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REVIEW: PART OF A SPECIAL ISSUE ON DEVELOPMENTAL ROBUSTNESS AND SPECIES DIVERSITY

Genetic assimilation: a review of its potential proximate causes and evolutionary consequences

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• **Background** Most, if not all, organisms possess the ability to alter their phenotype in direct response to changes in their environment, a phenomenon known as phenotypic plasticity. Selection can break this environmental sensitivity, however, and cause a formerly environmentally induced trait to evolve to become fixed through a process called genetic assimilation. Essentially, genetic assimilation can be viewed as the evolution of environmental robustness in what was formerly an environmentally sensitive trait. Because genetic assimilation has long been suggested to play a key role in the origins of phenotypic novelty and possibly even new species, identifying and characterizing the proximate mechanisms that underlie genetic assimilation may advance our basic understanding of how novel traits and species evolve.
• **Scope** This review begins by discussing how the evolution of phenotypic plasticity, followed by genetic assimilation, might promote the origins of new traits and possibly fuel speciation and adaptive radiation. The evidence implicating genetic assimilation in evolutionary innovation and diversification is then briefly considered. Next, the potential causes of phenotypic plasticity generally and genetic assimilation specifically are examined at the genetic, molecular and physiological levels and approaches that can improve our understanding of these mechanisms are described. The review concludes by outlining major challenges for future work.
• **Conclusions** Identifying and characterizing the proximate mechanisms involved in phenotypic plasticity and genetic assimilation promises to help advance our basic understanding of evolutionary innovation and diversification.

Key words: Genetic accommodation, genetic assimilation, phenotypic plasticity, cis and trans regulatory evolution, canalization, developmental robustness, species diversity.

INTRODUCTION

Phenotypic plasticity – the ability of an individual organism to change its phenotype in direct response to stimuli or inputs from its environment (Nijhout, 2003; West-Eberhard, 2003) – is commonplace. Yet the evolutionary significance of such developmental flexibility remains controversial (Laland *et al*., 2014; Wray *et al*., 2014). Although many evolutionary biologists have long held that plasticity has no relevance for evolution (other than to perhaps act as a stabilizing force by dampening any diversifying effects of selection; Huey *et al*., 2003; Price *et al*., 2003; de Jong, 2005), a growing number of researchers have suggested that plasticity can actually act as a diversifying force in evolution. Indeed, plasticity might play a key role in fostering new traits and species, and may even fuel adaptive radiation (West-Eberhard, 1986, 1989, 2003; Pigliucci and Murren, 2003; Schlichting, 2004; Pfennig *et al*., 2010; Moczek *et al*., 2011).

Although several routes have been proposed for how plasticity might impact evolutionary innovation and diversification (Bradshaw, 1965; Stearns, 1989; West-Eberhard, 1989, 2003; Pigliucci and Murren, 2003; Price *et al*., 2003; Schlichting, 2004; Pfennig and McGee, 2010; Pfennig *et al*., 2010; Moczek *et al*., 2011; Thibert-Plante and Hendry, 2011; Fitzpatrick, 2012), one widely cited pathway involves an evolutionary process known as 'genetic assimilation' (*sensu* Waddington, 1952, 1953). Genetic assimilation occurs when a trait that was originally triggered by the environment loses this environmental sensitivity (i.e. plasticity) and ultimately becomes 'fixed' or expressed constitutively in a population. Another way of wording this phenomenon is that an induced trait loses its environmental sensitivity and thereby becomes robust to the environment. Not only might this process represent a common way for new traits to arise (reviewed in West-Eberhard, 2003; Pfennig *et al*., 2010; Moczek *et al*., 2011), but if some populations undergo genetic assimilation and others do not – and if the affected traits influence the likelihood that these populations can exchange genes – then the differential loss of plasticity might represent a crucial step in the formation of new species (West-Eberhard, 1986, 1989, 2003, 2005; Pigliucci and Murren, 2003; Pfennig *et al*., 2010; Schwander and Leimar, 2011).

In this review, we explore this potential pathway to innovation and diversification. We begin by examining how the evolution of phenotypic plasticity, followed by its loss via genetic assimilation, might facilitate genetic evolution and thereby

promote the origins of new traits and even new species. We then discuss the various proximate (i.e. genetic, molecular and physiological) mechanisms that potentially underpin both phenotypic plasticity and genetic assimilation. We conclude by outlining future directions that promise to help illuminate plasticity's role in the origins of biodiversity.

THE FLEXIBLE – AND INFLEXIBLE – ORGANISM

All organisms can alter some aspect of their phenotype in response to changes in their environment; if not their morphology, then their physiology, behaviour and/or gene expression (Nijhout, 2003; Sultan, 2007; Gilbert and Epel, 2009). For example, temperature often influences phenotype because nearly all enzyme activity is temperature-dependent; food contains potent chemical signals that can dramatically alter phenotypes; light can stimulate plants to produce different-shaped leaves and shoots; pressure can cause plant stems and roots and animal muscles and bones to grow differently; and other organisms (be they pathogens, predators or competitors) can cause an individual plant or animal to release hormones, which can alter the phenotype that the individual produces (reviewed in Gilbert and Epel, 2009). Such phenotypic plasticity is so prevalent that it can be considered a defining feature of living things (Pfennig, 2004).

