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Vegetative phase change and shoot maturation in plants

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Abstract

As a plant shoot develops, it produces different types of leaves, buds, and internodes, and eventually acquires the capacity to produce structures involved in sexual reproduction. Morphological and anatomical traits that change in coordinated fashion at a predictable time in vegetative development allow this process to be divided into several more-or-less discrete phases; the transition between these phases is termed vegetative phase change. Vegetative phase change is regulated by a decrease in the expression of the related microRNAs, miR156 and miR157, which act by repressing the expression of SBP/SPL transcription factors. SBP/SPL proteins regulate a wide variety of processes in shoot development, including flowering time and inflorescence development. Answers to long-standing questions about the relationship between vegetative and reproductive maturation have come from genetic analyses of the transcriptional and post-transcriptional regulatory networks in which these proteins are involved. Studies conducted over several decades indicate that carbohydrates have a significant effect on phase-specific leaf traits, and recent research suggests that sugar may be the leaf signal that promotes vegetative phase change.

Keywords

Heteroblasty; phase change; miR156; miR172; SPL; SBP; flowering time; shoot morphogenesis; heterochrony

1. Introduction

During its post-embryonic development, a plant becomes established as a young seedling, increases in size and complexity, undergoes sexual reproduction, and eventually senescences and dies. This process involves gradual quantitative changes, as well as more rapid qualitative changes that occur at particular times in shoot development. The most obvious and best understood of these transitions is the switch from vegetative to reproductive development, which is accompanied by the production of novel reproductive structures, such as flowers or cones (Amasino, 2010; Andres and Coupland, 2012; Huijser and Schmid, 2011; Wilkie et al., 2008). This transition is preceded by a change in the competence of the shoot to respond to stimuli that induce reproductive development, and by changes in a variety of other traits, including leaf and stem morphology, growth rate and orientation, branching patterns, and disease or herbivore resistance. Variation in these latter traits has been described in many different species, starting with the observations of Goethe and Knight in the 18th century (Goethe, 1790; Knight, 1795). But—in contrast to the vegetative-to-reproductive transition—the molecular mechanism of these vegetative changes is still

largely unknown. Here, I present some of the major questions about this process that remain to be answered, focusing on the phenomenon of vegetative phase change in herbaceous plants. The extensive literature on this topic in vines and woody plants has been discussed in several excellent reviews (Day et al., 2002; Doorenbos, 1965; Greenwood, 1995; Hackett, 1985; Lee and Richards, 1991; Schaffalitzky de Muckadell, 1954; Zotz et al., 2011), and readers should consult these for other perspectives on this problem.

2. Terminology

Hildebrand (Hildebrand, 1875) and Goebel (Goebel, 1889) were the first to recognize that shoot development can be divided into juvenile and adult stages on the basis of species-specific vegetative traits such as leaf shape, the orientation of branch growth, and the capacity for sexual reproduction. Goebel noted that the degree of variation in these traits varied considerably between species, and coined the term “heteroblasty” to describe species that undergo major morphological changes, and “homoblasty” to refer to plants that display more modest changes (Goebel, 1900). Heteroblasty has since acquired a broader meaning, and is now often used to describe any morphological variation along the length of a shoot, independent of the nature or degree of this change (Zotz et al., 2011). An additional terminological complication is that the terms “juvenile” and “adult” were initially employed to describe different stages of vegetative development (Goebel, 1889; Hildebrand, 1875), but are now more often used to refer to vegetative vs. reproductive (flowering) shoots. Indeed, some authors have criticized the use of these terms to describe changes in vegetative morphology (Jones, 1999; Jones, 2001; Zotz et al., 2011), arguing that they should be used exclusively to describe a change in reproductive competence. As described below, reproductive competence is controlled by multiple pathways, which interact with the pathway controlling vegetative maturation at various points. Because vegetative maturation and reproductive maturation are equally important aspects of shoot development and are regulated in concert, it is not obvious why the developmental state of the shoot should only be defined by one of these processes. To avoid confusion it has been suggested that vegetative maturation and reproductive maturation be described using terms specific to each process, e.g. juvenile and adult phases of vegetative development, and juvenile and adult phases of reproductive development (Poethig, 1990). In this review the terms “juvenile” and “adult” will refer to specifically to phases of vegetative development, i.e., developmental changes that occur prior to floral induction.

3. Heteroblasty and vegetative phase change

Variation in the morphology or physiology of a shoot can occur for many reasons. Traits that change in a coordinated fashion at a predictable time prior to flowering and which are not readily modified by environmental conditions, are the basis for the division of shoot development into juvenile and adult vegetative phases. The process responsible for this type of variation has been termed “ontogenetic maturation” (Wareing, 1959) or “phase change” (Brink, 1962; Poethig, 1990). A second type of variation is evident in traits that change gradually over the life of the shoot, and which can be modified by re-rooting or by grafting shoots onto a more vigorous root stock (Day et al., 2002; Greenwood et al., 2010; Mencuccini et al., 2007). This type of heteroblasty has been termed “physiological aging”

(Wareing, 1959), and is thought to be a consequence of an increase in the size of the shoot (Day et al., 2002; Mencuccini et al., 2007). A third type of variation, termed “seasonal heterophylly” (Godley, 1985), is represented by the reproducible changes in shoot morphology that occur during a growing season along the primary axis of the shoot, or in newly-formed branches of herbaceous and woody perennials (Kozlowski, 1971; Moriuchi and Winn, 2005; Winn, 1999). These changes sometimes resemble those that occur early in the growth of the shoot (Critchfield, 1960), but differ in that they occur regularly, rather than only once. Changes in vegetative morphology may also arise from environmental heterogeneity, such as variation in light quality, temperature, growth substrate, and humidity (Bruni et al., 1995; Cutri et al., 2013; Deschamp and Cooke, 1985; Fisher et al., 2002; Goliber and Feldman, 1990; Jones, 1995; Lee and Richards, 1991; Ray, 1987), and may also be induced by damage from herbivory or disease (Boege and Marquis, 2005). Changes produced by these environmental conditions can resemble the changes that occur during phase change, but it is unclear if they are mediated by the same regulatory mechanism.

