Populus*) are known for extensive clonal reproduction that can have a strong effect on local genetic structure (Mock et al. 2008), and once established, a set of clones can dominate an area for long periods (Imbert and Lefevre 2003). Early studies in black poplar (*Populus nigra*) assessed the extent of clonal diversity remaining among two remnant populations along the Rhine River in the Netherlands (Arens et al. 1998). Using SSRs, they identified 13 distinct clones among the 71 ramets sampled, suggesting extensive clonality. Recent studies of the highly clonal quaking aspen (*P. tremuloides*) in the western U.S. also used SSRs to identify and map the spatial extent of clones on the landscape (Mock et al. 2008). Mock et al. (2012) was also able to document the widespread existence of triploid aspen clones in western U.S. landscapes, which represent a unique source of genetic diversity relative to the more recently colonized populations in the glaciated northern regions of the continent (Callahan et al. 2013). The molecular estimates of clone age in *P. tremuloides*, ranging from 175 to 10,000 years (Ally et al. 2008), suggest that once established, *Populus* clones may shape the genetic diversity of landscapes for millennia.

At larger spatial and temporal scales, the Pliocene and Pleistocene glacial cycles had a substantial impact on the genetic diversity of many North American (Soltis et al. 2006) and European trees (Hewitt 1999) through waves of range expansion and contraction, although not all species experienced population size changes (Grivet et al. 2006). Post-glacial migration rates from pollen cores predicted fast tree migration rates out of refugia (100–1000 m/year), but estimates from genetic data are contradictory (<100m/year) (McLachlan et al. 2005; Feurdean et al. 2013), and the increasing recognition of high latitude glacial refugia may refine our estimates of tree migration rates downward (Petit et al. 2008; Breen et al. 2012). Studies in trees revealed population size changes and genetic isolation in allopatric refugia create the conditions for speciation (Sorrie and Weakley 2001) as well as opportunities for adaptive introgression upon secondary contact (Bragg et al. 2015; Suarez-Gonzalez et al. 2016). For example, range shifts associated with Pleistocene climate change have been implicated in speciation between *Populus trichocarpa* and *P. balsamifera* (Levsen et al. 2012), as well as driving adaptive introgression during admixture of divergent European populations of *P. tremula* (De Carvalho et al. 2010).

The development of genomic resources in *Populus* (Tuskan et al. 2006) allowed for genotyping large numbers of individuals at hundreds to thousands of physically mapped loci (Ingvarsson 2008; Keller et al. 2010; Slavov et al. 2012). Applications of coalescent demographic modeling and Bayesian clustering to population genomic datasets revealed a general pattern of strong latitudinal gradients of population structure and geographic gradients in genetic diversity (Keller et al. 2010; Slavov et al. 2012). For example, in *P. angustifolia*, STRUCTURE analyses revealed southern, mid-latitude, and northern demes (Evans et al. 2015). These confounding effects of population structure (Fig. 1a) introduce challenges for finding adapted loci to under environmental selection from variables that covary with latitude (Fig. 1b), and hence understanding the demographic history of a sample became essential for any association genetic study using natural genomic diversity.

Candidate Gene Studies of Local Adaptation

Heritable phenotypic clines in phenology, growth, and other physiological traits are widespread in nature (Haldane 1948; Ingvarsson et al. 2006; Hall et al. 2007; Kempes et al. 2011), suggesting an intimate relationship between adaptive evolution and the landscape (Linhart and Grant 1996). In temperate and boreal forests trees, locally adapted clines of bud phenology (spring bud flush and late summer bud set) allow trees to coordinate their growth and dormancy with the available growing season, and achieve cold hardiness before low temperatures or drought become damaging (Ingvarsson et al. 2006). These phenotypic clines are the hallmark signatures of tree adaptation to climate, exhibiting strong heritability and genetically-based population differentiation, which have made them attractive targets for dissecting the genetic basis of local adaptation (Savolainen et al. 2007).

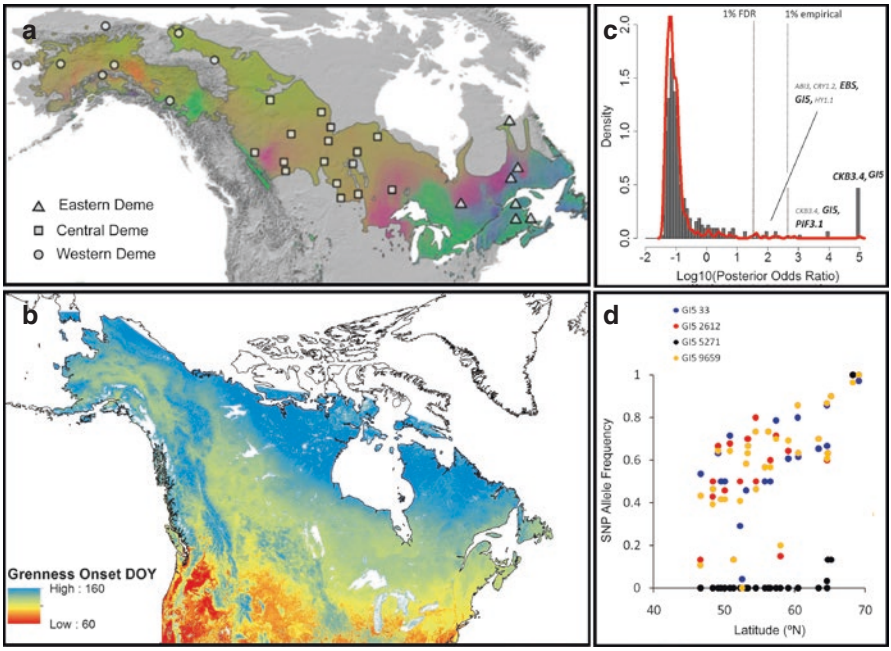

Fig. 1 Populations for landscape genomic studies are frequently sampled out of a context of spatially varying genomic structure and environmental change. The genetic structure of *P. balsamifera* is represented from a sample from 412 reference SNPs genotyped by Keller et al. (2010) with three demes observed: low diversity central and western demes, and a higher diversity eastern deme (**a**). Environmental gradients abound in nature, and greenness onset is a classic example of a biological phenomenon, controlled by both photoperiod and temperature, that changes across large geographic areas (**b**). Eliminating false positives in selection scans is always challenging; however, fewer false discoveries are observed from the tail of empirical *p*-value distributions derived from putatively neutral reference SNPs (**c**). This approach is demonstrated with the results of a BayeScan analysis (Foll and Gaggiotti 2008) of 412 reference SNPs and 335 candidate SNPs from 27 flowering-time genes that were sampled from the localities in (**a**). Setting a threshold conditioned with empirical *p*-values identifies local selection in SNPs from *GIGANTEA 5* (*GI5*), *EARLY BOLTING IN SHORT DAYS* (*EBS*), *PHYTOCHROME-INTERACTING TRANSCRIPTION FACTOR 3* (*PIF3*), and *CASEIN KINASE II BETA CHAIN 3* (*CKB3.4*). Allele frequency variation in four SNPs from *GI5* are associated with latitude (**d**). *GI5* is involved in light signaling to the circadian clock and local adaptation of these alleles may allow precise timing of phenology (Figures reproduced, with permission, from Fitzpatrick and Keller (2015) (**a**); Elmore et al. (n.d.) (**b**); Keller et al. (2012) (**c**, **d**))

The increased knowledge of gene function from model plants, especially *Arabidopsis*, coupled with the development of growing genomic resources in emerging angiosperm model trees (e.g. *Populus*, *Quercus*, *Eucalyptus*, and *Castanea*; Neale and Kremer 2011) facilitated a wave of local adaptation studies in tree bud phenology primarily focused on candidate genes from the flowering time and circadian clock associated pathways. Early efforts to associate adaptive clines in bud phenology with candidate genes were conducted in *P. tremula* by Ingvarsson and colleagues. *PHYB2* senses red to far-red light and regulates several important

traits in *Arabidopsis*, including hypocotyl elongation, meristem development, and flowering-time (Sheehan et al. 2007). It was hypothesized that changes in allele frequency in *PHYB2* would associate with clines in bud set driven by latitudinal variation in critical photoperiod. Latitudinal clines in *PHYB2* SNPs were detected, and, consistent with local adaptation, paralleled bud set clines; however, analytical methods were not capable of disentangling whether this was due to local selection or neutral processes (Ingvarsson et al. 2006). Follow-up studies including SNPs flanking the *PHYB2* region found allele frequencies did not deviate from neutral expectations, even though two nonsynonymous SNPs in *PHYB2* explained 1.5–5 % of clinal variation in bud set (Ingvarsson et al. 2008). Hall et al. (2007) leveraged the release of the *P. trichocarpa* genome (Tuskan et al. 2006) to design 18 neutral SSRs (7 near candidate genes) and sequenced 50 SNPs from five candidate genes (*PHYB2*, *COL2B*, *GA20OX1*, *ABI1B*, and *HYPO312*) for phenology responses. While phenology traits showed strong quantitative genetic differentiation among populations (Q_{ST}), candidate SNPs and SSRs near candidate genes did not show elevated F_{ST}, nor did the genetic data covary strongly with latitude (Hall et al. 2007). Ma et al. (2010) broadened the focus to include SNPs from 25 genes across the *P. tremula* photoperiod pathway. Using F_{ST} outlier tests for local adaptation, they were unable to differentiate adaptive from neutral patterns of population differentiation, even though several candidate SNPs were associated with phenotypes and showed allele frequency clines with latitude. Using alternative tests for positive selection based on comparing synonymous vs. nonsynonymous mutations (MK test) and intraspecific polymorphism vs. interspecific divergence (HKA test), (Hall et al. 2011) did find evidence for adaptive evolution in several photoperiod and circadian clock associated genes, including *PHYB2*.

These early studies in aspen revealed a complex reality that the genetic basis of local adaptation would be difficult to unravel for highly polygenic traits, and helped usher in two important realizations for the field of adaptive landscape genomics: (1) finding genes involved in local adaptation requires testing candidate genes against samples of loci from across the genome, using statistical models that account for the confounding effects of demographic history (Platt et al. 2010); and (2) deducing the genetic architecture of local adaptation would be a difficult road, as the average effect of a locus on a polygenic trait is small (Rockman 2012), and selection at the phenotypic level is distributed across many QTLs in the genome (Tiffin and Ross-Ibarra 2014). An enduring lesson for landscape genomics thus became limiting false discoveries while searching for local adaptation by developing, testing, and applying methods that explicitly modeled the demographic history of the sample (Lotterhos and Whitlock 2014). These new analytical methods included tests for clinal gene-environment associations (GEA), supported by programs such as Bayenv (Günther and Coop 2013) and latent-factor mixed models (LFMM) (Frichot et al. 2013), as well as F_{ST} outlier programs like BayeScan (Foll and Gaggiotti 2008) that detected diversifying selection against the background of neutral population structure.

