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Heteroblasty in the temperate rainforest tree *Gevuina avellana* Mol. (Proteaceae): Natural variation and regulation

Heteroblastia en *Gevuina avellana* Mol. (Proteaceae), un árbol del bosque templado lluvioso: Variación natural y regulación

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True wisdom is to recognize our own ignorance

(Socrates)

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CONTENTS

I. ABSTRACT

Heteroblasty is defined as drastic shifts in the morphology and/or physiology of leaf and stem units (phytomers) associated to the interaction between the ontogenetic program and environmental signals (*e.g* light, water, and nutrient availabilities). Heteroblasty has been proposed to have an adaptive value when changes in environmental conditions are predictable and may exert constraints on plant growth and development. *Gevuina avellana* Mol. (Proteaceae) is a tree species growing in the Chilean temperate evergreen rain forest. This species shows the greatest heteroblasty compared with others coexisting tree species, changing from simple to compound leaves during development. In this thesis, I studied heteroblastic development of *G. avellana* under a natural setting with the goals to understand: a) the effect of light availability on the natural variation of the heteroblastic trajectory of *G. avellana*, and b) the integration of external and internal cues driving the progression of the heteroblastic developmental trajectory. I found that leaf size and complexity showed a significant ontogenetic increase under high light availability. This could reflect a functional advantage to cope with environmental constraints of the high light micro-environment (e.g., high vapor pressure deficit, light energy overload). Regarding the integration of external and internal cues (i.e., habitat with ontogeny) driving heteroblasty, for *G. avellana*, heteroblastic development is most likely regulated by the transcriptional dynamics and heterochronic effects of *SQUAMOSA PROMOTER BINDING Like* genes (*SPL*) over leaf and flower regulatory factors, such *NAC* and *AGAMOUS Like* (*AGL*) genes. I propose that the phenotypic expression of the heteroblastic trajectory is a light regulated mechanism, in which phytochrome B has a pivotal role in the external environmental effect being communicated to the cellular machinery, where jasmonate and auxin along with the gene module *SPL-AGL-NAC* have a key role in regulating the difference in leaf complexity

observed in the field for contrasting light availabilities. In summary, this thesis work provides a comprehensive molecular, physiological and developmental framework for the underlying mechanism regulating how compound leaves are produced in the heteroblastic species *Gevuina avellana*.

II. RESUMEN

La heteroblastia alude a cambios muy drásticos en la morfología y/o fisiología de unidades consecutivas compuestas por entrenudo y hojas (fitómeros) asociados a la interacción entre el programa ontogenético y la información entregada por señales ambientales (ejemplo: disponibilidad de luz, agua o nutrientes). Se ha discutido y propuesto un valor adaptativo de la heteroblastia cuando, durante el desarrollo de la planta, cambios en las condiciones ambientales son predecibles y podrían resultar en limitaciones para el crecimiento y desarrollo. *Gevuina avellana* Mol. (Proteaceae) es una especie arborea típica del bosque del bosque templado lluvioso de Chile y Argentina. Esta especie presenta el desarrollo heteroblastico más marcado y dramático entre todas las especies arbóreas con las que cohabita, cambiando la forma de sus hojas durante el desarrollo vegetativo, de simples a altamente compuestas. En esta tesis, me enfoqué en dos objetivos principales respecto al desarrollo heteroblastico de *G. avellana* en su contexto natural: a) El efecto de la disponibilidad lumínica sobre la variación natural de la trayectoria heteroblastica de *G. avellana*, y b) los mecanismos de integración de señales del ambiente externo e interno de *G. avellana* que conducen la progresión y trayectoria de su desarrollo heteroblastico. Se encontró que bajo condiciones de alta disponibilidad lumínica se observa un aumento significativo en el tamaño y complejidad de las hojas. Esto podría ser el reflejo de implicancias funcionales útiles frente a restricciones ambientales propio de micro-

ambientes de alta disponibilidad lumínica (ejemplo: mayor déficit de presión de vapor, exceso de energía lumínica). En lo que respecta a la integración de señales externas e internas (hábitat más ontogenia) que conducen el desarrollo heteroblastico, este estaría regulado por la dinámica transcripcional de los genes *SQUAMOSA PROMOTER BINDING Like* (*SPL*) y su efecto como señal heterocrónica sobre la actividad de factores reguladores de la formación de hojas y flores, como los genes *NAC* y *AGAMOUS Like* (*AGL*). A su vez, se sugiere que la expresión fenotípica de la trayectoria heteroblastica es un mecanismo regulado por la luz, con el fitocromo B cumpliendo un rol fundamental en establecer la comunicación de entre las señales ambientales y celulares. Entre estas señales, la actividad hormonal de jasmonato y auxina, más el módulo genético compuesto por *SPL-AGL-NAC* cumplen un papel clave para establecer las diferencias en el grado de complejidad que presentan la hojas de *G. avellana* en su ambiente natural bajo condiciones contrastantes de disponibilidad lumínica. En resumen, esta tesis proporciona un marco conceptual integrado de los mecanismos moleculares, fisiológicos y de desarrollo acerca de cómo se van formando las hojas compuestas en la especie heteroblastica *G. avellana*.

CHAPTER I. INTRODUCTION

The high diversity of leaf shapes has caught the attention of naturalist for centuries (e.g., Goethe 1790; Goebel 1900; nowadays). Broadly all Angiosperm show a certain degree of variation in leaf shape inter and intraspecifically. The diversity in leaf shapes found among Angiosperm plants reveals a polygenic, mostly additive, and highly heritable regulation that has occurred independently and several times during evolutionary time (Langlade *et al.*,2005; Nicotra *et al.* 2011; Chitwood *et al.*,2014). Also, changes in plant morphological and physiological traits between successive leaves are intrinsic to development (Maherali *et al.*, 2009). With respect to the phytomeric nature of plant leaves, each unit is added sequentially in patterns determined by the underlying genetic architecture (constructional view). Its initiation occurs within the physiological environment established by the previous developmental history of the plant (developmental view). Therefore, the value of an individual organ, such as the leaf, depends on how functionally optimal it is and how long it persists during whole plant growth (Jones, 2001). Because of this, leaf phenotypes are highly contextual to the ontogenetic drift which induces modular changes in conjunction with prevailing environmental conditions (Diggle 1994; de Kroon *et al.*, 2005).

In most species, variations of leaf shape within a single individual are usually gradual and subtle. However, some species exhibit marked changes among successive phytomers as part of their ontogenetic programming, a phenomenon called heteroblasty (Goebel 1900; Diggle 2002; Zotz *et al.*, 2011). Although the answer as to why and how this strategy arose in plants remains unknown, it is thought that the level of heteroblasty depends on the evolutionary history, ontogenetic program, plasticity levels, and environmental factors (e.g., water or light availability) (Diggle, 1999; Kerstetter & Poethig, 1998). Several

authors have studied heteroblasty from the molecular, morpho-anatomic and evolutionary perspectives; however, several questions about the biological and functional advantages of heteroblasty still remain unanswered (Gould, 1993; Day, 1998; Diggle, 1999; Darrow *et al.*, 2002; Burns & Dawson, 2009; Gamage 2011; Zotz *et al.*,2011).

Historically, the heteroblasty syndrome has been attributed to the intrinsic expression of the ontogenetic programming, and to lesser extent to environmentally driven responses (Zotz *et al.*,2011). However, a crucial feature of the sessile life of plants is the ability to anticipate the forthcoming environmental conditions and initiate developmental responses accordingly, often because of the perception of subtle environmental cues (Casal *et al.*, 2004). Under this premise, heteroblasty has been reported as an adaptive response to environmental heterogeneity (Gould, 1993; Gamage & Jesson, 2007; Forster & Bonser, 2009). During the last decade, technological advances have allowed an integral view of this phenomenon (Tsukaya 2005, 2014); however, regulatory mechanisms that induce heteroblasty are just beginning to be elucidated for model species (Costa *et al.*, 2012; Chitwood *et al.*,2014). Genome and other omics scale research on morpho-physiological processes suggests that heteroblasty could be regulated by molecular clues which include: heterochronic genes, phytohormones, and interactions of small RNAs and organ regulatory factors in shoot apical meristem, during primary and secondary leaf morphogenesis (Kerstetter & Poethig, 1998; Chitwood & Sinha, 2014). Accordingly, understanding the ecological, physiological, and molecular basis underlying heteroblasty, as well as the adaptative strategy of this phenomenon and its role in the ecological distribution and evolutionary diversification of plants are issues that have acquired significant interest for different plant disciplines, especially for plant evolutionary biology and plant physiological ecology.

1.1. Environmental cues as source of information for plant development and performance and their effects.

Light, water and nutrient availability are the main requirements for growth and development of plants (Fitter & Hay, 2002). However, all of these factors are heterogeneous in natural environments, especially light (Pearcy, 2007). Plants have evolved to finely tune phase changes in response to both environmental and endogenous clues. This synchronicity in the life cycle of plants is a critical trait that ensures fitness (Jarillo & Piñeiro 2011). In plants, the ontogenetic program determines modular responses (on meristems, leaves, stem, and roots) which have an impact on morpho-physiological plasticity of traits when subjected to environmental changes (Schlichting, 1986; Givnish, 1988; De Kroon *et al.*, 2005).

For purposes of this thesis, I will mainly use forest ecosystems in most of the examples. In forest ecosystems, among the concurrent variation in multiple abiotic and biotic factors, light is the most important limiting factor (Valladares *et al.*, 2000). Canopy shade changes light quality and quantity, and the latter can be far more severe in the closest part of canopy. Canopy shade can create a mosaic of habitats that strongly affect plant growth and fitness. For this reason, plants are able to adjust their morphological and physiological traits to increase their carbon gain, survival, and competitiveness (Givnish, 1988; Takahashi, 1996; Fitter & Hay, 2002). Structural plasticity of shoots, mainly perpendicular light orientation and higher leaf area, are traits that favor light capture efficiency under low irradiance environments. On the other hand, under high irradiance environments, the architectural arrangement of branches favors self-shading, avoiding excessive radiation on leaves, which will have smaller leaf area and foliage than shade acclimated plants (Pearcy

et al., 2004). This ultimately constitutes a critical aspect of ecological breadth for forest species.

From a mechanistic point of view, the phytochrome pathway is used by plants to monitor various aspects of their light environment (Franklin 2009). The perception of the light environment provides information to modulate biochemical and developmental responses. One of the more studied responses is photomorphogenesis. As the term implies, plant development is profoundly influenced by light (e.g. etiolation and de-etiolation). Complete reversal of light-induced responses on development involves major long-term alterations in metabolism that can be brought about only by changes in gene expression (Wettberg & Schmitt 2005; Chitwood *et al.*, 2012a). The best understood sub-pathway involves a direct interaction of phytochrome B with bHLH-domain transcription factors, which results in the activation of multiple genes (e.g. *SENSITIVE TO RED LIGHT REDUCED 1, SRR1*). *SRR1* is involved in the phyB signaling, and is highly conserved between plants and animals as a regulator of the circadian clock. *SRR1* affect phyB-mediated signaling rather than stability or biosynthesis of the photoreceptor (Staiger *et al.*, 2003).

Several reports describe the magnitude of ontogenetic variations and its relation with environmental changes. This interaction provides mechanisms of morphological integration to environmental variables. However, the timing of these mechanisms is variable, depending on environmental signals perceived and developmental rate of plants (Day, 1998; Diggle, 2002). In keeping with these findings, De Kroon & Hutchings (1995) reported that, for example, in clonal plants, leaf bud activation occurrs earlier in ramets growing under high light than in those growing in low light environments.

In addition to light, other environmental factors which fluctuate within forests may influence the morphological and physiological responses with different magnitude; these factors include: variations in temperature and wind speed (Gurevitch, 1988), nutrients availability, mainly phosphorus and nitrogen (Hikosaka *et al.*, 1998; Givnish *et al.*, 2004; Niinemets & Sack, 2006), relative air humidity, and relative soil water content (Bjorkman & Powles, 1984; Pearcy, 2007). Under this heterogeneity, leaf morphology and the pattern of shoot architecture determine to a large extent the growth habit of seed plants. Leaves share common features such as being flat structures and play an essential role in plant photosynthesis, but also show an impressive variability in their size and shapes. Being determinate organs, they are subjected to the physical laws imposed by environmental pressures that constrain size and thickness. Thin leaves loose water and heat more rapidly when exposed to light (Ehleringer 2000). Large leaves require more support tissues. Broad leaves also heat rapidly when the ambient airflow is low. Increasing the level of dissection helps to cool down the leaf (Voegel 1970), but affects the surface area available for light capture. On one hand, it reduces the surface area of an individual leaf, whereas on the other hand, it may increase light interception at the whole plant level by reducing self-shading (Hasson *et al.*. 2010). Lambers *et al.* (2008) reported that small and thin leaves show high water vapor conductance; therefore, they dissipate much more heat by convection. They cool rapidly, reaching temperatures close to air which is favorable in open sites with high irradiance. Also, the authors indicate that having compound or highly lobed leaves is functionally similar to having small leaves with regards to heat dissipation. Ultimately, the effects on leaf size and shape have implication on plant acclimation and fitness to prevailing environmental conditions.

Key aspects of the synchronicity of developmental responses to environment are: (i) the integration of multiple external and internal cues; (ii) the uses of shared regulatory elements such as transcription factors, target proteins and hormones in diverse developmental cascades; and (iii) the role of physiological feedbacks to provide indirect environmental information (Sultan 2010). Distinct environmental cues and initial transduction events can converge on shared hormonal pathways to elicit common responses. For these reasons, plants have developed strategies, among which heterophylly or heteroblasty are striking mechanisms of acclimation to both prevailing and predictable environmental conditions (Kerstetter & Poethig, 1998).

1.2. Heteroblasty

Leaves are the easiest plant organ in which to observe plastic responses to environmental clues (Strauss-Debenedetti & Berlyn, 1994). Among the wide range of developmental plastic responses, heteroblasty has been postulated as an adaptive mechanism (Gould, 1993; Forster & Bonser, 2009). In this thesis, I will use the definition of heteroblasty as proposed by Goebel, which describes it as a drastic change in shoot and/or leaf morphology responding to an ontogenetic program (Goebel, 1900; Jones, 1999; Gamage & Jesson, 2007; Burns & Dawson, 2009). The other definition implies a whole-plant drastic change in morphology, which would not be restricted to individual components of the shoot (Allsop, 1965; Kaplan, 2001). Several species from different latitudes show heteroblastic development (Jones, 1999). Some of these have been studied from morpho-anatomical, molecular and evolutionary approaches (Gould, 1993; Brodribd *et al.*, 1993; Day, 1998; Diggle, 1999; Jordan *et al.*, 2000; Burns & Dawson, 2006; Gamage & Jesson, 2007; Forster & Bonser, 2009); however, biological and functional advantages, as well as internal regulatory mechanisms which induce heteroblasty are still not clear.

Recent studies have pointed out that although strongly driven by ontogenetic programming, heteroblastic expression could be affected by prevailing environmental conditions (Day, 1998; Burns, 2005; Forster & Bonser, 2009). For example, James & Bell (1999) reported that different light quantities (100, 50 and 10% sunlight) affect the time at which heteroblastic changes occur. They observed that in *Eucalyptus globulus* ssp *globulus* (Myrtaceae) ecotype of early developmental stage, low light quantity (10% sunlight) caused a delay in the time to achieve heteroblastic shifts.

In New Zealand forests, there is a striking abundance of species with heteroblastic development (Burns & Dawson, 2006). Day (1998) proposed that evolution of heteroblasty in New Zealand forests response to a process triggered by climate changes during Pleistocene rather than low light acclimation. In another study, Gould (1993) attempted to explain the functional significance of heteroblastic changes in *Pseudopanax crassifolius* (A. Cunn) C. Koch, a New Zealand native tree. He characterized changes in morphological and anatomical attributes in the five types of leaf produced by this species during its development. Gould infers that heteroblastic series may be associated with changes in leaf construction costs and/or light interception and heat dissipation, although he had no quantitative data to prove it. Darrow *et al.* (2002) studied water loss resistance in several New Zealand heteroblastic species. They examined hydraulic relations and the $\delta^{12}C$ y $\delta^{13}C$ ratio in sapling and adult leaves with strong heteroblasty, comparing with sapling and adult leaves from homoblastic species. They found that some heteroblastic species have water-wasteful behavior during early stages; but in adult stage this condition is reversed. On the other hand, other heteroblastic species are resistant to water loss during sapling stage and water-wasteful in the adult stage like homoblastic species. This response can be explained by the interaction between the shape of leaves (simple or divaricate) developed during adult

stage and the prevailing light condition in which the species grows (sun or shade environments).

In the temperate rain forest of Chile, although several species show ontogenetic variation in leaf shape (e.g., *Citronella mucronata*, *Aextoxicon punctatum*, *Myrceugenia planipes*), only *Gevuina avellana* is recognized as a strongly heteroblastic species. This species changes leaf shape from simple leaves in seedling stage to compound leaves along development. It is considered a semi-shade tolerant species, although it can be found inhabiting both densely closed and open canopies. Empirical observations suggest that in high light environments (e.g., forest edge), *G. avellana* shows highly pinnate leaves, while in shade environments leaves show a decrease in complexity (Donoso, 2006; Nicotra, personal communication).

Currently, in addition to classical morphological and ecophysiological approaches, the advance in molecular tools offers new opportunities to study heteroblasty and leaf morphogenesis in a more comprehensive way. Recently, Tsukaya (2006) reported the molecular mechanisms determining leaf shape, where mutation of the gene *SERRATA* (*Se*) accelerates heteroblastic changes in serrated-border of *Arabidopsis* plants. Champagne & Sinha (2004, 2007) analyzed leaf morphogenesis in simple and compounds leaves. They described several genes involved not only in leaf morphogenesis but also described other genes interacting and controlling normal development and identity of simple and compound leaves. The next section summarizes the main genetic components involved in leaf morphogenesis.

1.3. From genes to leaves: components and regulation of leaf morphogenesis

The variability of leaf shape is the result of highly divergent morphogenetics and gene regulatory foundations of plant developmental systems (Townsley and Sinha 2012). The

most conspicuous characteristic of leaf shape is the degree to which the leaf is subdivided into smaller segments. Leaves lacking subdivision are termed simple, whereas divided leaves are termed compound. A possible explanation is that mutations affecting the expression levels of transcription factors can modify the function of a major developmental regulatory complex in some organs without interfering with its other essential roles in morphogenesis. Such dosage-sensitive interactions may be broadly responsible for evolutionary change and provide a relatively simple mechanism for the generation of natural variation of leaf shape (Kimura *et al*. 2008). The complex process underlying leaf morphogenesis shows flexibility between highly conserved and evolving components in a comprehensive fashion in their responses to natural selection (True and Haag 2001). As a consequence, phenotypic differences of leaf traits even among phyllogenetically close individuals can vary widely regarding ontogeny and/or plastic responses (Chitwood *et al.*,2014). Recent advances in high-throughput phenomic techniques make it possible to integrate merely observational phenotypic studies with, for instances, molecular studies of the underlying mechanisms of natural variations in leaf morphology.

A thorough study of leaf development is required if we want to understand heteroblasty in a comprehensive context. Many recent studies have contributed toward improving our knowledge of leaf development using molecular, mechanistic, and evolutionary approaches (Nakayama *et al.*. 2012, 2014). In spite of the great variety of leaf forms found in nature including, simple, lobed or compound leaves (Champagne & Sinha, 2004), all of them share basic characteristics in their development from shoot apical meristems, which are resumed as follows:

a) *Leaf initiation*: In this phase, lateral cells from the stem apical meristem show increasing periclinal cell divisions and primordial zones are initiated.

b) *Primary morphogenesis*: In this phase the morphological domains are delimitated (also called suborgan identities) and primordia acquire identity as specific parts of leaf. These domains are abaxial-adaxial (dorsiventral), apical-basal (proximodistal), and margin-blade-midrib (lateral).

c) *Acquisition of tissue identity*: Coordinated processes of cell division, expansion, and differentiation occur in this phase. Here, leaf develops its photosynthetic capacity, vascular system, and epidermal tissues. Secondary morphogenesis at this stage due to variations in cell division or cell expansion rates can change the shape acquired during primary morphogenesis.