In higher plants, the transition from vegetative to reproductive development is marked by the production of a completely new structure specialized for gamete production (e.g., a flower or cone). In contrast, vegetative phase change involves heterogeneous and sometimes quite subtle changes in the character of leaves, stems, and buds. These changes are species-specific and include leaf shape and size, branching patterns, epicuticular wax, patterns of trichome production, cell shape, vascular patterns, histological staining, phyllotaxy, the capacity for adventitious root production, and the presence or absence of anthocyanin or other phytochemicals, as well as disease or insect resistance (Allsopp, 1967; Boege and Marquis, 2005; Greenwood, 1995; Hackett, 1985; Kerstetter and Poethig, 1998; Poethig, 1990; Whalen, 2005). Depending on the species and the trait under investigation, vegetative transitions may occur quickly and encompass only a few nodes, or occur gradually and encompass many nodes. This greatly complicates the analysis of vegetative phase change because different temporal patterns and traits may be controlled by different mechanisms in different species (Borchert, 1976; Jones, 1999). Fortunately, this problem has been resolved by the discovery of major regulators of vegetative phase change—the microRNAs, miR156 and miR157, and their direct targets, the SBP/SPL family of transcription factors. Recent studies demonstrating that these evolutionarily conserved genes regulate vegetative phase change in a range of plants, and are differentially expressed in juvenile and adult tissues of herbaceous as well as woody plants, makes it possible to study the underlying molecular mechanism of vegetative phase change across the plant kingdom.

4. miR156 and miR157: master regulators of vegetative phase change

miR156 was initially discovered as a regulator of vegetative phase change in *Arabidopsis* (Wu and Poethig, 2006) and maize (Chuck et al., 2007), and has since been shown to regulate this process in a number of other herbaceous (Fu et al., 2012; Salinas et al., 2012; Shikata et al., 2012; Xie et al., 2012; Xie et al., 2006; Zhang et al., 2011) and woody (Wang et al., 2011) species. miR156 is present in all land plants including moss (Axtell and Bowman, 2008), where it has been implicated in the transition from the protonemal to the leafy gametophore stage of development (Cho et al., 2012). miR157 differs by three nucleotides from miR156, but is frequently mis-annotated as miR156 so it is difficult to

determine its taxonomic distribution from the literature. Sequence searches of various databases demonstrate that it is not present in the moss *Physcomitrella patens*, but is present as a single nucleotide variant in the lycopod *Sellaginella moellendorffii*. It is also present in the gymnosperm *Taxus chinensis* (Qiu et al., 2009), in some monocots, and in most if not all eudicots (www.mirbase.org). The over-expression phenotype of miR157 is quite similar to that of miR156 (Shikata et al., 2012), but its expression pattern and function have not been as well characterized.

Evidence for the involvement of miR156 in vegetative phase change has come primarily from the phenotype of plants over-expressing this miRNA. Plants expressing miR156 under the regulation of a strong constitutive promoter have a common phenotype consisting of a prolonged juvenile phase, increased branching, accelerated leaf production, and delayed flowering. This phenotype has been observed in *Arabidopsis* (Schwab et al., 2005; Shikata et al., 2009; Wang et al., 2009; Wang et al., 2008; Wu et al., 2009; Wu and Poethig, 2006), maize (Chuck et al., 2007), rice (Xie et al., 2006), switchgrass (Chuck et al., 2011; Fu et al., 2012), poplar (Wang et al., 2011), tomato (Zhang et al., 2011) and *Torenia fournieri* (Shikata et al., 2012). In *Arabidopsis*, inactivation of miR156 with a target site mimic (35S::MIM156) (Franco-Zorrilla et al., 2007; Todesco et al., 2010; Wu et al., 2009), as well as loss-of-function mutations of *MIR156A* and *MIR156C* (Yang et al., 2013; Yu et al., 2013), produce the opposite phenotype: these plants express adult leaf traits precociously, are slow growing, and flower with many fewer leaves than normal. These results demonstrate that miR156 is both necessary and sufficient for the expression of the juvenile vegetative phase, but has other functions as well.