To understand why plasticity is ubiquitous, consider that all organisms encounter variation in their environment, and these fluctuations can destabilize development and thereby disrupt the match between the organism's phenotype and its environment (Whitman and Agrawal, 2009). Phenotypic plasticity can lessen such mismatches and thereby enhance fitness (Ghalambor *et al*., 2007). Generally, phenotypic plasticity is favoured when organisms confront environmental variation, when no fixed trait is best suited for all environmental conditions, when cues are available that reliably signal change in local conditions, and when the fitness benefits outweigh the costs of expressing plasticity (Berrigan and Scheiner, 2004; Travis, 2009; Whitman and Agrawal, 2009).

When the above conditions favouring plasticity are met, plasticity is generally thought to evolve because selection favours genotypes that are more responsive to the changes in the environment (Schlichting and Pigliucci, 1998). Indeed, in nearly every natural population surveyed, different genotypes are typically found to vary not only in *whether* they respond to a particular change in their environment but also in the *manner* in which they respond – in other words, different genotypes typically express different environmentally contingent phenotypic responses (Gupta and Lewontin, 1982; Sultan and Bazzaz, 1993; Kingsolver *et al*., 2004). Such 'reaction norms' provide the heritable variation on which selection can act to promote an evolutionary change in plasticity (Schlichting and Pigliucci, 1998; Windig *et al*., 2004).

Once it evolves, however, plasticity can subsequently be reduced or even *lost* evolutionarily, and this loss can have profound evolutionary consequences. Indeed, for more than a century various researchers have hypothesized that the gain and subsequent loss of plasticity can facilitate genetic evolution and thereby fuel the origins of new, ecologically relevant traits

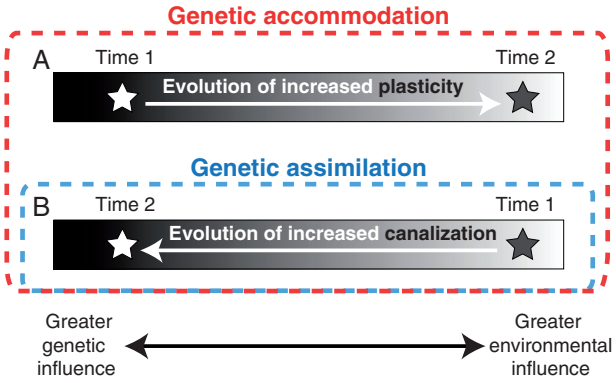

FIG. 1. A diagram illustrating the distinction between genetic accommodation and genetic assimilation. Genetic accommodation is any adaptive genetic change in the environmental regulation of a phenotype. For example, a trait may evolve either (A) increased or (B) decreased environmental sensitivity (i.e. phenotypic plasticity). The complete loss of phenotypic plasticity (i.e. increased canalization) is an extreme form of genetic accommodation known as genetic assimilation. Reproduced with permission from Pfennig (2015).

(Baldwin, 1902; Schmalhausen, 1949 [1986]; Waddington, 1953; West-Eberhard, 2003). According to one widely cited model for how this process might unfold (West-Eberhard, 2003), when selection acts on quantitative genetic variation regulating the expression of an initially environmentally induced trait, it can promote the evolution of either increased or decreased plasticity through the process known as 'genetic accommodation' (Fig. 1). Formally, genetic accommodation is defined as a mechanism of evolution wherein a novel phenotype, generated by either a mutation or an environmental perturbation, is refined into an adaptive phenotype through a series of quantitative genetic changes (West-Eberhard, 2003). In this sense, genetic assimilation represents a specific form of genetic accommodation in which plasticity decreases to the point that a trait becomes constitutively expressed (Waddington, 1953) (Fig. 1).

Genetic accommodation/assimilation can occur because most (if not all) traits are influenced by both genes and the environment (Gilbert and Epel, 2009). These two influences on phenotype are potentially evolutionarily interchangeable, meaning that selection can slide trait regulation anywhere along a continuum from total environmental control to total genetic control (Fig. 1). Thus, when genetic variation for the degree of environmental influence is present (as is nearly always the case; see above), selection can act on this variation and promote the evolution of either increased or decreased environmental sensitivity (West-Eberhard, 2003). If selection favours the elimination of all environmental influences – i.e. genetic assimilation – the end result is a genetically 'fixed' or 'canalized' trait (Waddington, 1942); i.e. a trait that is invariably produced regardless of normal changes in the environment (Fig. 2).