The temporal progression of each event suggests independent activity and regulation of several genetic programs (Dengler & Tsukaya, 2001). Sinha (1999) describes several genes actively participating in the first two phases for both, simple and compound leaves. Some of these are:

KNOTTED-LIKE GENES (*KNOX*): These genes encode homeodomain-containing proteins and have been subdivided into two classes. Class I (*I KNOX*) participate in leaf initiation. When these genes are down-regulated in the lateral zone of stem apical meristem, cells start to differentiate into the leaf primordium.

CLAVATA (*CLV*): These genes encode for a kinase receptor, which suggest a role for signal transduction events in stem meristem function, regulating the balance between cell division and cell differentiation. Also these genes are shown to controll meristem size in *Arabidopsis*.

TERMINAL EAR (*TE*): These genes are implicated in determining both the insertion and initiation zone of leaf primordia.

PHANTASTICA (*PHAN*): Expression of these genes causes differentiation of dorsiventral domains in both, leaves and flowers. These genes also participate in down-regulation of *KNOX-1* genes.

LEAFY/FLORICAULA (*FLO/LFY*): In simple leaved plants, expression of these genes is necessary for floral meristem establishment. In compound leaved plants, these genes regulate leaflet development, interacting with other genes, such as *KNOX-1*.

NO APICAL MERISTEM/ATAF/CUP SHAPED COTYLEDON (*NAC*): These transcription factor genes are essential for proper specification of lateral organ boundaries at the SAM and of leaflet boundaries. They also have a role in the timing of leaf maturation, spatial and temporal positioning of leaflets, secondary leaflet initiation and separation, and leaf margin elaboration (Berger *et al.*, 2009).

In the case of compound leaf development, particular mechanisms of gene interactions are more complex than in simple leaves. Champagne & Sinha (2007) performed a detailed description of compound leaves organogenesis, suggesting that interaction of class *I KNOX*, *PHANTASTICA* (*PHAN*), and *FLORICAULA/LEAFY* (*FLO/LFY*) are actively involved in leaf morphogenesis regulation in compound leaves of of Solanaceae and Fabaceae species. This variation is illustrated by the fate of differential leaf outgrowth during the secondary morphogenesis stage resulting in simple or compound leaves. The development of a compound leaf requires a prolonged maturation process, during which leaflets are reiteratively initiated from regions at the leaf margin. Several mechanisms have been shown to act within this developmental window to promote leaf elaboration, many of which also play a role in shoot apical meristem (SAM) function. Class I KNOTTED1-LIKE HOMEOBOX (KNOXI) transcription factors are essential for SAM maintenance (Hake *et al.*, 2004), and also play a central role in the modulation of

compound leaves (Bharathan *et al.*,2002; Hareven *et al.*,1996; Hay and Tsiantis, 2006; Parnis *et al.*,1997). In some legume species, such as pea and *Medicago*, the orthologous genes *UNIFOLIATA* and *SINGLE LEAFLET*, respectively, are also involved in leaf elaboration (Hofer *et al.*,1997; Wang *et al.*,2008). Likewise, several plant hormones, such as auxin and gibberellic acid, have also been implicated in leaf elaboration, either via regulation of maturation or through mediation of localized growth (Barkoulas *et al.*,2008; Jasinski *et al.*,2008; Berger *et al.*,2009).

In the third phase of foliar morphogenesis (identity phase), phytohormones acquire an essential role, generating several transduction signals for up and down gene-regulation, defining histological and morphological traits (Bai & DeMasson, 2006) which finally determine leaf identity at different ontogenetic stages (Kerstetter & Poethig, 1998).

From a molecular point of view, leaf identity regulation is subdivided into two categories:

a) Genes involved in transition stages of plants (seedling-sapling; sapling-adult).

b) Genes involved in sapling and adult traits establishment.

In both cases, the importance of genes involved in cytokinin (VIVIPAROUS (*VP8*) and gibberellins (DWARF 1, DWARF 3, DWARF 5 and ANTHER EAR) synthesis for phase transitioning has been described. Activity of phytohormone gibberellin and *VP8* gene expression promote change from sapling to adult (Kerstetter & Poethig, 1998). More recently, a conserved molecular mechanism for phase change has been described, in which *SQUAMOSA PROMOTER BINDING Like* transcription factors play important roles driving heterochronic signals for developmental events in plants, in coordination with small RNA regulatory pathways involving miRNA156 and miRNA172 (Chen *et al.*,2010). Specifically, *SPL*s are target of miR156, decreasing the overall amount of *SPL*, which in turn correlates

with juvenile-like traits. With the progress of vegetative growth, the level of miRNA156 decreases, and *SPL* genes increases in expression and amount. *SPL* binds to miR172 promoter and triggers the expression of several genes involved in adult-like traits. This is a process that seems to be highly conserved in higher plants, from annual herbs to woody perennial (Chen *et al.*, 2010; Wang *et al.*,2011).

For juvenile and adult trait establishment, Bai & DeMasson (2006) reported that expression of *TEOPOD 1, TEOPOD 2*, and *TEOPOD 3/ CORNGRASS* genes confer juvenile-like traits and their over-expression prolongs these juvenile characteristics, even during adult stage. On the other hand, expression of *GL 15* gene promotes adult traits (trichome density or cuticle thickness) and also is regulated by gibberellins and abscisic acid (Kerstetter & Poethig, 1998). Gibberellins, cytokinins, and auxins play a pivotal role in control of both simple and compound leaf morphologies (Champagne & Sinha, 2004). Hay *et al.* (2002) reported that an increment in endogenous concentration of gibberellins down-regulates the expression of *KNOX-1* genes, inducing leaflet differentiation in tomato plants. In *Arabidopsis thaliana*, GA acts as the main regulator in the expression of *FLO/LFY*. This phytohormone acts on MYB family transcription factors, producing differentiation and development of floral meristems (Gocal *et al.*, 2001). Recent findings link MADS-domain transcription factors encoded by floral organ identity genes to genetic pathways that control leaf development (Pajoro *et al.*, 2014). Direct links have emerged between floral organ identity and genes involved in abaxial-adaxial domains, organ boundary formation, tissue growth, and cell differentiation (Sablowski, 2015).

Finally, another conserved mechanism contributing to leaf shape variation is the genetic pathway driven by NAC-domain genes, briefly mentioned above. Proteins of this family contain a highly conserved N-terminal DNA-binding domain and a variable C-terminal

domain (Xie *et al.*, 2000; Duval *et al.*, 2002; Ernst *et al.*, 2004; Olsen *et al.*, 2005). The importance of these genes is that they are the master regulators of alterations in leaf margin in both simple and compound leaves. Moreover, their activity occurs in a broad range of plant species, constituting a conserved molecular framework for margin alteration and compound leaf development (Blein *et al.*, 2008, 2010).

1.4. Heteroblastic development of *Gevuina avellana* Mol.: Problem statement

Despite the increasing accumulation of our knowledge on basic leaf developmental mechanisms in model angiosperm species, comparative studies with non-model species are imperative to complete our knowledge of the basis underlying the diversity of leaf shapes, i.e. leaf development may be too complex to be understood in the light of modifications deduced only in model species. Also, there is little knowledge of how the control of the direction of organogenesis in compound leaves occurs; moreover, control of the ontogenetic change from a simple to highly lobed/compound leaf is poorly understood.

The attempt to understand comprehensively the link between gene expression, hormonal action, and morphogenetic events is an increasing trend in the study of ontogenetic variation, such as the heteroblastic syndrome. Ideally, studying mechanisms involved in the regulatory network and signal cascades driving the morphological changes in heteroblastic species requires an organism that undergoes the ontogenetic changes defined for the heteroblastic syndrome. Despite this, the few studies that address heteroblasty under a comprehensive approach have been conducted with species that are not heteroblastic *sensu stricto* (e.g., Arabidopsis, maize). This is because usually, heteroblastic plants (*sensu stricto)* are not the best suited for molecular analyses as the genomic resources available for them are still limited, and experiments with these large and

slow growing species are often space demanding and time consuming (Huijser and Smith 2011; Zotz *et al.*, 2011), compared to *Arabidopsis* or *Zea mays*. In addition, morphological differences between the juvenile and adult phase tend to be more pronounced in woody perennial plants than in annuals such Arabidopsis or maize. Given their slow development and sparse data on the molecular nature of factors that regulate phase transition in trees, none of these studies have been addressed in heteroblastic trees *sensu stricto*. Nevertheless, the recent development and advances in Next Generation Sequencing techniques provide an excellent opportunity to study the genetic basis of morphological traits and the underlying molecular dynamics in non model species, even without a reference genome.

Gevuina avellana Mol. (Proteaceae) is a typical forest tree species that belongs to an endemic and monospecific genus growing in the evergreen temperate rain forest in Chile and Argentina (Halloy *et al.*, 1996). As general characteristics, this species species can reach 20 m of height, and 60 to 80 cm of diameter. It has a straight trunk and narrow canopy under semi shade condition, and bushy appearance when grows in open and sunny sites (Donoso, 2006). In particular, this species shows the greatest heteroblasty leading to ntogenetic changes in leaf shape, compared with others species with which it shares common micro-environments. In this species heteroblastic development goes from simple to compound leaves during early development, with the change continuing until reproductive stage. In addition, it presents great plasticity in leaflet size, depending on light environment under which it develops, being smaller in sun than shade (Donoso, 2006; Nicotra, personal communication).

In accordance with the background presented above, *G. avellana* is a good model to study the heteroblastic developmental trajectory under an ontogenetic contingency context. This implies the study of a developmental trait and its plasticity within the context of a

comprehensive integration of environmental and cellular signals that preceded the formation of a new phytomer. To carry out this approach, we address the following questions: 1. Given the wide range of light conditions this species inhabits, what is the magnitude of the effect of light availability on the heteroblastic development and trajectory of *G. avellana*, and what would be the advantages of changing leaves morphology (from simple to compound) with respect to the micro-environmental constrains of low light and high light niches?, and 2. How exogenous and endogenous signals that regulate gene expression that lead the heteroblastic development and trajectory in *G. avellana* integrated at the molecular level?

This thesis determines factors that induce and regulate the heteroblastic changes, the physiological implications of this change and the relation with *G. avellana* performance in an ecological context.

1.5. HYPOTHESES

Hypothesis 1

Although in the secondary forest setting *G. avellana* mostly regenerate in shaded understory microsites (Donoso 1978), saplings can inhabit widespread light conditions. Given this, it is postulated that the heteroblastic development of *G. avellana* is positively modulated by light availability, i.e. at high irradiance, heteroblastic development responds plastically to constraints associated to this light condition modifying the phenotypic trajectory of the leaves, increasing the degree of pinnation.

Hypothesis 2

Along vegetative development in *G. avellana*, an increase in auxin concentration in newly formed leaf primordia induces significant changes in the transcriptional dynamics of genes related with heterochronic cues and leaf morphogenesis, increasing the expression of genes involved in the formation of compound leaves. These could be up-regulation of *KNOX-1* genes as seen in a multitude of species, or alternatively, up-regulation of the floral homeotic gene *FLO/LFY* with the simultaneous down regulation of class *KNOX1* genes, as occurs in compound leafed Fabaceae species.

1.6. OBJECTIVES

General Objective

To determine factors inducing and regulating heteroblastic changes, their physiological implications and effect on performance in seedlings and saplings of *G. avellana*.

Specific Objectives

1. To disentangle the effect of light availability on variations of the heteroblastic trajectory of *G. avellana* established and growing under natural conditions.

2. To determine the underlying patterns of gene expression dynamics that regulates heteroblastic development and growth trajectory of *G. avellana*.

CHAPTER II. EXPERIMENTAL APPROACH

For this study, I used leaves and apical buds from *Gevuina avellana* growing in a temperate rain forest area of Katalapi Park, located in Pichiquillaipe, Región de Los Lagos, in South Central Chile (41°31′07.5″ S, 72°45′2.2″ W). This Park has an extraordinary diversity and endemism of flora and fauna, been part of Valdivian temperate rain forest ecoregion (WWF Chile). Several biodiversity and ecophysiology studies have been done in this park for the last decade (www.parquekatalapi.cl). The climate on the Park is temperate, highly humid with oceanic influence (Di Castri & Hajek, 1976) but with winter frosts. Annual precipitation is near to 2200 mm and maximum mean temperatures are 10°C in winter and 22°C in summer (Reyes-Díaz *et al.*, 2005). Also, this Park has research facilities for sample treatments, analysis and assays.

A total of 135 individuals growing under different light environment were selected. In these individuals, leaf shape, size traits, and light environment were analyzed to determine the effects of light availability and ontogeny on the degree of pinnation. We used the last fully expanded leaf from the last cohort displayed on the shoot. Additionally, samples of leaf primordia from plants with different heteroblastic degree and light environments were collected and placed immediately in liquid nitrogen. Subsequently, I performed a *de novo* assembly of the transcriptome of each sample by using a high throughput RNAseq protocol to evaluate the range of transcriptional responses associated with the ontogenetic contingency of *G. avellana* (i.e., developmental responses arose from the interaction of the ontogenetic program and environmental cues). Details of the experimental, methodological, and data analyses procedures are extensively described in chapters III and IV.

CHAPTER III

LIGHT ENVIRONMENT HAS LITTLE EFFECT ON HETEROBLASTIC DEVELOPMENT OF THE TEMPERATE RAINFOREST TREE *GEVUINA AVELLANA* MOL. (PROTEACEAE)

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Light Environment Has Little Effect on Heteroblastic Development of the Temperate Rain Forest Tree *Gevuina avellana* Mol. (Proteaceae)
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LIGHT ENVIRONMENT HAS LITTLE EFFECT ON HETEROBLASTIC DEVELOPMENT OF THE TEMPERATE RAIN FOREST TREE *GEVUINA AVELLANA* MOL. (PROTEACEAE)

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Premise of research. Heteroblastic species are those that show an abrupt change in shape and/or size among individual metamers during ontogeny. *Gevuina avellana* Mol. (Proteaceae) is a typical tree species in the temperate rain forests of Chile and Argentina. This tree shows drastic heteroblasty, changing from simple leaves at the seedling stage to pinnate leaves during development. It regenerates mostly in shady understories, but juveniles can be found growing under a wide range of light conditions (5%–50% canopy openness). Thus, considering that light has been proposed as a driver of the heteroblastic strategy, *G. avellana* is an interesting model to study the potential environmental modulation of its ontogenetically programmed heteroblasty. Therefore, the aim of this study was to determine the effect of light availability on *G. avellana*'s heteroblastic trajectory. We postulated that *G. avellana*'s ontogenetic changes in leaf complexity (i.e., heteroblasty) increase under high light availability.

Methodology. Saplings along most of the light availability gradient were sampled. Plant height was used as a proxy for ontogeny. We measured several leaf traits (leaf area), shape (aspect ratio), pinnation (leaf dissection index), and complexity (fractal dimension index). First, we evaluated the change in each leaf trait with height by means of Pearson's correlation. Then we tested for differences in leaf traits along the ontogeny between two light environments (higher and lower than 10% canopy openness) by the line-fitting standardized major axis method.

Pivotal results. We found positive correlations between each leaf trait and plant height ($P < 0.001$), while only leaf size and complexity showed higher ontogenetic increases under high light.

Conclusions. Light environment has a small but significant effect on the heteroblastic trajectory of *G. avellana*. In particular, saplings inhabiting microsites with high light availability have larger and more complex leaves. While allometry would explain the ontogenetic trajectory of leaf size, the increased complexity could reflect functional advantages for large leaves in tall plants, especially under high light conditions.

Keywords: canopy openness, Chile, temperate rain forest, heteroblasty, ontogeny.

Online enhancements: appendix figure and tables.

Introduction

Many plant traits change spatially and temporally along environmental gradients, thus optimizing the use of available resources for growth, survival, and competition (Fitter and Hay 2002). Accordingly, interspecific trait variation contributes to explaining species segregation along environmental gradients (e.g., Wright 2002; Lusk et al. 2006). However, intraspecific variation in plant traits has also been observed with environmental changes, not only between and within populations but also within a single individual (Gould 1993; Zotz et al. 2011). The capacity of a single individual to modify its physiology and/or morphology in response to environmental changes is referred to as phenotypic plasticity (Schlichting 1986).

Plant traits usually change gradually during development (Evans 1972; Diggle 1997; Ishida et al. 2005). The ontogenetic program itself induces modular changes in meristems, roots, stems, and leaves (Schlichting 1986; Givnish 1988; De Kroon et al. 2005); these changes occur in conjunction with prevailing environmental conditions (Diggle 1994; Strauss-Debenedetti and Berlyn 1994). In some species, plant traits change dramatically during ontogeny, even in a relatively constant environment, a phenomenon known as heteroblasty (Goebel 1900; Diggle 2002; Zotz et al. 2011). Unlike phenotypic plasticity, heteroblasty has been preferably attributed to the intrinsic ex-

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pression of the ontogenetic programming and, to a lesser extent, environmentally induced changes (Jones 1999; Zotz et al. 2011). It has been proposed that heteroblasty provides an adaptive advantage when a predictable change in the environmental conditions occurs during plant ontogeny (Day 1998; Winn 1999; Gamage 2010; Zotz et al. 2011). For example, many *Acacia* species initially produce compound leaves, which are replaced by phyllodes in later developmental stages; true leaves and phyllodes differ in morphology and physiology so that the respective traits provide resistance to the prevailing environmental stress at the corresponding developmental stages (shade and drought, respectively; Brodribb and Hill 1993; Pasquet-Kok et al. 2010). Despite the genetic control of heteroblasty, recent studies indicate that heteroblastic responses can be modified by environmental conditions. For instance, both the trajectory and the timing of *Acacia implexa*'s heteroblastic development respond to nutritional and light status (Forster and Bonser 2009).

In closed forests, plants are exposed to progressively higher irradiances as they grow taller (Kira and Yoda 1989; Coopman et al. 2011). Consequently, most species change their light requirements throughout ontogeny (Lusk et al. 2008; Poorter et al. 2010). For example, gradual ontogenetic changes in a suite of anatomical, morphological, and physiological leaf traits in the tree species *Nothofagus nitida* allow this species to adjust the photosynthetic apparatus to the new light environment (Coopman et al. 2008, 2011). While in most trees phenotypic plasticity allows an acclimation response to predictable changes in light availability during ontogeny, heteroblasty could be an alternative programmed response (Zotz et al. 2011). The fact that seedling leaves of the heteroblastic species *Pseudopanax crassifolius* resemble those of adults living in shaded environments, whereas adult leaves have traits of sun-acclimated plants, indirectly suggests that changes in the light environment throughout ontogeny could be a selective pressure for the evolution of heteroblasty (Gould 1993). However, some studies contradict this hypothesis. First, several heteroblastic species occur in open and highly disturbed forests (Day 1998). Second, photosynthetic performances do not always change between early and late forms in heteroblastic species (Zotz et al. 2011).