In a study of local adaptation for phenology in *P. balsamifera*, Keller et al. (2012) used 412 putatively neutral reference SNPs to model effects of demographic history

and test for local adaptation in 27 candidate genes in the flowering-time pathway (Fig. 1c, d). F_{ST} outlier tests and GEA convergently identified 11 candidate SNPs that had signatures of excessive population structure or association with climate. Interestingly, phytochromes (e.g. *PHYA*, *PHYB1*, *PHYB2*) showed no signs of local adaptation in *P. balsamifera* despite their signature of adaptation along a latitudinal gradient in the related *P. tremula* (Ingvarsson et al. 2006; Ma et al. 2010), nor did phytochromes show evidence of species-wide positive selection, as in *P. tremula* (Hall et al. 2011; Keller et al. 2011). Instead, local selection in *P. balsamifera* was primarily targeted at the circadian clock controlled gene *GIGANTEA* (*GI*) (Oliverio et al. 2007), a master regulator of multiple developmental processes such as germination, flowering, and osmotic stress (Mishra and Panigrahi 2015). *GI* is known to mediate daylength signaling from the phytochromes to regulate expression of the genes *CONSTANS* and *FLOWERING LOCUS T* involved in flowering and vegetative phenology in *Populus* (Böhlenius et al. 2006). Thus, an important comparative insight from these candidate gene studies was that convergent phenotypic clines in related species may result from selection targeting the same overall functional pathway (e.g. light-regulated circadian responses) but involving different genes within the pathway. Indeed, this trend also appears to be true for the evolution of sex-determination in *Populus* section *Populus* and *P*. section *Tacamahaca* despite the ancient origin of dioecy in the genus (Geraldes et al. 2015).

More recently, Menon et al. (2015) took advantage of *P. balsamifera*'*s* extensive distribution across boreal and arctic latitudes to study natural variation in adaptation to freeze tolerance. Menon et al. combined physiological assays of freeze tolerance using electrolyte leakage tests with gene expression and population genetic analyses on candidate genes in the poplar C-repeat binding factor (*CBF*) gene family. Their findings indicated that northern genotypes were physiologically more cold tolerant than southern genotypes, and that *CBF* gene expression was strongly induced by cold in balsam poplar. One gene (*CBF2*) also showed population genetic evidence of selection in the form of an excess of derived alleles, elevated F_{ST}, and clinal allele frequency variation. While transcriptional regulation and sequence polymorphisms in *CBF* genes were clearly associated with cold adaptation, *CBF* candidate genes only explain a small fraction of cold-induced changes in gene expression, suggesting the involvement of additional pathways.

Outside of *Populus*, investigations of the genomics of local adaptation in *Quercus* and other Fagaceae were underway by the mid-2000s. Foundational work studying QTLs and gene expression of various tissues identified a number of candidate genes involved in landscape variation in bud phenology and drought response (Porth et al. 2005; Casasoli et al. 2006; Derory et al. 2006, 2010; Soler et al. 2008). These studies spurred a growing body of work in *Quercus*, and provided an important resource for comparison to *Populus* and other taxa. In the European oak (*Q. petrea*), Alberto et al. (2013) integrated F_{ST}-outlier, GEA, and genotype-phenotype association tests based on SNPs from 106 phenology-related candidate genes to test for molecular signatures of local adaptation along altitudinal and latitudinal gradients. While 34 genes were significant in at least one of the tests, only 4 were identified in more than one: endochitinase class I, seed maturation protein, ribosomal protein L18a, and plastocyanin A. Some candidate genes were significant in both altitudinal and latitudinal

gradients, suggesting convergent selection at different spatial scales (ribosomal protein L18a, *GI*, Photosystem II polypeptide, 5′-adenylylsulfate reductase, ribosomal protein S11). Sork et al. (2016) also started with candidate genes from other studies and combined multiple tests for signatures of local adaptation in *Q. lobata* in California, including F_{ST}-outlier and univariate and multivariate environmental association tests. Five of seven genes with significant SNPs were significant in more than one test: auxin-repressed protein and α-amylase/subtilisin inhibiton, which are involved in bud phenology; and heat shock protein 17.4 and elongation factor 1-α, which are involved in temperature stress response. The F_{ST}-values of outliers in *Q. lobata* are substantially higher than those in *Q. petraea*, suggesting that differences in population history and climate heterogeneity may lead to very different degrees and patterns of local adaptation. Furthermore, several candidate genes for cold hardiness in *Pseudotsuga menziesii* (Krutovsky and Neale 2005) and bud phenology in European oaks (Casasoli et al. 2006; Derory et al. 2010) also show evidence of local adaptation in the Californian oak, *Q. lobata* (Sork et al. 2016). For the most part, however, candidate genes identified across a limited number of studies were different, leaving it unclear how common convergent adaptation is among species and between angiosperm clades (Martins et al. n.d.; Peñaloza-Ramírez et al. n.d.; Alberto et al. 2013; Sork et al. 2016; Gugger et al. 2016a)

The recent publication of the *Eucalyptus grandis* genome (Myburg et al. 2014) promises to be a useful tool to study adaptive evolution in the species rich and ecologically diverse *Eucalyptus*. For example, *E. pauciflora* occurs from sea-level to tree-line and seems like a particularly well-suited species for studying adaptation to temperature, cold hardiness, and drought stress. Adaptation studies in *Eucalyptus* are in the nascent stages (Dillon et al. 2015), but some traits, e.g. adaptation to aridity (Steane et al. 2014), are better understood. A recent candidate gene study of local adaptation in *E. camaldulensis* identified associations between climate (especially temperature and evaporative demand) and two genes -- *ERECTA* (an LRR receptor-like kinase) and *PIP2* (an aquaporin), both of which also showed excess population differentiation in F_{ST} outlier tests (Dillon et al. 2015). Additional work in *E. tricarpa* has uncovered extensive evidence of phenotypic plasticity and local adaptation for ecophysiological traits related to drought avoidance, although no associations with molecular data were sought (McLean et al. 2014). We eagerly await further results from landscape genomic studies in *Eucalyptus* that expand on our current knowledge of candidate genes involved in local adaptation to environmental gradients, especially drought and other forms of osmotic stress, and facilitate comparison with the existing body of work in northern hemisphere forest trees.

Genome-Wide Studies of Local Adaptation

While candidate gene studies show promise for understanding local adaptation on the landscape, they are still biased by the choice of genes to investigate. The development of new SNP arrays (Geraldes et al. 2013) allowed for genotyping tens of thousands of loci across the genome, while whole genome, exome, or transcriptome

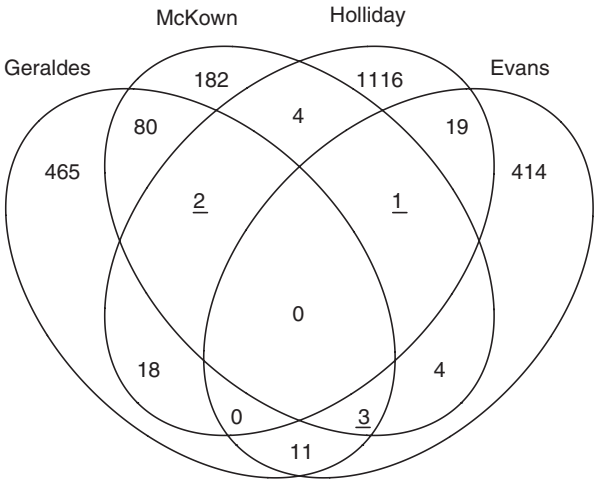

Fig. 2 Overlap of candidate genes for local adaptation in *Populus trichocarpa* identified in selection scans by Geraldes et al. (2014), McKown et al. (2014a, b, c), Holliday et al. (2016), and Evans et al. (2014). No overlap was detected by all studies, but six genes overlapped among three studies (*underlined*) (Version 2.2 Gene annotations from Geraldes et al. (2014) were updated to 3.0 for comparisons)

re-sequencing studies enabled testing millions of SNPs and identification of locally adapted genomic regions without any *a priori* specification of the number or types of genes involved (Evans et al. 2015; Holliday et al. 2016). To date, genome-wide association studies (GWAS) have received the most attention in *P. trichocarpa* (Zhou et al. 2014; McKown et al. 2014a, b, c; Evans et al. 2015; Holliday et al. 2016; Porth et al. 2013). Loci associated with adaptation to climate (Evans et al. 2014), aridity (Steane et al. 2014), adaptation across latitude and elevational gradients (Holliday et al. 2016), and associations with adaptive ecophysiological traits (Mckown et al. 2014a, b, c) have been discovered.

It is interesting to consider the repeatability of the genomic regions implicated by local adaptation scans in *P. trichocarpa*, given that they have involved different research labs, independent germplasm collections across a similar landscape gradient, and different statistical approaches to testing for adaptation. We compared local adaptation outliers from four studies in *P. trichocarpa* based on a range-wide sampling: two studies (McKown et al. 2014a, b, c; Geraldes et al. 2014) generated genomic data from genes discovered in the Geraldes et al. (2013) SNP array, while Holliday et al. (2016) sequenced whole exomes and Evans et al. (2014) sequenced whole genomes. Gene annotations from Geraldes et al. (2014) were updated from the *P. trichocarpa* genome version 2.2 to 3.0. Our comparisons were based on unique *P. trichocarpa* gene IDs identified as locally adapted in each study. These include 579 genes from Geraldes et al. (2014), 276 from McKown et al. (2014a, b, c), 1160 from Holliday et al. (2016), and 452 from Evans et al. (2015) (Fig. 2). No gene was identified as a selection outlier by all four studies, however six genes were

Table 1 Annotations for six genes implicated in local adaptation from range-wide samples of *Populus trichocarpa* by four separate studies

Gene model	*Arabidopsis* homolog	Gene function	Overlap
Potri.010G212900	AT5G13560	NA	M, H, E
Potri.005G073000	AT5G65270.1	GTP binding	G, M, E
Potri.006G257000	NA	Membrane protein	G, M, E
Potri.010G165700	AT3G01140	Transcription factor	G, M, E
Potri.006G225600	AT2G26200	Methyltransferase	G, M, H
Potri.009G017400	AT2G35940.1	Transcription factor HOX domain proteins	G, M, H

Annotations retrieved from www.popgenie.org
M McKown et al. (2014a, b, c), *G* Geraldes et al. (2014), *H* Holliday et al. (2016), *E* Evans et al. (2014)

identified as outliers in common across combinations of three studies (Table 1). Two of these shared outliers were transcription factors, while a third was a methyltransferase, possibly indicating an important role for transcriptional and epigenetic regulation of gene expression in local adaptation. One gene, Potri.010G165700, a MYB-like DNA binding protein, showed upregulated expression in dormant and pre-chilling vegetative and floral buds (www.popgenie.org), a profile supportive of this gene's association with bud phenology in GWAS (McKown et al. 2014a, b, c).