Although genetic accommodation can occur whether a novel trait is mutationally or environmentally induced (West-Eberhard, 2003), environmentally triggered novelties are likely to have greater evolutionary potential than mutationally induced ones, for at least three reasons (West-Eberhard, 2003). First, changes in the environment often impact many individuals simultaneously, in contrast to genetic mutations, which

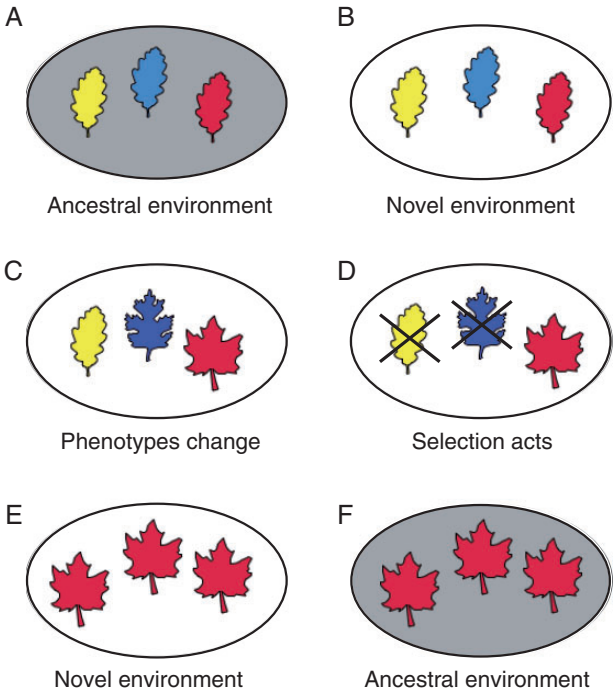

FIG. 2. Phenotypic plasticity, followed by genetic assimilation, may facilitate the evolution of a new, canalized trait regardless of the environment through the following steps (here, the trait is a new leaf shape; different colours represent different genotypes). (A) A genetically variable population (B) experiences a novel environment (indicated here as a change from a shaded to an unshaded background). (C) Consequently, the environment induces novel phenotypes (different leaf shapes), but different genotypes respond differently (by producing different-shaped leaves). (D) Selection disfavours those genotypes that produce maladaptive phenotypes (leaf shapes) in the novel environment (indicated here by an X). (E) Such selection may result in the evolution of a novel, canalized trait (a novel leaf shape) that is expressed regardless of the environment. (F) That is, the novel trait is produced even when the environment changes back to the original, ancestral state.

initially affect only one individual and its immediate descendants (not to mention that the vast majority of mutations are deleterious; Kassen and Bataillon, 2006; Halligan and Keightley, 2009). This widespread impact of environmental change enables a newly induced trait to be tested among diverse genotypes, thereby providing fertile ground for selection to act and increasing the chances that genetic accommodation will occur (West-Eberhard, 2003).

Second, although the chance that a particular mutation will occur is not influenced by whether or not the organism is in an environment where that mutation would be advantageous – i.e. adaptively directed mutation does not occur (Sniegowski and Lenski, 1995) – the situation is quite different for an environmentally triggered novel trait. Such a trait is always associated with a particular environment – the one that triggered it. Therefore, environmentally induced traits are more likely than mutationally induced novelties to experience consistent selection and directional modification (West-Eberhard, 2003). This allows new environments to immediately produce and select among new phenotypes and rapidly refine their expression (Badyaev, 2005).

Third, plasticity promotes the storage and release of 'cryptic genetic variation', i.e. variation that is expressed only under atypical conditions (Gibson and Dworkin, 2004; Hermisson and Wagner, 2004; Dworkin, 2005*a*; Schlichting, 2008; Ledón-Rettig *et al*., 2010; Paaby and Rockman, 2014). The release of such variation ultimately makes genetic accommodation possible (Moczek, 2007; Moczek *et al*., 2011). Phenotypic plasticity facilitates the build-up of cryptic genetic variation, both because the effects of novel genetic variants are buffered by compensatory plastic responses (Moczek, 2008) and because environment-specific genes experience relaxed selection in the non-inducing environment (Lahti *et al*., 2009; but see Hunt *et al*., 2011; Leichty *et al*., 2012). Essentially, because living systems are robust to mutations and minor environmental perturbation (Masel and Siegal, 2009), they can accumulate genetic variation without that variation having any phenotypic effects and thereby being removed by selection. However, if the system is disturbed (e.g. following exposure to a novel environment and the stress that often accompanies such situations; Badyaev, 2005), robustness breaks down, and the formerly cryptic genetic variation is revealed phenotypically and exposed to selection. Because this storage and release of cryptic genetic variation is the biological analogue of an electric capacitor (which stores and later releases electric charge), it has been dubbed 'evolutionary capacitance' (Masel, 2013). Evolutionary capacitors – which have been implicated in the heat shock protein Hsp90 (Rutherford and Lindquist, 1998), yeast prions (True and Lindquist, 2000) and complex gene networks (Bergman and Siegal, 2003) – represent molecular switch mechanisms that can shift genetic variation between cryptic and exposed states (Masel, 2013).

In sum, there are multiple reasons to expect environmentally triggered novelties to have greater evolutionary potential than mutationally induced ones. Indeed, genetic mutations might contribute not so much to the *initial origins* of phenotypic novelties as to the pool of genetic variation that ultimately makes genetic accommodation/assimilation possible. Or, as West-Eberhard (2003, p. 158) put it, 'genes are followers, not necessarily leaders, in phenotypic evolution'.