The expression pattern of miR156 is consistent with its role in promoting juvenile development. In *Arabidopsis* (Jung et al., 2012; Jung et al., 2011; Wahl et al., 2013; Wang et al., 2009; Wu et al., 2009; Wu and Poethig, 2006), maize (Chuck et al., 2007), and rice (Xie et al., 2006), miR156 is expressed at high levels in young seedlings and at lower levels in older plants. An analysis of miR156 levels in fully expanded leaves of species that undergo significant changes in leaf morphology during vegetative phase change (specifically, *Acacia confusa*, *Acacia colei*, *Eucalyptus globulus*, *Hedera helix*, and *Quercus acutissima*) demonstrated that juvenile leaves have significantly higher levels of miR156 and miR157 than adult leaves (Wang et al., 2011), and showed that variation in leaf morphology in the transition zone between these two phases was correlated with intermediate levels of these miRNAs (Figure 1). Along with the results of the genetic analyses mentioned above, these results strongly suggest that vegetative phase change is mediated by a decline in the level of miR156/miR157. From a practical standpoint, these observations also indicate that the relative levels of miR156/miR157 can be used as molecular markers for shoot identity in situations in which it is difficult to identify these phases on the basis of morphological criteria.

5. miR156 targets

miR156 acts by repressing the expression of *SQUAMOSA PROMOTER BINDING PROTEIN (SBP/SPL)* genes, which encode plant-specific transcription factors (Klein et al., 1996; Cardon et al., 1999). Three of the 13 *SBP* genes in moss (Axtell et al., 2007; Riese et

al., 2007), 11 of the 19 *SBP* genes in rice (Xie et al., 2006), and 11 of the 17 *SPL* genes in the Columbia accession of *Arabidopsis thaliana* (Gandikota et al., 2007; Rhoades et al., 2002) have sequences complementary to this miRNA. miR156 represses the expression of *SPL* genes by directing the cleavage of their transcripts and by promoting translational repression. *SPL* transcripts with sequences complementary to miR156 are cleaved at the expected position within this sequence (Addo-Quaye et al., 2008; German et al., 2008), and increased levels of miR156 typically lead to a decrease in the abundance of these transcripts (Chuck et al., 2007; Kim et al., 2012; Sanchez et al., 2011; Schwab et al., 2005; Wu and Poethig, 2006; Xie et al., 2006). Conversely, mutations that block miRNA activity (Ronemus et al., 2006; Smith et al., 2009), as well over-expression of a miR156 target site mimic (Franco-Zorrilla et al., 2007; Wang et al., 2008; Wu et al., 2009), produce an increase in *SPL* transcript levels. Evidence that miR156 is also capable of inhibiting translation initially came from the observation that the abundance of the *SPL3* protein was not correlated with the abundance of the *SPL3* mRNA in lines transformed with *35S::SPL3*; in particular, it was noted that transgenic seedlings with elevated levels of the *SPL3* mRNA displayed no increase in the *SPL3* protein (Gandikota et al., 2007). Subsequent studies demonstrating that mutations in the microtubule-binding protein, katanin (Brodersen et al., 2008), and the GW-repeat protein, *SUO* (Yang et al., 2012), increase the abundance of the *SPL3* and *SPL9* proteins without affecting the level of their mRNAs provided additional evidence that plant miRNAs are capable of mediating translational repression. Although the mechanism by which katanin promotes miRNA function is still unknown, GW-repeat proteins in animals have well-characterized roles in miRNA-mediated translational repression and mRNA turnover (Braun et al., 2013; Ding and Han, 2007) and it may be that *SUO* acts in a similar fashion. In any case, it is significant that the phenotype of *suo* mutations is largely attributable to a reduction in the activity of miR156 (Yang et al., 2012). This result demonstrates that translational repression is important for the normal function of miR156, and implies that activity of *SPL* genes cannot be accurately predicted from the levels of their transcripts in cells in which miR156 is expressed.

Evidence that the effect of miR156 on vegetative phase change is attributable to its effect on *SPL* expression comes from the phenotype of *SPL* genes with mutations in the miR156 target site. Transgenic plants constitutively expressing wild-type *SPL3*, *SPL4* and *SPL5* transcripts have a nearly normal phenotype, whereas plants transformed with miR156-resistant versions of these transcripts flower extremely early, and express some adult vegetative traits precociously (Cardon et al., 1997; Gandikota et al., 2007; Jung et al., 2011; Wang et al., 2009; Wu and Poethig, 2006; Yamaguchi et al., 2009). Similarly, transgenic plants expressing miR156-resistant *SPL9*, *SPL10* or *SPL13* transcripts have a much stronger phenotype than plants expressing the corresponding wild-type versions of these transcripts (Martin et al., 2010; Shikata et al., 2009; Wang et al., 2008; Wu et al., 2009). However, the best evidence that miR156 regulates phase change through its effect on *SPL* gene expression is provided by an EMS-induced mutation of *SPL15*, whose precocious phenotype is attributable to a single nucleotide change in the miR156 target site that leads to an increase in the expression of the *SPL15* transcript (Usami et al., 2009). These results demonstrate that miR156 has a major effect on the expression of *SPL* genes and suggest that it acts primarily, if not exclusively, by repressing the expression of this gene family.

Phylogenetic analyses indicate that *SBP/SPL* genes with miR156 target sites can be grouped into 5 clades (Guo et al., 2008; Salinas et al., 2012). The phenotypes of plants transformed with miR156-resistant forms of these genes, as well as their loss-of-function phenotypes, suggest that they regulate many different aspects of plant development. These include glume architecture (Wang et al., 2005), inflorescence branching/bract development (Chuck et al., 2010), and ligule differentiation (Moreno et al., 1997) in maize; panicle (Jiao et al., 2010; Miura et al., 2010) and grain morphology (Wang et al., 2012) in rice; fruit ripening in tomato (Manning et al., 2006); and leaf initiation (Martin et al., 2010; Wang et al., 2008), embryo development (Nodine and Bartel, 2010), pollen development (Xing et al., 2010), and trichome (Gan et al., 2011) and anthocyanin patterning (Gou et al., 2011) in the inflorescence in *Arabidopsis*. Thus, *SBP/SPL* genes regulate a variety of processes in addition to those associated with vegetative phase change.