In heteroblastic species, changes occurring throughout ontogeny could be partially modulated by environmental changes because, for both heteroblastic plants and homoblastic plants, developing plant primordia are highly plastic (Goebel 1908; Mulkey et al. 1992). In fact, heteroblastic species' leaf morphology and anatomy have been found to be more plastic in response to changes in light availability than homoblastic congeners (Gamage 2010). Since the beginning of the twentieth century, several studies have described examples of juvenile-like leaf production in heteroblastic species when grown under low light (e.g., Goebel 1908; Njoku 1956; Jones 1995). To explain this pattern, some authors have hypothesized that the heteroblastic trajectory is delayed under low light conditions (Allsopp 1965; Lee and Richards 1991; Gould 1993). Alternatively, the phenotypic differences found in heteroblastic species under low and high light conditions may be attributed to individual leaves' plastic response to the prevailing condition; these differences would not then affect the progression of ontogenetic programming (Jones 1995 and references therein). Few studies about environmental modulation of heteroblastic trajectory have been conducted in tree species, and those that exist commonly lack complementary field studies that may shed light on the relevance of this process in natural populations (Zotz et al. 2011).

In the temperate rain forests of Chile and Argentina, *Gevuina avellana* Mol. (Proteaceae) is a native, short-lived, and fast-growing tree species (Donoso 2004). It occurs in both primary forests and secondary forests (Rodríguez et al. 1983). Interestingly, among the three other members of the Proteaceae family and the ca. 13 coexisting tree species in this forest type (Lusk et al. 2008), *G. avellana* is the only known strongly heteroblastic species, changing drastically from simple to highly compound leaves during early development (fig. 1). Although this species regenerates mainly in closed-canopy microsites (Donoso 1978), juveniles can be found growing under a wide range of light conditions, from less than 5% canopy openness (%CO) to ca. 50 %CO (fig. 2; Lusk 2002 and references therein). Considering the wide range of light conditions in which *G. avellana* recruits, this species is a convenient model to examine the relationship between the ontogenetic programming of leaf morphology and its environmental modulation. In particular, we hypothesized that heteroblastic trajectory of *G. avellana* will be accentuated by light availability. In this sense, leaves produced at more illuminated microsites would resemble leaves unfolded by older individuals growing in more shaded microsites.

Material and Methods

Study Area and Species

The study site corresponds to a 30-ha secondary temperate rain forest stand located in south central Chile (Katalapi Park: lat. 41°31′07.5″S, long. 72°45′2.2″W). Forest structure corresponds to a coastal regenerating forest with patches of remnant trees that have been protected from cattle grazing for 20 years. The area presents ca. four adult reproductive *Gevuina* avellana trees per hectare, ensuring a homogeneous and sufficient seed supply. The composition of the canopy includes *Nothofagus nitida* (Phill.) Krasser, *Laureliopsis philippiana* (Looser) Schodde, *Luma apiculata* (DC.) Burret, *Amomyrtus luma* (Molina) Legr. et Kaus, *Aextoxicum punctatum* R. et P., *Eucryphia cordifolia* Cav., and *Drimys wintery* J.R. et G. Forster (Saldaña and Lusk 2003; Lusk and Corcuera 2011). The climate is humid temperate with an oceanic influence (Di Castri and Hajek 1976), although frost events do usually occur from early fall to spring (Reyes-Díaz et al. 2005). Annual precipitation is close to 2000 mm concentrated between April and November (Escandón et al. 2013). The mean monthly minimum air temperature (5°C) occurs in August, and the mean monthly maximum air temperature (22°C) occurs in February (for further climatic details, see Coopman et al. 2010).

Regeneration Abundance along the Light Gradient

In order to evaluate sapling distribution in relation to light availability, we established three 100-m-long and 4-m-wide areas, starting from the edge of the forest and continuing into the closed forest. These areas were separated by ca. 10 m and included reproductive *G. avellana* individuals. Cleared areas previously used for cattle were excluded. Within these areas, we

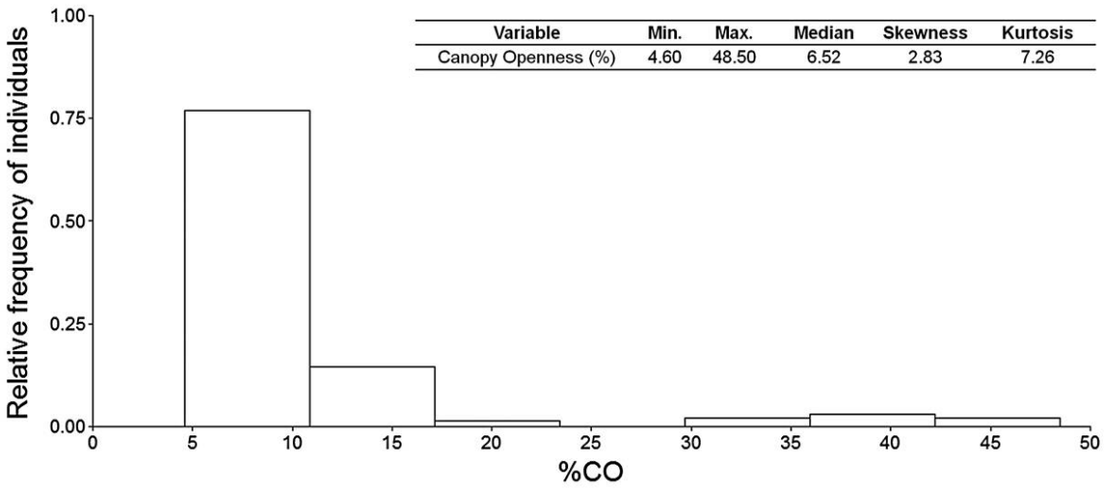

Fig. 1 Regeneration of *Gevuina avellana* along a gradient of canopy openness (%CO). Columns show the relative frequency of individuals from three sampled areas covering a total of 120 m². Table inset shows the coefficient of skewness and kurtosis.

quantified the light availability for all young *G. avellana*, including seedlings up to individuals reaching ~1.3 m in height; this resulted in 135 individuals. Plants were carefully selected in order to avoid sprouts. The light environment of each *G. avellana* plant was characterized with hemispherical photographs taken on homogeneous overcast days using a Nikon Coolpix 4500 digital camera equipped with an FC-E8 fish-eye lens (Nikon, Tokyo). The camera was hand leveled above the last developing metamer of each plant and oriented so that the top of the image faced north (Chazdon and Fields 1987). Photographs were analyzed for %CO using Gap Light Analyzer 2.0 software (Frazer et al. 1999).

Plant Age

In order to evaluate the ontogenetic trajectory of leaf traits, we used plant height as a proxy for age, since estimating plant age in small trees utilizes a destructive method involving counting stems' annual growth rings (i.e., in cross sections taken above the root:shoot transition zone). Previously, we tested whether plant height was a good proxy for plant age in 20 saplings with heights ranging from 20 to 120 cm, growing in a similar light environment (12–17 %CO). Annual growth rings were counted with a trinocular microscope (Nikon, model C-DS) in 1% safranine-stained fresh cross sections. We found a strong linear relationship between the two variables (age = 0.07height + 0.57; $R^2 = 0.96$, $P < 0.0001$), supporting the use of height as a surrogate for plant age, at least for the light environment tested. To evaluate to what extent this relationship might be extrapolated to the whole light environment gradient in the studied area, we evaluated whether the ontogenetic trajectory of the relationship between plant height and the diameter of the stem base changed in relation to light availability for all studied plants ($n = 135$). For this, we analyzed changes in plant height with the diameter of stem base across four %CO classes (corresponding to the interquartile ranges) by means of one-way ANCOVA. The %CO was considered as a categorical variable due to the high skewness of its distribution (skewness = 2.83, $P < 0.001$). We found that plant height did not differ between %CO classes, nor did the relationship between stems' height and stem bases' diameter (table A1; tables A1, A2 available online). That is, the ontogenetic trajectory of the relationship between plant height and the stem base diameter was found to be independent of the %CO, allowing us to use height as a proxy for age along the whole light availability gradient.

Leaf Traits

Leaf size, shape, pinnation, and complexity were estimated in all 135 *G. avellana* plants. Specifically, one current-year fully expanded leaf from each plant was collected, arranged on a white board under a transparent cloth, and photographed. The images were binarized, and the area, perimeter, length, and width of each leaf were obtained using ImageJ software (Wayne Rasband/National Institutes of Health, Bethesda, MD). From these data, we calculated the aspect ratio as the leaf length:width ratio within a fitted ellipse and the leaf dissection index as perimeter(area)$^{-0.5}$. While aspect ratio is an indicator of leaf shape (Chitwood et al. 2013), the leaf dissection index is commonly considered as a quantifiable descriptor of the pinnation degree (McLellan 1993; McLellan and Endler 1998). Finally, we evaluated how jagged and complex the leaflets were by means of the fractal dimension index. For this purpose, we used the box-counting method available in the FracLac plug-in for ImageJ (Borkowski 1999; Sisó et al. 2001; Karperien 2012).

Statistical Analyses

Because of the high skewness of the regeneration abundance distribution toward the shadier extreme of the light gradient (fig. 2), the effect of light availability would have been difficult to detect with raw data. Therefore, the %CO was divided into two classes. We then looked for differences

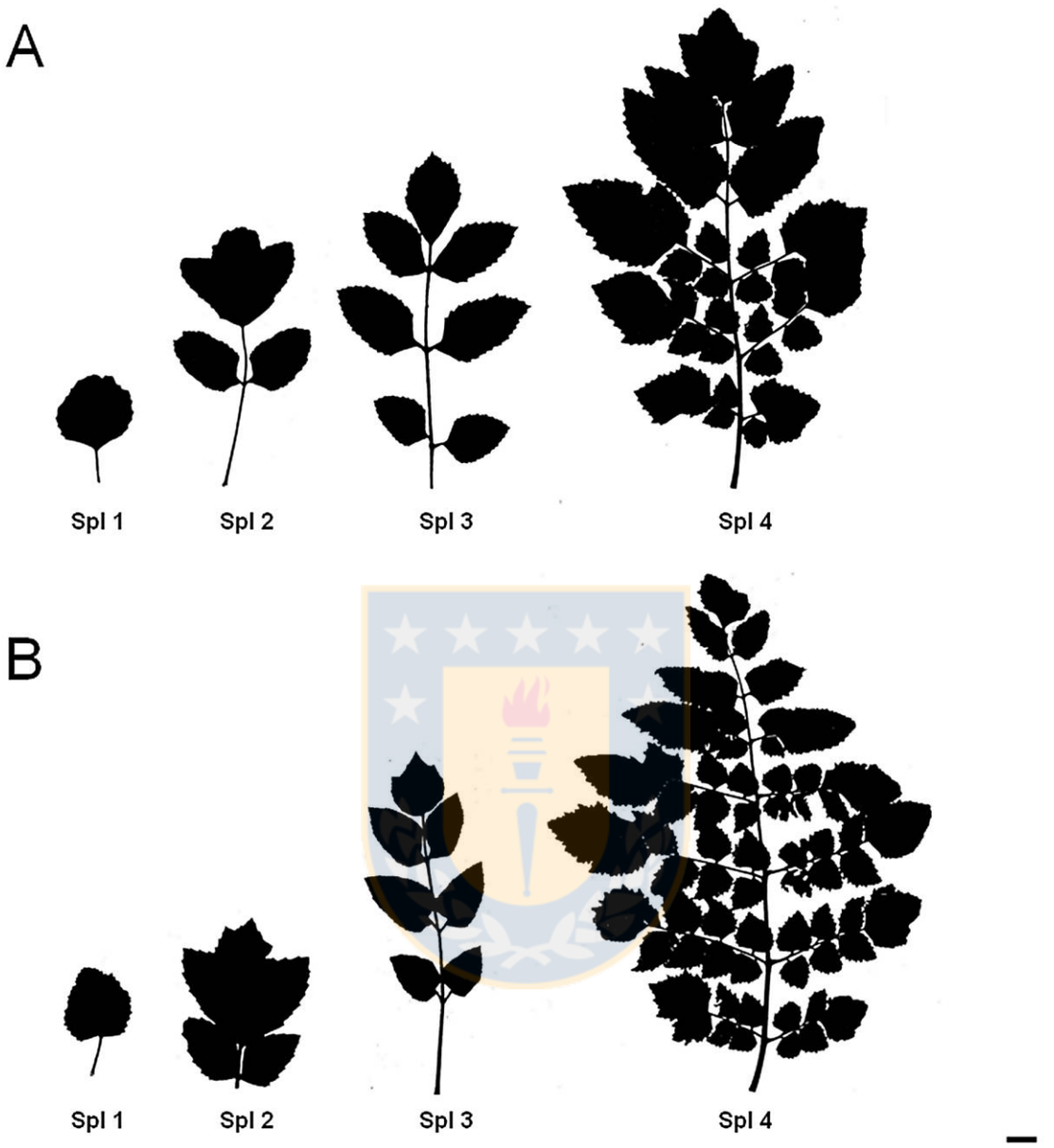

Fig. 2 Visual scheme of the heteroblastic trajectory of *Gevuina avellana* leaves under contrasting light availabilities. Ontogenetic stages were selected, dividing plant height (in centimeters) by quartiles: Spl = saplings; Spl 1 = 4–12.5; Spl 2 = 12.5–27; Spl 3 = 27–47.2; Spl 4 = 47.2–134. Lower and upper quartiles of %CO: *A* = 4.6–5.8; *B* = 10–48.5, respectively. Scale bar = 2 cm.

in the ontogenetic trajectory of leaf traits between these classes. Specifically, we considered low-light plants as those growing at %CO less than 10 and high-light plants as those growing at %CO greater than 10. Previous studies have indicated that leaf traits such as longevity and gas exchange parameters in *G. avellana* differ between this %CO threshold (Lusk 2002; Lusk and Corcuera 2011). We used Pearson's correlations to evaluate the relationship between leaf traits and plant height within these two %CO classes. To compare the slopes and the elevation of the linear relationships between these two light environments, we used standardized major axis estimation (SMA) instead of ordinary least square regression (Warton et al. 2006). Specifically, common slope was tested using the likelihood ratio and then compared to a χ^2 distribution; when line fitting shared a common slope, its elevations were compared by calculating the Wald statistic and comparing the χ^2 distribution between the two %CO classes. These analyses were performed by means of the R

library *smatr* (Warton et al. 2012). Leaf traits and plant height were log transformed to meet parametric assumptions, while the normality, heterodasticity, and independence of the residuals were evaluated visually (Warton et al. 2006).

Results

Regeneration along the Light Gradient

Gevuina avellana occupied most of the light gradient available in the forest (ca. 5–50 %CO), although it was absent in deep shade conditions (<4 %CO). Plant density along the light gradient showed a leptokurtic distribution with a positive skew (kurtosis = 7.26, skewness = 2.83; fig. 2). Thus, 75% of plants occurred between 4.6 and 11.4 %CO and drastically declined as the canopy opened. Plant height did not differ between %CO classes (i.e., higher and lower than 10 %CO; $F_{1, 133} = 2.20$, $P = 0.140$), allowing us to compare leaf traits' ontogenetic trajectory in these two light environments.

Leaf Traits, Ontogeny, and Light

All leaf traits were strongly correlated (fig. A1, available online). They scaled positively with plant height for the two light availability ranges (fig. 3; see also fig. 1). Each trait was strongly correlated with plant height ($r > 0.75$) with the exception of the aspect ratio, which showed the weakest correlation (table 1). Nonsignificant differences were found in the slopes of the SMA-fitted lines of low- and high-light plants.

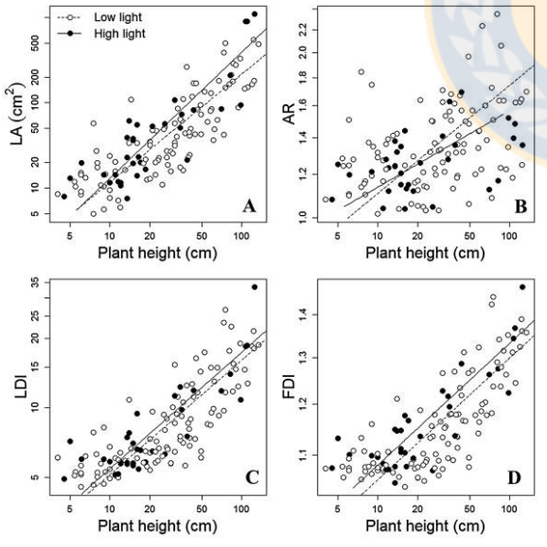

Fig. 3 Ontogenetic trajectory of *Gevuina avellana* leaf traits for contrasting light availabilities. Relationships between plant height and leaf area (LA; *A*), between plant height and aspect ratio (AR; *B*), between plant height and leaf dissection index (LDI; *C*), and between plant height and fractal dimension index (FDI; *D*). Lines represent the fitted values of the standardized major axis in low- and high-light environments (≤10 %CO and >10 %CO, respectively). Plant height denotes plant ontogeny ($P < 0.0001$, $R^2 = 0.96$).

Taking into account the common slopes of all measured traits for the two light environments, we compared the elevation of the SMA lines between high %CO and low %CO (Warton et al. 2006). For the same plant height, plants growing in high light had larger and more complex leaves (table 1; fig. 3). No differences in aspect ratio or the leaf dissection index were found between %CO classes (table 1; fig. 3). The former results were not due to an artifact of the apparently arbitrary categorization of %CO classes, since similar results were found when changes in leaf traits with plant heights were compared along the continuous light gradient by means of two-way ANOVA (table A2).

Discussion and Conclusions

Gevuina avellana is the only Chilean temperate rain forest species that exhibits strong heteroblasty. As it grows, its leaves become larger, more elongated (reflected by an increasing aspect ratio), dissected, and complex (figs. 1, 3). This relationship can be explained based on Sinnot (1921), who argued that the smaller leaf size of juvenile plants when compared to their adult counterparts is due to limitations of plant resource availability and vasculature development in young plants, as well as differences in the meristem size of young plants compared to their adult counterparts (Evans 1972). We also observed highly dissected leaves and larger leaf lamina in basal resprouts of *G. avellana* compared to plants grown from seeds of the same height. Considering the temporary increase in the root:shoot ratio of resprouting plants (Kruger and Reich 1993), the difference in leaf size between resprouting plants and saplings is expected under the hypothesis of resource constraints in early ontogenetic stages.

The inherent increase in leaf size throughout ontogeny has been shown to improve light interception, which is particularly relevant in closed canopies (Zotz et al. 2011). However, the benefit of large leaf display has proven to be counterproductive in tall saplings because plants are progressively exposed to higher irradiances as they grow taller, thus increasing the excitation pressure on photosystem II (PSII), which can lead to photoinhibition risk and/or photodamage of the photosynthetic apparatus and, ultimately, reduction of carbon gain (Coopman et al. 2011). Concomitant with higher irradiances, leaves at higher elevations within the canopy are subjected to a greater evaporative demand (i.e., a greater vapor pressure deficit [VPD]; Kira and Yoda 1989; Saldaña et al. 2013). This increase in VPD decreases stromal CO_2 concentration (by reducing both stomatal conductance and mesophyll conductance), thus leading to overexcitation of PSII and photodamage (Huner et al. 1996). Electron sink limitation induced by the combined effects of high irradiance and water stress has proven to be exacerbated in large leaves (Givnish 1979; Lambers et al. 2008), leading to a potentially chronic photoinhibition (Coopman et al. 2008). Considering the semishade regeneration strategy of *G. avellana* (this article; for old-growth forest, see also Lusk 2002; Donoso et al. 2004), this species should be sensitive to high-light-induced VPD (Escandón et al. 2013), especially considering the large leaves of taller plants. In this sense, the higher degree of leaf pinnation of large leaves has been found to diminish light overload and increase the convective heat dissipation capacity (Givnish 1979). Specifically, the interleaflet

Table 1

Analyses of the Linear Relationship between Plant Height and Several Leaf Traits in Low- (≤10 %CO) and High- (>10 %CO) Light Environments (n = 101 and n = 34, Respectively) in *Gevuina avellana*

	Pearson's correlation		Common slope test			Common elevation test		
Leaf trait and light	R	P	Estimate [95% CI]	Likelihood ratio	P	Estimate [95% CI]	Wald statistic	P
LA:								
Low	.82	<.001	1.288 [1.150, 1.444]	1.643	.200	−.316 [−.511, −.121]	5.08	.024
High	.86	<.001	1.478 [1.234, 1.771]	...	...	−.183 [−.386, .020]	...	...
AR:								
Low	.41	<.001	.197 [.165, .237]	3.509	.061	−.129 [−.173, −.085]	.42	.516
High	.44	<.001	.139 [.101, .191]	...	...	−.138 [−.184, −.093]	...	...
LDI:								
Low	.80	<.001	.497 [.441, .561]	.023	.879	.211 [.134, .287]	1.61	.205
High	.85	<.001	.506 [.419, .609]	...	...	.239 [.161, .317]	...	...
FDI:								
Low	.76	<.001	.091 [.080, .103]	1.3×10^{-4}	.991	−.068 [−.083, −.053]	6.73	.010
High	.84	<.001	.091 [.075, .111]	...	...	−.057 [−.072, −.042]	...	...

