Evolution of morphology includes evolutionary shifts of developmental processes in space or in time. Heterochrony is defined as a temporal shift. The concept of heterochrony has been very rewarding to investigators of both animal and plant developmental evolution, because it has strong explanatory power when trying to understand morphological diversity. While for animals, extensive literature on heterochrony has been elucidated along with the field of evolution of development, in plants the concept has been applied less often and is less elaborately developed. Yet novel genetic findings highlight heterochrony as a developmental and evolutionary process in plants. Similar to what has been found for the worm Caenorhabditis, a heterochronic gene pathway controlling developmental timing has been elucidated in flowering plants. Two antagonistic microRNAs miR156 and miR172 target two gene families of transcription factors, SQUAMOSA PROMOTOR BINDING PROTEIN-LIKE and APETALA2-like, respectively. Here, we propose that this finding now allows the molecular investigation of cases of heterochronic evolution in plants. We illustrate this point by examining microRNA expression patterns in the Antirrhinum majus incomposita and choripetala heterochronic mutants. Some of the more beautiful putative cases of heterochronic evolution can be found outside flowering plants, but little is known about the extent of conservation of this flowering plant pathway in other land plants. We show that the expression of an APETALA2-like gene decreases with age in a fern species. This contributes to the idea that ferns share some heterochronous gene functions with flowering plants.

Keywords: microRNA156, microRNA172, choripetala, incomposita, Ceratopteris, heterochrony, APETALA2, SPL.

INTRODUCTION

Time is a fundamental aspect of all developmental processes. It plays a role in different types of development, such as growth or differentiation and at different scales, whether it be cellular, at the tissue or at the organ level (Moss, 2007). In the discipline of evolution and development, evolutionary changes in the regulation of developmental time or “heterochrony” were once proposed to explain much of morphological diversity (Gould, 1977). To test whether this is indeed the case, it is necessary to be able to unambiguously identify cases of heterochrony. Developmental time is now known in several organisms to be controlled by endogenous mechanisms that interact with endogenous and environmental stimuli (Slack and Ruvkun, 1997, 1998; Moss, 2007; Huijser and Schmid, 2011). Also in plants, a “heterochronic pathway” has been elucidated (Chuck et al., 2007a,b; Wu et al., 2009). For both plants and animals, several classic examples of morphological evolution have been proposed to be heterochronic in nature, suggesting that the regulation of a heterochronic pathway has evolved in these instances. The discovery of a heterochronic pathway contributes to the testability of these hypotheses of regulatory evolution. Ultimately, it should become possible to answer such questions as, “Is evolution of developmental timing frequent in plants?” “Can it explain most of morphological diversity?” “What types of morphological consequences can evolution of developmental timing have?” Or more generally is heterochrony indeed such an important developmental process in the evolution of morphology? Here we mostly review some of the elaborate literature on heterochronous evolution and how it can be applied in the field of plant evolution and development.

TIME AND RATE IN PLANT DEVELOPMENT

In contrast to animal development, plant development entails the continuous development of new organs as time progresses. This open developmental shoot system generates different organs depending on the age of the plant. The different types of above ground leaf-like organs that develop result in a “heteroblastic” sequence observable in the mature plant (Allopp, 1967; Zott et al., 2011). This sequence starts with embryonic leaves or cotyledons, then juvenile leaves develop, these transition into adult leaves and finally inflorescence leaves or bracts develop. The floral organs can be seen as a continuation of this sequence of different leaf types, with sepals and petals still resembling leaves. While stamens and carpels do not resemble leaves in most species, they can still be interpreted as such (Figure 1). von Goethe, 1790, Arber, 1937, Takhtajan, 1976). A similar sequence can be observed in monococious inflorescences, in which lower positioned unisexual male flowers develop earlier than unisexual female flowers. Also in dioecious species, the different floral types are associated with a timed change in identity resulting in either closed flowers that obligately self or open flowers that can outcross (Lord and Hill, 1987). Regular time obviously progresses at a constant rate. However, what is called developmental time or age can be fast or slow, meaning that development can be accelerated or retarded relative...
TYPES OF HETEROCHRONY