The divergent functions of *SPL* genes are apparent from the phenotypes of plants over-expressing these genes (Cardon et al., 1997; Martin et al., 2010; Usami et al., 2009; Wang et al., 2008; Wu et al., 2009; Wu and Poethig, 2006). However, their normal functions have been difficult to establish because of the high degree of functional redundancy within this family. *SPL8* (Unte et al., 2003; Zhang et al., 2007), *SPL14* (Stone et al., 2005), and *SPL9* are the only members of this family in *Arabidopsis* that have a loss-of-function phenotype on their own, and of these only *SPL9* is regulated by miR156. *SPL9* and *SPL15* are members of the same clade. *spl9* mutations produce a slight increase in the rate of leaf initiation and a very weak juvenilized phenotype, and *spl15* mutations are nearly wild-type in appearance. However, plants mutant for both genes have a prolonged juvenile phase, are late flowering, have an increased number of branches, and an increased rate of leaf initiation (Schwarz et al., 2008; Wang et al., 2008). *spl9* and *spl15* also enhance the male sterility phenotype of *spl8*, demonstrating that they act redundantly with this gene to promote pollen development (Xing et al., 2010). *SPL2*, *SPL10* and *SPL11* are also members of a single clade in *Arabidopsis*. *spl2* mutations have no apparent loss-of-function phenotype, but slightly enhance the delayed phase change phenotype of *spl9 spl15* (Schwarz et al., 2008) and the male sterile phenotype of *spl9 spl15 spl8* (Xing et al., 2010). *SPL10* and *SPL11* are closely related, tandemly duplicated genes, and have little or no loss-of-function phenotype alone, or in combination with *spl2* (Shikata et al., 2009). To get around this problem, Shikata and colleagues (Shikata et al., 2009) fused *SPL10* to the SRDX transcription repression domain, and expressed this protein in transgenic plants using both the constitutive 35S promoter and the endogenous *SPL10* promoter. This approach was expected to produce a phenotype similar to the loss-of-function phenotype of *SPL10* on the assumption that *SPL10* normally acts to promote gene expression. Transgenic plants had narrow rosette leaves, stunted inflorescences, reduced apical dominance, defective flowers and siliques, and abnormally round cauline leaves with excessive numbers of trichomes, but had a normal pattern of abaxial trichome production and flowered normally. Unfortunately, this phenotype is largely inconsistent with the phenotype of plants expressing miR156-resistant *SPL10* transcripts, whose major feature is the accelerated expression of adult traits (Wu et al., 2009). Given that both these phenotypes are produced by mutant transgenes, it is difficult to know which phenotype more accurately reflects the normal function of *SPL10*.

6. Molecular insights into the phenomenology of vegetative phase change

How are vegetative phases specified?

Vegetative development is commonly divided into two phases—a juvenile and adult phase. However, patterns of heteroblastic variation are often more complex than this. For example, in *Eucalyptus* shoot development is typically divided into seedling, juvenile, transitional, and adult phases (Blake, 1953; Boland et al., 2006), and similar categories have been described in several species native to New Zealand (Day et al., 1997; Gould, 1993). Even in weakly heteroblastic species, such as *Arabidopsis* (Telfer et al., 1997) and maize (Bongard-Pierce et al., 1996), the first two leaves differ from other juvenile leaves in a variety of ways, and could easily be considered a distinct leaf type. Can these patterns be reconciled with a model of shoot maturation based on changes in the abundance of miR156?

Northern analysis shows that miR156 is present throughout shoot development, but is present at different levels at different times in development. In *Arabidopsis*, miR156 declines rapidly within two weeks after germination (Jung et al., 2012; Wu et al., 2009; Wu and Poethig, 2006), and then declines slowly over several weeks in plants grown in short days to delay flowering (Wahl et al., 2013; Wang et al., 2009). If quantitative variation in the abundance of miR156 produces corresponding variation in *SPL* gene expression, it is not difficult to imagine how continuous variation in the expression of this miRNA could lead to multiple, apparently discrete leaf types. Preliminary evidence for this scenario is provided by *Acacia colei*, where the abundance miR156 and miR157 have been measured in individual juvenile, transition, and adult leaves (Figure 1). In this species, miR156 and miR157 are present at high levels in juvenile leaves, at intermediate levels in transition leaves, and at low levels in adult leaves (Wang et al., 2011). In other species, miR156 expression has only been measured in entire plants, in shoot apices containing multiple leaves, or in completely juvenile or adult leaves, so it is unknown how tightly its expression is correlated with the expression of the morphological traits that are used to define vegetative phases. The precise relationship between miR156 levels, target gene expression, and leaf morphology will need to be assessed in order to determine if this regulatory system is capable of generating the diversity of heteroblastic patterns seen in nature. It is of particular interest to determine how this system operates to specify intermediate developmental states. At present, the juvenile phase is probably best defined as the period during which miR156 expression is sufficiently high to completely, or nearly completely suppress the production of *SPL* proteins.