Note. The strength of the relationship was evaluated by means of Pearson's correlations, and lines were described by their standardized major axes (SMA). The SMA slopes and elevations are also shown. CI = confidence interval; LA = leaf area; AR = aspect ratio; LDI = leaf dissection index; FDI = fractal dimension index.

distance has proven to be a key feature of compound leaves, which, in combination with the angle of air flow's interception, determines the leaf heat dissipation capacity (Balding and Cunningham 1976). In addition, it has been proposed that compound leaves as well as leaf lobbing have functional advantages in terms of avoiding self-shading and increasing crown light interception (Niklas 2013). Thus, the heteroblastic trajectory of *G. avellana* could be an adaptive response to constraints exerted by a programmed enlargement of leaf size, the vertical change in environmental variables (i.e., increasing light availability and VPD), and the need to optimize crown light capture.

If heteroblasty in *G. avellana* is a response to changes in the light environment along the ontogeny, the remaining questions are why this is the only strongly heteroblastic species in this ecosystem type and why the uncommonness of its heteroblastic trajectory (i.e., changing from simple to highly compound leaves), which has only been reported for some climber species (Zotz et al. 2011). Several nonexcluding hypotheses might explain it. First, *G. avellana* is the tree with the largest leaves in the Chilean temperate rain forest; thus, the negative effect of the programmed enlargement of the leaf size in the photosynthetic apparatus (see above) might be exacerbated more in *G. avellana* compared to other coexisting species. Second, changes in leaf complexity could be a consequence of the expression of floral homeotic genes during the vegetative phase. For instance, the expression of the *FLORICAULA/LEAFY* (*FLO/LFY*) gene controls flower development in a wide range of species but also the development of compound leaves of *Pisum sativum* during the vegetative phase (Champagne et al. 2007). The pleiotropic effect of floral regulatory networks is a plausible explanation for the increased leaf pinnation of *G. avellana* throughout ontogeny, considering that the architecture of its long axillary inflorescences resembles the highly compound leaves of taller plants.

Regarding modulation of the heteroblastic trajectory in relation to light, we found that leaves are smaller and less complex under low light (fig. 3). Specifically, in shady conditions, *G. avellana* saplings showed more juvenile-looking leaves compared to individuals of the same age growing under higher light availability. Similar results have been found in both herbaceous and woody plants (see Jones 1995 and references therein), although the underlying mechanisms are still controversial (see "Introduction"). In our study system, the effect of light availability in modulating heteroblastic trajectory was small compared to the effect of height (i.e., age). This finding backs up the idea of a strong ontogenetic program in the development of leaves of *G. avellana* and little morphological plasticity in relation to current or predictable changes in environmental conditions. Hence, the principal mechanism of acclimation to changes in light availability along the ontogeny should be mediated most likely by physiological changes, as described for concurring tree species (Coopman et al. 2008, 2011).

In summary, the overall early heteroblastic trajectory of *G. avellana* is almost completely driven by plant ontogeny. The common ontogenetic trajectory of all studied leaf traits could be the result of their coordination by genetic programming (McLellan 1993). However, the ontogenetic trajectories of leaf size and complexity were modulated by light availability. Thus, we agree with Barthélémy and Caraglio (2007), who argued that environmental factors may modulate organ differentiation (plasticity) but rarely modify inherent ontogenetic programming. On the other hand, light availability's effect on leaf complexity may reflect functional advantages to deal with the ontogenetic enlargement of leaf size. To our knowledge, this is the first study evaluating the natural variation of heteroblastic development influenced by light availability in the Chilean temperate rain forest. Clearly, further ecophysiological studies focusing on the functional implications of heteroblasty per se and its environmental modulation are necessary, for which *G. avellana* is an effective study system. These further studies must cover multifactor environmental modulation, as pointed out by Zotz et al. (2011). They would most likely help to resolve the question of why *G. avellana* is the only strongly heteroblastic species in this Neotropical temperate rain forest.

Acknowledgments

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Appendix from Ostria-Gallardo et al., "Light Environment Has Little Effect on Heteroblastic Development of the Temperate Rain Forest Tree *Gevuina avellana* Mol. (Proteaceae)" (Int. J. Plant Sci., vol. 176, no. 3, p. 000)

Supplementary Material

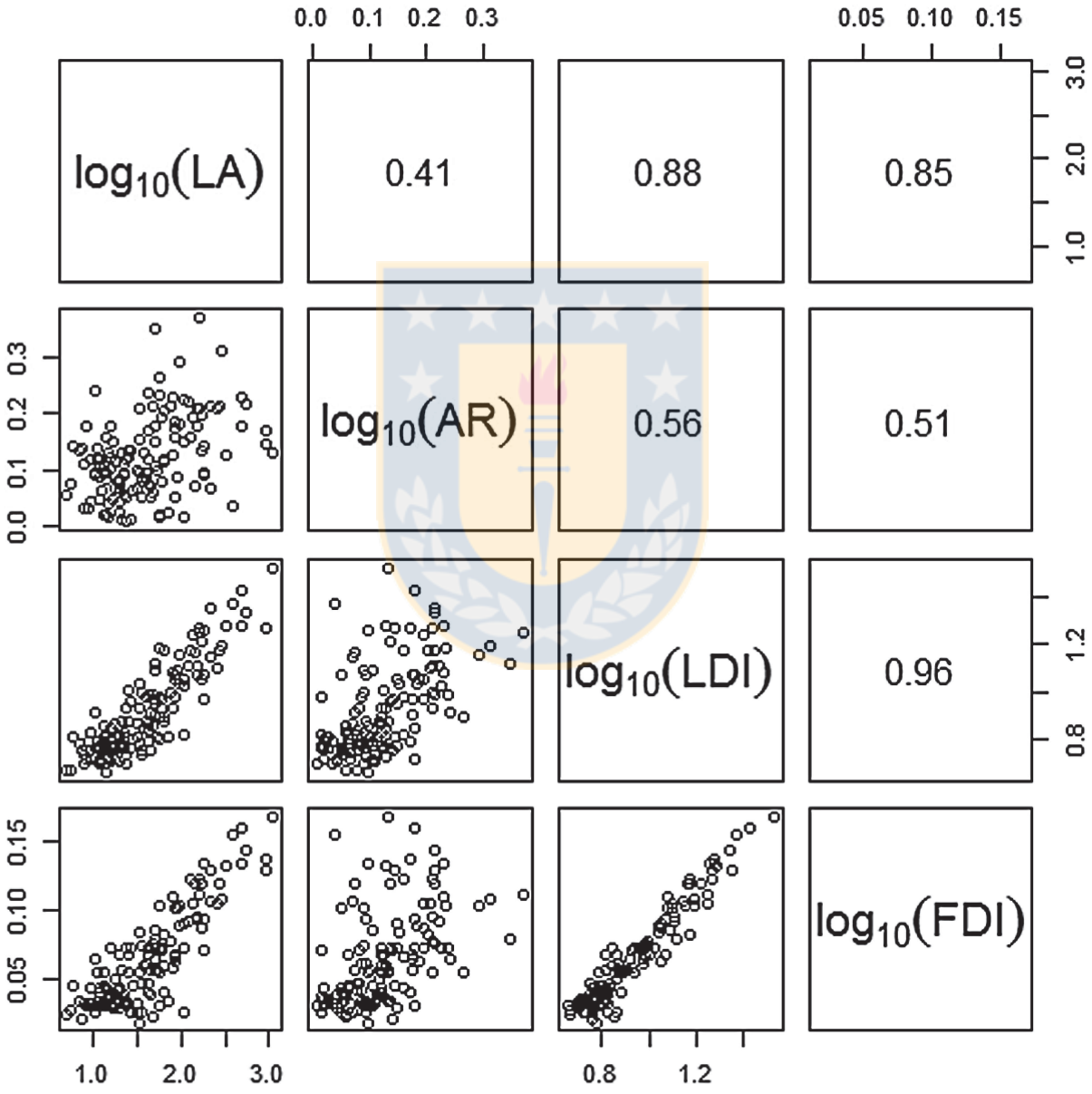

Fig. A1 Correlation between log-transformed leaf traits: LA = leaf area; AR = aspect ratio; LDI = leaf dissection index; FDI = fractal dimension index. In the lower panel, there are scatterplots between variables. The upper panel shows the corresponding Pearson's correlations.

Table A1. Results of the ANCOVA Conducted to Evaluate Changes in the Relationship between Plant Height and Basal Stem Diameter among Canopy Openness Interquartile Ranges

	df	SS	MS	*F*	*P*
Stem diameter (SD)	1	15.62	15.616	440.22	>.001
Canopy openness (CO)	3	.14	.045	1.28	.284
SD × CO	3	.03	.011	.30	.827
Residuals	130	4.61	.036	. . .	. . .

Note. Plant height and basal stem diameter were log transformed prior to the analysis.

Table A2. Results of the ANOVA Conducted to Evaluate the Effect of Light Availability on Leaf Traits along Its Ontogenetic Trajectory Defined by Plant Height

	df	SS	MS	*F*	*P*	Explained variance (%)
Leaf area:						
Height (H)	1	23.98	23.980	308.59	<.0001	68.3
Canopy openness (CO)	1	.617	.617	7.94	.006	1.8
H × CO	1	.361	.361	4.65	.033	1.0
Residuals	131	10.180	.078	. . .	. . .	. . .
Aspect ratio:						
H	1	.122	.122	28.24	<.0001	17.7
CO	1	.001	.001	.29	.589	.2
H × CO	1	.001	.001	.12	.734	.1
Residuals	131	.567	.004	. . .	. . .	. . .
Leaf dissection index:						
H	1	3.220	3.220	260.99	<.0001	65.6
CO	1	.045	.045	3.63	.059	.9
H × CO	1	.028	.028	2.31	.131	.6
Residuals	131	1.616	.012	. . .	. . .	. . .
Fractal index:						
H	1	.098	.098	210.50	<.0001	60.0
CO	1	.004	.004	7.84	.006	2.2
H × CO	1	.001	.001	1.34	.249	.4
Residuals	131	.061	.001	. . .	. . .	. . .

Note. The response variables and plant height were log-transformed.

CHAPTER IV

TRANSCRIPTOMIC ANALYSIS SUGGESTS A KEY ROLE FOR *SQUAMOSA PROMOTER BINDING PROTEIN LIKE*, *NAC* AND *YUCCA* GENES IN THE HETEROBLASTIC DEVELOPMENT OF THE TEMPERATE RAINFOREST TREE *GEVUINA AVELLANA* MOL. (PROTEACEAE)

Manuscript submitted for publication

Transcriptomic analysis suggests a key role for *SQUAMOSA PROMOTER BINDING PROTEIN LIKE, NAC* and *YUCCA* genes in the heteroblastic development of the temperate rainforest tree *Gevuina avellana* Mol. (Proteaceae)

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ABSTRACT

Heteroblasty is a fascinating mechanism that contributes to the diversification of leaf shape in angiosperms. *Gevuina avellana* Mol. (Proteaceae) is the only tree species of the temperate rain forest of Chile and Argentina that shows strong heteroblastic changes in leaf shape during ontogeny, transitioning from simple leaves during juvenile development to highly pinnate leaves during the adult stage. Light availability within the forest canopy also modulates leaf size and complexity within the species. Here, we used RNA-seq on the Illumina platform to compare the range of transcriptional responses in leaf primordia of *G. avellana* at different heteroblastic stages and growing under different light environments. We identify differential expression of key genes involved in: i) phase change; ii) leaf and flower morphogenesis; and iii) light-related signal cascades. Our results show a significant and steady up-regulation of *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*), *NAC*, *YUCCA*, and *AGAMOUS-LIKE* floral activator/repressor genes associated with increases in age, leaf complexity, and light availability. By contrast expression of *TCP*, *TPR*, and *KNOTTED1* homeobox genes showed a sustained down-regulation in relation to the three variables mentioned. In addition, genes involved in auxin synthesis/transport and jasmonate activity were differentially expressed indicating an active regulation of processes controlled by these hormones. Our analysis of the transcriptional profile of *G. avellana* sheds light on the integration of internal and external cues in the progression of heteroblastic development in this species.

INTRODUCTION

Leaf shape is a conspicuous trait among angiosperms, exhibiting the greatest diversity and variability at all taxonomical categories (Nicotra *et al.*, 2011). One of the most fascinating mechanisms contributing to the diversification of leaf form is heteroblasty. Since Goebel (1900), plant scientists have continued to describe drastic and abrupt changes in leaf size and shape throughout ontogeny that constitute heteroblasty (Jones 1999; Zotz *et al.*, 2011). In addition, recent studies show that the phenotypic expression of heteroblastic trajectories is highly contextual and responds plastically to prevailing environmental conditions (Jones 1995; Burns 2005; Gamage 2011; Chitwood *et al.*, 2014). Because of their sessile life, plants anticipate forthcoming environmental conditions through a suite of mechanisms and initiate appropriate developmental responses (Diggle 1997; Casal *et al.*, 2004). In this sense, it has been proposed that heteroblasty provides adaptive advantages to the predictable changes in the environment during ontogeny (Day 1998; Winn 1999; Gamage 2010). For example, *Acacia* trees show heteroblastic progression by transitioning from compound leaves to simple phyllodes. Interaction between the morphological/physiological stage in the trajectory from compound to simple leaves and prevailing environmental stresses (shade and then drought) faced throughout the development of the tree constitutes an adaptive mechanism utilized by plants in their natural habitat (Brodribb and Hill 1993; Pasquet-Kok *et al.*, 2010).

A central core of the genetic regulatory circuit underlying age-dependent changes in plants is the accumulation of SQUAMOSA PROMOTER BINDING PROTEIN LIKE transcription factors (SPLs) which act as a timing cue for phase change transitions and for the developmental trajectory of the plant (Chen *et al.*, 2010). Recent findings suggest that

miRNAs, specifically the opposing expression patterns of miR156 and miR172, regulate a conserved framework of phase changes in many, if not all, angiosperms (Wang *et al.* 2011). miR156 represses the expression of *SPL* transcription factor genes and shows high correlation with juvenile-like vegetative leaf traits. As the expression of miR156 decreases, the expression of *SPLs* increases paralleled with adult-like vegetative traits (Poethig 2013). For example, the ontogenetic increases of leaf serration in simple leaves of *Arabidopsis thaliana* and the number of leaflets in compound leaves of *Cardamine hirsuta* are regulated by the destabilization of TCP-CUC transcription factors mediated by *SPL*s (Rubio-Somoza *et al.*, 2014; Chitwood and Sinha 2014). The *CUP SHAPE COTYLEDON* (*CUC*) genes belong to the NAC family of transcription factors which operate as a conserved boundary-specification program that modulate the sites of leaf and leaflets initiation in Eudicots (Blein *et al.*, 2008). In early leaves of *A. thaliana* and *C. hirsuta*, TCP transcription factors interfere with the activity of CUC proteins. As leaves progress in ontogeny, accumulation of SPL proteins acts as a heterochronic cue that destabilizes TCP-CUC complexes. Thus, the active CUC proteins become available to promote the increase in leaf complexity (i.e. serration and leaflet formation) in the newly formed organs of both species.

Despite increasing knowledge of the molecular control of phase change, the gene regulatory networks driving the phenotypic trajectory of phase change within the context of the natural variation in plant development are largely unknown (Chitwood *et al.*, 2014). Moreover, the regular use of “model systems”, although effective in defining different molecular pathways because of the ease of genetic transformation and experimental manipulation in these species, cannot provide specific considerations for regulation of leaf shape in all other non-model species. Therefore, in order to understand how

developmentally programmed processes (e.g., heteroblasty) and environmental signals are combined to generate the resulting phenotype across species, information should be derived from comparative studies between model and non-model species within a natural environmental framework (Rowan *et al.*, 2011). Fortunately, the recent technical advances in functional genomics studies through next generation sequencing have revolutionized the study of the functional complexity of gene regulatory networks and their role in biological processes in non-model species, even those with uncharacterized genomes (Grabherr *et al.*, 2011). The enormous potential of these new sequencing techniques in extending the range of studied species provides the opportunity to reach a large-scale, comparative overview of the regulation of phenotypes in plants.

Because of their perenniality, forest trees are excellent experimental systems for the comparative study of the relationship between naturally occurring genotypes and phenotypic diversity (Neale and Ingvarsson 2008). Understanding of gene function and genetic variation controlling complex traits in forest trees can provide new clues about how climate, and micro-climate variations, shape geographic and population genetic structure, as well as the evolution of unique arboreal adaptations (e.g., heteroblasty) to the seasonal cycles along life history trajectories (Sork *et al.*, 2013). In the temperate rainforest of Chile and Argentina, *Gevuina avellana* Mol. (Proteaceae) is a native tree, and the only species among coexisting trees that shows strong heteroblasty (Ostria-Gallardo *et al.*, 2015). It grows in a wide range of light environments along the forest canopy, ranging from 5% canopy openness to ca. 50% of canopy openness (Lusk 2002). In addition, when growing under high light conditions, the species produces larger and more complex leaves (Fig.1; Ostria-Gallardo *et al.*, 2015). The molecular regulation of the architecture of simple and

compound leaves is increasingly well understood (Barathan and Sinha 2001; Champagne *et al.*,2007; Blein 2010; Townsley and Sinha 2012, Tsukaya 2014; Ichihashi *et al.*,2014). However, the mechanism behind the ontogenetic change from simple to highly compound leaves is less known, probably because it is not a common ontogenetic change and has been reported mainly for some vines (Zotz *et al.*,2011), and is rarer among trees. Nakayama *et al.* (2014) described how the semiaquatic heterophyllic species *Rorippa aquatica* (Brassicaceae) produces both types of leaves, simple and compound, responding to changes in the surrounding environment (e.g., above or below water, ambient temperature, light intensity). The different leaf shapes and degrees of complexity are achieved by the effect of multiple environmental cues on the regulation of gibberellin and cytokinin levels via the *KNOX1* and *CUC* genes. Although this study was in the context of heterophylly, in which the resulting phenotype of individual leaves is highly sensitive to changes in environmental conditions, one would expect heteroblastic development to use similar gene modules, but with a strong age-dependent control on differential gene expression. Thus, given the contingent nature in which the heteroblastic development of *G. avellana* is deployed, this species is an excellent model to study the molecular basis of the ontogenetically driven change from simple leaf to highly compound leaf within a natural setting.

Taking into account the advances in knowledge of genetic mechanisms leading to the architecture of simple and compound leaves, the control of age-dependent changes in leaf shape/complexity, and the availability of new sequencing techniques to study non-model species, we used RNA-seq on the Illumina platform to study the transcriptome of leaf primordia of *Gevuina avellana* Mol. (Proteaceae) from individuals with different degrees of heteroblastic development, and growing under different light environments.