GENETIC ASSIMILATION'S ROLE IN DIVERSIFYING EVOLUTION

Genetic assimilation has long been regarded as potentially crucial in the origins of novel traits (Waddington, 1952, 1953, 2003; Pigliucci and Murren, 2003; Aubret and Shine, 2009; Lande, 2009; Moczek *et al*., 2011). With genetic assimilation, the origin of a new, canalized trait does not require new genes; instead, selection can promote the origins of a novel trait by acting on existing genetic and epigenetic variation in a population (Schlichting and Pigliucci, 1998; e.g. see Emlen *et al*., 2007; Ledón-Rettig *et al*., 2008; Aubret and Shine, 2009; Pfennig and Martin, 2009, 2010; Scoville and Pfrender, 2010). In other words, a plastic trait can be converted into a canalized trait through evolutionary adjustments in *the regulation of a trait's expression*.

Although laboratory studies have demonstrated that genetic assimilation can promote novelty (Waddington, 1952, 1953), and there are several suggestive field studies (e.g. Losos *et al*., 2000; Pfennig and Murphy, 2000; Wund *et al*., 2008; Scoville and Pfrender, 2010; Robinson, 2013; reviewed in Schlichting

and Wund, 2014), ascertaining whether or not genetic assimilation actually *has* contributed to the evolution of any complex, novel trait in any *natural* population has long been questioned (Simpson, 1953; Williams, 1966; Orr, 1999; de Jong, 2005; Futuyma, 2013; Wray *et al*., 2014). A chief difficulty with establishing whether genetic assimilation has promoted novelty is that, once a novel trait has evolved, its evolution cannot be studied *in situ* (Hall, 1999). One way around this problem is to study lineages (i.e. populations or species) that are thought to be ancestral to the lineage possessing the novel trait in question (West-Eberhard, 2003; Badyaev, 2005; Ghalambor *et al*., 2007; Ledón-Rettig *et al*., 2008). Using such an approach, one can then test whether: (1) ancestral species express the trait only through plasticity; (2) novel environments uncover cryptic genetic variation; and (3) trait expression has been refined in derived species. This approach has recently been applied in, among other systems, spadefoot toads and has revealed that a novel ecomorph (the 'carnivore' morph) has likely arisen through a 'plasticity-first' scenario (Ledón-Rettig *et al*., 2008, 2010). Moreover, a recent meta-analysis has uncovered several convincing cases in which genes appear to be 'followers' in the origins of novel traits (Schwander and Leimar, 2011). For other possible examples in which genetic assimilation might have promoted novelty, including in plants, see the recent review by Schlichting and Wund (2014).

Genetic assimilation might even play a role in speciation. When an induced phenotype becomes expressed constitutively, environmentally induced variation within populations or species can be translated into diverse phenotypes between populations and species. Thus, genetic assimilation generates diversity because it produces fixed (genetic) differences among populations due specifically to the shift from a plastic to a non-plastic phenotype. As noted in the Introduction, if a trait that is involved in mating choice, habitat use or reproductive mode undergoes genetic assimilation in some populations but not in others (e.g. Diggle and Miller, 2013), then this differential loss of plasticity might lead to the evolution of reproductive isolation between these populations and, possibly, speciation (West-Eberhard, 1986, 1989, 2003; Pfennig *et al*., 2010). Theory has demonstrated genetic assimilation's capability for promoting diversification (Lande, 2009), and empirical studies find that phenotypic plasticity produces intraspecific variation that parallels interspecific variation within the same clade, suggesting that the former might often form the basis for the latter (Badyaev and Foresman, 2000; Losos *et al*., 2000; Pfennig and Murphy, 2000; Gomez-Mestre and Buchholz, 2006; Bull-Herenu and Arroyo, 2009). Moreover, there are numerous examples in which a formerly plastic trait has undergone canalization (i.e. lost its plasticity) in a particular lineage, and such shifts are typically accompanied by speciation (Schwander and Leimar, 2011). However, further study is needed to ascertain what role, if any, genetic assimilation plays in speciation (Pfennig *et al*., 2010; Nosil, 2012).

Phenotypic plasticity, followed by genetic assimilation, might also promote adaptive radiation, influencing both the likelihood of occurrence and the patterns of diversity that emerge (reviewed in West-Eberhard, 2003; Wund *et al*., 2008; Pfennig *et al*., 2010). In adaptive radiation, a single ancestral lineage diversifies rapidly in response to divergent selection pressures across numerous environments (Schluter, 2000). According to the 'flexible stem' hypothesis (West-Eberhard, 2003), an adaptive radiation arises when ecological circumstances favour diversification in an ancestral taxon that expresses phenotypic plasticity in the types of traits that characterize the adaptive radiation. Under such circumstances, when individuals are exposed to the same selective environments, plasticity in the ancestral lineage repeatedly reveals the same sets of phenotypes. This model might explain 'replicate' adaptive radiations (i.e. the situation in which many descendant species evolve parallel ecotypic variation in response to similar selection pressures) in a number of systems (Meyer, 1987; Losos *et al*., 2000; Wund *et al*., 2008). However, further study is needed to test this model more rigorously.