New candidates for local adaptation have also been found using whole-transcriptome sequences (ignoring expression) from natural populations to associate coding SNPs with environmental variation on the landscape. Using this approach in 22 *Q. lobata* individuals produced 220,427 SNPs for analysis (Gugger et al. 2016a). Of these, 79 SNPs from 50 genes were significantly associated with climate gradients, especially minimum temperature and precipitation variables, and most of these genes were differentially expressed after drought stress (Peñaloza-Ramírez et al. n.d.), suggesting they are likely involved in local adaptation. A few SNPs are in genes with known roles in response to stimulus or stress (e.g., SAUR-like auxin-responsive protein family), cold shock protein binding (zinc knuckle family protein), light response or photosynthesis (e.g., cryptochrome 1), and trichome development (Myosin family protein with Dil domain) in *Arabidopsis* (Swarbreck et al. 2008). Furthermore, the 50 candidate genes have elevated levels of genetic diversity, consistent with balancing selection or other mechanisms favoring different alleles in different contexts. In a similar population RNASeq study in *Populus balsamifera*, transcriptome-wide gene expression was compared between northern and southern genotypes grown under common garden conditions (Wang et al. 2014). A total of 4,018 of 32,466 genes showed differential expression between northern and southern genotypes. Genes that were more highly expressed in southern genotypes included homologs of the *Arabidopsis* flowering time pathway (*EARLY FLOWERING 3*, *FLOWERING LOCUS C*, *PHYA* and *CAULIFLOWER*). However, unlike in the *Quercus* study of Gugger et al. (2016b), the differentially expressed genes did not harbor SNPs that showed signatures of local selection (based on F_{ST}

or GEA outlier analyses), suggesting that expression divergence is largely uncoupled from nucleotide divergence in *P. balsamifera*.

While the underlying goal of these studies is to identify and understand patterns of adaptive genomic evolution, they also simultaneously address fundamental questions of how genomic diversity is shaped in geographic space. For example, Geraldes et al. (2014), found that diversifying selection along a latitudinal gradient principally was enriched for phenology and photoperiod related genes. At the same time, they addressed a classic landscape genetic question about geographic isolation along riparian zones by demonstrating that populations in connected drainages are more genetically similar than between drainages. Similarly, Holliday et al. (2016) identified genes associated with climatic differences in growing season length by testing whether adaptation involves selection targeting the same genomic regions between latitudinal and altitudinal gradients in *P. trichocarpa*. Their finding of significant overlap in the outlier regions associated with latitude and altitude provided rare support for the hypothesis of parallel adaptation along similar environmental gradients in different geographic regions of the species range.

Merging the unique biology of angiosperm trees with the landscape genetics/genomics program has generated a deep understanding for the connection between landscapes, biodiversity, and the evolutionary processes of gene flow, genetic drift, and natural selection. The initial goals of the field, as outlined by Manel et al. (2003), continue to be relevant and guide research today. Despite analytical hurdles that still exist, applications of new sequencing technologies combined with more refined analysis methods are generating exciting discoveries.

Challenges and Solutions for Landscape Studies in Trees

Many landscape genomic studies have borrowed sampling schemes from phylogeography, the study of population lineages across large (e.g. continental) landscapes (Fig. 1a). This approach has yielded much information about how genetic and phenotypic variation aligns along environmental gradients (e.g. photoperiod, temperature, and precipitation) that vary across large geographic regions. However, range-wide sampling can produce methodological challenges, false positive associations due to uncontrolled population structure, and biases in our expectations of which genomic regions are locally adapted. In this section, we review some of the challenges currently facing landscape genomics and highlight potential solutions.

Collinear Environmental, Phenotypic, and Genotypic Axes

The biological characteristics of trees that make them good candidates for landscape and ecological genomic studies also generate challenges for studies of natural variation that require clever study designs and/or novel methodologies to solve. The

collinear variation of environmental, phenotypic, and genomic variation across landscapes is a major challenge to overcome (Fig. 1a, b). For many temperate and boreal species, the structure of neutral loci following population expansion from glacial refugia mimics the signal of adaptive selection searched for by association methods (Lotterhos and Whitlock 2014). Disentangling false positives arising from demographic history against true positives arising from selection creates a problem for treating landscape samples as 'natural experiments'.

Algorithms accounting for demography in a sample of loci have been developed to reduce the false-discovery rate (FDR) due to population structure, while maintaining power to avoid false negatives (Wen-Yun Yang et al. 2012; Frichot et al. 2013; Günther and Coop 2013, Berg and Coop 2014). However, deciding where to place a cut-off value for significance tests remains difficult, given the very large number of tests in most population genomic datasets. Recently, it was recommended that researchers create empirical *p*-values from test statistic distributions derived from putatively neutral loci (Lotterhos and Whitlock 2014). While it is challenging to know what kind of locus is truly neutral (e.g. the promoter for *TB1*, a gene in the QTL that reorganizes teosinte-like to maize-like branching, is 41 kb upstream of the gene; Clark et al. 2006), selecting intergenic loci and conditioning *p*-values of candidate loci from them should decrease the FDR markedly (Lotterhos and Whitlock 2014). Additionally, sampling individuals from regions of the landscape that have experienced the same demographic history and have low F_{ST} can greatly minimize false positives generated by demography, particularly if covariation of the ecological and environmental variables of interest is present. Thus, future studies on the genomics of local adaptation will benefit from careful consideration of sampling design and generation of appropriate null expectations, both in terms of individuals on the landscape and SNPs across the genome.

Validating Adaptive Associations Discovered in Natural Populations

Local adaptation studies are generating many associations increasing our knowledge of gene functions (McKown et al. 2014a, b, c); however, attempts to validate these loci using independent evidence is still limited (Ćalić et al. 2016). For the most part, the candidate genes identified across even the most robust studies are different. Our comparison of four local adaptation studies in *P. trichocarpa* (McKown et al. 2014a, b, c; Evans et al. 2014; Geraldes et al. 2014; Holliday et al. 2016) using broadly overlapping genomic data sets find few loci in common (Fig. 2). What portion of the many associations not shared between studies result from false positives, differences in the biology of the samples, or methodological approaches employed? This brings into focus the growing need for functional validation of adaptation outliers.

Functional validation of genes identified from GWAS can be difficult, however, when successful, the results are compelling (e.g. Meijón et al. 2013). Genetic approaches to gene validation in trees is possible (Kim et al. 2011), and the advent

of CRISPR/Cas9 make editing putatively adaptive and non-adaptive alleles of candidate genes even more feasible (Fan et al. 2015; Bono et al. 2015). Thus, we are optimistic that validation of the most promising candidates using transgenic methods will be an increasing trend in the future.

Another validation approach for local adaptation studies is to replicate the association, for example by testing the phenotypic effect of the candidate locus segregating within a pedigreed mapping family (Stinchcombe and Hoekstra 2008). Trees typically have long generation times and waiting upwards of 4–8 years (for example in *Populus*, Difazio et al. 2011) for selected lines to reach reproductive maturity is not uncommon, and producing advanced generation mapping families is difficult. Nevertheless, concerted efforts have produced a handful of advanced generation pedigrees for linkage trait mapping in angiosperm trees in *Populus* (Taylor 2002) and several members of the Fagaceae, notably *Quercus*, *Fagus*, and *Castanea* (Kremer et al. 2012). Long-term mapping populations comprised of hundreds of progeny are now available for *Q. alba* at the University of Tennessee, Knoxville and University of Missouri Center for Agroforesty (http://hardwoodgenomics.org/), as well as for *Q. robur* in Europe (Bodénès et al. 2012; Plomion et al. 2016).

However, researchers remain limited when established mapping families do not segregate for the alleles or traits of interest. Nature, however, has provided additional solutions to some of these problems, particularly in taxa that extensively hybridize with congeners, allowing for strategic admixture mapping from natural hybrid zones. In these zones, naturally occurring F1s can be selected to produce F2 or backcross recombinant generations. In *Populus*, a well known tri-hybrid zone exists in southern Alberta between *P. balsamifera*, *P. deltoides*, and *P. angustifolia* (Floate 2004). Aided by diagnostic SNP genotyping arrays that identify *Populus* species and their hybrids (Isabel et al. 2013), hybrid F1s as well as every combination of backcrossed F2s can be found there (Floate et al. 2016). Hybrid zones between *P. balsamifera* and *P. trichocarpa* are also well known (Geraldes et al. 2014), and in Wyoming and Colorado another tri-hybrid zone has been observed between *P. balsamifera*, *P. trichocarpa*, and *P. angustifolia* (Chhatre et al. n.d.). Using the diverse array of interspecific hybrids as parents in targeted crosses has the potential to accelerate the time to production of advanced generation mapping families.

Investment in Phenotyping

The exponential decline of genotyping costs (Manel et al. 2015) has shifted the data collection bottleneck in ecological and landscape genomic studies towards phenotyping. Phenotyping traits representative of the diversity of developmental stages and tissue types can be difficult for trees, given their large size, delayed reproductive maturity, massive root systems, and the high cost of establishing and maintaining long-term common gardens. Increased attention is being devoted to developing standardized methods for high-throughput phenotyping (Brown et al. 2014), and

automated leaf morphology software developed for *Populus* (Bylesjö et al. 2008) has been successfully applied to other species (Ivetić et al. 2014). Similarly, researchers are beginning to investigate the complexity and adaptive contribution of below-ground tissues (Madritch et al. 2014) and their symbiotic interactions (Hacquard and Schadt 2015). However, renewed attention needs to be paid to funding and maintaining common gardens, sharing and integrating data across gardens for purposes of meta-analysis, and innovating new methods and approaches to capturing phenotypes from common gardens and natural landscapes.

Common gardens play a critical role in landscape genomic studies and connect genotype to phenotype to environment (Porth et al. 2013; Evans et al. 2014). Gardens with diverse landscape-scale sampling have been established in *Populus*, including extensive collections of *P. trichocarpa* by different research groups (461 individuals from McKown et al. 2014a, b, c, >1,000 individuals from Evans et al. 2015, and 391 individuals from Holliday et al. 2016), as well as the AgCanBap collection of *P. balsamifera* (525 individuals; Soolanayakanahally et al. 2009, 2013), the Swedish Aspen collection (SwAsp) of *P. tremula* (116 individuals; Luquez et al. 2007), collections of *P. fremontii*, *P. angustifolia*, and their hybrids (hundreds of individuals; Holeski et al. 2013), and the Chinese *P. tomentosa* collection (460 individuals; Du et al. 2014). In oaks, a comprehensive provenance trial for *Q. lobata* was established in 2013 at two sites from seeds sourced from 95 locations throughout its distribution (Delfino-Mix et al. 2015). Funding cycles, however, are too short to support the long-term maintenance needed for tree studies, and diverse collections of provenance trials established in the past century have been abandoned and overlooked. Few trials are still maintained, but interest is rebounding in taking advantage of these historical resources. For example, a mature provenance trial of approximately 300 trees from 30 populations sampled across the range of the tuliptree (*Liriodendron tulipifera*) was planted in the mid-1980s in Chapel Hill, NC, but was orphaned for much of its existence (Fetter and Weakley n.d.). Older, but smaller, trials are also available for *Q. alba*, including one comprised of 23-year-old trees from 17 populations planted in three locations in Indiana (Huang et al. 2015).