Specifically, we asked if in *G. avellana*, up-regulation of genes related to phase changes (e.g. *SPL*) correlates with the up-regulation of genes related to leaf complexity and, to what extent the light environments contribute to modulation of expression of genes related to the heteroblastic leaf trajectory. Our analysis identified key genes correlated with plant development, leaf dissection and light availability, and provides a comprehensive mechanistic hypothesis for the molecular and physiological basis underlying heteroblastic development of *G. avellana*.

MATERIALS AND METHODS

Study site, experimental design and sampling

The study site corresponds to 30 hectares of a secondary temperate rainforest stand located in South Central Chile (Katalapi Park: 41°31′07.5″ S, 72°45′2.2″ W). The forest structure corresponds to a coastal regenerating forest with patches of remnant trees that have been protected from cattle grazing for 20 years. The area presents ca. 4 adult reproductive *Gevuina avellana* Mol. trees per hectare ensuring a homogeneous and sufficient supply of seeds. The canopy is almost exclusively composed by evergreen angiosperms including *Nothofagus nitida* (Phill.) Krasser, *Laureliopsis philippiana* (Looser) Schodde, *Luma apiculata* (DC.) Burret, *Amomyrtus luma* (Molina) Legr. et Kaus, *Aextoxicum punctatum* R. et P., *Eucryphia cordifolia* Cav., and *Drimys winteri* J.R. et G. Forster (Saldaña and Lusk 2003; Lusk and Corcuera 2011). The climate is temperate with a humid oceanic influence (Di Castri and Hajek 1976) although frost events occur from early autumn to spring (Reyes-Díaz *et al.*. 2005). Annual precipitation is near to 2000 mm with

maximum mean temperatures of 10°C in winter and 22°C in summer (for further climatic details see Coopman *et al.*. 2010). The growing season is concentrated between December to March coinciding with a mild dry period where the lowest air relative humidity ranges between 45% and 55% (Escandón *et al.*,2013).

Three areas of 100 m long and 4 m wide randomly distributed across the park, starting at the edge of the forest and continuing through the closed forest, were chosen in order to include most of the light gradient (Fig. 2A). We characterized the light availability along the areas by the analysis of hemispherical photographs taken under homogeneous overcast days using a Nikon Coolpix 4500 digital camera equipped with a FC-E8 fisheye lens (Nikon, Tokyo, JP). The camera was hand leveled and oriented so that the top of the image faced north (Chazdon and Fields 1987). Photographs were analyzed for the percentage of canopy openness (%CO) using the Gap Light Analyzer 2.0 software, which allows a quantitative description of the canopy structure and amount of transmitted light through the canopy (GLA, Frazer *et al.*. 1999). Then, we established four light environment classes by inter-quartile ranges of %CO, hereafter called deep-shade (DS); shade (SH); semi-shade (SS); and sun (S). Within each light environment, the healthiest seed-grown *G. avellana* plants were carefully selected ranging from 4 to 200 cm in height, ensuring different developmental stages within the vegetative phase. Given height differences of plants within each light environment, we characterized the light availability above each individual and then the leaf primordia from the apex were collected in liquid nitrogen and stored at -80°C. For statistical analyses we ranked plant height, number of leaflets, and light availability by quartiles to establish four classes each (Supplemental Table S1).

RNA-seq library preparation and sequencing

RNA-seq libraries were prepared from the collected leaf primordia using a custom high-throughput protocol for Illumina RNA-seq library preparation (Fig. 2B; Kumar *et al.*,2012). Because of the high content of secondary metabolites (interfering substances) that co-precipitated during our first attempts to get nucleic acids from the samples, we modified the step of direct mRNA purification by adding 120 µl of Polyvinylpyrrolidone (PVP-40) 20% per 1 ml of Lysis/Binding buffer (LBB) that has the 2-Mercaptothanol and Antifoam A added. After bead beating, samples went through incubation at 65°C, shaking for 20 minutes. The supernatants were transferred to a Qiashredder column and spin, maximum speed for 30 minutes. Subsequent steps were as described in Kumar *et al.* (2012). We obtained a total of 24 libraries which were pooled and sequenced at the UC-Berkeley Genomics Sequencing Laboratory on a single lane of HiSeq 2000 platform (Illumina Inc. San Diego, CA, USA) obtaining reads in 100 bp paired-end format.

Preprocessing of Illumina reads

Reads were preprocessed as described in Ranjan *et al.*. (2014) and detailed here. The preprocessing of reads involved a quality filter trimming to remove all the reads with average Phred quality scores < 20 and low-quality bases from the 3' end of the reads (Supplemental figure S1). Then we removed adapter/primer contamination and duplicated reads using custom Perl scripts. After quality filter processes, the reads were sorted into individual samples based on barcodes using fastx_barcode_splitter.pl and then barcodes were trimmed using the FASTQ/A Trimmer script fastx_trimmer from Fastx_toolkit (hannonlab.cshl.edu/fastx_toolkit/).

***De-novo* transcriptome assembly and prediction of Open Reading Frames (ORFs)**

We used the Trinity software package (version r2013-02-25) for efficient and robust *de-novo* assembly of a transcriptome without a reference genome from RNA-seq data (Grabherr *et al.*,2011). The assembly was performed at The Lonestar Linux Cluster at Texas Advance Computing Center (TACC, University of Texas) as described in Ranjan *et al.* (2014). All the subsequent bioinformatics and statistical analyses were performed either on our local servers or in the iPlant atmosphere and Discovery computing environment (Goff *et al.*,2011).

In order to filter out transcriptional artifacts, such as chimeric or poorly supported contigs, original reads were mapped to assembled transcripts using Bowtie2 with parameters: -a –rdg 6,5 –rfg 6,5 –score-min L,-6,-.4, followed by SAMtools usage to generate a bam alignment file (Li *et al.*,2009; Langmead and Salzberg, 2012). Subsequently, express software was used to calculate abundance estimation for each transcript in terms of FPKM (Fragments per kilobase per transcript per million mapped reads) and transcripts with ≥ 1 FPKM were retained for downstream analysis (Roberts and Pachter, 2013). Highly similar/redundant contigs were clustered using CD-HIT clustering algorithm based on similarity threshold of 95%, and representative contig from each cluster was retained (Fu *et al.*,2012). Subsequently ORFs were predicted from the filtered and clustered contigs using TransDecoder (https://transdecoder.github.io/), and these predicted and clustered ORFs were used for annotation and downstream analysis.

Functional annotation of the transcriptome

We compared predicted ORFs from the final transcriptome to the NCBI nr (non-redundant) database and to the Arabidopsis protein database (TAIR10 database) using BLASTX with an e-value threshold of 1e-3 in both cases (Altschul *et al.*,1997). The BLASTX output file was used for Blast2GO analysis to annotate the contigs and ORFs with GO terms (e-value filter 1e-6) describing biological processes, molecular functions and cellular components (Götz *et al.*,2008), and then the ANNEX and GO slim files were used to enrich the annotation (Ranjan *et al.*,2014). EC numbers were also generated from the KEGG pathway (Kanehisa and Goto 2000). Finally, we obtained a sequence description file with arbitrary nomenclature based on degrees of similarity identified in both nr and TAIR10 databases according to e-value and identity to genes in BLAST references.

Differential expression analysis and GO enrichment analysis

The reads from each sample were mapped to the predicted ORFs using default RSEM parameters as described by Ranjan *et al.* (2014), and the abundances of the ORFs were extracted (Li and Dewey 2011). To ensure a reliable differential gene expression analysis, we removed transcripts with very low estimated counts and then normalized the RSEM-estimated abundance values. Next, we used the run_DE_analysis.pl script based on the EdgeR Bioconductor package on the R statistical language programming to identify differential expressed transcripts (Robinson and Oshlack 2010; R Development Core Team 2011; Haas *et al.*,2013). Differentially expressed genes were identified for all pair-wise comparisons between each class of height, light and degree of pinnation, with a *P*-value <0.01. The RSEM and Perl scripts used are bundled in the Trinity software package.

GO-enrichment analysis of differentially expressed genes was conducted with the GOSeq Bioconductor package and GO-terms and GOslim-terms generated by Blast2GO (Young *et al.*,2010).

Principal Component Analysis (PCA) and Self-Organizing Maps (SOM) Clustering

Normalized RSEM-estimated counts were used for clustering assembled ORFs based on expression patterns (Chitwood *et al.*,2013). In order to detect the effects of light availability and ontogeny on gene expression, we selected genes from the upper 75% quartile of coefficient of variation for expression across plant development and light environments. The scaled expression values within samples were used to cluster these genes for a multidimensional 1 x 4 hexagonal SOM for plant height, number of leaflets, and light environment using the Kohonen package on R (Wehrens and Buydens 2007). 100 training interactions were used during clustering with a decrease in the alpha learning rate from ca. 0.0060 to 0.0040 (Supplemental figure S2). SOM outcome was visualized in PCA space where PC values were calculated based on the gene expression of samples across height, number of leaflets, and light environments. In addition, we clustered genes for height, number of leaflets, and light environment in a single 1x3 hexagonal SOM to look at the distribution of clusters and interaction among factors, resulting in groups of genes with common properties between factors (e.g., height and light environment; Supplemental figure S3).

RESULTS

***De-novo* assembly and transcriptome annotation**

A total of 99,642,474 high quality 100-bp paired-end reads were obtained after sequencing the RNAseq libraries. *De novo* transcriptome assembly using pre-processed reads and subsequent clustering of the assembled contigs at the threshold of 95% sequence identity yielded 185,301 *G. avellana* contigs (> 200bp). The N50, which is the largest contig length such that using equal or longer contigs produces half the bases of the transcriptome, was 686 bp length while the average contig length was 538 bp (Fig. 3A; Supplementary dataset 1). The prediction of coding sequences from the 185,301 transcripts resulted in 48,074 predicted open reading frames (ORFs) (Figure 3B), which were used for downstream analysis of differential expression. BLAST searches of predicted ORFs against the nr database and the TAIR10 Arabidopsis protein database resulted in the annotation of 43,341 and 38,975 ORFs, respectively. An insight into the taxonomic distribution of top blast hits of ORFs against nr database revealed 73% of top hits to model woody tree-species (Fig. 3C).

Among predicted ORFs, the functional annotation resulted in 97,258 (52.48 %), 42,858 (21.48 %), and 39,815 (23.13 %) annotated counts for biological process (BP), molecular function (MF), and cellular component (CC), categories respectively (Supplemental fig. S4). Additionally, 12,963 ORFs were annotated as enzymes, with transferases being the most abundant class, followed by hydrolases and oxidoreductases (Supplemental fig. S5).

Transcript expression patterns across ontogeny and light availability.

A major focus of this study was to investigate the ontogenetic expression of genes correlated with height, leaf pinnation, and light availability. A Self-Organized Map (SOM)

clustering was used to describe the accumulation pattern of transcripts related to these factors, each partitioned into four clusters (nodes). We first studied the pattern of accumulation of transcripts related to plant height, which is the proxy for plant age (for details see Ostria-Gallardo *et al.*. 2015). For plant height, the nodes (hereafter node H*n*) were organized along height classes 1 to 4, showing accumulation patterns of transcripts through the progression of plant height from node H1 to node H4, as visualized using a combination of SOM and PCA (Fig. 4A). Each node was highly enriched for specific Gene Ontology terms (GO). Node H1 transcripts were enriched for response to red and far-red light, meristem activity, monooxygenase activity and auxin transport/signaling GOs. Transcripts in nodes H2 and H4 were enriched for ribosome and translation GOs, whereas node H3 transcripts are enriched for flower development, cell plate formation and cell proliferation, and epigenetic GOs (Supplemental dataset 2).

Next, we studied the accumulation pattern of transcripts for the number of leaflets. As in plant height, the nodes (hereafter node Lf*n*) were organized by increases in leaf pinnation (Fig. 4B). Transcripts in nodes Lf1 and Lf3 were highly enriched for ribosome and translation GOs. Transcripts in node Lf2 were enriched for meristem initiation, monooxygenase activity and electron transport GOs, whereas transcripts in node Lf4 were highly enriched for cell proliferation, regulation of meristem growth, flower development, and epigenetic GOs (Supplemental dataset 3). Finally, the accumulation pattern of transcripts related to light availability behaved similar to the pattern in plant height and number of leaflets, explaining prominent densities of transcripts along increasing light availability (Fig. 4C). For example, node L4 exhibits high transcript accumulation in deep shade (DS) and progresses along light environments to node L1 transcripts, which

accumulate at high levels in sun (SU). Transcripts in node L1 were enriched for ribosome and translation GOs. Transcripts in nodes L2 and L3 were enriched for cell proliferation, flower development, and epigenetic GOs. Transcripts in node L4 were highly enriched for circadian rhythm and flowering GOs (Supplemental dataset 4).

Differential transcript expression and GO-enrichment analysis

Three independent differential transcript expression analyses were conducted using annotated ORFs for plant height, number of leaflets, and light environment to identify candidate genes that would be involved in heteroblastic development of *G. avellana* (Supplemental datasets 5─19). First, we studied gene expression changes (FDR < 0.05) related with ontogeny by comparing among height classes. As expected, increases in the number of differentially expressed genes were detected with increases in plant height (Supplemental dataset 5─10). Among all plant heights comparisons, H1 *versus* H4 showed the largest number of differentially expressed genes, with 669 and 1514 down-regulated and up-regulated transcripts, respectively. GOslim categories enriched in up-regulated transcripts included transcription factor complex and sequence-specific DNA binding transcription factor activity, oxidation-reduction process, catalytic activity, transporter activity, transmembrane transport and cell wall GOs. Transcripts of special interest under those GO terms were: transcription factors, monooxygenase activity and hormone synthesis and signaling, kinases, and cell wall formation/expansion (Table 1).

Next, we examined changes in gene expression associated with the degree of leaf pinnation. The highest number of differentially expressed transcripts was found between leaflet1 *versus* leaflet3 classes. From a total of 929 differentially expressed transcripts, 486

and 443 showed down-regulation and up-regulation, respectively. GOslim categories in up-regulated genes included catalytic activity and metabolic process, oxygen binding, sequence-specific DNA binding transcription factor activity, cell wall and translation regulator activity. Transcripts of interest under these GO-terms for degree of pinnation were: members of transcription factors families, the argonaute RNA silencing family, hormone responses genes, kinases, and cell wall expansion (Table 1).

In addition, considering that the progression of height and number of leaflets are part of the ontogenetic program, we found that 3 members of the *SPL* transcription factors family, *SQUAMOSA PROMOTER BINDING-LIKE* genes 4, 8, and 12 (*SPL4*, *SPL8*, *SPL12*) are up-regulated steadily as plants grow taller and leaves become more complex. Also, *NAC* and *AGAMOUS*-like genes show respectively about twofold and sixfold up-regulation in sequential comparisons of plant height classes and between the extremes of leaflets' number (L1 vs L3).

Finally we quantified the changes in expression of transcripts associated with light availability by comparing expressed transcripts in deep-shade to those in shade, semi-shade and sun. The highest number of differentially expressed genes was found in Deep Shade *versus* Sun, with 574 and 2632 down-regulated and up-regulated transcripts respectively. GOslim categories for up-regulated genes included catalytic activity, transcription factor activity, oxidation-reduction process, monooxygenases, and tryptophan metabolic process. Transcripts of particular interest were: transcription factors families, kinases coding genes components of enzymatic reactions, phytochrome B-mediated light signal, hormone synthesis and signaling, and genes related with shade avoidance syndrome. Interestingly, we observed a sustained up-regulation of *NDPK1* genes from 3 to 8 fold along the light

gradient. Also *NAC*, *AGAMOUS*-like and *JAZ* genes show steady up-regulation in sequential comparisons of light environments classes (Supplemental datasets 14─19).

DISCUSSION

This is the first study for *Gevuina avellana* that not only reports a comprehensive shoot transcriptome of the species, but also identified key genes underlying the characteristic heteroblasty in the species. Other studies have described 24 EST regions, representing the only data currently available at the NCBI database for this basal Eudicot tree (Riegel *et al.*,2010). The robustness of our transcriptome data*et al.*lowed us to identify the gene expression dynamics in the leaf primordia of *G. avellana*, unraveling the regulation of ontogenetic leaf pinnation under natural environmental conditions. We show progressive enrichment in up-regulated genes related with phase change (Chen *et al.* 2010), floral development (Kaufman *et al.*,2009; Fernandez *et al.*,2014), organ separation and leaf complexity (Olsen *et al.*,2005; Blein *et al.*,2008) as plant development progressed and light availability increased. Surprisingly, genes reported as key components in development of compound leaves such class *I KNOX* or the *FLO/LFY* genes (Bharathan & Sinha, 2001; Champagne *et al.*,2007; Ge *et al.*,2014) turned out to be either down-regulated or absent in our *G. avellana*'s transcriptome dataset. One plausible explanation for these findings is that *KNOX1* genes do not have a pivotal role in the heteroblastic increase of leaf complexity in *G. avellana* and that this role is assumed by other regulatory factors such as *SPL*, *NAC*, and *AGAMOUS*-like genes (*AGL*). In turn, the absence of *FLO/LFY* could be most likely due to assembly or sampling methods as the gene is known to be expressed at very low levels (Coen *et al.*,1990). This is plausible given the presence of *AGL* genes and their progressive up-regulation in relation to height and light availability. We assume that *AGL* genes have a

direct effect on the regulation of heteroblastic development of *G. avellana*. If our assumptions are correct, then instead of a common mechanism regulating all compound leaves, these leaf-type arose through divergent developmental paths and rewiring of gene regulatory networks (GRNs) that have converged to generate compound leaves (Hasson *et al.*,2010; Townsley & Sinha 2012). It is also possible the up-regulation of *AGL* genes in sequentially older plants represents a more mature state that is verging towards reproductive development. Further analyses and experimental studies will be required to discriminate between these two hypotheses.

Differential gene expression and the heteroblastic trajectory of *Gevuina avellana.*

Among the differentially expressed genes, we focused our attention on the up-regulation of *SQUAMOSA PROMOTER-BINDING LIKE* (*SPL*) and *NAC* transcription factors genes with the increase of both developmental age and light availability. The transcriptome of *G. avellana* showed up-regulation of the *SPL* genes 4, 8, and 12 in relation to ontogeny and *SPL* 4, 7, 9, 12 and 13 in relation to light availability. *SPL* 4, 8 and 12 are involved in GA signaling, floral induction/fruit ripening, and programmed cell death (Chen *et al.*,2010). *SPL* 7 and 9 participate mainly in regulating shoot maturation and plant architecture. This suggests that the up-regulation of *SPL* genes has an important role in the heteroblastic development of *G. avellana* during the vegetative phase. Probably *SPL* genes drive the timing and progress of heteroblasty but not leaf pinnation *per se*. The up-regulation of NAC transcription factors has been shown to facilitate the sequential change from simple to compound leaves. Specifically, the requirement of *NAM/CUC* activity is essential in promoting leaflet separation locally and leaflet formation at a distance by interacting with other regulators of compound leaf development. This dual role is likely to

be a conserved molecular framework for compound leaf development within eudicots (e.g., *C. hirsuta*, *Solanum lycopersicum*, and *Pisum sativum*; Blein *et al.*,2008). In all the studied species with compound leaf development, the *NAM/CUC3* genes are required for proper expression of *KNOXI/LFY*-like genes and vice-versa, indicating a coordinated regulation of leaflet formation through a positive feedback loop (Blein *et al.*,2008). Interestingly, the *G. avellana* transcriptome shows down-regulation of *KNOXI* members *KNAT6*, *BP*, and *STM* and the absence of *LFY*-like genes. Nonetheless, we found up-regulation of several *AGL* genes such *AP1*, *SEP1* and *SEP3* as well as the floral repressor *SHORT VEGETATIVE PHASE* (*SVP*) correlated with ontogeny. We assume that during the vegetative phase of *G. avellana*, the expression of *AGL* floral promoter/repressor genes in leaf primordia would be components of the mechanism regulating its leaf pinnation. For example in Arabidopsis mutants, the expression of *AGL* genes in leaf tissues promotes changes in leaf morphology producing upward curling in rosette and cauline leaves, and also blocks the mechanism of floral induction during vegetative phase (Fernández *et al.*,2014). We speculate that the mechanism that controls compound leaf development in *G. avellana* underwent GRN rewiring, selecting *AGL* genes for an analogous role to those of *KNOXI* genes seen in almost all compound leafed species.