Having discussed the potential evolutionary consequences of phenotypic plasticity and genetic assimilation, we now turn to the important issue of the possible proximate mechanisms that underpin both processes.

POTENTIAL PROXIMATE MECHANISMS OF PHENOTYPIC PLASTICITY AND GENETIC ASSIMILATION

Mechanisms of phenotypic plasticity

Given the potential importance of genetic assimilation in evolutionary innovation and diversification (as outlined above), understanding the molecular mechanisms involved in this process might improve our knowledge of how novel traits evolve; however, such an understanding can also help in assessing whether genetic assimilation is likely to be common or rare in nature. Given that genetic assimilation represents a loss of phenotypic plasticity, a natural starting point for thinking about the mechanisms that might underlie genetic assimilation is the molecular causes of phenotypic plasticity.

Phenotypic plasticity typically involves the following three general steps. First, an individual's sensory system detects and transduces information about its external environment. In some cases, the environmental stimulus and any response that it elicits may be very general, as with phenotypic changes wrought by changes in an individual's temperature or nutrition (reviewed in Gilbert and Epel, 2009). In other cases, the requisite stimuli and responses are highly specific. For example, certain plants possess receptor proteins that detect only the plant's most common natural enemies (Zhao *et al*., 2005). Second, the signal detected by the sensory system is transduced into a molecular response at the biochemical level that alters the activities within cells. In the case of multicellular organisms, this information may be conveyed elsewhere in the organism's body via hormonally mediated signals. Indeed, hormones underlie nearly all instances of plasticity in animals and likely also play an important role in plasticity in plants (Gilbert and Epel, 2009). Third, the target cells, organs or tissues respond accordingly by altering phenotype.

Ultimately, phenotypic plasticity is nearly always accompanied by changes in gene expression (Aubin-Horth and Renn, 2009), an observation that has provided key insight into the molecular mechanisms of phenotypic plasticity (Gilbert and Epel, 2009). In particular, differential gene expression (and, hence, phenotypic plasticity) often involves alterations in the binding of transcription factors to a gene's promoter or other regulatory

elements. Transcription factors differ in the sequences that they recognize, their abilities to activate or repress transcription and their responsiveness to external signals. Thus, one proposed mechanism of phenotypic plasticity is that the individual's sensory system transduces information from its external environment by recruiting different transcription factors, thereby activating different genes. These newly activated genes may then trigger different hormones, which ultimately cause alternative phenotypes to be produced (Nijhout, 2003).

Any particular plastic response may involve many steps, potentially encompassing numerous genes and environmental and physiological factors. This complexity provides copious targets on which selection can act, from the types of signals that an individual's sensory system can detect to the threshold amount of a particular hormone needed to trigger a phenotypic response (Pfennig *et al*., 2010; Moczek *et al*., 2011).

Potential mechanisms of genetic assimilation

For genetic assimilation to take place, the reaction norms that underlie a phenotype's expression in a population must undergo an evolutionary shift, such that genotypes that express a phenotype robustly across a range of different environments become fixed in the population (Pigliucci *et al*., 2006). Experimental evidence indicates that such changes in reaction norms may be driven by variation in signalling pathways that mediate the relationship between genotype, environment and phenotype. One way such variants might act is by changing developmental thresholds for environmentally influenced hormones and other key signalling molecules (Moczek and Nijhout, 2002; Suzuki and Nijhout, 2006).

Although there have yet to be case studies reported in which genetic assimilation has been linked to specific genetic changes (but see Scoville and Pfrender, 2010), work on related topics – including the molecular basis of gene expression variation, genotype–environment interaction, and robustness – provides valuable insights into possible causes of genetic assimilation. As with phenotypic plasticity in general, genetic variants that alter *gene regulation* are likely important contributors to genetic assimilation (Pfennig and Ehrenreich, 2014). Populations commonly harbour large numbers of genetic variants that affect gene expression (e.g. Brem *et al*., 2002; Schadt *et al*., 2003; Morley *et al*., 2004). Additionally, many new mutations that arise within populations impact gene regulation (Landry *et al*., 2007). Thus, ample genetic diversity in gene expression exists within populations that affects gene regulation and may serve as a reservoir of cryptic phenotypic effects (Gibson and Dworkin, 2004; Moczek, 2007; Le Rouzic and Carlborg, 2008; Paaby and Rockman, 2014).

There are multiple plausible ways in which changes in gene regulation might facilitate canalization of a plastic trait (Fig. 3). For example, the regulation of genes that control a plastic trait might become decoupled from their environmental cue (Fig. 3A) or might evolve regulation by a secondary pathway that makes their expression robust to the environment (Fig. 3B; e.g. Matsui *et al*., 2015). Describing such changes in gene regulation in a binary manner is helpful in considering possible models for genetic assimilation, but it is important to note that real cases of genetic assimilation are likely to be