While the challenge of establishing and maintaining common gardens will likely remain in landscape genomics, we need to start thinking more strategically about how to use and combine these resources, and where applicable, study tree phenotypes *in natura*. Aggregating trait data from many common gardens would prove useful for meta-analyses of adaptive phenotypic trends across spatial scales, times, and even closely related species. Comparative work of this type has begun in *Populus* (Soolanayakanahally et al. 2015), but more awaits. Finding new opportunities to collaborate and integrate data across research labs hosting common gardens is needed, and tree biologists should target funding mechanisms to build research coordination networks around common garden resources.

Landscape genomics would also benefit from testing the transferability of genotype-phenotype associations from common gardens to the field. The challenge of landscape phenomics will define the next generation of studies that seek to close the genotype-phenotype-environment triangle of adaptive association. The hurdles, while logistically challenging, are largely technological, and progress in this area is

essential to make meaningful applications of tree management and conservation of genetic resources under environmental change (Pettorelli et al. 2014). Exciting advances in remote sensing are now being applied to phenotyping forest trees at scales of clonal stands to entire landscapes (Homolová et al. 2013). For example, remote sensing of seasonal phenology is being deployed at fine (Toomey et al. 2015) to continental (Graham et al. 2010) scales, and satellite-based phenology products such as MODIS provide coarse landscape-scale phenotyping of phenology that correlates with ground-based measurements in *Populus* (Elmore et al. n.d.). This opens doors to connecting landscape variation in forest phenology with adaptive gradients in allele frequencies in genomic regions associated with phenology under controlled experimental conditions. At finer scales of individual trees, measurement of several key phenotypes (e.g. spring bud flush and plant water balance) may be possible through use of field deployed sensors that detect differences in tree mass using acceleration or reflectance using light-emitting detectors (e.g., Kleinknecht et al. 2015). While still in need of further development, landscape-scale phenotyping holds the potential to connect adaptive genomic variation to forest productivity in natural stands, and is a challenge that future studies will surely begin to address.

Future Research Directions for Forest Tree Landscape Genomics

Refinements in genomic sequencing, association methods accounting for demography, and inclusion of broader evolutionary questions has driven the steady increase of the impact of landscape genomic studies in angiosperm trees. We make the following recommendations and predictions for the future of the field:

Growing Genomic Resources Outside of Populus

Genomic resources in *Populus* are now well-developed and easily accessible (e.g. www.popgenie.org), but are still lacking for many other angiosperm trees. Increasing genomic resources in other taxa will allow for more comparative studies that will increase the impact of what is known from *Populus* (see Sollars and Buggs, this volume). Genomic resources in *Quercus* are steadily increasing and are most developed for the white oaks (*Quercus* section *Quercus*). Recently, the first draft oak genome, based on a combination of Roche 454, Illumina, and Sanger sequences, was announced for the ecologically and economically important European white oak, *Quercus robur* (Plomion et al. 2016). The genome contains 18 k scaffolds (>2 kb) totaling about 1.3 Gb, which is about 1.7 times the haploid genome size (Kremer et al. 2007b). To enable research in ecological genomics, there are several important improvements underway. These include the development of a genome sequence

with collapsed haplotypes to facilitate SNP calling, further assembly that utilizes linkage maps and additional sequencing to determine gene order, and structural and functional annotation (Plomion et al. 2016) that will leverage abundant expressed sequence tags (EST) and RNA-Seq libraries from a variety of tissues (Ueno et al. 2010; Tarkka et al. 2013; Lesur et al. 2015).

A draft oak genome is now available for *Q. lobata* (Sork et al. n.d.), a threatened western North American species with a population history that stands in contrast to many studied trees (Grivet et al. 2006; Gugger et al. 2013). Based entirely on Illumina sequencing, two complementary assemblies were produced. The first (v0.5) aggressively collapses haplotypes to optimize its use for SNP calling (Gugger et al. 2016b). The second (v1.0) retains haplotypes and is composed of 18.5 k scaffolds (>2 kb) with total length of 1.15 Gb (Sork et al. n.d.). This latter assembly is similar in both size and sequence to that of *Q. robur*, with 93% sequence identity and 97% of scaffolds reciprocally aligning. Functional and structural annotations are derived from an annotated reference transcriptome assembly for *Q. lobata* (Cokus et al. 2015) in addition to *de novo* annotations.

Other resources for eastern North American trees are available from the Hardwood Genomics Project (http://www.hardwoodgenomics.org/) and from the Fagaceae Genomics Web (http://www.fagaceae.org) for many important tree species, including oaks, chestnuts, sweet gum, beech, and tuliptrees, among others. These include EST libraries and transcriptome assemblies for *Q. alba* and *Q. rubra* that were generated from a variety of tissues and sequencing platforms, as well as a low-coverage genome assembly for *Q. alba* that was constructed for identifying SSRs. Early analyses suggest high similarity with other oaks and broad synteny within Fagaceae (Kremer et al. 2007a; Bodénès et al. 2012; Cokus et al. 2015).

Along with the development of sequence resources, there has been an explosion in the number of SNPs and SSRs available. Over one million SNPs were discovered in California oaks, especially *Q. lobata* (Cokus et al. 2015; Gugger et al. 2016a). Tens of thousands of SNPs are known in *Q. robur* (Ueno et al. 2010), and recently, a cross-species genotyping array with over 7k SNPs was developed for European oaks (Lepoittevin et al. 2015). In addition, thousands of SSRs have been identified in *Q. alba* and *Q. robur* (Durand et al. 2010; Bodénès et al. 2012).

Incorporating Gene Expression Studies

Advances in transcriptomics (Lee et al. 2015) and isoform detection (Bernard et al. 2015) are making it possible to ask new questions about the genomic mechanisms of local adaptation and the evolution of phenotypes. Integrating gene expression into selection scans can provide complementary information to SNP genotypes when conducting selection scans and GWAS. Experimental designs that analyze differential expression dependent on both treatment and source population can produce excellent candidates for inferring local adaptation. In a small greenhouse experiment that exposed *Q. lobata* seedlings from three source populations to a

drought treatment, whole-transcriptome gene expression changes identified 56 genes that had significant interaction terms (Peñaloza-Ramírez et al. n.d.). These genes are primarily involved in metabolic processes, stress response, transport/transfer of molecules, signaling, and transcription regulation. Given the prevalence of molecules involved in metabolism, it was hypothesized that local adaptation to drought in oaks may involve different abilities to metabolize or different ways of altering metabolic activities in the face of drought.

Differentially expressed genes or isoforms may experience different forms of selection (Hartfield 2016). Some of these expression differences may be linked to sex function in dioecious or monoecious species with imperfect flowers (Geraldes et al. 2015). Analysis of differential expression during the development of imperfect flowers in *Betula* revealed a transcription factor, *BPMADS2*, involved in specifying petal and stamen identity and is expressed only in developing male flowers (Järvinen et al. 2003). Local adaptation in the expression of this transcription factor may allow for precise timing of male flower development.

Testing the Role of Epigenetics in Local Adaptation

NGS is enabling research on the potential role of epigenetic mechanisms of local adaptation in trees. Cytosine methylation varies among individuals and populations (Schmitz et al. 2013). It can arise through random mutation (Becker et al. 2011), be induced by the environment (Verhoeven et al. 2010), can be stably inherited (Becker and Weigel 2012), and may be an additional source of variability for local adaptation. Early evidence in oaks shows that single methylation variants (SMVs) in CpG contexts are exceptionally differentiated among populations (Platt et al. 2015). In addition, CpG SMVs have strong associations with climate and specific variants show evidence of local adaptation to temperature stress (Gugger et al. 2016b). Therefore, it is hypothesized that either CpG SMVs are markers for loci involved in local adaptation or they are under divergent natural selection themselves. Additional research is needed to better understand the mutation process, assess heritability, and link methylation to phenotypes.

Genomic Predictions of Climate Change and Local Adaptation

Understanding the genomics of local adaptation to current environmental conditions allows us to ask how populations might respond when climates change (Sork et al. 2013; Wang et al. 2014; Fitzpatrick and Keller 2015). For example, research in *Arabidopsis thaliana* suggests that the fitness of populations under novel conditions can be predicted from SNPs in genes involved in local adaptation (Hancock et al. 2011). Prediction of fitness from SNPs might be powerfully applied to tree genomics in studies that pair genotypes with growth traits from

common garden trials. Furthermore, the future genetic composition of populations under climate change can be modeled, and the degree of mismatch between current and projected genomic composition can be considered in management planning (Fitzpatrick and Keller 2015). Identifying adaptive genotypes under projected climate change may be critical for conservation of biodiversity and economically important genotypes, core arguments in favor of assisted migration (Aitken et al. 2015).

Assessing the Prevalence of Adaptive Introgression

Oaks, poplars, and many other angiosperm trees are notorious hybridizers with large effective population sizes readily capable of generating adaptive molecular variation. An important question for tree genomics is how species are able to maintain their ecological, morphological, and genomic identities in the face of widespread hybridization, and whether hybridization contributes actively to adaptation in hybrid zones and outside of them. Genomic tools offer unprecedented opportunities to investigate the genes relevant to speciation (Wu 2001; Bawa and Holliday, this volume), whether hybridization facilitates the transfer of beneficial alleles across species boundaries (Suarez-Gonzalez et al. 2016), the ecological factors that promote or restrict hybridization (Ortego et al. 2014), and how important these processes are for evolution in trees (Gailing 2014; Gailing and Curtu 2014).

Evidence from European poplars suggests that advanced generation hybrids accumulate genetic incompatibilities that not only decrease their fitness, but are frequently lethal (Christe et al. 2016). Nevertheless, unidirectional or biased introgression of genes occurs frequently in angiosperm trees (Thompson et al. 2010; Geraldes et al. 2014) and fragments of nDNA can introgress across hybrid zones (Martinsen et al. 2001). In *P. tremula*, adaptive introgression from Russian to Swedish populations may have contributed to adaptive shifts in the phenology of Swedish trees (De Carvalho et al. 2010). Similarly, the high rate of hybridization in oaks has long been a topic of interest (Muller 1952), and has influenced the development of the ecological species concept (Van Valen 1976).

One promising area for future study is to investigate the role of introgression in responses to pathogens or other sources of plant stress (e.g., cold, drought, soil-metal tolerance). What role does adaptive introgression play when interspecific populations meet in an environment where hybridization increases pathogen or herbivore pressures (Holeski et al. 2013; Busby et al. 2013)? Do genetic incompatibilities accumulate in hybridizing populations that limit the ability for adaptive immune responses to track the movement of pathogens/herbivores as they 'step' across species boundaries (Floate et al. 2016)? Some evidence from *Populus* protease inhibitor genes hints that introgression may accompany adaptive responses to herbivore enemies (Neiman et al. 2009). When multiple hybrid zones exist between the same set of species, does unidirectional gene flow create a "sink

species", biasing the pattern of introgression, or does local selection play a dominant role regulating which genomic regions preferentially introgressed? Geraldes et al. (2014) hypothesize that hybridizing populations of *P. trichocarpa* and *P. balsamifera* in Alaska may be adaptively introgressing cold tolerance or drought tolerance genes that would experience negative selection in the southern hybrid zone. Trees seem to be ideal systems studying the interaction between hybridization and natural selection.