Integration of internal-external cues for proper heteroblastic development

At the incipient stage, leaf development requires local auxin maxima in cells at the flanks of the SAM and the rachis, allowing expression of the genes controlling initiation and separation of both leaves (from the SAM) and leaflets (from each other; Blein *et al.*,2010; Townsley and Sinha 2012). Whether correlated with developmental stage and/or light availability, we found both up-regulated and down-regulated transcripts related with

auxin transport and signaling, which suggests an active regulation of processes controlled by this hormone. However, *YUCCA* genes, particularly *YUC7*, and *YUC9* showed steady up-regulation with increasing light availability and plant development. Also, we found up-regulation of *JAZ*-domain protein coding genes, a key component of JA signaling, with the increase in both age and light availability. *YUCCA* genes have a pivotal role in the tryptophan-dependent auxin biosynthesis pathway. In turn, the activation of *YUCCA* promoter is modulated by jasmonic acid (JA) which is also known to be involved in the distribution of the auxin-exporter *PIN-FORMED 2* (*PIN2*) (Hentrich *et al.* 2013; Brumos *et al.* 2014). Given that JA usually acts at long distances from the source to the target tissue (Lucas and Lee 2004) an increase of *JAZ* genes serves as an indirect evidence of JA activity. JAZ are ubiquitinated in presence of JA which triggers JA-responsive gene expression (Chini *et al.*,2009). This process is balanced by a positive feedback loop between JA and JAZ avoiding exacerbated responses (Kazan and Manners 2008; Pauwels and Goossens 2011). Thus, the up-regulation of JAZ genes in *G. avellana* may reflect a rapid turnover of this protein by JA and an increase in JA-mediated responses such as the activation of *YUCCA* promoters. Several JA-mediated responses (e.g., shade avoidance, defense mechanisms) are driven by light quality mediated by phytochromes (Ballaré 2009). As the plant perceives an increment in the red to far-red ratio (R:FR) a signal cascade mediated by phytochrome B induces the conversion of inactive JA to the active JA-Ile (Moreno *et al.*,2009; Radhika *et al.*,2010). Given the contingent nature of development in *G. avellana*, as the plant grows there is a vertical increase in light availability concomitant with an increase in red light. It is known that phytochrome B (phyB) mediates several photomorphogenic responses under enriched red-light through direct interaction with bHLH-domain transcription factors resulting in the activation of multiple genes (Alabadi *et*

al.,2001). Therefore, for this tree, we hypothesize that the JA-responsive expression of *YUCCA* genes progresses coordinately with the increase of plant development and light availability.

Together with our previous finding of the significant effects of light availability on leaf size and complexity (Ostria-Gallardo *et al.*,2015), the morphological and physiological fate of leaf primordia is closely linked to the interaction between ontogeny and the prevailing light environment. Thus, we propose a model that links the ontogenetic contingency of *Gevuina avellana* with the mechanism underlying its heteroblastic development (Fig. 5). We hypothesized that the resulting heteroblastic development in *G. avellana* has been shaped by gene interactions in GRNs that were selectively maintained as they were functionally advantageous in allowing the plant to cope with the predictable environmental changes throughout the ontogeny of the plant (e.g., increasing vapor pressure deficit and light availability). These interactions may have undergone stochastic rewiring during the course of evolution from the basal Angiosperms to the well characterized model taxa. Thus, our analysis sheds light on the integration of internal and external clues in the progression of heteroblastic development in *G. avellana*. Clearly, further molecular and ecophysiological studies focusing on candidate genes proposed in this study plus their functional implications on heteroblasty are necessary. These further studies must be pursued using approaches that cover development within an environmental context to identify the signal perception and transduction mechanisms through which the environment informs and modulates development, as pointed out by Sultan (2010). These studies might help explain why *G. avellana* is the only strongly heteroblastic species in this

temperate rain forest and how the resulting phenotypes influence its natural morphological variation along the gradient of light availability.

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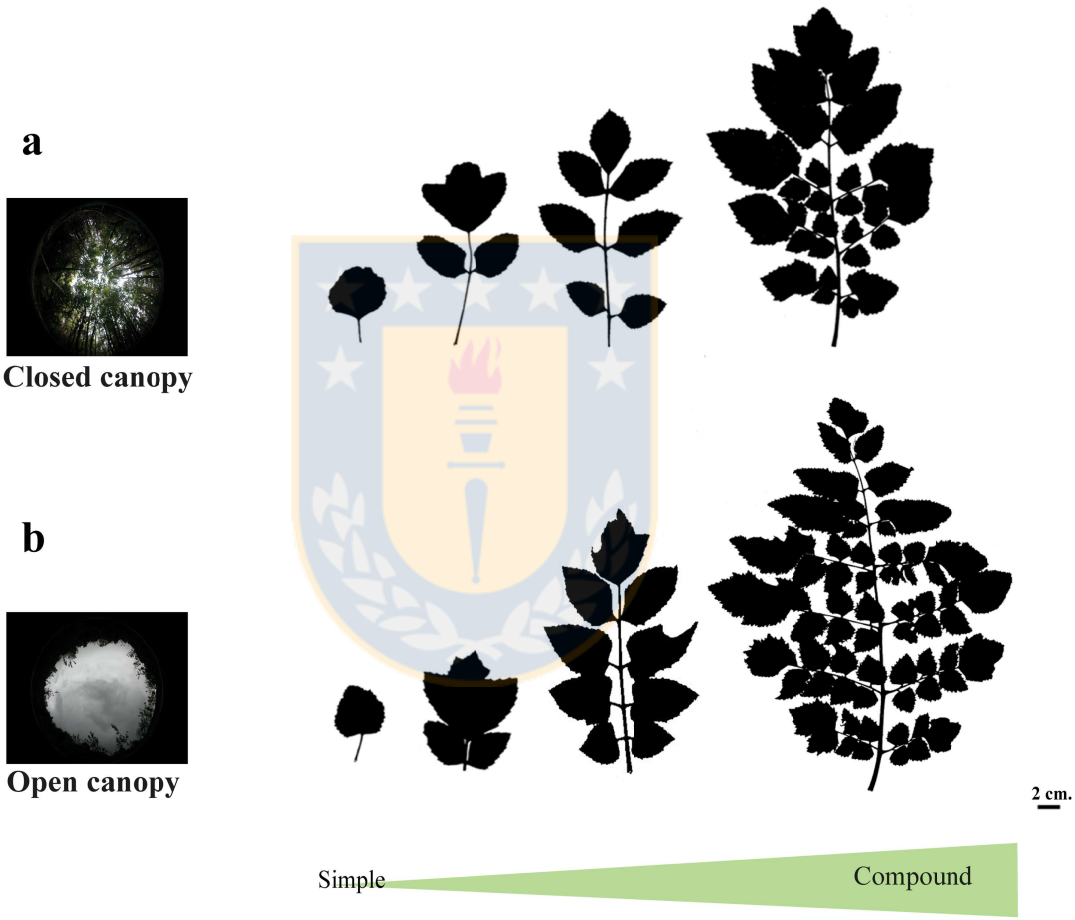
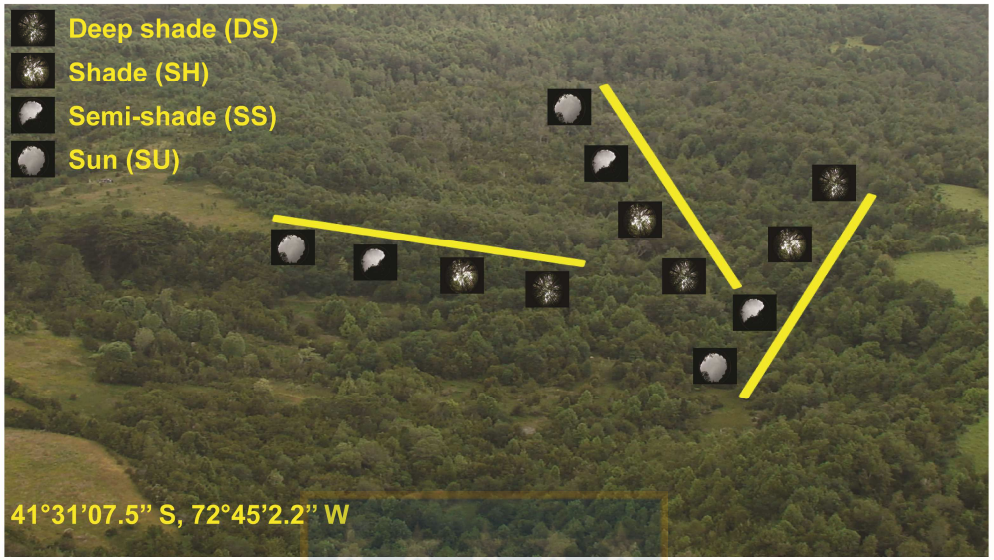


Fig. 1 Heteroblastic leaf development in *Gevuina avellana*. This species occupies a wide range of light environments, which has a small but significant effect on the phenotypic trajectory of leaves. Plants inhabiting closed canopy microsites have smaller and less complex leaves (a), compared with plants inhabiting in more opened canopy microsites (b).

a



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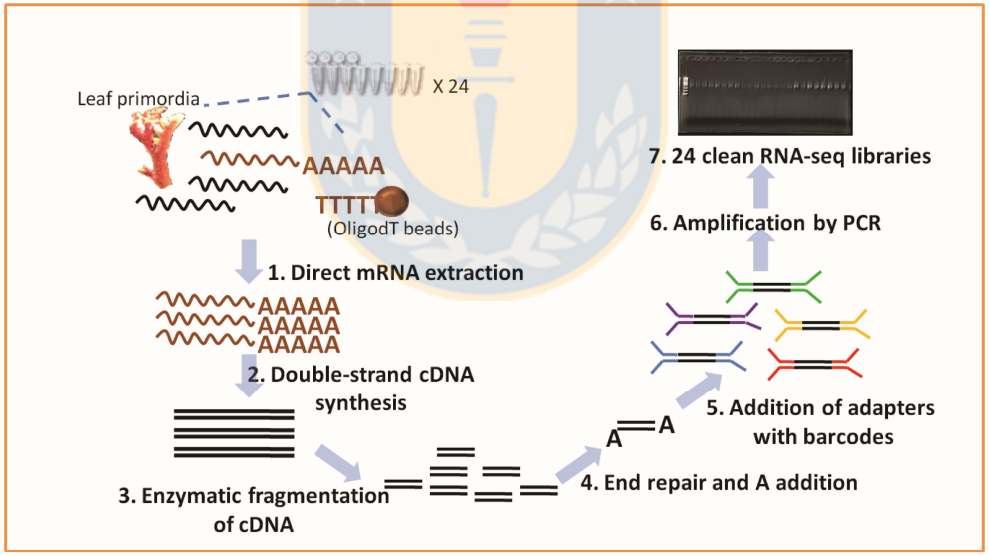


Fig. 2 a) Study site with the three areas for collection of leaf primordia from *G. avellana* trees of different sizes, under different canopy openness. b) High throughput RNA-seq libraries preparation. (Modified from Kumar *et al*., 2012).

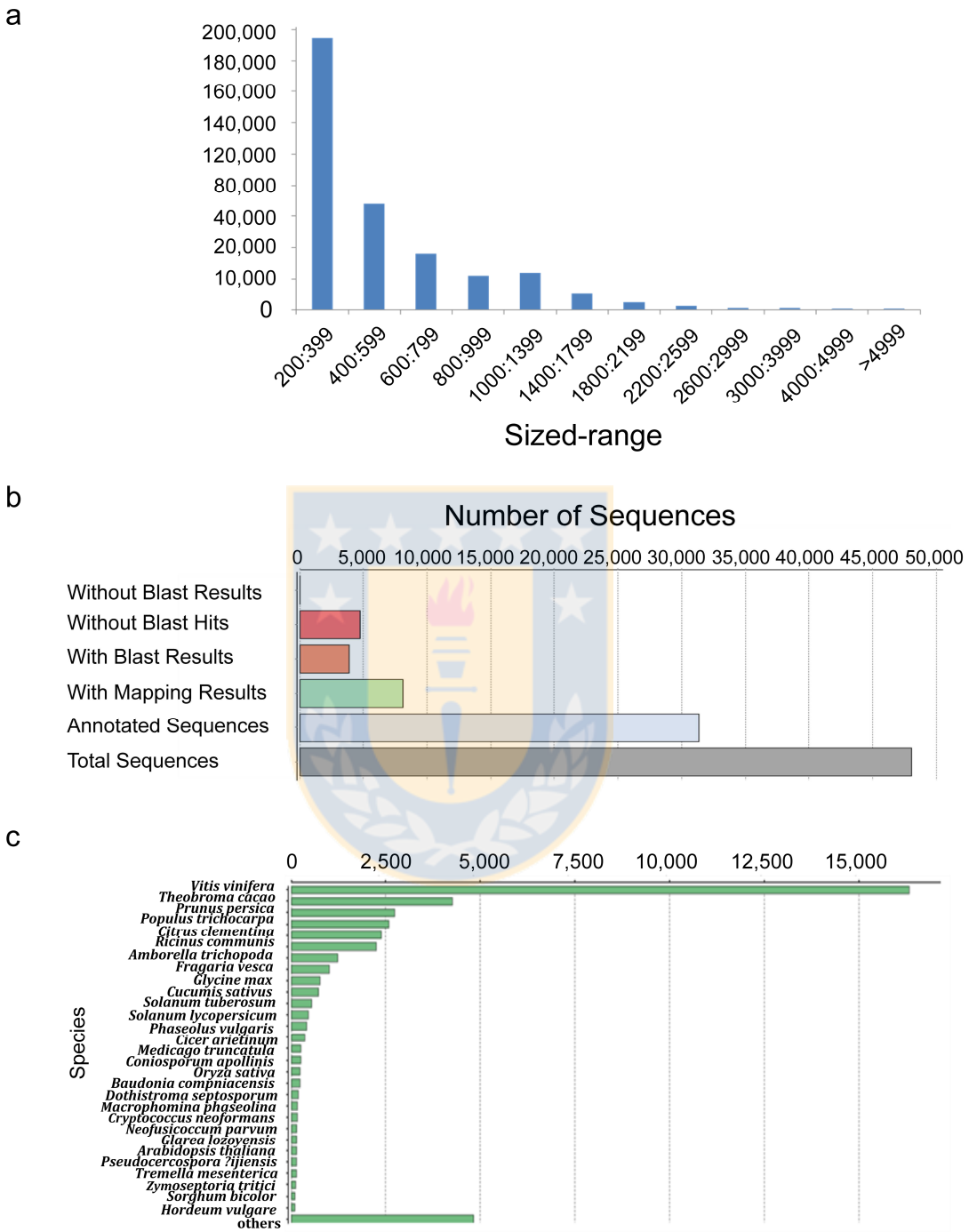


Fig. 3 Transcript size distribution showing high proportion of small sized transcripts in the final transcriptome assembly of *G. avellana* (a). Sequences distribution after Blast2GO analysis showing 70% of total sequences with annotation for predicted ORFs (b), and Top-Hit species distribution of *G. avellana*'s final transcriptome showing abundance of top-hits to the sequences of *Vitis vinifera* and tree species (c).

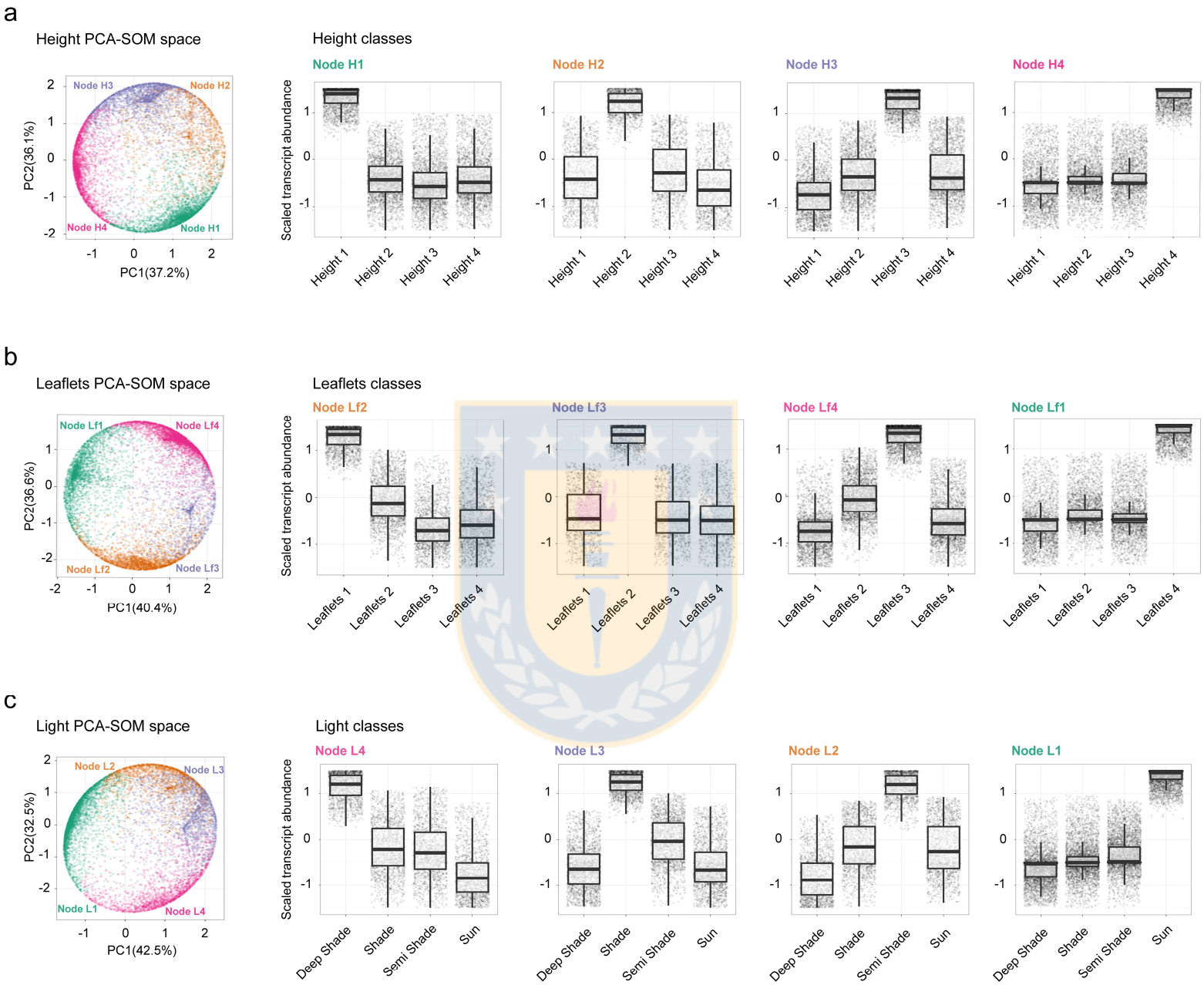


Fig. 4 Principal component analysis (PCA) with self-organizing maps (SOM) clustering of gene expression for the different plant height (a), number of leaflets (b), and light environment (c) classes. Each PCA-SOM space represents the expression profile of transcripts indicating node membership by different colors, and node number plus sufix for each evaluated factor. A total of four clusters was defined for each factor showing the expression pattern specific to each class of *G. avellana*'s size, pinnation, and light availability.