The relationship between vegetative and reproductive maturation

One of the most confusing issues in shoot maturation is the relationship between vegetative and reproductive aspects of this process (Jones, 1999). Most plants only flower when they are in an adult vegetative phase, but the timing of flower production varies widely relative to the timing of vegetative phase change: some plants flower immediately after this transition, others remain in an adult vegetative phase for a long time before flowering, and some species routinely flower in a juvenile vegetative phase (Brown et al., 2006; Hopper and Maslin, 1978; Potts and Wiltshire, 1997; Wiltshire et al., 1991; Zimmerman et al., 1985). Furthermore, genetic analyses of vegetative phase change and floral induction indicate that

these developmental transitions are inherited independently in *Eucalyptus* (Jordan et al., 1999; Wiltshire et al., 1998), *Pisum* (Wiltshire et al., 1994), maize (Abedon et al., 1996), and *Arabidopsis* (Telfer et al., 1997).

Although these observations suggest that vegetative phase change and floral induction may have little to do with each other, they are readily explained by the architecture of the regulatory pathways that control these transitions. Genetic analyses of flowering time in *Arabidopsis* have shown that floral induction is regulated by multiple inputs (“pathways”) that regulate the expression of genes involved in the transformation of the vegetative meristem into an inflorescence meristem [reviewed in (Amasino, 2010; Wellmer and Riechmann, 2010)]. In *Arabidopsis*, these inputs include photoperiod, prolonged cold temperature (vernalization), ambient temperature, gibberellic acid (GA), sugar, the autonomous pathway, and the vegetative phase change pathway. The ability of a plant to respond to any one of these pathways depends on the state of the other pathways. A good example is the interaction between the vernalization and photoperiod pathways. Under laboratory conditions—but not necessarily in the field (Wilczek et al., 2009)—plants that contain functional alleles of *FRI* and *FLC* require several weeks of exposure to cold in order to respond to floral inductive long-day (LD) conditions; i.e., in the absence of a cold treatment, the vernalization pathway over-rides the photoperiod pathway. On the other hand, vernalized plants only flower early if they are grown under LD, demonstrating that vernalization by itself does not induce flowering. Rather, it creates a permissive state in which other factors can operate.

The vegetative phase change pathway resembles the vernalization pathway in the sense that it regulates the competence of the shoot to respond to various conditions that promote flowering rather than by directly mediating flower production. High levels of miR156 presumably set a threshold that buffers fluctuation in the abundance of SPL transcripts, preventing premature floral induction. Evidence that this pathway impacts reproductive competence was initially obtained in maize, where it was found that *Teopod2*—a hypermorphic mutation of miR156—delays the photosensitive period for floral induction (Bassiri et al., 1992). In *Arabidopsis*, over-expressing miR156 delays flowering, whereas reduced levels of miR156 accelerate flowering under LD (Schwab et al., 2005; Schwarz et al., 2008; Wang et al., 2009). However, plants with reduced levels of miR156 do not flower earlier than normal in a non-inductive short day (SD) photoperiod (Wang et al., 2009). These results suggest that miR156 represses flowering early in development when it is highly expressed, and that the decline in its expression creates a permissive state for floral induction. During the adult phase, floral induction is dependent on other factors—such as photoperiod, vernalization or GA signaling—which regulate the transcription of *SPL* genes and other genes involved in flowering, but have no effect on the expression of miR156 (Jung et al., 2012; Jung et al., 2011; Wang et al., 2009; Yu et al., 2012) (Figure 2).

One of the ways in which miR156 represses flowering is through its effect on miR172, a miRNA that represses several AP2-like transcription factors that inhibit flowering. High levels of miR172 promote flowering by repressing the expression of these floral repressors [reviewed in (Zhu and Helliwell, 2011)]. miR172 is strongly up-regulated when adult plants are transferred from SD to LD (Jung et al., 2007; Schmid et al., 2003), but its expression

also increases early in development in the absence of a LD stimulus (Wu et al., 2009). This early increase in miR172 is complementary to the decrease in miR156 expression, and is thought to be a consequence of this decrease because plants over-expressing miR156 have reduced levels of miR172, whereas plants with reduced levels of miR156 have elevated levels of miR172 (Jung et al., 2011; Wu et al., 2009). miR156 represses miR172 through its effect on the expression of *SPL9*, *SPL10* and related *SPL* genes. Transgenic plants expressing miR156-resistant *SPL9* transcripts under the regulation of the endogenous *SPL9* promoter have elevated levels of miR172 and flower early (Wang et al., 2009; Wu et al., 2009). This phenotype is attributable to the increased transcription of *MIR172B*, which is a direct target of *SPL9* (Wu et al., 2009). A reasonable hypothesis is that high levels of miR156 during the juvenile phase reduce the sensitivity of plants to a photoperiodic stimulus (i.e., reduce reproductive competence) by repressing the expression of *SPL9* and other *SPL* genes that promote the transcription of *MIR172B*. This hypothesis is supported by the observation that *spl9 spl15* double mutants are late flowering and display a delayed sensitivity to photoperiod (Schwarz et al., 2008).