Seeking Out Understudied Systems and Questions

The studies we have discussed offer initial insights into how tree genomes evolve in response to ecological selection pressures, but much work remains to thoroughly understand the molecular basis of local adaptation. Much of the research to date has emphasized local adaptation with regard to bud phenology, growing season length, and other sources of abiotic stress. Biotic interactions and non-climate-related environmental adaptation have received far less attention in landscape studies, particularly in the area of pathogen responses, which is an area of critical importance for human economies and culture (e.g. coffee trees and leaf rust, Avelino et al. 2012; the banana and Panama disease, Ordonez et al. 2015). Thus, additional research is needed to understand local adaptation in a broader array of contexts, especially those focused on co-evolution and reciprocal selection driven by species interactions (Whitham et al. 2008). Trees, as foundational species that structure biodiversity, are ideal subjects for community-level genomic studies.

We must also expand our geographic and taxonomic focus to include understudied regions, environments, and taxa. A quick survey of the angiosperm (and non-angiosperm) tree landscape genetics literature reveals a strong geographical bias for boreal forest, and, increasingly, temperate forest trees. Although trees from these regions hold much of the Earth's biomass, a broader taxonomic and geographic sampling of forest trees is needed to understand the diversity of adaptation in natural landscapes. Tropical forests harbor incredible ecological diversity (Gentry 1988) and the evolutionary findings from boreal and temperate trees regarding the selective environments and the genomic pathways of adaptation in trees may be the exception, not the rule. Negative density dependent selection, principally from fungal pathogens (Augspurger 1984), acts to keep tropical tree diversity high and abundance low (Bagchi et al. 2014). What effect does negative density dependent selection from pests and pathogens have on patterns of adaptive genomic diversity in tropical trees? How is diversifying selection for genes involved in an immune response achieved in the face of limited gene flow and low abundance (Hamilton 1999)? Are large effective population sizes sufficient to maintain the supply of standing variation and new mutations required for trees to keep pace in the arms race against pathogens and herbivores? Are the spatial scales of local adaptation to pathogens small, moderate, or large in tropical forests?

Revisiting the Scale and Scope of Landscape Genomic Studies

Understanding the genetic determinants of locally adaptive clines is a long-standing goal of landscape genetics (Manel et al. 2003). The large-scale, often range-wide sampling schemes used to investigate clines have become standard in the tree genomics literature. Multiple studies have discovered adaptive evolution of genes associated with phenology clines (Keller et al. 2012; McKown et al. 2014a, b, c; Evans et al. 2014; Geraldes et al. 2014), and indeed, given the high broad-sense heritability of phenology traits (Mckown et al. 2014a, b, c) and their direct connection to fitness, one expects strong local selection. Additional ecological dimensions to local adaptation should receive greater focus, including different soil chemistries and biotic interactions that vary across landscapes. A modified scope and sampling design is needed to detect local adaptation that does not vary clinally. Designing studies to more precisely isolate different environmental gradients can help disassociate correlated predictors that are common in range-wide studies. For example, temperature and photoperiod both typically covary with latitude, and a researcher interested in temperature adaptation may have trouble interpreting results from a sampling design oriented along a north–south gradient. However, a sample from different elevations along a gradient, while keeping latitude constant, will remove effects of photoperiod. Similar designs have been employed recently to detect parallel adaptation to climate across altitudinal transects in *P. trichocarpa* (Holliday et al. 2016). Smaller scale, targeted landscape genomic studies have much to offer and can unravel new dimensions of local adaptation.

Conclusions

The challenge of understanding genomic variation responsible for adaptive phenotypes on the landscape has been fully engaged for more than a decade by scientists working with angiosperm forest trees. Sequencing and methodological advances have enabled a deeper understanding of neutral and adaptive variation, but the field stands short of proving causative relationships between genotype, phenotype, and the environment. The breadth of evolutionary questions continues to grow, as does the taxonomic sampling, and the field is expanding the availability of genomic resources for a broader array of economically and ecologically important forest trees. Identifying unique geographic hotspots of genomic diversity on landscapes, even before we fully understand their adaptive significance, is a valuable contribution to conservation of tree germplasm during climate change. However, careful consideration of research questions and the sampling design most likely to yield interpretable results is critical and will allow for more diverse studies across the many ecological dimensions of local adaptation. Angiosperm trees will remain important models for studies of adaptive evolution in the coming years, and the combination of landscape genomics with validation methods have the potential to make huge contributions to conservation, sustainable development, and our understanding of the natural world.

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Pathogenic and Mutualistic Symbiotic Interactions in Angiosperm Trees

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Abstract Plants and microbes have formed intimate associations since the emergence of plants onto dry land. The impact of these associations on the plant can range across a spectrum from parasitic to beneficial. In all cases, the interaction between plant and microbes has necessitated the development of a properly balanced plant immune system. We investigate here how the advent of the genomics era has impacted our understanding of plant:microbe symbioses for angiosperm trees.

Keywords Angiosperm trees • Tree pathogens • Mutualistic fungi • Endophytes • Transcriptomic analysis

Introduction

Before there were plants on land, and long before the appearance of forest-like ecosystems during the mid-Devonian, there were fungi inhabiting early soil-like crusts. Associations between basal plants and fungi has been proposed as the way by which plants were first able to colonize land (Selosse and Le Tacon 1998; Bonfante and Selosse 2010; Selosse and Strullu-Derrien 2015). Supporting this theory is a rich fossil record rife with examples of fungal structures found within plant tissues (Strullu-Derrien et al. 2014; Taylor et al. 2014) and the fact that extant members of basal land plants are able to form associations with fungi (Desiro et al. 2013; Field et al. 2014; Rimington et al. 2015). Concurrent with the development of beneficial plant:microbe interactions, hence-forth referred to as 'mutualistic' interactions, was the development of detrimental plant associations with microbes typically referred to as plant 'parasites' or 'diseases'. One such class of microbe was the oomycetes; filamentous heterotrophic stramenopiles that were extant around the time of land-based plant evolution. Present-day members of this group include the infamous pathogenic genus *Phytophthora,* which can be devastating to forest ecosystems and crops globally. The interaction between oomycetes and plants has been dated using the fossil record to the Devonian (Taylor et al. 2006) with the earliest evidence for their role in plant pathogenesis found

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in fossils from the Carboniferous (Strullu-Derrien et al. 2011). Therefore, given the long-term association between plants and microbes, we must take into account how these small, but significant, organisms have impacted the evolution of plant genomes and how extant plants signal with, and respond to, microbes.

It has now been one decade since the release of the first angiosperm tree genome (Tuskan et al. 2006). The *Populus trichocarpa* genome has enabled a shift away from using annual plant model systems as proxies for tree biology, and has led to a revolution in our understanding of how long-lived trees function at the genetic level. It has also enabled the research community to begin to uncover how trees interact with mutualistic organisms (e.g., ectomycorrhizal fungi) as, for once in plant biology, the oft-used *Arabidopsis thaliana* could not be used as a model. This is due to the evolutionary loss in *A. thaliana,* and the Brassicaceae in general, of the genes necessary to enable symbiosis between the plant and mycorrhizal fungi (Delaux et al. 2014). Therefore, the advent of the genomic age in angiosperm trees has enabled us to begin the mammoth task of decoding how trees interact with their mutualistic fungal partners. The genomic era in angiosperm trees has, likewise, facilitated the study of pathogenic plant:microbe interactions. These studies seek to understand the modification in gene signalling occurring in plants during disease, with the ultimate goal of managing or preventing the spread of costly diseases. Since 2006, through the tireless work of many groups around the world, there have also been a number of new genome sequencing efforts that have led to annotated draft genomes for numerous angiosperm trees such as *Carica papaya* (Ming et al. 2008), *Citrus* spp. (Wu et al. 2014), *Eucalyptus grandis* (Myburg et al. 2014), and *Quercus robur* (Plomion et al. 2015), as discussed by Sollars and Buggs (Chap. XX, this volume).

This chapter will synthesize the current research that is ongoing in the field of microbial interaction with angiosperm trees. We focus, to a large degree, on studies that seek to analyze the transcriptomic events surrounding either plant:microbe mutualistic interactions or pathogenic interactions in both the root system of the plant as well as the aerial tissues. We conclude with some future outlooks in this area.

Tree Defences Against Pathogenic Invasion

Angiosperm trees are exposed to a plethora of organisms including bacteria, fungi and micro-arthropods through the contact between roots and soil. Despite the fact that these organisms number in the millions of individuals per gram of soil, a relatively small number are able to enter the root or form an on-going relationship with the plant. This would suggest that plants have evolved a very highly tuned perception/immune response signalling network to tightly control which microbes gain access within their tissues. In some cases, the interaction between the plant root and soil microbes can be considered to mutually benefit both partners – this is mutualistic symbiosis. In other cases, the interaction is pathogenic and detrimental, or even deadly to the plant.

Genomics research in the area of plant pathogens has concentrated on determining what kind of defensive response the tree uses to fend off pathogenic invaders

and what traits cause some trees to be more resistant than others. A tree's degree of success against pathogenic invasion is dependent on the speed and effectiveness of its defensive response upon detection of a pathogen. 'Compatible' pathogenic plant:microbe interactions are typically referred to in a case where a host plant is not able to successfully mount a defence against an invading organism and the microbe is able to colonize the plant tissues and complete its life cycle. This usually leads to the death, or reduced fitness, of the plant. 'Incompatible' interactions are those where the host plant is able to identify the presence of an invading organism and, through localized defence responses, limit or kill off the foreign tissues. While this may lead to localized cell or tissue death in the plant host, it usually does not affect the greater fitness of the host plant nor does the host plant usually die. Complicating the defence is the fact that different pathogen lifestyles (e.g., biotrophic versus necrotrophic) require a different defensive strategy on the part of the plant and that many pathogens have evolved specialized effector proteins to overcome host defences. Effectors are small proteins (<300 amino acids) secreted by an invading organism in response to the presence of a host. Found in bacteria, fungi and oomycetes, these effectors enter the plant cell and reprogram cellular functions, often relating to defence, to facilitate pathogen colonization. Effectors typically have no homology to other known proteins and are specific to a pathogen:host pair and help define the host range of a given pathogen (Martin and Kamoun 2012). The plant must therefore develop an immunity or countermeasure to the effectors (through the evolution of resistance (R) proteins) or defend itself in other ways.

Plant defences fall into two main categories: general stress/pathogen responses and phytohormone based responses. General responses may include the production of reactive oxygen species, activation of the hypersensitive response pathways, production of heat shock proteins or proteins designed to strengthen plant cell walls (ex, proline rich proteins). Plants undergoing invasion will also typically express a variety of pathogenesis-related (PR) proteins. This diverse grouping of proteins are so named as their transcription is triggered by the presence of a pathogen, abiotic stress or activation of the hypersensitive response (Veluthakkal and Dasgupta 2010). They include proteins such as β-1,3-glucanases, chitinases, thaumatin-like proteins and peroxidases. Phytohormone based defences involve the regulation of salicylic acid, jasmonic acid or ethylene pathways, and to a lesser extent abscisic acid or auxin pathways.