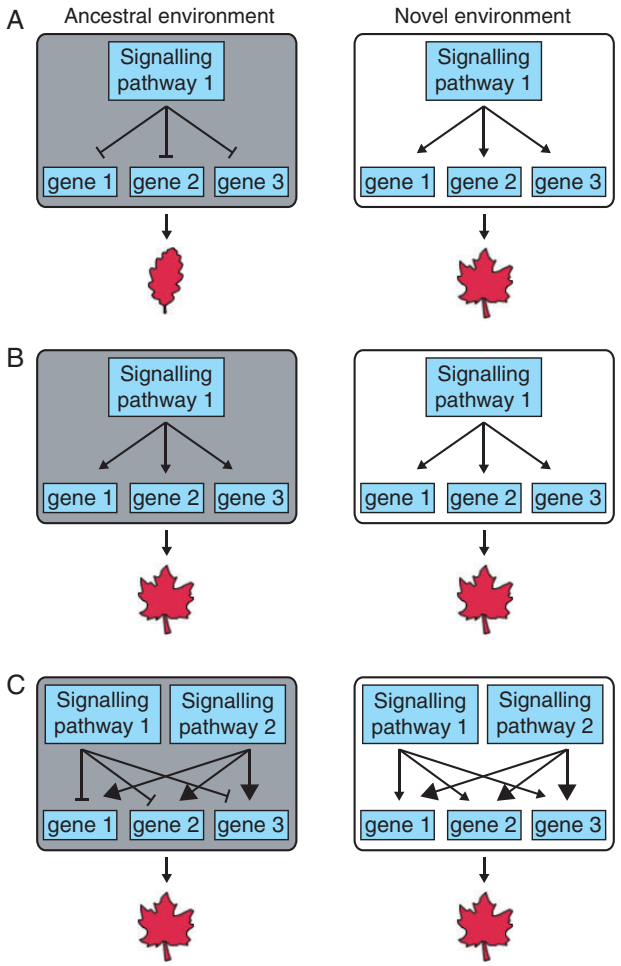

FIG. 3. Potential causes of phenotypic plasticity and genetic assimilation. (A) A new trait arises due to a change in gene regulation triggered by a novel environment. Two plausible mechanisms for genetic assimilation of the novel trait are illustrated: (B) evolution of the pathway underlying the novel trait such that it is no longer environmentally responsive; and (C) rewiring of development such that a new, environment-independent pathway causes the novel phenotype to be constitutively expressed. Note that genes 1, 2 and 3 represent genes that must be transcribed for the novel phenotype to be expressed, while signalling pathways 1 and 2 are pathways that can activate these genes. The thicker arrows in (C) indicate that signalling pathway 2 has a stronger effect on genes 1, 2 and 3 than signalling pathway 1.

caused by *quantitative* genetic changes in gene regulation, i.e. changes involving one or more loci with quantitative effects on transcript levels (Ledón-Rettig *et al*., 2014). These genetic changes that facilitate genetic assimilation may occur in phenotypically important genes themselves or, alternatively, they may occur in the upstream regulators of these genes. These two classes of variants are referred to as *cis* and *trans* regulatory polymorphisms, respectively (Albert and Kruglyak, 2015).

Strong arguments can be made for both *cis* and *trans* regulatory evolution contributing to genetic assimilation. With regard to *cis* regulatory polymorphisms, because they generally exhibit lower sensitivity to the environment than *trans* regulatory polymorphisms (Smith and Kruglyak, 2008; Cubillos *et al*., 2014), *cis* regulatory variants have the potential to rapidly canalize the

expression of individual genes. The most important types of *cis* regulatory variants within the context of genetic assimilation are likely to be those that create or disrupt transcription factor binding sites (Wittkopp and Kalay, 2012). For example, loss of a binding site for a conditionally active transcription factor might eliminate a gene's sensitivity to the environment. Alternatively, gain of a binding site for a constitutively active transcription factor might also result in the decoupling of a gene's expression from the environment by leading to increased redundancy in a gene's regulation and a corresponding higher robustness in the gene's expression across conditions.

Although the rewiring of gene regulation by *cis* regulatory polymorphism is a plausible mechanism for genetic assimilation (as described above), variants that influence gene expression in *trans*, or through a combination of *cis* and *trans* effects, might also be capable of causing genetic assimilation. This is particularly true when considering novel traits that depend on many genes having their expression altered, as *cis* variants only affect their cognate gene (and potentially its downstream targets if it is a transcription factor), while *trans* variants can potentially impact the regulation of *many* genes (e.g. Brem *et al*., 2002; Yvert *et al*., 2003).

There is at least one additional important difference between *cis* and *trans* regulatory variants with respect to their potential to contribute to genetic assimilation: their mutational target spaces. While genes usually have one promoter, their expression is often influenced by a large number of *trans* factors. Despite transcription factors being the most direct regulators of gene expression, many other factors can influence the abundances of transcripts and proteins within a cell (Yvert *et al*., 2003; Albert and Kruglyak, 2015). These include, but are not limited to, environmental sensors and other components of signalling cascades that modulate the activities of transcription factors in response to environmental cues, as well as proteins and non-coding RNAs that directly determine transcript stability and translation. Because so many factors affect the expression of a gene in *trans*, the mutational target space for *trans* effects is often one or more orders of magnitude larger than for *cis* effects (Denver *et al*., 2005; Landry *et al*., 2007; Gruber *et al*., 2012).