Several attempts have been made to classify types of morphological heterochrony based on the possible outcomes of allometric variation of morphology and inferences of heterochrony. Heterochrony is not limited to morphological observations though and a developmental viewpoint of the concept was elaborated in Raff and Wray (1989). More recently, Smith (2001) untangled the different historical meanings of the term heterochrony by recognizing two identifiable types: “growth heterochrony,” following Gould, and “sequence heterochrony,” more in line with the original usage of Haeckel and de Beer that focuses on the relative timing of developmental events. Here we wish to mainly consider the heterochronic morphological consequences of certain developmental control genes that can also be viewed as heterochronic. Such a direct link between a heterochronic underlying mechanism and a morphological result can, in our view, contribute to the testability of putative morphological cases of heterochrony in either mutants or evolutionary examples. Therefore, we aim to provide the term heterochrony with a clear molecular basis, without aiming to limit or redefine its meaning.

THE RELATION OF HETEROCHRONY TO OTHER MODIFICATIONS OF DEVELOPMENT

At first, it appears easy to contrast heterochrony to other modifications that can occur in the evolution of development. Heterotopy for instance, is defined as a developmental process affected in location, while heterochrony is a process affected in time. However, a shift in timing of development can also result in a change in location. For instance a petal primordium could develop later than usual and as a consequence also shift in position. This illustrates that strictly using morphological observations, it is difficult to distinguish modifications in ontogeny. In previous discussions of heterochrony in plants, there was no mention yet of heterochronic genes or a pathway identified. However, now that a pathway has been identified, by investigating the mechanistic (molecular) process behind a morphological change, a distinction could be made between heterochrony and heterotopy based on the underlying genes affected. A further problem is how to distinguish heterochrony and heterotopy from homeosis, another important category in evolution of development which entails the transformation in evolution of the identity of an organ. We would argue that homeosis can be the result of both heterochrony and heterotopy. For instance a sepal to petal transformation can result from a spatial shift of the petal identity program, but it could also result from a heterochronic shift.

One explicit criticism is that heterochrony is unable to explain the origin of new structures in evolution, as only a shift in time of an existing process is meant by the term (Hoelder, 2013). However, the same criticism could be voiced against homeosis and heterotopy and relates more to the effect of the shift being dynamic or static (Webster and Zelditch, 2005). When dynamic, a modification in size, shape, or identity of the structure occurs during the shift, while when static the structure is only repositioned in time or location.

TYPES OF HETEROCHRONY

Several attempts have been made to classify types of morphological heterochrony based on the possible outcomes of allometric to regular time or relative to other developmental events (Poethig, 2003). Developmental rate of, e.g., plastochron length in case of leaves, is the time that passes between the development of two successive leaves. Developmental rate in plants is counted in numbers of organs that develop per unit of time. Plants are special in this sense because developmental rate can be easily measured in the adult form of the plant as an average number of organs that has developed in a certain period of time, which makes plants a good system to study developmental time.