Three other targets of miR156 that have been implicated in flowering are *SPL3*, *SPL4* and *SPL5*. Indeed, the first evidence that the miR156-SPL interaction plays a role in shoot maturation was the observation that plants over-expressing miR156-resistant forms of these genes flower early (Cardon et al., 1997; Gandikota et al., 2007; Wu and Poethig, 2006). In addition to being directly repressed by miR156, *SPL3/4/5* are transcriptionally repressed by the AP2-like proteins targeted by miR172, and are thus indirectly repressed by miR156 through its effect on *miR172B* expression (Jung et al., 2011; Wu et al., 2009) (Figure 2). *SPL3/4/5* are expressed at relatively low levels in SD, and at much higher levels in LD (Cardon et al., 1999; Schmid et al., 2003). This response to photoperiod is independent of miR156 because miR156 levels are identical in plants grown in LD and SD (Jung et al., 2012); furthermore, mutations in genes required for the photoperiodic response do not affect miR156 levels (Wang et al., 2009). Photoperiodic regulation of *SPL3/4/5* is mediated by FT (Schmid et al., 2003) through its effect on the transcription of *SOC1* and *FD*, both of which directly promote the transcription of *SPL3* (Jung et al., 2012). *SPL3*, in turn, directly regulates the transcription of several genes involved in flowering, including *FUL*, *LFY*, *API*, and *FT* (Wang et al., 2009; Yamaguchi et al., 2009).

Given that photoperiod is capable of inducing *SPL3/4/5* expression independently of miR156, what role does miR156 play in the regulation of these genes during shoot development? One possibility is that miR156 over-rides the inductive effects of LD during the juvenile phase, and this hypothesis is supported by the observation that plants over-expressing miR156-sensitive *SPL3/4/5* transcripts flower only slightly earlier than normal in LD (Wu and Poethig, 2006). Apparently, miR156 levels are sufficiently high at this stage that even elevated levels of *SPL3/4/5* transcripts cannot overcome repression by these miRNAs. miR156 has also been postulated to play a role in floral induction in SD (Wahl et al., 2013; Wang et al., 2009). Under these conditions, miR156 levels decline gradually at the shoot apex, and *SPL3/4/5* transcripts increase in a reciprocal fashion. Unfortunately, there is still no evidence that decline in miR156 is responsible for the increase in *SPL3/4/6* transcripts under SD, nor is there conclusive evidence that these genes play a role in floral

induction under either LD or SD conditions. For example, the phenotype of plants expressing miR156-resistant versions of *SPL3/4/5* transcripts under the regulation of their endogenous promoters has yet to be described. If miR156 indeed plays an important role in the regulation of *SPL3/4/5*, these plants should have an early flowering phenotype. More importantly, plants with reduced levels of miR156 do not flower early under SD (Wang et al., 2009). They produce fewer leaves than wild-type plants because they initiate leaves more slowly (Wang et al., 2008), but they produce flowers at the same time as wild-type plants. Another major problem is that the loss-of-function phenotype of *SPL3*, *SPL4* and *SPL5* is still unknown. The only existing loss-of-function mutation of *SPL3* has no obvious phenotype (Wu and Poethig, 2006). Whether this is because the mutation is hypomorphic (Kim et al., 2012), because of functional redundancy with *SPL4* and *SPL5*, or because *SPL3* has only a minor role in shoot development remains to be determined. Viral-induced gene silencing of a homolog of *SPL3/4/5* in *Antirrhinum majus* delays flowering (Preston and Hileman, 2010), which is consistent with the early flowering phenotype of plants over-expressing *SPL3/4/5*. But it will take additional work to establish the function of these genes in *Arabidopsis*.

How is timing of vegetative phase change regulated?

A remarkably comprehensive but little known study of vegetative phase change in *Eucalyptus tenuiramis* (Wiltshire and Reid, 1992) illustrates some of the common features of this process. One long-standing question is whether the timing of this process is dependent on the age or the size of the shoot (Day et al., 2002; Greenwood et al., 2010; Mencuccini et al., 2007; Robinson and Wareing, 1969; Vanderklein et al., 2007). Wiltshire and Reid (1992) showed that the node at which vegetative phase change occurs is more closely correlated with the total number of nodes produced up until the transition point than with the height of the shoot or the duration of shoot growth, and is more strongly correlated with the cumulative amount of light received by the shoot than with day length or temperature. These results suggest that vegetative phase change is controlled by a factor that changes with leaf number, and suggest that the production of this factor is light dependent. Wiltshire and Reid addressed the question of whether phase change is regulated by factors from the root system by reciprocally grafting *E. tenuiramis* and *E. risdonii*. These cross-compatible sister species differ primarily in the timing of vegetative phase change; *E. tenuiramis* undergoes vegetative phase change at the 36th node, on average, whereas *E. risdonii* remains permanently juvenile. Grafting adult shoots from *E. tenuiramis* onto an *E. risdonii* root stock or juvenile shoots of *E. risdonii* onto an *E. tenuiramis* root stock had no effect on the timing of vegetative phase change in the scions, suggesting that the timing mechanism is localized to the shoot.

In *E. tenuiramis* (Wiltshire and Reid, 1992), as in other plants (Schaffalitzky de Muckadell, 1954; Telfer et al., 1997), the phase identity of a branch matches the phase identity of the primary node from which it arises. Branches at juvenile nodes produce approximately the same number of juvenile leaves as the primary shoot subsequently produces, whereas branches at adult nodes immediately produce adult leaves. Because the lateral buds that produce branches arise *de novo* from stem/leaf tissue, this phenomenon suggests that the developmental identity of a lateral bud is determined by the identity of the cells from which

it originates, i.e., that the identity of leaf or stem cells determined the fate of a lateral bud. An alternative possibility is that the factors responsible for vegetative phase change act globally throughout the shoot, simultaneously transforming both the primary shoot apex and lateral buds and immature branches. For this to be true, branches at different positions on the shoot would have to be at different growth stages at the time of vegetative phase change in order to account for the gradient in the number of juvenile leaves produced by these branches.