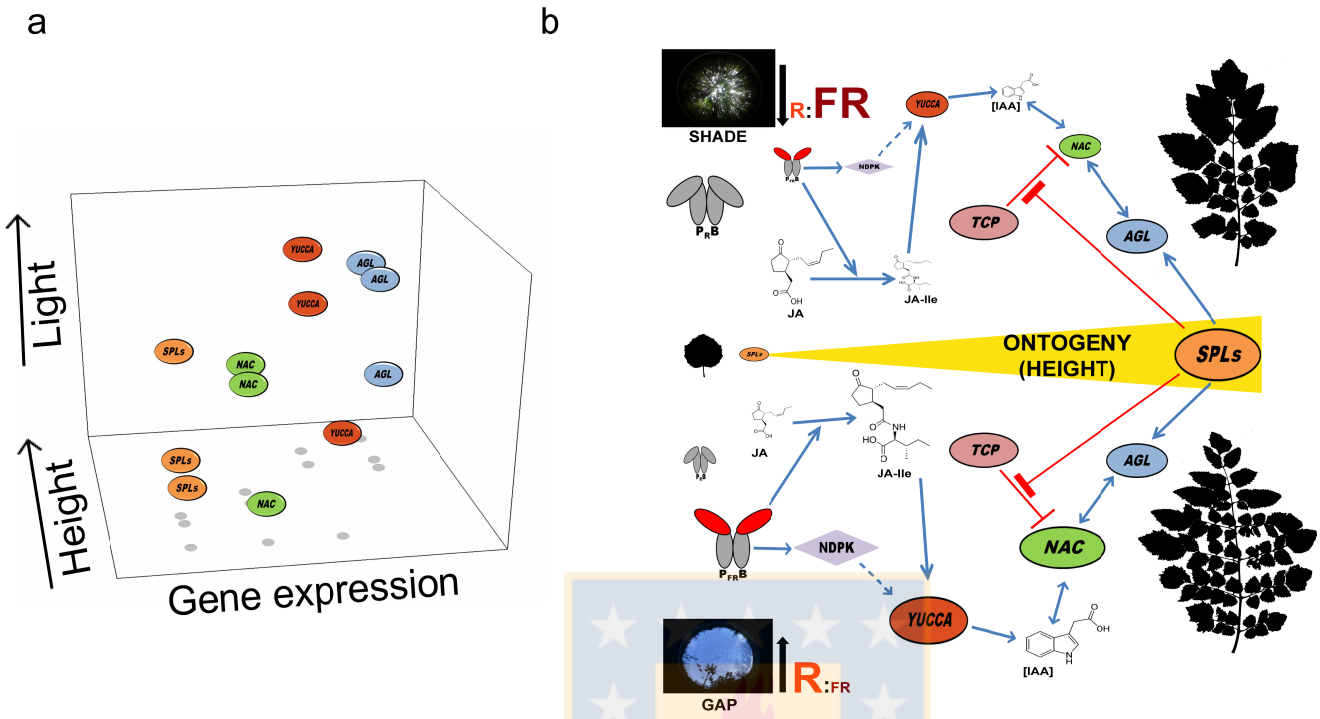


Fig. 5 a) Up-regulation of *SPL*, *YUCCA*, and *AGL* genes in relation to increases of height and light availability. Relative values for gene expression derives from the maximum value of fold change (logFC) between quartile 1 vs 2, 3, and 4, for both, height and light. b) Proposed model for the age-dependent changes of leaf shape and contribution of light, during the vegetative phase of *G. avellana*. The chnge in leaf shape is regulated by the progressive expression of *SPL* genes during ontogeny, which achieve two pivotal roles: First, the up-regulation of *SPL* genes drives the destabilization of TCP-NAC complex, and the active NAC proteins promote the increases in leaf complexity. Second, *SPL* genes acts upstream for the expression of *AGAMOUS-like* genes (*AGL*) in a coordinated fashion, involving floral promoters and repressors. For the sequential changes in leaf complexity, *NAC* genes undergo an increased expression due to a positive feedback loop with *AGL* together with local auxin maxima along the rachis, controlled by the activity of nucleoside phosphate kinase and the Ja-mediated expression of *YUCCA* genes. This is a process highly coordinated by phytochrome B in the context of light availability and the enrichment of red light sensed by the plants as they grow taller. (Arrow = induction/activation; dashed arrow = signal cascade; perpendicular line = repression).

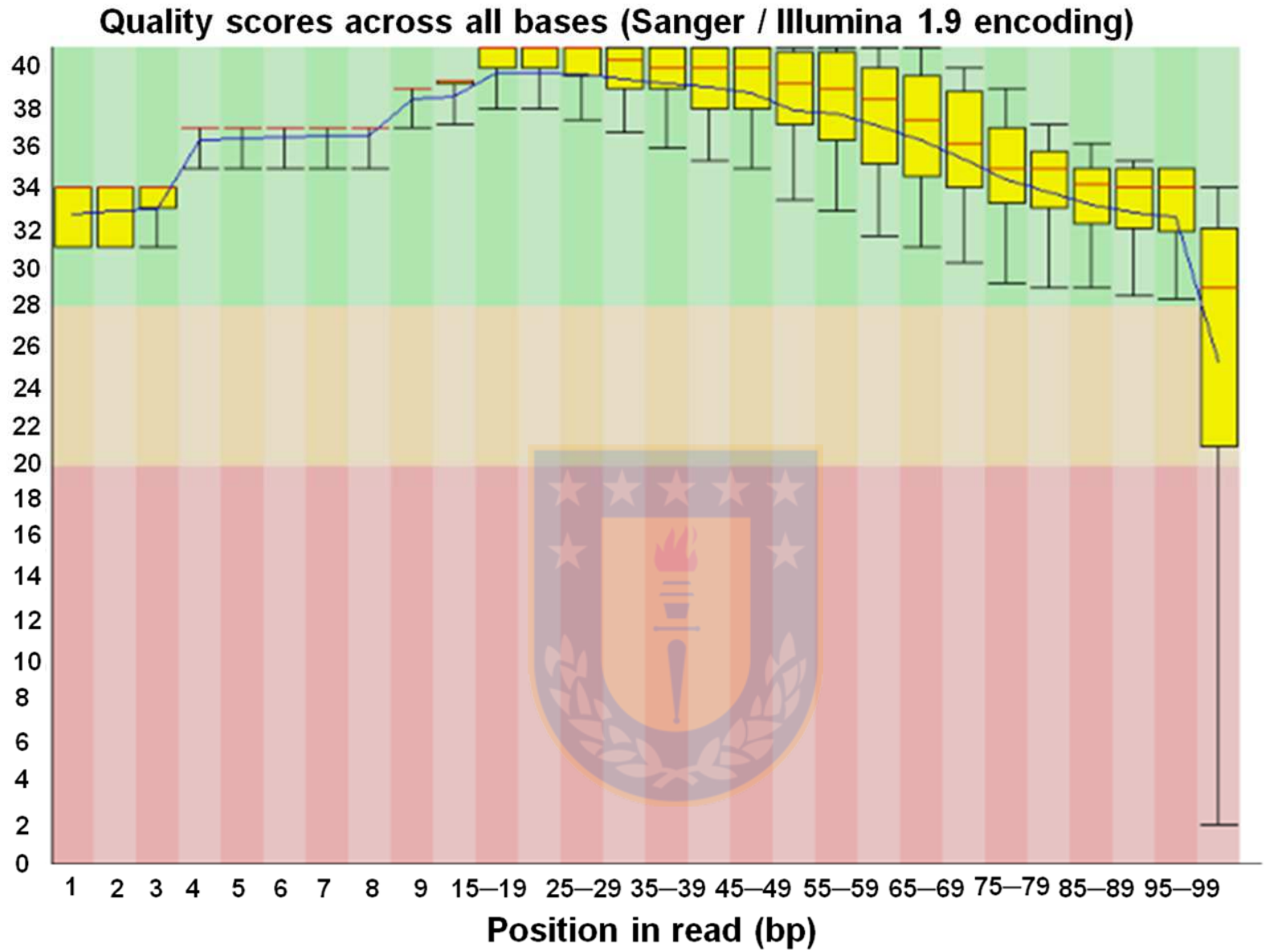


Fig. S1 Quality scores and accuracy of Illumina Hiseq 100-bp paired end-reads. After a series of quality filters, we discarded all those reads that showed values below 20 on the histogram that indicates bad quality reads. Values between 20 to 30 indicate 99% of accuracy, and above 30 indicate 99.9% of accuracy regarding to bases correctly read by the sequencer.

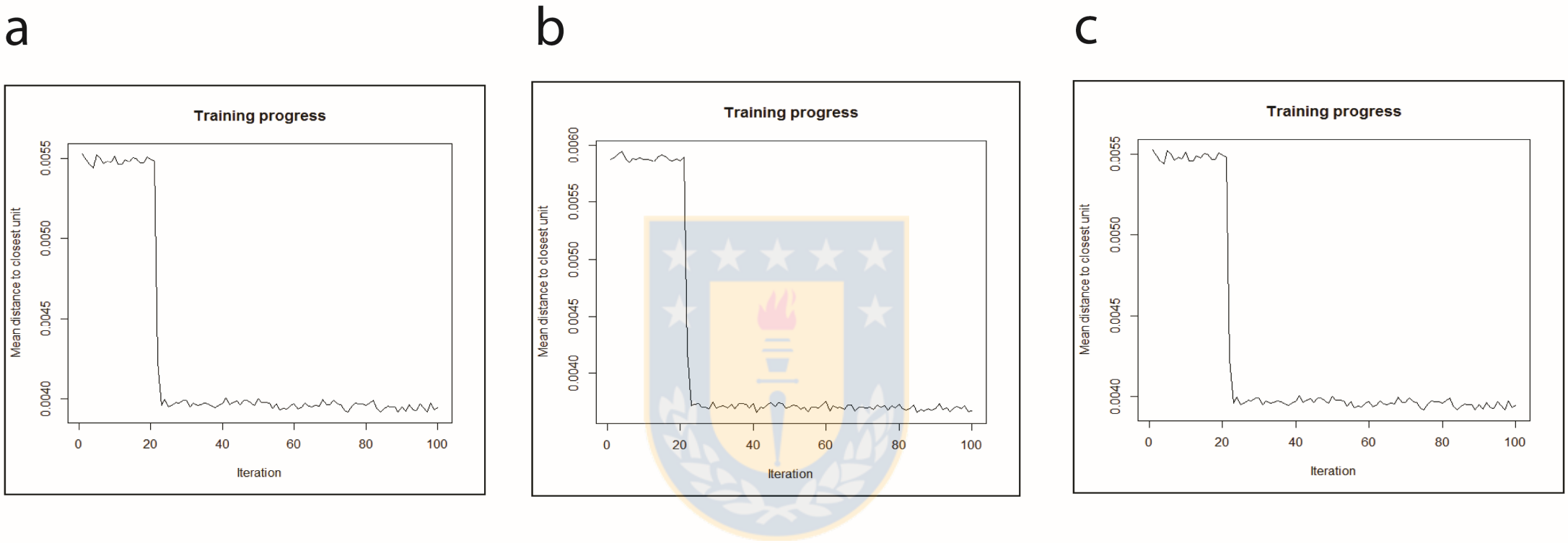


Fig. S2 Training progress of the average distances of genes using Self Organizing Maps, showing the effect of the neighbourhood shrinking to include the winning unit for height (a), leaflets (b), and light (c) factors.

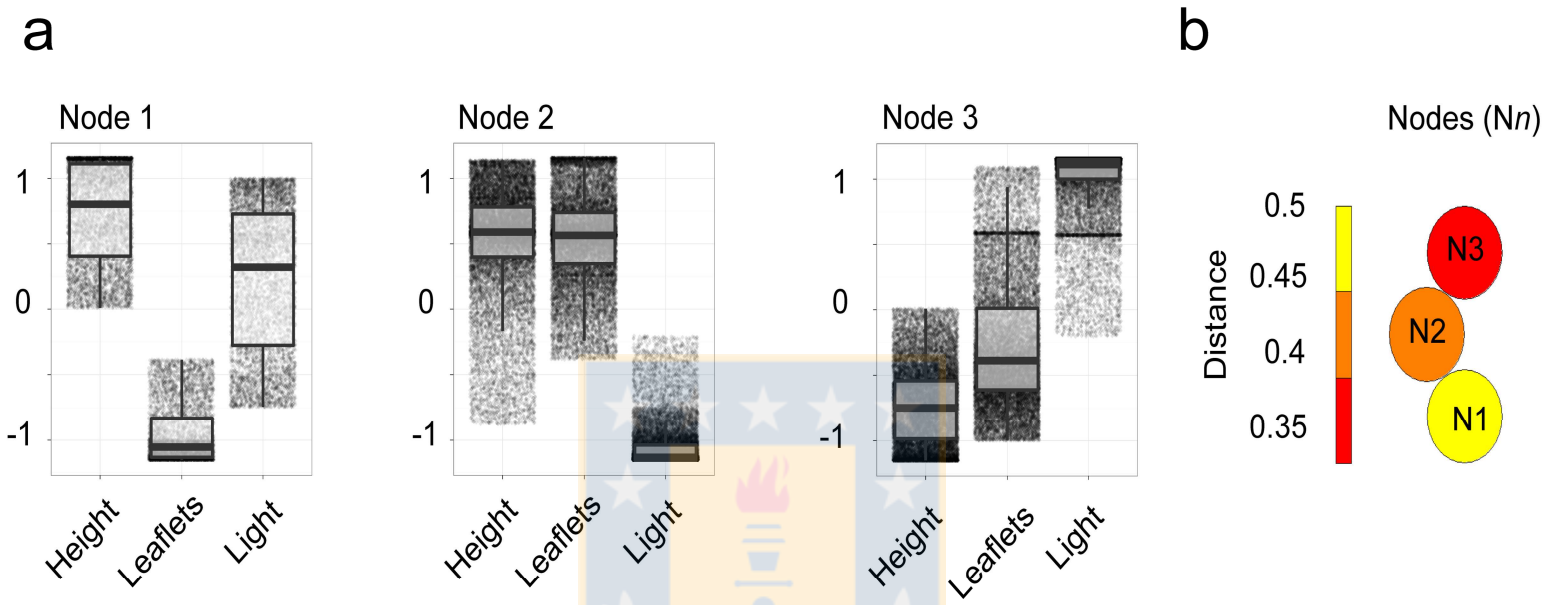


Fig. S3 Pattern of clustering comparing the expression of all transcripts among plant height, number of leaflets, and light environment (a). Each node shows densities of transcripts with similar accumulation patterns between factors; height and light (node 1), height and leaflets (node 2), and leaflets and light (node 3). (b) Quality of transcript mapping, showing the distance between the nodes members. The closest distances are found in node 3 whereas the biggest distances are for node 1.

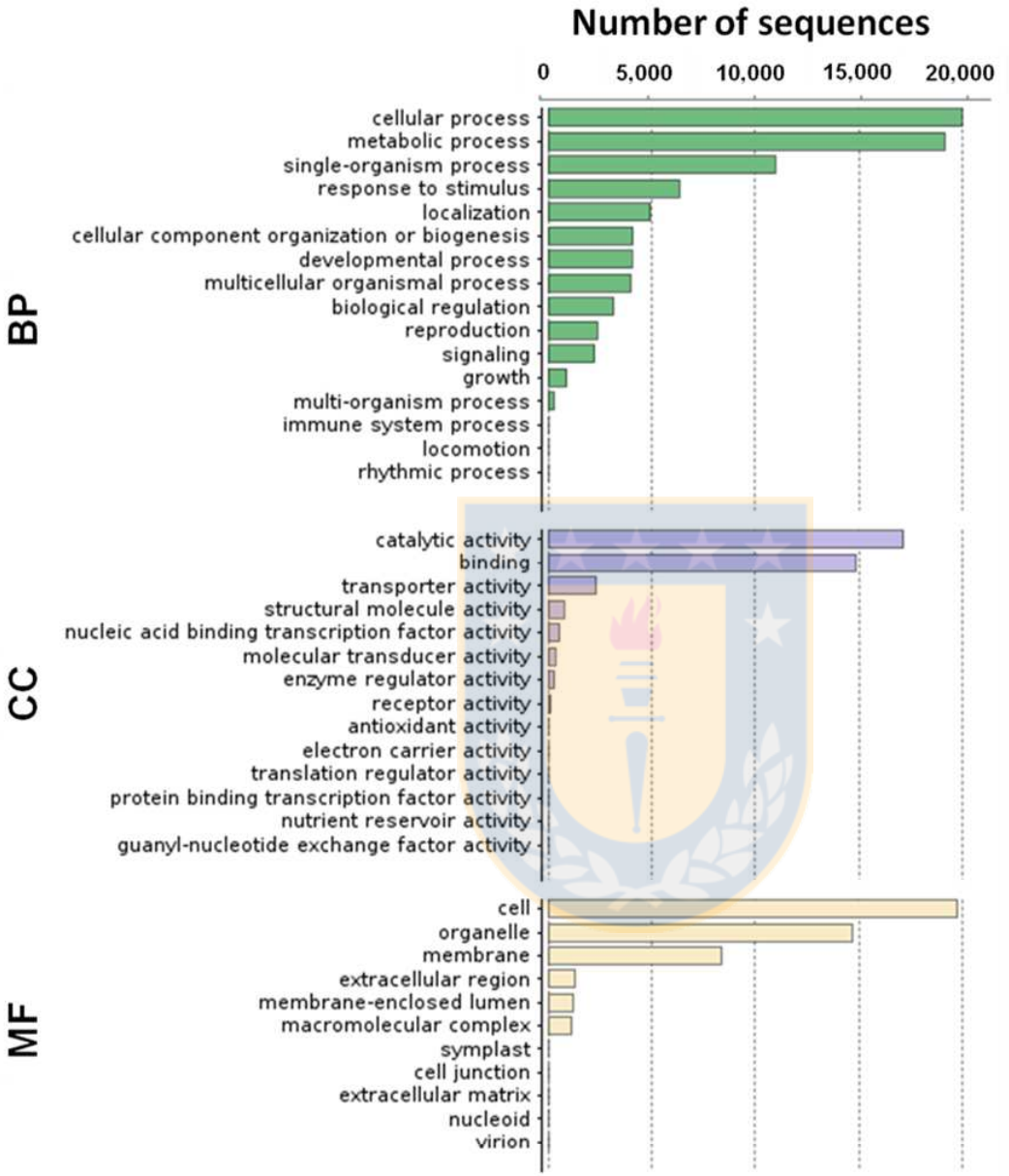


Fig. S4 GO-category distribution of *G. avellana* transcripts among level 2 GO categories: biological process (BP), celular component (CC), and molecular function (MF).