DEFINITIONS OF HETEROCHRONY

The term heterochrony was first introduced by Ernst Haeckel in the second half of the 19th century (Smith, 2003). It was used to describe deviations from his well-known “Biogenetic Law” which states that the sequence of developmental events largely recapitulates the sequence of events in the evolutionary history of the species. In several books, de Beer uncoupled heterochrony from recapitulation and used the term to denote a relative displacement of a character in its timing of development when comparing two related species (De Beer, 1951). Gould (1977), in his reevaluation of the concept, focused heterochrony again on parallels between or reverse relations of ontogeny and phylogeny and emphasized size and shape as the measures to detect heterochrony. The way the concept has most often been applied and tested is therefore through morphological measurements. Because in development, size and shape tend to change through growth, cases of heterochrony have been documented through a quantitative analysis of size and shape, called allometry (Gould, 1977; Klingenberg, 1998; Webster and Zelditch, 2005; Box and Glover, 2010). This resulted in detailed descriptions of quantitative
Where Gould (1988) used to believe that the cleistogamous flower was a paedomorphic “progenetic dwarf” version of the chasmogamous flower (progenesis), several studies showed that different heterochronous processes are involved in the resulting precocious, but unopened and smaller, flower (Mayers and Lord, 1983a,b; Li and Johnston, 2000). In cleistogamous flowers of Viola odorata for example, pollen maturation initiates earlier compared to the ancestral chasmogamous flower (pre-displacement). But not only the early onset of meiotic processes will lead to precocious flowers, an increased leaf initiation rate and flower formation (acceleration) and a repressed cell expansion (progenesis) will contribute to the final phenotype. Finally, enlarged sepals have been interpreted as vegetative characters displaced into reproductive development (Figure 2C, d). A good example is the inflated calyx of Physalis species after fertilization. This could be interpreted as hypermorphosis, as the organ develops beyond its normal growth and the vegetative character extends into the reproductive phase.

From these examples it becomes clear that identifying the exact type of heterochronous evolution in more realistic examples is often difficult and it can be expected that more than one type is involved in many cases of heterochronous evolution (Li and Johnston, 2000). The identified type of heterochrony can also depend on the chosen point of reference. In animals, the most often chosen point of reference is sexual maturity. In plants, several other points of reference have been used like the initiation of primordia or anthesis as offset (Li and Johnston, 2000; Box and Glover, 2010).

A HETEROCHRONIC PATHWAY IN FLOWERING PLANTS

While extensive literature is available on heterochrony, its definition and typology, at least for plants this does not take into account a now known mechanistic basis of developmental timing. In this paragraph, we provide an updated brief introduction to a basic mechanism of developmental timing, which is extensively reviewed elsewhere (Chuck et al., 2009; Huijer and Schmid, 2011; Zhu and Helliwell, 2011; Schwab, 2012; Yamaguchi and Abe, 2012). What we denote here as one recently discovered “heterochronic pathway” is more specifically the sequential and antagonistic function of two microRNAs, their upstream regulators and downstream effectors or targets.

It has been established that two microRNAs, miR156 and miR172, act as the main players in the regulation of developmental timing in flowering plants (Chuck et al., 2009a,b; Wang et al., 2009; Wu et al., 2009). In early stages of development, miR156 levels are high and they decrease during plant development, while miR172 shows the opposite pattern. These microRNAs contribute to both the juvenile–adult phase transition and the transition to flowering through their sequential and antagonistic actions (Wu et al., 2009). miR156 represses targets of the SQUAMOSA PROMOTOR BINDING PROTEIN-LIKE (SPL) gene family and maintains juvenile features of the plant (Schwab et al., 2005). When miR156 levels decline, the SPL proteins increase and will activate miR172, activate flowering genes, and induce adult leaf features (the functional evolution of SPL genes is reviewed in Preston and Hileman, 2013). miR172 targets 6 members of the AP2-like transcription factor family in Arabidopsis (Aukerman and Sakai, 2003). During the stages when miR172 levels are increasing, the APETALA2-like (AP2-like) genes are progressively silenced and adult leaf traits and flowering is induced.
FIGURE 2 | Illustrative examples of types of heterochronic evolution. (A) Schematic overview of the different types of heterochrony. Developmental time is the time span required to reach a certain developmental stage. In normal ancestral development (a) an organism requires time span $t$ to reach development stage $x$. In Paedomorphosis development is reduced. Depending on the cause three subtypes can be distinguished. Delayed onset is the cause in post-displacement (b). In neoteny (c) development rate is slowed down. In progenesis (d) the normal time span is shortened, development will stop prematurely. In Peramorphosis an extended level of development is achieved. Again, we distinguish three subtypes. In pre-displacement (e) an earlier onset will result in a prolonged time span of development or in early maturation. In acceleration (f) developmental rate is increased. Finally, in hypermorphosis (g) development is continued after normal offset. (B) Hypothetical examples of heterochrony. Figure (a) shows a reference plant with different types of plant organs. Peramorphic develop "beyond" this, while paedomorphic plants retain juvenile features. In post-displacement (b), onset is later and an additional pair of cotyledons develops, in pre-displacement (e), onset is later and no cotyledons develop. In neoteny, less organs develop with larger internodes (c), while in acceleration (f), more organs develop with shorter internodes. In progenesis (d), offset is earlier and no bract develops, while in hypermorphosis (g), offset is later and additional bracts develop. (C) Examples of heterochrony taken from the literature. See text for explanation.
Even though much progress is made in understanding the regulation of phase transitions through microRNAs and their downstream effectors, the upstream molecular mechanisms are just starting to be understood only in *Arabidopsis thaliana*. Recently it was shown that sugars control the miR156 age-dependent decrease (Proveniers, 2013; Yang et al., 2013; Yu et al., 2013). When growing older, the plant accumulates sugar through increasing photosynthesis activity. Sugar in turn represses miR156 expression at the transcriptional and post-transcriptional level, causing miR156 to decrease (Yang et al., 2013; Yu et al., 2013). miR172 levels can also be influenced by other environmental factors. SHORT VEGETATIVE PHASE (SVP) binds directly to the pri-miR172a promoter and represses transcription at low ambient temperatures (Cho et al., 2012b). Much about its functional origins in sporophyte development may be learned from studying this microRNA in lycopsids, ferns, and gymnosperms. Yet precisely for these plant lineages researchers are confronted with strong methodological limitations, such as the inability to genetically modify species.