Angiosperm Tree Responses to Common Pathogens

Root pathogens: Phytophthora

One of the main pathogens of plant roots are oomycetes from the genus *Phytophthora.* Oomycetes are one of the earliest pathogenic organisms of plants. Part of the kingdom Stramenopila, these parasites are related to the ocean dwelling diatoms and brown algae and have caused some of the most devastating plant disease epidemics in recent history (e.g., *Phytophthora* induced Irish Potato Blight; Goss et al. 2014).

They are also known to attack woody shrubs and trees, leading to root rot or leaf drop, water starvation and death. In fact, the extent of forest and nursery dieback due to *Phytophthora* infection has led to this genus being considered as one of the most destructive groups of pathogens globally (Brasier 2008). *Phytophthora* is a hemi-biotrophic organism, meaning that the initial stages of plant host colonization can only occur in live tissues, while the reproductive stages of the oomycetes life-style is in dead tissue (Fig. 1a). While the majority of studies with *Phytophthora* concentrate on annual plant hosts, a few transcriptomic studies with angiosperm trees including *Fagus sylvatica* (beech; Schlink 2010), *Persea americana* (avocado; Reekstring et al. 2014), *Notholithocarpus densiflorus* (tanoak; Hayden et al. 2014), citrus (Boava et al. 2011) and *Carica papaya* (Porter et al. 2009) have been performed. Infection by *Phytophthora* typically induces defensive pathways in the tree, including the production of PR proteins such as chitinases, thaumatin proteins and glycosyl hydrolases in tanoak (Hayden et al. 2014) or peroxidases and β-1,3-glucanases in papaya or avocado (Porter et al. 2009; Reekstring et al. 2014). Despite these defenses, these diverse species remain susceptible to *Phytophthora. Fagus sylvatica* is highly susceptible to infection by *Phytophthora citricola* and transcriptomic study shows that it does very little by way of defense against the pathogen (Schlink 2010). In fact, the salicylic acid pathways are actually down regulated in infected tissues. This could be either because the pathogen manages to evade detection, or, more likely, that the pathogen suppresses the immune response of the plant, possibly through the use of effectors. In another study comparing interactions of *Phytophthora* with the incompatible (*Poncirus trifoliata*) and compatible (*Citrus sunki*) varieties of citrus and their hybrids, Boava and colleagues (2011) show that a set of 24 genes are differentially regulated between all combinations of incompatible/compatible interactions at 48 h post infection. A number of the genes upregulated in resistant plants are involved in defense, specifically in the hypersensitive response, such as disease resistance protein RPS4 and a TIR-NBS-LRR resistance protein. Induction of the hypersensitive response in the plant causes localized cell death, which is disadvantageous to the *Phytophthora* while still in its biotrophic phase. These studies demonstrate that the degree of pathogen resistance is linked to the ability of the tree to mount a timely and effective defense, for the upregulation of these same hypersensitive response genes at a later time point (i.e., when the pathogen has begun its necrotrophic phase) would likely be detrimental to the plant while benefiting the pathogen.

Leaf and Stem Pathogens

Roots in contact with soil are not the only plant tissues exposed to, or colonized by, microbes. The aerial parts of the tree including the leaves and stems are also in contact with a diversity of pathogenic microbes. Interactions between aerial tissues and pathogenic microbes have been best studied in annual plants, as they are tractable for study using traditional genetic methods, and pathogens cause billions of dollars

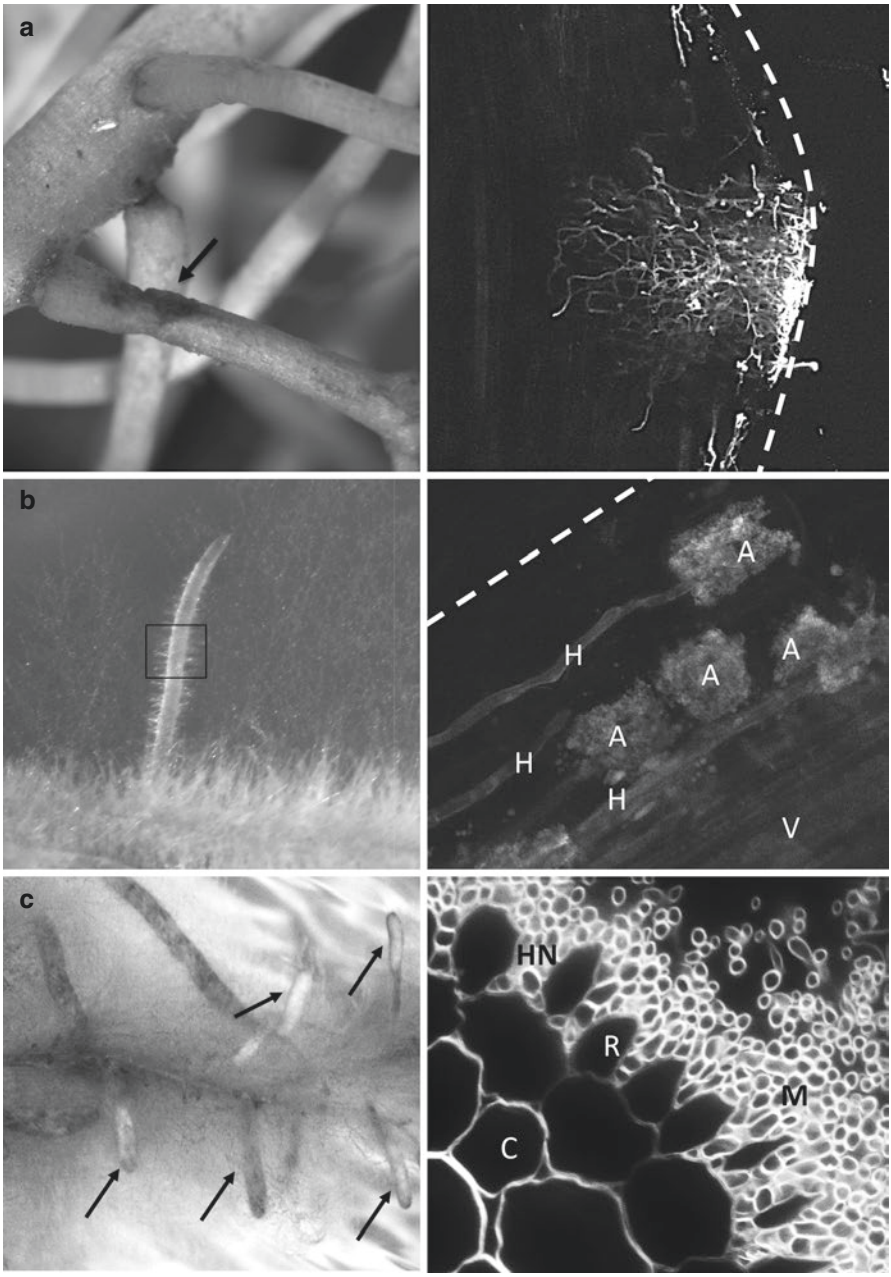

Fig. 1 Microbes at home in plant tissues. (**a**) *Phytophthora* sp. infect roots causing blackened lesions (*arrow* in left panel) which are made up of dense groupings of hyphae that explode from the root surface (fluorescently labelled hyphae in the right panel, root surface indicated by *dashed white line*). (**b**) Young roots are colonized by arbuscular mycorrhizal fungi (*boxed area* in left panel is magnified in the right panel where fluorescently labelled arbuscules [*A*] and hyphae [*H*] are visible). (**c**) Roots colonized by the ectomycorrhizal fungus *Pisolithus.* Colonized roots (*arrows*, left panel) appear swollen due to the fungal hyphae surrounding them. The right panel is a fluorescently stained transverse cross section of a colonized root. *M* mantle, *HN* Hartig net, *R* rhizodermal cell, *C* cortex cell

in losses to the global agricultural community annually. With the advent of the genomic age for angiosperm trees, we can now begin to investigate how different microbes impact forest environments and to genetically identify the basis for disease resistance in ecologically, economically, and culturally important trees.

Common leaf pathogens of trees include *Melampsora, Phytophthora* and *Sphaerulina.* Rust pathogens (e.g., *Melampsora* sp.) and other spot-causing pathogens affect the leaves of a wide variety of angiosperm trees. *Populus* is an excellent platform for studying disease resistance and susceptibility to leaf pathogens. The host tree *P. trichocarpa* x *P. deltoides* 'Beaupre' forms both compatible and incompatible interactions with different strains of the fungal rust *M. larici-populina.* Rinaldi and colleagues (2007) and later Petre and colleagues (2012) investigated the early and late stage transcriptomic response of the host plant. They found that under compatible interactions very few genes were significantly regulated (Rinaldi et al. 2007; Petre et al. 2012). This was especially true during the early stages of the interaction, with only a sulphate transporter (*PtSultr3;5*) found to be induced. No pathogenesis-related, defence signalling genes or hormone associated genes were significantly regulated. In contrast, strains of *M. larici-populina* that formed an incompatible interaction with *P. trichocarpa*×*P. deltoides* 'Beaupre' induced a more significant shift in the transcriptome of the leaf (1,730 transcripts ≥2-fold differentially accumulated). Genes exhibiting differential expression included pathogenesis-related proteins 1, 2 and 5, ethylene signalling genes (e.g., ACO1, EREBP), glutathione *S*-transferases and receptor-like kinases while genes related to photosynthesis were repressed (Rinaldi et al. 2013). Different metabolic enzymes and cell wall precursors (e.g., I3PS) had elevated expression profiles in response to incompatible *Melampsora* strains resulting in an increase in lignin-based defences. Globally, angiosperm tree leaves respond in a similar fashion when challenged with different pathogens such as *Sphaerulina musiva* and *S. populicola* (Foster et al. 2015), *Microsphaera alphitoides* (Kurth et al. 2014), *Marssonia brunnea* (Chen et al. 2015) or *Stemphylium vesicarium* (Pereira et al. 2015). There were some differences between the *Melampsora* and the other pathosystems, however: colonization of poplar leaves by *Sphaerulina* sp. led to increases in the transcription of genes annotated as chitinases, β-glucosidases, WRKY transcription factors and NBS-LRR resistance proteins as well as the involvement of abscisic acid signalling pathways (Foster et al. 2015). Similarly, *S. vesicarium* produced a novel induction of cytochrome P450's – a response notably absent from other analyses described in this section (Pereira et al. 2015).