The larger mutational target space for *trans* regulatory variants than *cis* regulatory variants may suggest there is a greater chance for polymorphisms that occur *in trans* to decouple a trait from its environmental cue. In particular, *trans* regulatory variants that cause signalling activity in conditionally active pathways, even in the absence of their inductive cues, might result in genetic assimilation. Examples of such environmentally insensitive variants have been described. For instance, laboratory strains of *Saccharomyces cerevisiae* possess an allele of *GPA1*, a component of the mating pheromone responsive mitogen activated protein kinase (MAPK) pathway, that shows high activity even in the absence of mating pheromone (Yvert *et al*., 2003). MAPK pathways are evolutionarily conserved across eukaryotes and play diverse roles in development and physiology (Seger and Krebs, 1995; Schaeffer and Weber, 1999; Hamel *et al*., 2006), suggesting that similar phenomena could occur in other species.

Our description of the role of changes in gene regulation *in cis* and *trans* in genetic assimilation is an oversimplification in that we have largely focused on the mechanisms by which *individual* genetic polymorphisms might contribute. However, it is well known that heritable changes in most traits are influenced by *numerous* genetic variants (Mackay *et al*., 2009) and that these variants can show complicated non-additive effects on gene expression due to their collective influence on gene regulatory *networks* (Omholt *et al*., 2000; Gjuvsland *et al*., 2007; Nuzhdin *et al*., 2012). Furthermore, genetic variation in other molecular processes, such as post-translational regulation of proteins or changes in protein–protein interactions, might contribute to genetic assimilation. Moving forward, it will be important to develop case studies in which genetic assimilation has been conclusively shown and the involved genetic variants identified and functionally characterized. In the following section we discuss approaches that can be used to explore this problem.

APPROACHES FOR INVESTIGATING THE MOLECULAR MECHANISMS THAT UNDERLIE GENETIC ASSIMILATION

Current genetic and genomic approaches provide powerful tools for characterizing the mechanisms underlying genetic assimilation. Admittedly, these techniques will work best for traits that have well-understood evolutionary histories and can be studied in model organisms, which typically have high-quality genomes and transcriptomes. However, as new sequencing technologies make it easier to generate high-quality genomes and transcriptomes in other species, these approaches should increase in their applicability to non-model organisms. Here, we first describe methods for examining how canalization of a phenotype might arise due to changes in gene regulation across genotypes and environments. We then discuss mapping techniques that can be used to identify genes and genetic variants that cause regulatory changes that result in genetic assimilation. We also mention how systematic mutagenesis screens in model organisms might improve understanding of genetic assimilation.

All organisms exhibit modular gene expression throughout development (e.g. Arbeitman *et al*., 2002; Schmid *et al*., 2005). Alterations in how these gene modules are expressed across developmental stages and tissues is known to play an important role in phenotypic evolution (as discussed in Peter and Davidson, 2011; Ichihashi *et al*., 2014 and elsewhere), and likely also contributes to phenotypic plasticity (Schneider *et al*., 2014) and genetic assimilation (Pfennig and Ehrenreich, 2014) (Fig. 4). RNA-Seq, at multiple stages of development, can be used to quantify the abundances of all transcripts in an organism's genome, even for non-model species (Wang *et al*., 2009), providing a foundation for defining modules of co-expressed genes and examining the regulatory relationships among these modules (Nuzhdin *et al*., 2012; Schneider *et al*., 2014).

A strategy that will likely help in characterizing the molecular basis of genetic assimilation is comparing expression patterns among canalized and plastic genotypes in both their ancestral and novel environments (Fig. 4). Of course, this approach requires knowledge of the evolutionary history of plasticity in the study organisms, and the number of systems in which this is known may be limited (but see examples in