ORIGIN AND EVOLUTION OF HETEROCHRONIC GENE FUNCTIONS IN LAND PLANTS

The pathway described above has been best studied in *Arabidopsis thaliana* and maize, and its basic function in controlling developmental timing is likely to be conserved in flowering plants, gymnosperms, and to some extent in ferns (Axtell and Bartel, 2005; Shigyo et al., 2006; Axtell et al., 2007; Floyd and Bowman, 2007; Huijser and Schmid, 2011). In addition the interaction of miR156 with its targets is probably also conserved in mosses (Arazi et al., 2005; Axtell et al., 2007). However, in the moss model system *Physcomitrella patens*, miR156 promotes the development of leafy gametophores, suggesting that its function in flowering plant sporophytes evolved from an opposite function in moss gametophytes (Cho et al., 2012b). The biological significance of these sequences therefore remains unclear. Also our own cloning efforts did not result in miR172 sequences from selected fern species. This is in contrast to the miR172 binding site in an APETALA2-like putative target which is present in ferns but not in lycopsids. In the AP2-like sequences of the lycopod Selaginella, no miR172 binding site is present (Floyd and Bowman, 2007).

While little evidence is available for the presence of miR172 in ferns, in *Ceratopteris thalictroides*, a putative miR172 target sequence has been cloned (Axtell and Bartel, 2005). In the absence of a convincing sequence or expression pattern for a mature miR172 microRNA in ferns, an open question is whether the cloned APETALA2-like genes with miR172 binding site is progressively downregulated in *Ceratopteris* development. To investigate whether this APETALA2-like putative target displays an expression decrease during sporophyte development in ferns, we investigated its expression using qRT-PCR in a developmental time series of the fern *Ceratopteris richardi* sporophyte (Figure 3). We indeed observed a decrease in expression levels with an increased developmental age. Interestingly, we did not find expression to be detectable in the *Ceratopteris* gametophyte, suggesting that this target gene only functions in sporophyte development. These data add to the idea that developmental timing is regulated by AP2-like genes in ferns. The data together suggest that the binding site in AP2 likely evolved before the origin of the cognate microRNA, but that AP2-like genes already are involved in developmental timing in ferns. Because of the likely absence of miR172 in ferns, an alternative mechanism may be responsible for the progressive down regulation of AP2.
Complementary, constitutive expression would result in upward identity shifts along the floral axis. Partially consistent with this prediction, knock-down of miR172 in stamens results in a partial transformation to petals in Arabidopsis (Wollmann et al., 2010). A problem with miR172 knock-down is that one is not necessarily able to generate slightly different levels that result in predictable transformations (Todesco et al., 2010). For this, the genetic analysis of the different miR172 genes will be illuminating. While miR172 ectopic expression in Nicotiana benthamiana results in the transformation of sepals into petals (Mishiba et al., 2008a), miR172 ectopic expression in Arabidopsis results in sepals transformed into carpels and the absence of petals, strongly resembling the ap2 mutant (Chen, 2004). It could be that in Arabidopsis, constitutive expression under the 35S promoter attains too strong level to obtain the expected series of organ identity transformations dependent on the expression level of miR172. Consistent with this idea is that escopic ovules also develop on the leaves in these plants and entire gynoecia in the axil of leaves (Aukerman and Sakai, 2003).