Stems and trunks of trees are not immune to pathogenic microbes. A number of important stem canker blights have had devastating impacts on a number of important forest trees. Chestnut blight, caused by the fungus *Cryphonectria parasitica,* led to the widespread loss of American chestnut in the early twentieth century. This fungus attacks stems of trees, girdling the cambium and leading to plant death. Interestingly, Chinese chestnut is resistant to *C. parasitica.* This has led to a number of breeding programs attempting to introgress blight resistance from Chinese chestnut into American chestnut stock, as well as a number of studies attempting to identify the key differences in defensive compounds deployed by resistant varieties of chestnut as opposed to susceptible species. Using comparative transcriptomics, Barakat and

colleagues (2012) studied the response of Chinese and American chestnut to *C. parasitica.* American chestnut:*C. parasitica* (compatible interaction) had 1,715 differentially regulated genes during this interaction, while Chinese chestnut:*C. parasitica* (incompatible interaction) was found to have 720 differentially regulated genes. Despite the differences in the total number of genes differentially regulated, the classification of the genes expressed was almost identical between the two species of chestnut. Genes involved in lignin synthesis (e.g., *4CL, CAD, CCR*), the hypersensitive response and the phytohormones ethylene, jasmonic acid, salicylic acid and abscisic acid were upregulated in both species. In some cases (e.g., laccases), the amplitude of gene regulation was greater in the resistant Chinese chestnut. The authors of this study concluded that the resistance in the Chinese chestnut is likely due to the timing and amplitude of gene regulation rather than to specific genes (Barakat et al. 2012).

Other examples of pathogens that affect tree stems are poplar stem canker caused by *Botryosphaeria dothidea* (Liao et al. 2014) and Dutch elm disease caused by *Ophiostoma* spp. (Perdiguero et al. 2015). In these two different pathosystems, there is a consistent core transcriptional response by the host involving the induction of WRKY controlled hypersensitive response and genes associated with cell wall reinforcement, as well as a number of LRR protein kinases and calcium binding proteins (Liao et al. 2014). As mentioned above, chestnut canker induces nearly all of the major phytohormone pathways while *B. dothidea* only affected the jasmonic acid pathway. *Ophiostoma* spp. colonization of elm leads to a different panel of phytohormones again, with induction of brassinosteroid and auxin signalling predominating in this pathosystem (Perdiguero et al. 2015). Thus there is variation in both the type and intensity of defence used by the plant in response to different pathogens.

Despite these differences, overall the different pathosystems covered here have two main similarities: (1) the host plant responds with different transcriptomic dynamics between compatible and incompatible plant:microbe interactions and, (2) the plant's response to microbial interaction is generally fairly narrow with the same gene classes being induced in most all pathosystems considered. These broad findings are very important in how we further study these interactions, as the former finding would suggest that the timing and level of reaction by the host plant is the basis of disease resistance, while the latter would suggest that there is likely a huge evolutionary pressure for disease-causing microbes to overcome these stock-standard host disease response parameters.

Mutualistic Interactions

Root Mutualists: Mycorrhizal Fungi

Not all microbes attempting to colonize plant tissues are detrimental. Mycorrhizal fungi such as arbuscular mycorrhizal (AM; Fig. 1b) and ectomycorrhizal (ECM; Fig. 1c) fungi benefit trees by providing the plant with growth limiting nutrients in exchange for photosynthate, while also increasing the plants' ability to resist

environmental and biotic stress. AM fungi are the extant representatives of what are thought to be the fungi that enabled plant development on land. These fungi are obligate biotrophs: they cannot grow, live or reproduce without a viable association with a host plant. In these interactions, fungal hyphae grow into the apoplastic space of the root and then penetrate through the cell wall of individual plant cells to form "arbuscules" – tree-like or coil structures of highly branched hyphae that act as the interface with the plant cell where nutrients are exchanged between the two partners (Fig. 1b). It should be noted that the AM fungal hyphae never penetrate the cellular membrane of the host cell. Rather, the host plasma membrane invaginates to surround the incoming hyphae and form a new, symbiotic membrane with the fungal cell wall called the periarbuscular membrane. ECM fungi are much less ancient than AM fungi, appearing in the fossil record around the time of the evolution of gymnosperm trees. All ECM fungi have evolved from saprotrophic free-living fungi. As opposed to AM fungi, there appears to be no common ancestor to ECM fungi. Rather, this lifestyle has arisen many times in the fungal tree of life through convergent evolution (Kohler et al. 2015). As opposed to AM fungi, which are obligate biotrophs, ECM fungi can live without a host as they can grow slowly on decomposing plant material. In the presence of a host, individual ECM hyphae grow around young, developing lateral roots forming a dense covering on the outside of the root called a 'mantle' (Fig. 1c). Once the mantle is formed, the hyphae then grow into the apoplastic space of the root forming a structure called the 'Hartig net'. Unlike AM fungi, ECM hyphae remain in the apoplast and the nutrients exchanged between the plant and the fungus happen at the interface of cell walls. Through genomic techniques and analyses now available to us, we are able to delve into the molecular mechanisms that promote symbiosis. Understanding the genetic traits that control the formation of successful mycorrhizal partnerships can help determine ecological fitness and management strategies. The formation of mycorrhizal symbiosis, however, is a complex procedure, requiring a carefully balanced cascade of transcriptomic changes on the part of both partners.

Over the years a number of studies have considered plant transcriptomic changes in response to colonization by mycorrhizal fungi. Studies using AM fungi have concentrated on non-tree hosts, though one study with *Causuarina glauca* as a host has been published (Tromas et al. 2012). A number of genes were identified that are upregulated in *C. glauca* as it interacts with *Glomus intraradices* (now *Rhizophagus irregularis*), including a number of cytochrome P450s and nutrient transporters (high affinity phosphate transporters, ABC transporters and aquaporins). Transcriptomic studies in trees undergoing colonization by ECM fungi are more varied, including hosts *Eucalyptus globulus* (Duplessis et al. 2005; Plett et al. 2015a), *Betula pendula* (Johansson et al. 2004; Le Quere et al. 2005), *Quercus robur* (Frettinger et al. 2007; Tarkka et al. 2013; Sebastiana et al. 2014), *Castanea sativa* (Sebastiana et al. 2009), *Populus tremuloides* (Larsen et al. 2011) and *Populus trichocarpa* (Plett et al. 2015b). Across the ECM plant:host combinations, some general trends are consistently observed, including an early induction of host defensive pathways and upregulation of nutrient transporters.

In early stages of ECM colonization, the plant mounts a general defensive strategy, with the production of reactive oxygen species, activation of the hypersensitive

response or production of chitinases (Le Quere et al. 2005; Duplessis et al. 2005; Frettinger et al. 2007; Sebastiana et al. 2009; Plett et al. 2015a, b). Metallothioneins, cysteine-rich metal binding proteins that aid in oxidative stress tolerance, are often upregulated in roots at this stage in ECM:tree interactions (Duplessis et al. 2005; Frettinger et al. 2007; Johansson et al. 2004). Interestingly, in a study of poplar roots colonized by AM fungi this trend is reversed, with transcription of all tested metallothioneins being repressed in mycorrhized roots (Pallara et al. 2013). Another commonly upregulated set of defensive proteins are pathogenesis-related (PR) proteins. Upregulated members of this family in ECM:tree interactions include chitinases, peroxidases and thaumatin-like proteins (Le Quere et al. 2005; Duplessis et al. 2005; Frettinger et al. 2007). One of the areas of curiosity in mutualistic plant:microbe interactions is attempting to understand how the tree is able to differentiate between a beneficial or a pathogenic invasion. The initial defence response of the plant to mutualistic fungi is quite similar to that raised against a pathogen. Even at early stages of colonization, however, the defensive response of the plant is fairly mild. It is possible that some yet unknown mechanism is used by the plant to determine that the invading fungus is beneficial. Current research, however, would indicate that mutualistic fungi have evolved as efficient manipulators of the plant's defences and like pathogens, use effectors to subdue plant defences and gain access to the plant (Kloppholz et al. 2011; Plett et al. 2011, 2014a).

These initial defence responses on the part of the tree are reduced in later stages of colonization, allowing for the successful formation of symbiosis between tree and fungus. The plant's defensive strategy does not end, however. In later stages of colonization plant hormones such as ethylene or jasmonic acid begin to be differentially regulated. In a study on *Arabidopsis thaliana* colonized by the generalist fungi *Piriformospora indica*, Camehl and colleagues (2010) demonstrate that ethylene sensitivity is correlated with the degree to which plant roots are colonized by the fungus. Mutant plants with impeded ethylene sensitivity have the highest levels of fungal colonization. Similarly, in the interaction between *Populus trichocarpa* and the ECM fungus *Laccaria bicolor*, Plett and colleagues (2014b) demonstrate that both ethylene and jasmonic acid are upregulated in later stages of fungal colonization and that they impede the progress of the fungus. These studies show that hormone regulation in the plant serves to maintain a low level defensive strategy that prevents the fungus from overstepping its boundaries and overwhelming the plant. Ethylene related genes are also upregulated in oak (Tarkka et al. 2013) and *Eucalyptus* (Duplessis et al. 2005) at mid to late stages of colonization. Conversely, other hormone receptors such as for abscisic acid, also known for its role in stress response, are repressed (Duplessis et al. 2005; Tarkka et al. 2013).

At the heart of the mutualistic association between mycorrhizal fungi and their host trees is the exchange of nutrients. On the part of the plant, this requires the activation of sugar transporters to deliver photosynthate to the fungus, as well as nitrogen and phosphorus transporters to accept the nutrients delivered by the fungus. At mid- and late-stage colonization, once the mycorrhiza is established and functional, nutrient transporters in the plant are generally upregulated (Duplessis et al. 2005; Sebastiana et al. 2014), though some studies have shown that this

response may occur earlier, before the formation of the Hartig net (Plett et al. 2015b). These may include various sugar transporters, nitrogen and phosphate transporters, lipid transporters, more general ABC-transporters or aquaporins. AM and ECM fungi are capable of delivering both nitrogen and phosphorus to the plant, as well as other trace elements, but AM fungi are more commonly associated with phosphorus uptake while ECM fungi are associated with nitrogen delivery. Several phosphate transporters are upregulated in poplar in both AM or ECM interactions (Loth-Pereda et al. 2011). Members of the high-affinity ammonium importer family (AMT1) were upregulated in mycorrhized poplar roots (Selle et al. 2005). Amino acid transporters are also typically upregulated as this is one of the forms that fungi prefer to deliver nitrogen (Larsen et al. 2011). Additionally, as ECM fungi do not have sucrose transporters, they rely on plant cell wall bound invertases to produce hexose sugars for uptake (Casieri et al. 2013). One plant cell wall bound invertase was found to be upregulated in the interaction of oak with a fungal symbiont (Sebastiana et al. 2014).

In AM associations there is some evidence that the exchange of nutrients operates on a rewards system, where fungal symbionts providing the most nutrients are rewarded with the most carbon (Kiers et al. 2011). No similar level of control has been proven in ECM symbiosis, though plant hexose transporters – usually repressed in mycorrhizal roots – can control the concentration of hexose sugars in the apoplastic space, and thus the fungus' access to sugars (Casieri et al. 2013). If the fungus is not delivering sufficient nutrients to the plant, the plant can regulate its invertase and hexose transporter activity and limit the fungal carbon supply to avoid parasitism (Nehls et al. 2010).

While the idea of mutualism implies a friendly exchange, the host tree must balance both its defensive responses and the allocation of nutrient resources to maximize its benefit from the symbiosis but also to prevent parasitism on the part of the fungus.