The most striking result to emerge from this study (Wiltshire and Reid, 1992) concerns the behavior of epicormic buds. Epicormic buds are developmentally arrested accessory meristems that form at the base of primary lateral buds, and only emerge after the primary shoot is damaged or decapitated. In contrast to primary lateral buds, which produce branches with slightly more juvenile leaves than the primary shoot, epicormic buds from the base of mature trees produce fewer juvenile nodes than the primary shoot. Wiltshire and Reid noted that these buds were present in an arrested state while the shoot was undergoing phase change and concluded “although apical meristems have an innate timing...other meristematic tissue (such as accessory buds) can be influenced by the status of the whole plant”. This is the first indication that vegetative phase change is associated with changes in the character of organs and tissues throughout the entire shoot, not just within the shoot apex. It raises the question of whether the signals that regulate this process act specifically on or within the shoot apical meristem (SAM), or operate more generally on organs and tissues throughout the shoot.

This question has been addressed in maize, where adult shoots can be readily rejuvenated. Adult shoot apices with 1 or 2 leaf primordia undergo complete rejuvenation *in vitro* (Irish and Karlen, 1998). The plants that develop from these cultured shoot apices produce the same number of juvenile leaves and flower with the same number of leaves as seed-derived plants. In contrast, explanted shoot apices with 5 or more leaf primordia undergo partial rejuvenation (Orkiszewski and Poethig, 2000). The leaf primordia present on the apex at the time it was explanted into culture develop juvenile tissue at their base, and these partially rejuvenated leaves are followed by one or two completely juvenile leaves before the shoot reverts to the adult phase and produces flowers. Importantly, the number of leaves produced by explanted shoots is identical to the number of leaves they would have produced *in situ*. That is, the fate of the SAM is unaffected by this treatment. These results indicate that the phase identity of a leaf is specified independently of the phase identity of the SAM, and implies that the factors that regulate juvenile vs. adult leaf identity act directly on leaf primordia, and can modify their identity even after they have been initiated.

To identify the source of these factors, Yang and colleagues deleted the root system, cotyledons, and leaf primordia of juvenile *Arabidopsis* seedlings, and monitored the effect of these treatments on leaf identity and the expression of miR156 (Yang et al., 2011). They found that phase change occurred normally in the absence of the root system and in plants lacking cotyledons, but was delayed by leaf ablation. This effect was associated with an increase in miR156 expression, and was dependent on miR156. miR156 levels also increased significantly in adult shoot apices of maize cultured *in vitro*, which is consistent with the effect of this treatment on shoot identity (Irish and Karlen, 1998; Orkiszewski and

Poethig, 2000), and with the observation that *SPL* gene expression decreases in rejuvenating maize shoots (Strable et al., 2008). The conclusion of this study is that vegetative phase change is promoted by a factor or factors produced by leaves. This conclusion is consistent with earlier studies demonstrating that defoliation and severe pruning prolongs the production of juvenile leaves in both herbaceous (Njoku, 1956) and woody plants (Libby and Hood, 1976; Schaffalitzky de Muckadell, 1954).

Efforts to identify the endogenous factors that regulate vegetative phase change have focused on carbohydrates and on the hormone, gibberellic acid (GA). Depending on the species, GA promotes or suppresses vegetative phase change and flowering (Zimmerman et al., 1985). For example, exogenous GA promotes rejuvenation in English ivy (Rogler and Hackett, 1975) and in *Acacia melanoxylon* (Borchert, 1965), but accelerates vegetative phase change and flowering in maize (Evans and Poethig, 1995) and *Arabidopsis* (Telfer et al., 1997; Wilson et al., 1992). GA has no effect on the expression miR156 in *Arabidopsis*, but promotes the expression of some *SPL* genes (Galvao et al., 2012; Jung et al., 2012; Wang et al., 2009; Yu et al., 2012). This response contributes to GA-mediated flowering under SD conditions because plants over-expressing miR156 (i.e., plants in which *SPL* expression is suppressed) are less sensitive to GA than wild-type plants (Yu et al., 2012). The extent to which GA promotes vegetative phase change via its effect on *SPL* expression is less clear because this hormone has nearly the same effect on vegetative phase change in wild-type plants, plants over-expressing miR156, and plants doubly mutant for *spl9* and *spl15* (Schwarz et al., 2008). In this respect, it is interesting that GA levels do not increase in the shoot apical meristem within the first two weeks after germination (Eriksson et al., 2006); GA would be expected to increase during this period if it played a major role in vegetative phase change.

Evidence for the involvement of carbohydrates in vegetative phase change has been accumulating for over 100 years. Goebel (Goebel, 1900) was the first to propose that nutrients play a crucial role in this transition, and he performed many experiments to test this hypothesis (Goebel, 1908). A particularly comprehensive analysis of the effect of specific nutrients on leaf morphology was conducted by Allsopp using the water fern *Marsilea drummondii* (Allsopp, 1954, 1963). Allsopp showed that plants grown in the absence of exogenous sugar produced only simple, juvenile leaves (Allsopp, 1952), and found that supplementing the growth medium with any of several metabolizable sugars accelerated the production of adult leaves (Allsopp, 1953b). Adult shoots transferred to a medium lacking exogenous carbohydrates reverted to the juvenile form (Allsopp, 1953a). Similar results have been obtained with isolated shoot apices (Feldman and Cutter, 1970; Njoku, 1971), and isolated leaf primordia (Sussex and Clutter, 1960) cultured on media containing varying amounts of sugar.