A HETEROCHRONIC INTERPRETATION OF APETALA2 GENE FUNCTIONS

As APETALA2-like genes are under the direct control of miR172, their functions can also be interpreted as heterochronic. While originally, Arabidopsis APETALA2 function was interpreted as A-function in the ABC model (Coen and Meyerowitz, 1991), multiple functions for APETALA2-like genes can now be distinguished. Two functions are involved in the timing of identity transitions, either from the shoot apical meristem into a flower meristem or in the identity transitions of floral organ primordia. A third function is in floral determinacy, the end or offset of development.

A first function of APETALA2 is in timing the specification of the floral meristem by repressing vegetative characteristics from flowers. This function is clear from the phenotypes in several species in which flowers acquire vegetative characters such as the development of bracts and supernumerary sepals (Bowman et al., 1989). Similar phenotypes have been observed for Antirrhinum lpi/lpi2 mutants and also in the rice homolog supernumerary bracts (Litt, 2007). This is the case for Arabidopsis ap2 alleles, in which sepals are often transformed into bracts or leaf-like structures (Bowman et al., 1989). The heterochronic interpretation of these phenotypes is that because of a delayed transition from inflorescence meristem to floral meristem supernumerary bract-like organs develop.

A second function is in timing the identity of the floral organs. In the ABC model, it was proposed that a floral A-function exists that acts to repress C-function from the outer floral organ whorls and contributes to the establishment of sepal and petal identity. Recent findings in Arabidopsis show that the repressive function of APETALA2 is more general and that the outer boundaries of B-function (APETALA3 and PISTILATA) and C-function (SEPALLATA3) are marked by APETALA2 (Kogan et al., 2012). The classic ABC model with homoeotic functions may thus alternatively be viewed as a combination of heterochronic and heterotopic functions to specify floral
organ identity. Heterotopic functions would involve only B- and C-function added onto a ground state of floral meristem identity (Lotz, 2007).

APETALA2 mutants in different species also show other heterochronic phenotypes. A mutant with a weaker phenotype in a maize AP2-like gene is glossy5, which develops adult characteristics in juvenile leaves (Lauster et al., 2003). In barley, the cleistogamy2 mutant was positionally cloned and identified as an AP2-like gene (Nair et al., 2010) which is interesting considering that cleistogamous flowers are a classic example of heterochrony (Loed and Hill, 1987).