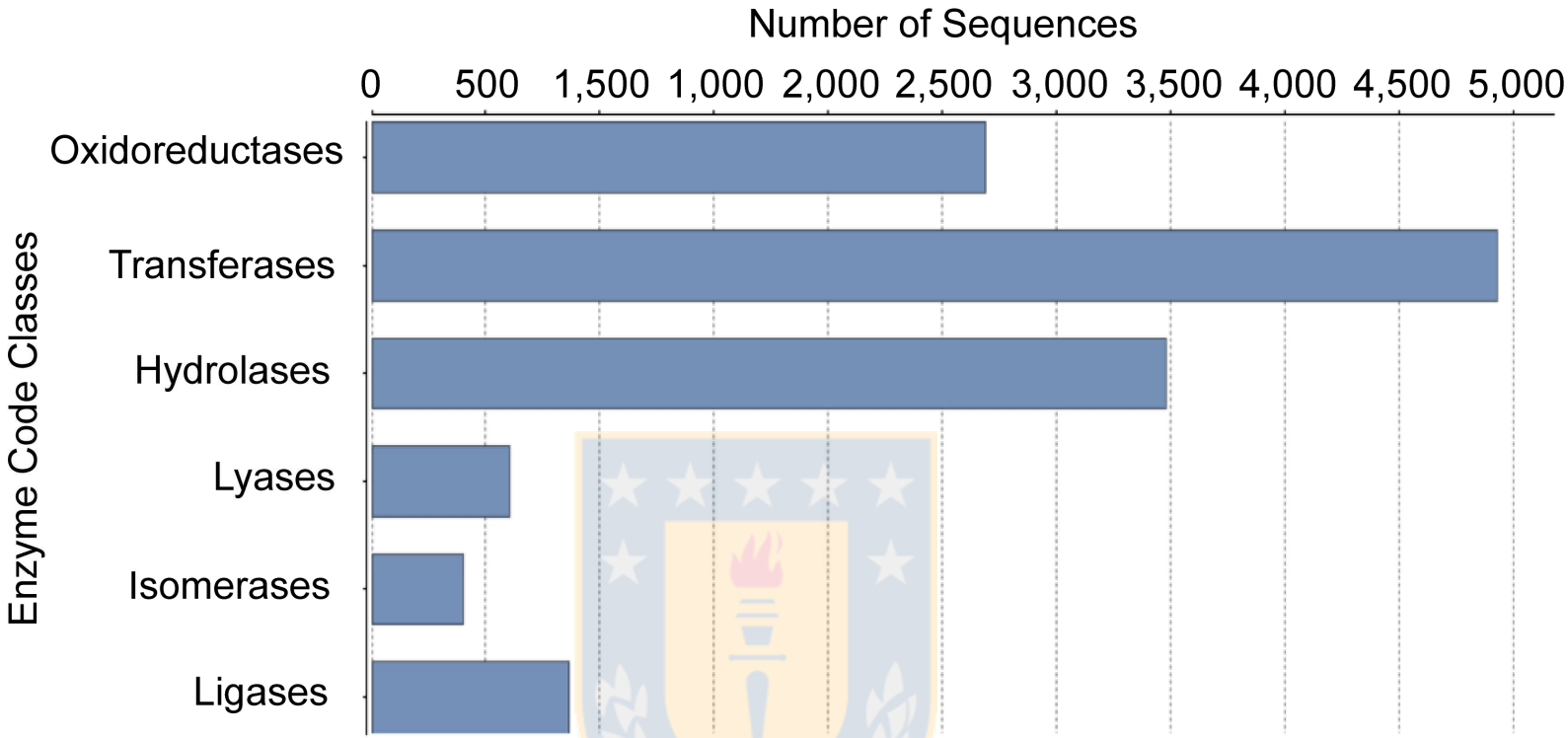


Fig. S5 Enzyme code distribution for the final *G. avellana* transcriptome. GO terms assigned for enzyme types shows high abundance for transferases, suggesting high metabolic activity involved in trasnferences of functional groups.

CHAPTER V. GENERAL DISCUSSION

The study of heteroblasty, or metamorphosis of leaves along development, comprises different approaches, from evolutionary ecology to molecular biology. To some degree, they have converged in the common agreement that, the phenotypic responses in the heteroblastic series are conditioned by the environmental information (Diggle 1994; Sultan 2010; Chitwood *et al.*,2014). This thesis combined a molecular-ecophysiological approach; that is, it used morphometric traits of leaf shape and a reliable quantitative analysis of the light environment of forest canopy, plus the assessment of a large-scale transcriptome dataset from leaf primordia sampled *in situ*. The goals were to determine: i) at what degree, and how the heteroblastic development of *G. avellana* is conditioned by light availability of the forest canopy, and ii) the underlying patterns of gene expression dynamics that regulates the heteroblastic development and trajectory of this species. Although the two hypotheses tested ─ *H1*. At high irradiance, the leaf pinnation and complexity increases; *H2*. During development, the increased expression of FLO and the decreased expression of KNOX genes produce the progressive formation of compound leaves ─ were partially true, the findings of this thesis provides basic elements for a comprehensive insight of the strategy used by this basal Eudicot tree for acclimation to vertical and horizontal changes in light availability during its earlier heteroblastic development.

Both hypotheses are discussed in detail below, which led into a mechanistic model about the integration of external/internal cues driving the heteroblastic development and trajectory of *G. avellana*.

5.1. Light as a conditioning factor of the heteroblastic trajectory

In forest ecosystems, light is the limiting factor driving many responses in forest species. Light determines plant establishment, growth, foliar display and morphology, crown architecture, and ultimately, survival and fitness responses (Valladares and Niinemets, 2008). Given the spatial and temporal heterogeneity of light availability within the forest canopy, it has been proposed that heteroblasty evolved in some forest species as an adaptive and functional strategy to predictable changes in light conditions experienced during ontogeny (Day 1998; Winn 1999). Due to the wide range of light availability that *G. avellana* exploits within the forest canopy (Lusk 2002; Ostria-Gallardo *et al.*,2015), it was postulated that the phenotypic trajectory of leaf pinnation in their heteroblastic series increases at high light availability as responses to constraints of this light micro-environment. The analysis of leaf shape descriptors showed a significant increase for the size and complexity of leaf outlines in responses to increases in light availability. However, not all shape descriptors showed significant differences between low and high light availability. Therefore, the first hypothesis of this thesis was partially true. Specifically, the leaf dissection index and the aspect ratio showed no significant response to light availability (Table I, Chapter 3). It has been reported that the phenotypic differences of heteroblastic species under low and high light are attributed to a plastic response of individual leaves to the prevailing condition without affecting the progression of ontogenetic program (Jones 1995). In an elegant experimental design, Jones (1995) studied leaves morphogenesis of the heteroblastic species *Cucurbita argyrosperma*. It changes from slightly lobed leaves to deeply lobed along ontogeny, being more pronounced under high light; under low light, leaves resemble a more juvenile-like type. She found that the morphological differences between shade and sun leaves did not arise until the latter stages of leaf morphogenesis. The author concluded that the less lobed and juvenile looking leaves

produced in shade were because of the plasticity of a genetically programmed ontogenetic change rather than a prolongation of the juvenile phase. We decomposed the heteroblastic series of *G. avellana* into two variables of different magnitudes; the leaf dissection index which indicates number of leaflets produced in a leaf, and the fractal dimension index, which describes how much complex and jagged are the leaf outlines. The effects of light availability on leaf dissection index were negligible, reflecting a strong ontogenetic dependency of leaf pinnation, i.e., the number of leaflets produced. In turn, the increases in the fractal dimension index at high light can be a plastic morphological acclimation to the prevailing light availability perceived by the developing leaf primordium. Gamage (2010) reported for the heteroblastic trajectory of two species from the New Zealand temperate forest a significant increase in leaf serration (more complex shape) in gaps compared with homoblastic congeners. The observed response was associated with a better anatomical plasticity, physiological performance and survival under high light. Therefore, our results suggest that heteroblasty of *G. avellana* helps to solve allometric constraints of leaf size, and improve the functional implications and plant performance under different light niches.

5.2. Genetic basis of heteroblastic development of *G. avellana*

Based on the current knowledge about the ontogenetic control of the heteroblastic expression (Diggle 2002; Zotz *et al.*,2011) and the recent increases in knowledge about the complex gene regulatory networks that shape simple and compound leaf development (Ishihashi *et al.*,2014; Tsukaya 2014; Chitwood and Sinha 2014), it was postulated that heteroblastic development in *G. avellana* will be governed by a similar molecular mechanisms as described by Champagne *et al.*. (2007) for legumes. Specifically, during *G. avellana* development, a progressive increase in the level of auxin in the newly formed leaf

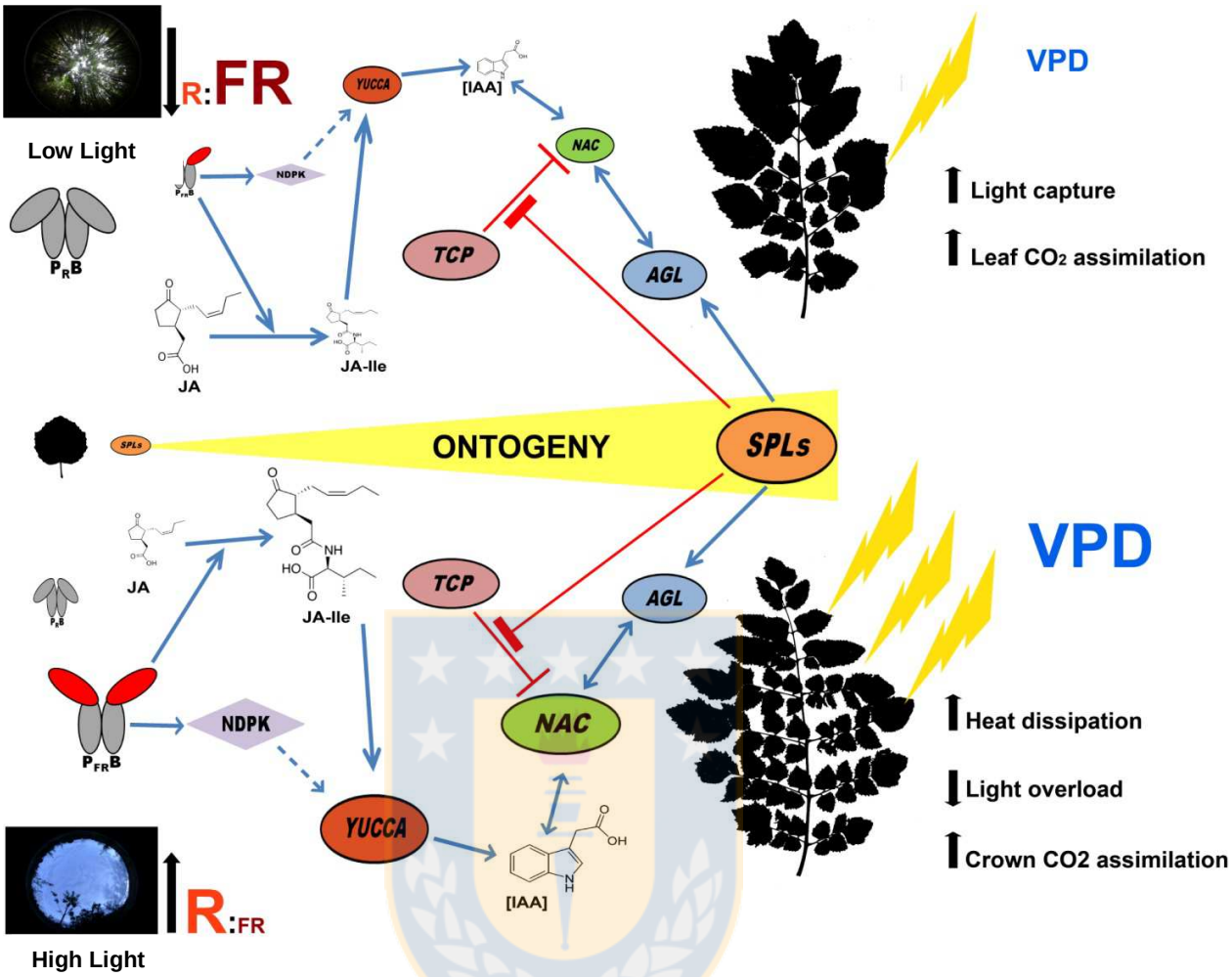
primordia lead to significant changes in the transcriptional dynamics inducing the up-regulation of the plant-specific floral homeotic gene *FLORICAULA/LEAFY* (*FLO/LFY*) and *NAC* genes, and the down-regulation of class *KNOX1* genes, underlying the genetic core of the heteroblastic development of *G. avellana*. This hypothesis was also partially true. As expected, along plant development, it was found a sustained down-regulation of class KNOX1 genes, and sustained up-regulation of *NAC* genes. However, instead of the expected up-regulation of *FLO/LFY*, we found that pattern for the expression of the MADS-box genes *AGAMOUS-Like* (*AGL*) (discussed in detail in chapter 4). Also, our results showed a sustained up-regulation of *SPL* genes, which are known as a master regulator of phase change (Chen *et al*.,2010). The interaction between floral repressors and the regulatory networks involved in phase changes are important components of the timing in developmental transitions (Yang *et al*.,2011). At the same time, the expression of this floral inductor/repressor during vegetative phase can induce alteration in leaf morphology. Fernández *et al.* (2014) reported for Arabidopsis that the expression of *AGL* genes in leaf tissues is needed to block floral induction during vegetative phase, but also produced changes in leaves morphology. Among the genes which *AGL* interact with, *NAC* genes are targets; for example, an *Arabidopsis NAC* gene, *NAP*, is activated by *AP3/PI*, an *AGL* gene. When activated, *NAP* controls the transition between cell division and expansion in stamen and petals (Sablowski & Meyerowitz 1998). Also, as a key component of a conserved molecular framework for compound leaf development (Blein *et al.* 2008), the relation between the sustained expression of *NAC* genes with age found in *G. avellana*, suggests a strong age-dependent control of its heteroblastic development. Thus, it is proposed for *G. avellana*, that its heteroblastic development is driven by an intricate network of genes, in

which *SPL*, *AGL*, and *NAC* form a gene module that coordinates the heterochronic progression of leaf pinnation.

5.3. Towards a comprehensive mechanistic model of the heteroblastic development and trajectory of *G. avellana.*

Given the wild provenance of the sampled individuals used in this thesis, it was expected that the collected samples were fully acclimated to the prevailing micro-environmental condition. The importance of light for the temporal and spatial configuration and interaction of forest communities, and as a source of information for the directionality of many traits in forest species has been discussed throughout this thesis. However, there are other factors, specific for the micro-environments within the forest, exerting constrains at different levels and degrees (e.g., changes in vapor pressure deficit, wind speed, litter degradation, mobilization and recycling of nutrients, etc). Different environmental cues trigger signal transduction events that can converge on shared gene expression and hormonal pathways to elicit common responses (Franklin 2009; Smékalová *et al.*,2014). Given this, it may be expected that a resulting phenotype will be a response to the additive effects of the limiting factor plus the micro-environmental constrains in which an individual grows and develops (Diggle 1997). In simple words, it is the ecological context that determines the functional success of developmental outcomes (Sultan 2010).

The combined results of this thesis support the above mentioned statement, and allowed us to propose a mechanistic model involved in the heteroblastic development and trajectory of *G. avellana* leaves, and at the same time, suggest hypotheses about the functional meaning of its heteroblasty (Model 1).



Model 1. Proposed interaction between ontogenetic programming and environmental cues for heteroblastic development and trajectory of *G. avellana* leaves growing under low and high light availability micro-sites.

The heterochrony of the heteroblastic series of *G. avellana* is a process highly dependent of the ontogenetic programming, controlled by the accumulation of *SPL* genes, which in turn promote the expression of *AGL* genes, and also interfere with the *TCP*-mediated inactivation of NAC transcription factors. On the other side, the phenotypic fate of the heteroblastic series in *G. avellana* depends on the prevailing light environment.

When it grows under a closed canopy, the plant perceives an environment enriched with diffuse and far red light. Under these conditions, phytochrome B is mostly inactivated (Ballaré 2009). Phytochrome B initiated a signal cascade that ultimately leads to an accumulation of *NAC* genes (Figure 5 of chapter 4). This accumulation is mediated by a proper cellular environments conferred by a feedbackloop with increases in auxin concentration (Rodriguez *et al.*,2013 and references therein). With inactive phytochrome B, there are a higher proportion of cells without an appropriate environment for *NAC* expression and activity. Therefore, it is expected that leaves have less boundary specification along the petiole and leaflets, leading to less complex leaves, as we found for those individual from low light environments. These low light leaves showed an increase in the length to width ratio (aspect ratio) compared to high light leaves when plants reach c.a. 20 cm. Typically, semi-shade tree species, such as *G. avellana*, respond to lower R:FR with the so called "shade-avoidance syndrome", inhibiting the leaf blade expansion and favoring petiole and main stem elongation (Kozuka *et al.*,2005; Wettberg and Schmitt, 2005). Hence, the heteroblastic trajectory of low light leaves with increasing aspect ratio would be functional to cope with self shading, improve light capture and carbon balance at whole plant level (Hasson *et al.*,2010), and minimize water loss due to thicker boundary layers of less complex leaflets/leaves.

Under high light conditions, the enrichment of red light promotes the activation of phytochrome B. A signal cascade involving the activation of Jasmonic acid (from JA to JA-Ile) and nucloside diphostphate kinases (NDPKs) induce the expression of *YUCCA* genes and metabolic activity. *YUCCA* genes are keys on the biosynthesis of auxin through the tryptophan pathway (Radhika *et al.*,2010). With the increases of auxin maxima alternated along the leaf morphospace, contiguous cells regions acquire a proper environment for

NAC expression and activity. This pattern has been reported for the increased serration and production of leaflets for *Arabidopsis* and *Cardamine hirsuta*, respectively (Rodriguez *et al.* 2013 and references therein). Therefore, the increases in auxin concentration mediated by phytochrome B signal cascade are a key component for the production of more complex and jagged leaves in *G. avellana* plants sensing an increase in light availability. The functional meaning of the increase in complexity under high light may be a response to light energy overload of the photosynthetic apparatus (PSII) (Huner *et al.*,1996). This hypothesis is detailed in the Discussion section of chapter 3. Depending on the magnitude, over-excitation of PSII can lead to chronic photoinhibition (Coopman *et al.*,2008) that would be exacerbated in un-pinnated large leaves (Lambers *et al.*,2008). With more dissected and jagged leaves, *G. avellana* improves heat convective dissipation due to thinner boundary layer in the inter-leaflets spaces, and avoids light overload for upper leaf cohort at the same time that allows the passage of light to the lower cohorts, which may improve whole crown light energy use.

5.4. CONCLUSIONS

This is the first study that provides a strong ecophysiological and molecular basis about the mechanism behind the heteroblasty of *G. avellana*. It is concluded that:

1) The heteroblastic development of *G. avellana* is a process highly dependent on the ontogenetic programming controlled by the transcriptional dynamic of the heterochronic genes *SQUAMOSA PROMOTER BINDING Like* and their effect on homeotic genes.

2) The heteroblastic trajectory of leaf size and complexity is conditioned to the prevailing light availability of the specific micro-environment where the individual plants are established and grows. This involves the coordination of light-mediated signals, hormone synthesis and signaling, and the heterochrony of the ontogenetic program.

Concluding Remarks: The results of this thesis support the multiple hypotheses proposing that, instead of a unique model for all compound leaves, these structures may arise through different developmental paths that converge to generate compound leaves. For example, the downregulation (*KNOX1*), as well as the absences (*FLO/LFY*) of genes, considered crucial for compound leaf development in most of model species, suggests a rewiring of gene networks in this basal eudicot tree, involved in the regulatory mechanisms controlling meristem identity and flowering transition.

The robustness of this thesis can serve for comparisons with model and non-model species to enhance the current knowledge about the mechanisms behind developmental plasticity of leaves, and how genetics, development, and environment interactively modulate a complex trait, which *a priori* confers functional advantages in the plants performance. More researches combining the power of observational field studies and high-throughput phenomic methods are needed to a better understanding of the ecological evolution of plant's developmental and functional strategies in their natural settings.

5.5. GENERAL REFERENCES

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