Recent studies in *Arabidopsis* suggest that the striking effect of sugar on heteroblastic development is attributable to its effect on the expression of miR156 (Yang et al., 2013; Yu et al., 2013). Mutations in the chlorophyll biosynthetic gene, *chl1*, as well as several other yellow-green mutations, prolong the juvenile phase (Röbbelen, 1957; Yang et al., 2013b; Yu et al., 2013b). *chl1* has elevated levels of miR156, and its effect on vegetative phase change is dependent on miR156, as demonstrated by the observation that a reduction in the level of

miR156 blocks the vegetative phase change phenotype of *chl*. The possibility that the phenotype of *chl* is attributable to reduced carbohydrates was tested by examining the effect of various sugars on the level of miR156 and vegetative phase change in mutant and wild-type plants. Glucose or sucrose accelerated vegetative phase change and reduced the abundance of miR156 in *chl* and wild-type plants when applied to whole seedlings, isolated leaf primordia, or to petiole stubs on defoliated seedlings (Yang et al., 2013; Yu et al., 2013). These studies also showed that *MIR156A* and *MIR156C* are the major sources of miR156 in Arabidopsis seedlings, and are specifically down-regulated by sugar. These results therefore support the long-standing hypothesis that vegetative phase change is mediated by an increase in the nutritional status of the shoot.

The identity of the endogenous sugars and signaling pathways involved in vegetative phase change is still largely unknown, although there has been some progress on this front. HEXOKINASE1 (HXK1)—a regulator of glucose signaling in Arabidopsis—promotes the accumulation of miR156 in seedlings grown in the absence of exogenous sugar and is required for the effect of glucose on miR156 expression, but does not block the decline in the abundance of this miRNA during shoot development (Yang et al., 2013b). This result implies that glucose promotes vegetative phase change via the HXK1 signaling pathway, but is not completely responsible for this developmental transition. Another sugar that may be involved in vegetative phase change is trehalose-6-phosphate (T6P). In Arabidopsis, T6P is synthesized from glucose-6-phosphate by TREHALOSE PHOSPHATE SYNTHETASE 1 (TPS1) (Blazquez et al., 1998). Null alleles of *TPS1* are embryo lethal, but this phenotype can be corrected by transiently expressing TPS1 during embryo development (van Dijken et al., 2004), or avoided by using hypomorphic alleles (Gomez et al., 2010). *tps1* plants are extremely late flowering due to the reduced expression of the floral inducer FT (Wahl et al., 2013). In addition to having low levels of FT, *tps1* mutants have elevated levels of miR156 and reduced levels of at least three miR156-regulated transcripts—SPL3, SPL4 and SPL5 (Wahl et al., 2013). Although the functional significance of the effect of *tps1* on miR156 and SPL3/4/5 expression has not been determined, this phenotype suggests that T6P may play a role in vegetative phase as well as in floral induction.

7. Conclusion

Genetic analyses of vegetative phase change and flowering in model organisms such as *Arabidopsis*, maize, and rice have produced significant advances in our understanding of the molecular mechanisms of shoot maturation in plants. These studies have shown that miR156/miR157 coordinate events in vegetative and reproductive development by post-transcriptionally repressing the expression of a group of functionally differentiated SBP/SPL transcription factors. This regulatory mechanism not only explains how temporal changes in phase-specific traits are coordinated, but also provides an explanation for the apparent independence of many of these traits. In particular, the identification of *SBP/SPL* genes that regulate both vegetative traits and floral induction reveals the basis for the association between vegetative phase change and reproductive competence. At the same time, the fact that *SBP/SPL* genes are regulated independently of each other at transcriptional level explains how traits that are normally coordinated can become dissociated in time or space. For example, increased transcription of *SBP/SPL* genes that promote floral induction

without a corresponding increase in the transcription of *SBP/SPL* genes that regulate vegetative phase change may explain the phenomenon of flowering during the juvenile phase. One major unanswered question is how the timing of vegetative phase change is regulated. Leaves produce a signal that promotes vegetative phase change, and there is good evidence that sugar is a component of this signal. But it remains to be determined if this leaf signal is an active or permissive regulator of vegetative phase change, and whether other factors also play important roles in the timing of this developmental transition.

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Figure 1.

The abundance of miR156, miR157, and miR172 is correlated with the vegetative phase of woody species. A–E, Juvenile and adult leaves of A) *Acacia confusa*, B) *Acacia colei*, C) *Eucalyptus globulus*, D) *Hedera helix* (English ivy), and E) *Quercus acutissima* (sawtooth oak). The leaves shown in A and B are successive leaves on the same shoot. F) Northern blots of small RNA from the leaves shown in A–E, hybridized with probes to the indicated transcripts. Scale bars correspond to 2 cm. Modified from (Wang et al, 2011).

Figure 2.

miR156 controls reproductive competence by repressing the expression of genes required for flower production. The pathways illustrated here are based on validated miRNA-target interactions and transcription factor binding studies described in the text. Black indicates genes that are expressed; gray represents repressed genes.