DETECTING HETEROCHRONY THROUGH HETEROCHRONIC PATHWAY GENES

Previous review literature on heterochrony in plants (Lord and Hill, 1987; Li and Johnston, 2000; Box and Clever, 2010), discusses the concept of heterochrony in terms of morphological changes in development and not in terms of heterochronic genes. A problem with a morphological definition of heterochrony is that it can become too broadly applicable. Any type of growth or induction at every scale has a time aspect attached to it and such heterochronies would likely have many different underlying causes. This would contribute little to the use of the term heterochrony. Examples of proposed heterochronic evolution may then seem naïve or the application of the term does not appear useful anymore. For instance, it is possible to use the term progenesis for a population of Arabidopsis thaliana, either mutant or natural, that flowers early. The question is whether applying such a term contributes much to our understanding of evolution. It soon becomes possible to call all evolution of development heterochrony when the term is not more strictly applied. However, classic examples of heterochrony that stand a more rigorous test may be present in the literature (see below). The question may thus become how to investigate putative cases using current methods. While flowering time per se may appear to be a phenotype plex. It would also be difficult to classify if their action has modified in comparison to the ancestral form or in comparison to the wild-type, this might be present in the literature (see below). The question may thus become how to investigate putative cases using current methods. While flowering time per se may appear to be a phenotype modification can be investigated using transgenic or genetic approaches. Thoroughly investigating heterochronic evolution at the molecular level should be helpful in correctly identifying the type of heterochrony and determining whether onset, rate or offset of developmental time has changed. In addition, the relative frequency of this heterochrony versus other modes of developmental evolution can be investigated and the types of morphological consequences can be described.

TWO HETEROCHRONIC MUTANTS: Antirrhinum incompesita and choriapeta

We illustrate these above points by investigating the Antirrhinum majus incompesita (inco) and choriapeta (cho) mutants. The inco mutant has been characterized both morphologically and genetically in Masiero et al. (2004) and Wilkinson et al. (2000). While inco is affected in an ortholog of the Arabidopsis MAD2-box gene SVP, the molecular basis of the cho phenotype remains unresolved. Inco flowers characteristically develop prophylls or bract-like structures at the base of their flowers, while these are absent from wild-type Antirrhinum flowers (Figure 4A). Some flowers also display a petaloid sepal phenotype, which inco has in common with cho (Figures 4B,C). The petaloid sepal phenotype in inco is strongly enhanced in a cho background, suggesting that INCO and CHO are functionally related in controlling sepal identity. At least for cho it has been shown that the petaloid sepals show ectopic B-class gene expression (Wilkinson et al., 2000).

From a morphological point of view, the ontogeny of inco and cho mutants has been compared to wild-type flower development using scanning electron microscopy. Such an analysis has the potential to reveal shifts in the relative timing of organ development, or heterochronies. Indeed for inco, the initiation of the lateral sepals is delayed and the primordia are displaced toward the center of the flower primordium. Probably as a consequence, the lateral sepals become fused and petaloid in inco (Masiero et al., 2004). While inco flowers characteristically develop prophylls or bract-like structures at the base of their flowers, while these are absent from wild-type Antirrhinum flowers (Figure 4A), some flowers also display a petaloid sepal phenotype, which inco has in common with cho (Figures 4B,C). The petaloid sepal phenotype in inco is strongly enhanced in a cho background, suggesting that INCO and CHO are functionally related in controlling sepal identity. At least for cho it has been shown that the petaloid sepals show ectopic B-class gene expression (Wilkinson et al., 2000).

To understand whether the heterochronic morphologies of inco and cho can be explained by modifications to the regulation of heterochronic pathway genes, we investigated in both wild-type and cho biological replicates the expression patterns of mature microRNAs 156 and 172 using stem-loop qRT-PCR relative to the housekeeping gene actin. While we retrieved the expected expression pattern in wild-type plants (Figure 4D), remarkably in cho and more strongly in inco mah172 expression is notably higher late in adult development only to strongly decrease rather than increase when flowers develop (Figures 4E,F). These expression patterns illustrate that modifications in developmental timing can be complex. It would also be difficult to classify cho as pre-displaced or neotenic because of the combination of rate effects. Furthermore, if the phenotypes can be (partially) explained by this changed expression pattern, our observations contribute to the notion that heterochronous phenotypes can be diverse.
While in the previous paragraphs we provided a heterochronic ectopic expression of B-function (Litt and Kramer, 2010; Ronse bracts. These studies inconsistently did, or did not observe correlate expression of B-function genes to petal identity in sepals whether the pathway is affected in these instances and how? A heterochronic pathway has been elucidated. The question is sic cases of heterochronic evolution can be investigated now that interpretation of mutant phenotypes, a number of putative clas-
is similar to what has been observed for wild-type Antirrhinum flowers. (C) Inco also occasionally develops petaloid sepals. (D) Petaloid sepals and an unfused corolla can also be observed in chorispetalae (E). The expression pattern of miR156 left axis and miR172 right axis in Antirrhinum is similar to what has been observed for wild-type Arabidopsis and other species. Both in meso (B) and in rho (F), an early increase in miR172 can be observed late in adult development and lower expression levels are present in inflorescence tissue. Error bars represent standard errors of three technical replicates. A second biological replicate gave similar results.