Leaf Mutualists: Endophytes

Leaf colonizing microbes classified as 'endophytes' are more enigmatic in both their definition and lifestyle than pathogenic or soil borne mutualistic microbes. Broadly speaking, endophytes are any organism that can inhabit plant tissues without inducing a visible symptom of death or disease. The vast majority of endophytes are commensal in their relationship with the plant, but a number have been identified that exhibit mutualistic aspects in their interaction with the plant (Hallman et al. 1997; Rodriguez et al. 2009). These 'mutualistic' relationships between the plant leaves and endophytes appear to have little to do with the mutual exchange of growth limiting nutrients as found in below-ground mutualisms. Rather, in return for plant photosynthate, the majority of studied endophytes appear to benefit the plant by improving the disease resistance of leaves (Arnold et al. 2003; Mejia et al. 2008; Rodriguez et al. 2009). A recent study found that endophytes are likely the secondary

line of plant pathogen defence, operating in tandem with the induction of major host genes encoding disease resistance factors (Raghavendra and Newcombe 2013). While a great deal of effort has gone into identifying, isolating and describing pathogens and endophytes of angiosperm trees, far fewer studies have looked at the genetic regulation of genes associated with the endophytic colonization process.

Throughout the tropics, cacao beans are a major agricultural product. As such, the trees producing these beans (*Theobroma cacao*) and their associated microbiota have been the topic of many studies (Crozier et al. 2006; Hanada et al. 2010; Konate et al. 2015). Dominating the endophytic community in the leaves of *T. cacao* are relatively few genera of fungi. One of the most dominant is *Colletotrichum tropicale,* an ascomycete from the Sordaromycetes (Rojas et al. 2010). Practically, inoculation of *T. cacao* plants with this endophyte reduces incidences of both *Phytophthora* rot as well as insect herbivory (Mejia et al. 2008; Rojas et al. 2010; van Bael et al. 2011, 2012). These phenotypes are likely due to the impact that inoculation has on the transcriptional regulation of the host: nearly 8 % of all *T. cacao* genes were differentially regulated in leaves undergoing colonization by *C. tropicale* (Mejia et al. 2014).

A large portion of the gene regulation was associated with plant defence. These included signalling receptor kinases, heat shock proteins, pathogenesis-related proteins, peroxidases as well as genes involved in the regulation of reactive oxygen species. Two competing plant hormone signalling pathways, that of ethylene and jasmonic acid, were also differentially regulated. Within the former pathway, genes associated with ethylene biosynthesis (e.g., *ETHYLENE OVERPRODUCER1* (*ETO1*)*, ACC oxidase*) and an ethylene response ethylene binding protein (*EREBP*) were upregulated while a negative regulator of ethylene signalling (*EBF1*) was downregulated. Induction of the ethylene pathway during the colonization process mirrors that seen during the late stages of colonization of both mutualistic and pathogenic root microbes (Duplessis et al. 2005; Camehl et al. 2010; Rinaldi et al. 2013; Tarkka et al. 2013; Plett et al. 2014a). Physical defence pathways were also induced in *T. cacao* leaves colonized by *C. tropicale.* Genes coding for proline rich proteins (involved in cell wall hardening) and tubulin sub-units (involved in cellulose deposition) were amongst the 20 cell wall biogenesis attributed genes. Consistent with the regulation of these genes was a concomitant increase in lignin and cellulose by 23 and 20 %, respectively (Meije et al. 2014).

Fungal colonization also altered many physiological properties of the leaf. These included alterations to nitrogen metabolism and photosynthesis. In the former case, 115 genes associated with leaf nitrogen metabolism were regulated. These included a number of glutamate synthases and resulted in a significant increase in $\delta^{15}N$ levels in leaves by 0.5 %. This increase in nitrogen was not due to fixation by the endophyte, but might be attributed to *C. tropicale* causing a reduction in the off-gassing of nitrogen rich compounds by the leaves, an increase in nitrogen uptake from the soil or due to remobilization from other parts of the plant. With relation to photosynthesis, many genes associated with chlorophyll binding, regulation of the photosystems and RuBisCO were downregulated. This translated into a significant decrease in light saturated rates of maximum photosynthesis by 32 % (Mejia et al. 2014).

A smaller study considering the impact of two leaf endophytes (*Penicillium* sp. and *Truncatella angustata*) on the expression of certain defence related genes in *Populus angustifolia* leaves was performed by Busby and colleagues (2013). As found in *T. cacao,* cell wall modifying and transcription-related genes were significantly upregulated during these interactions. Secondary metabolism genes *PtZOG1, PtCYP92A* and P450s associated with detoxification were found to be downregulated by 4–20× while *PtCAD9* was found to be upregulated in the interaction between *T. angustata* and *P. angustifolia*. Interestingly, the genes tested with roles in defence, signal perception and hormone metabolism were not significantly regulated during the interaction. Therefore, there are some overlaps in the results between molecular events occurring in host leaves of both tropical and temperate forest trees during fungal endophyte colonization.

Conclusion

Classically, studies considering pathogenic or mutualistic plant:microbe interactions have been performed independently of one another. Now, by speaking the same language of gene and genome, different science disciplines can begin to interact more freely and gain a richer understanding of these interactions. This advancement has led to an emerging field of research at the interface of pathogenic and mutualistic interactions. Crop scientists, especially, have made use of newly available genomic datasets to advance this field of research and to underscore the similarities in host response to pathogenic or mutualistic interactions (Ané et al. 2002; Arrighi et al. 2006; Genre et al. 2009; Czaja et al. 2012; Wang et al. 2012; Rey et al. 2013, 2015; Zhang et al. 2015). In the study of angiosperm trees, research groups globally have noted the striking similarities in the transcriptomic responses of the host plant in regards to defence (Fig. 2). Essentially, it would appear, the plant responds with a pre-set gene core to whatever microbe appears to be present in its tissues. So how does a particular microbe cope with this? Why are some microbes successful at entering into commensal or mutualistic interactions with the plant while others become parasitic? A likely explanation for successful interaction between microbes and plants is the evolution of the 'effector' protein as has been detailed in both mutualistic and pathogenic organisms (Martin and Kamoun 2012). Very few effectors, however, have been reported upon in detail in the interaction between microbes and angiosperm trees (Kloppholz et al. 2011; Plett et al. 2011; 2014a), leaving this an area of exploration that must be addressed.

The study of transcriptomics in plant disease progression has also demonstrated the importance of the type of defensive response from the plant, as well as the intensity and timing of that response. This was true in pathogenic compatible and incompatible interactions where the timing and amplitude of gene regulation was found to be one of the explanatory variables in determining plant resistance. The timing and intensity of defence programs induced in the plant may also be the key to understanding how mutualistic relationships are established. We have found that, while plant defences are induced in plants during an interaction between ECM

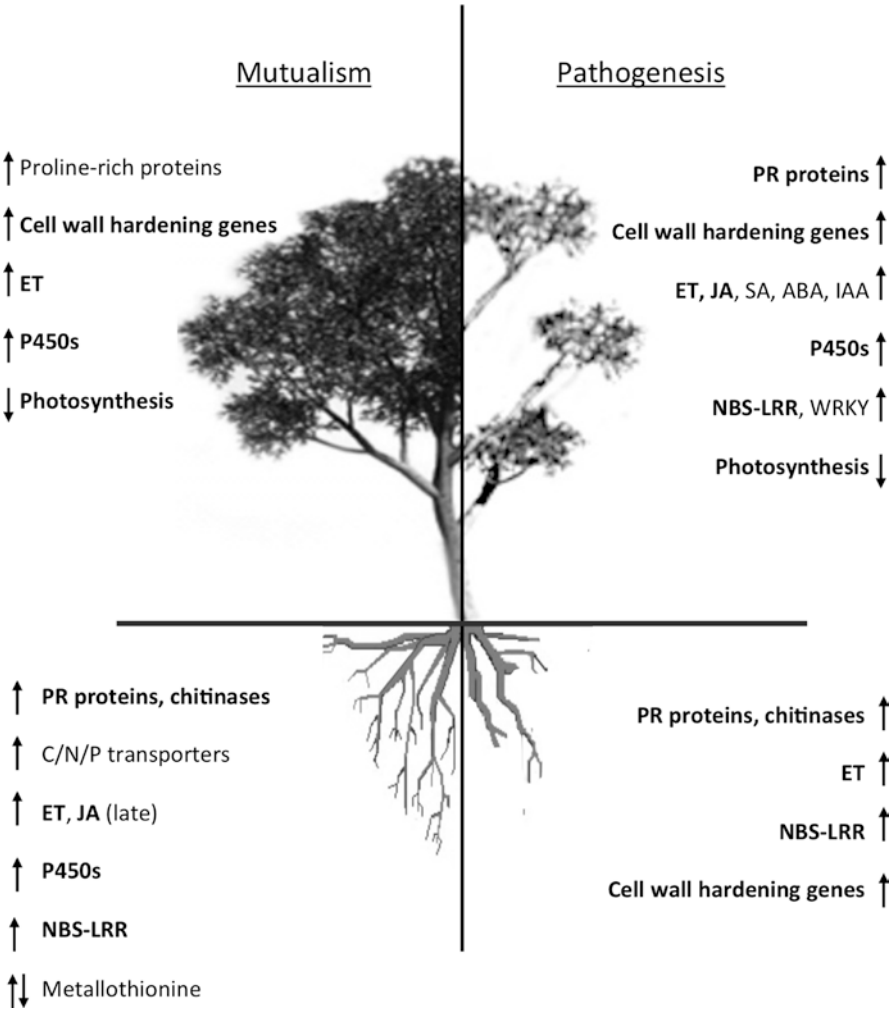

Fig. 2 Mirrored plant responses? A summary of the host plant pathways regulated during colonization by microbes from different lifestyles and in different parts of the plant. *Bold text* indicates elements that are regulated in a similar manner by more than one lifestyle of microbe. *ET* ethylene, *JA* jasmonic acid, *SA* salicylic acid, *ABA* abscisic acid, *IAA* auxin, *P450* cytochrome P450

fungi and their hosts, the induction of these pathways is always late in the establishment of symbiosis (Plett et al. 2015b). This late-stage induction likely only acts to limit fungal invasion to a sustainable level for the plant (Camehl et al. 2010; Plett et al. 2014b). Altogether, these findings highlight the need for additional transcriptomic studies to determine the pathways that are induced or repressed in successful defences, as well as host transcriptional regulation over the time course of infection. As the cost of sequencing continues to decrease, and as bio-informatic tools become more user friendly, we hope to see developments in this area of research in the near future.

In conclusion, we are in a very exciting time of research. We are no longer constrained by traditional model plants and can now begin to plumb the depths of true biological diversity in the plant kingdom. This is especially true in our understanding of how plants, and angiosperm trees in particular, interact with the microbial world around them. This will not only further our theoretical understanding of how the world around us works, but also will prove to be the foundation for new industrial innovations in our quest for more sustainable agro-forestry practices.

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Erratum to: *Populus* as a Model Tree

Carl J. Douglas

Erratum to : Plant Genetics and Genomics: Crops and Models

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The title for chapter ''Populus as a Model Tree'' was inadvertently set with the book title ''Comparative and Evolutionary Genomics of Angiosperm Trees''. This has now been updated with the correct chapter title to read as ''Populus as a Model Tree''.

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