**PUTATIVE CASES OF HETEROCHRONIC EVOLUTION IN PLANTS**

While in the previous paragraphs we provided a heterochronic interpretation of mutant phenotypes, a number of putative clas-
sic cases of heterochronic evolution can be investigated now that a heterochronic pathway has been elucidated. The question is whether the pathway is affected in these instances and how? A series of studies in evolution and development has tried to correlate expression of B-function genes to petal identity in sepals or bracts. These studies inconsistently did, or did not observe ectopic expression of B-function (Litt and Kramer, 2010; Ronse De Craene and Brockington, 2013). It will be interesting to re-investigate some of these studies in light of the idea that APETALA2 is able to repress floral homeotic functions from the outer whorls as shown in Arabidopsis (Kogan et al., 2012). In the two cases in core eudicots we investigated, Davida involucrata and Impatien

![FIGURE 4 | The heterochronic Antirrhinum mutant chorispetalae. (A) Incom-petite, prophylls develop which are absent in wild-type Antirrhinum flowers. (B) Inco also occasionally develops petaloid sepals. (C) Petaloid sepals and an unfused corolla can also be observed in chorispetalae. (D) The expression pattern of miR156 left axis and miR172 right axis in Antirrhinum is similar to what has been observed for wild-type Arabidopsis and other species. Both in meso (B) and in rho (F), an early increase in miR172 can be observed late in adult development and lower expression levels are present in inflorescence tissue. Error bars represent standard errors of three technical replicates. A second biological replicate gave similar results.]

**THE IMPORTANCE AND APPLICABILITY OF HETEROCHRONY**

Heterochrony in animal evolution and development has been rec-ognized as the major evolutionary mechanism contributing to diversity (De Beer, 1951; Gould, 1977). In comparison to ani-
mals, the role attributed to heterochrony in the evolution of plant development is historically smaller, not necessarily reflecting the biological significance of the concept. Indeed, notable exceptions, such as Armen Takhijta, acknowledged a major role for hete-rochrony in plant evolution. Heterochrony by these proponents has been used to explain major, still outstanding questions in botany, such as the neotenic origin of the flower (Takhijta, 1976).
We propose that the molecular study of putative cases of heterochrony will assist in assessing the relative frequency of this type of developmental evolution in comparison to other types. There are obvious and previously noted methodological difficulties when applying the idea of heterochrony to plants. The open development of plants was originally thought to be more difficult to study from a heterochrony point of view (De Beer, 1931). As plant development initiates in closed buds, the primordia are not easily visualized in a dynamic manner. Therefore, measuring the rate of development usually is indirect, through the use of a developmental rate over a prolonged period of time. For instance the counting of leaves that developed in a certain time. For flower development, most studies lack data on either growth rate or relative timing of events. Average developmental rate cannot be measured and needs to be estimated from comparative floral ontogenetic work. While difficult, several of these examples have been reported though not necessarily recognized as heterochrony. As the incompatibility and choricarpus mutants described above (Wilkinson et al., 2000; Masiero et al., 2004). A reason why heterochrony in leaf development (heteroblasty) is thought to be difficult to study is that many plants lack clear morphological markers for the transition from the juvenile to the adult and sexually mature stage. However, now that clear molecular markers are available to study the transition from the juvenile to adult phases in development, this difficulty can be overcome.

We would argue that plants, because they retain previous developmental stages in the adult form, are excellent models to study heterochrony. Even now, our current thinking about plant morphology could be named “heterochronic.” For example the idea of a carpel as essentially a folded young leaf reveals this (Arber, 1937; Takahashian, 1976).

MATERIALS AND METHODS

C-fem (Ceratopteris) spores were obtained from Carolina Biologicals (NC, USA). Antheridiun majus chropicarpus and wild-type seeds were obtained from IPTK Gatersdeben. For Ceratopteris richardii, spores were germinated in liquid (for gametophytes) or on solid (for sporophytes) Basic C-fern medium (NC, USA) in a Conviron Adapis growth cabinet at 25°C under 200 μmol photons per meter squared per second (photosynthetic photon flux density PPFD) of cool white light in a 16 h light, 8 h dark cycle. To sample gametophytes, spores were germinated in an erlenmeyer and harvested 10 days after inoculation by centrifugation. Sporophytes were germinated in Magenta jars and sampled after homogenization in liquid nitrogen. RNA was extracted using the Plant RNA Reagent (Invitrogen) for Ceratopteris and using TRIzol (Invitrogen) for Antirrhinum according to the manufacturers protocol. DNA present in the RNA prep was degraded using Turbo DNase (Invitrogen) and degradation was confirmed by PCR amplification of actin and evaluation using agarose gel electrophoresis. DNA free RNA was then reverse transcribed using the cDNA Reverse Transcription kit (Promega) according to the manufacturer’s procedures. Before using the cDNA for qRT-PCR, whether the cDNA was amplifiable was tested using regular PCR amplification of actin.

Expression analysis in Ceratopteris was performed using qRT-PCR and normalized relative to actin. Primers for APETAL2A expression were 5′-CAGCATCCTAGGATTTCTAGATAT-3′ and 5′-GGCATCTGTTAGTCCGGAGCCTGT-3′ and for actin 5′-TGGCGGTGTATTCTTCTGAAGAT-3′ and 5′-CCTCATTACACACCTGATACATT-3′. For Antirrhinum, cDNA was prepared using a combination of stem-loop and oligo-dT primers. Stem-loop primers were 5′-GTCCGTATCCAGTGACCGTCTGCATGCTC-3′ and 5′-GGTTCATTCAGTGACCGTCTGCATGCTC-3′ to amplify the mature microRNAs.

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CONTRIBUTIONS

Koen Geuten conceived the ideas in the manuscript. Koen Geuten and Helenen Coenen wrote the manuscript. Experiments were performed through technical assistance.

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# Heterochronic genes in plant evolution and development

Heterochrony is a fundamental concept within developmental biology, central to the way we understand the evolution of development and the origin of new body forms. The study of heterochronic genes in plants is particularly intriguing due to the unique developmental and evolutionary challenges faced by angiosperms, which have a complex life cycle involving both a vegetative and an reproductive phase. These two phases are defined by the presence of leaves and flowers, respectively, and the transition between them is a significant developmental event.

### Key Points
- **Neoteny** refers to the retention of juvenile characteristics into adulthood, which has been suggested to play a role in the evolution of flowering plants.
- The **AP2 subfamily** of APETALA2 and PISTILLATA genes is essential for the transition from vegetative to reproductive development in angiosperms.
- **miR156** and its target **SPL3** are key regulators of floral transition in Arabidopsis.
- **miR172** and its target **SPL16** regulate developmental transitions in Arabidopsis.
- **FMOOHA** is involved in the control of flower development in Arabidopsis and acts as a master regulator of floral meristem identity.
- **MIR172** gene family plays a crucial role in the transition to flowering.
- **miR156** and **miR172** are involved in the temporal regulation of shoot development in Arabidopsis.

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