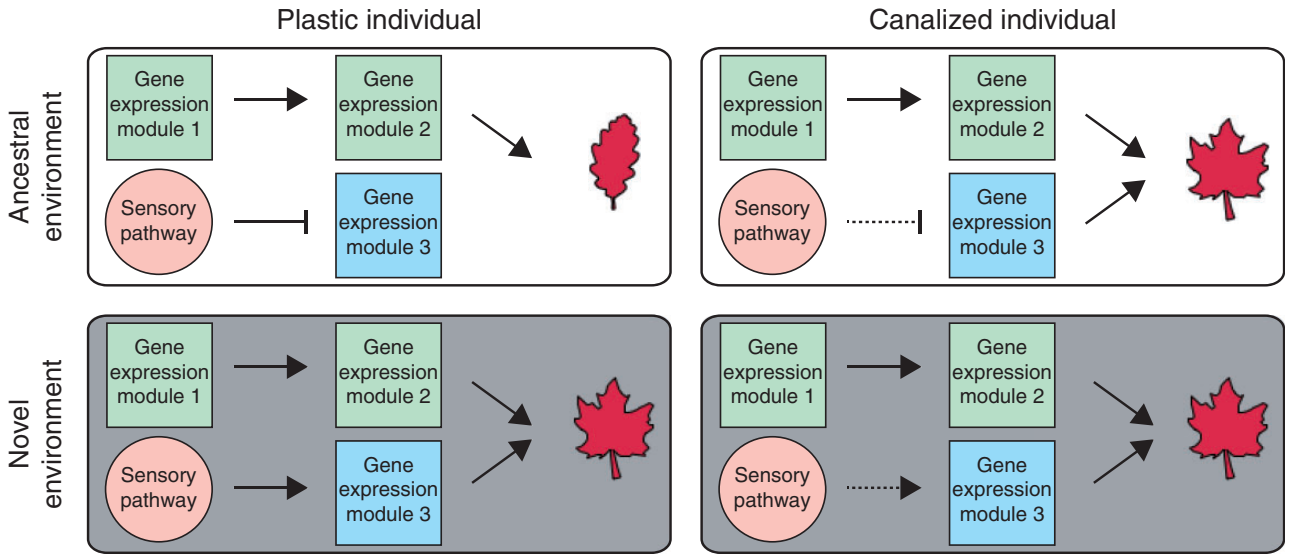

FIG. 4. Examining how changes in gene regulatory networks contribute to genetic assimilation. Expression analysis across development can be employed to determine transcriptional changes that enable constitutive expression of a phenotype. Comparison of plastic and canalized genotypes in the ancestral and novel environment can be used to identify gene modules that underlie canalization. Here, we have shown a hypothetical example of what might be found in such a study. In the example, the sensitivity of gene expression module 3 to regulation by an environmentally responsive sensory pathway has been reduced in canalized individuals (indicated by a dotted line). Due to this reduction, gene expression module 3 is expressed when individuals are not reared in the novel, inductive environment. This higher expression leads to constitutive expression of the new trait across conditions.

Schwander and Leimar, 2011; Schlichting and Wund, 2014). Nevertheless, through such an experiment, gene modules that are differentially expressed across conditions in plastic individuals but expressed at similar levels across conditions in canalized individuals can be identified. Bioinformatic analysis of genes in these modules (Schneider *et al*., 2014), potentially in combination with experimental techniques for studying the binding of proteins to DNA, such as ChIP-Seq (Kidder *et al*., 2011) and DNase-FLASH (Vierstra *et al*., 2014), can then be used to determine the changes in transcription factor activity that have resulted in canalization. Recent work has shown that these approaches for studying how protein–DNA interactions regulate chromatin structure, transcription and phenotypic outcome can be applied in non-model organisms (Simola *et al*., 2013).

Given that canalization requires the fixation of genetic variants that make a trait robust to the environment, this robustness itself can be viewed as a quantitative trait (e.g. de Visser *et al*., 2003; Dworkin, 2005*b*; Flatt, 2005; Levy and Siegal, 2012; Queitsch *et al*., 2012) and subjected to linkage mapping (Lander and Botstein, 1989) (Fig. 5). For this approach to work, it must be possible to mate individuals from a canalized population or species to individuals from an ancestral population or species that has remained plastic (Fig. 5). Furthermore, recombinants from these crosses must be viable.

Although historically it might have been difficult to use genetic mapping to identify loci that canalize a trait, next-generation sequencing has revolutionized genetic mapping by crosses (e.g. Ehrenreich *et al*., 2010; Bloom *et al*., 2013). Indeed, new sequencing technologies have made it cheaper and easier to identify markers for conducting linkage mapping, and have also simplified the process of genotyping cross progeny (Andolfatto *et al*., 2011). Recent studies have shown that by analysing large genetic mapping populations, high statistical power and precise mapping resolution can be achieved, facilitating detection and cloning of most, if not all, of the loci involved in a trait (Ehrenreich *et al*., 2010; Bloom *et al*., 2013; Taylor and Ehrenreich, 2014). Within the context of genetic assimilation, cloning of the genes and genetic variants underlying these loci can help to shed light on the molecular mechanisms that cause canalization.

Finally, much of what we know about genetic assimilation comes from model organisms. Indeed, Waddington's original work on genetic assimilation was conducted in *Drosophila melanogaster* (Waddington, 1952, 1953), and in recent years work in multiple model systems, including *Arabidopsis* (Queitsch *et al*., 2002; Sangster *et al*., 2008*a*, *b*), *Caenorhabditis* (Felix, 2007; Milloz *et al*., 2008; Duveau and Felix, 2012), *Drosophila* (Gibson and Hogness, 1996; Rutherford and Lindquist, 1998; Gibson *et al*., 1999; Dworkin *et al*., 2003) and yeast (Jarosz and Lindquist, 2010; Tirosh *et al*., 2010; Halfmann *et al*., 2012), has been used to explore the mechanisms that reveal cryptic genetic variation and may give rise to genetic assimilation. However, such work is still in its infancy, meaning that these model systems have great potential to advance our understanding of genetic assimilation in ways that would be very difficult in non-model systems. For example, saturating screens can be conducted in these organisms to identify mutations that decouple plastic traits from their environmental stimuli. When layered onto our knowledge of the gene regulatory networks of these species, general rules about how to manipulate the genotype–environment–phenotype relationship may emerge. As new genetic engineering approaches, such as CRISPR/Cas9 (Mali *et al*., 2013), gain increasing usage across species, comparable analysis techniques may be possible in non-model organisms as well.

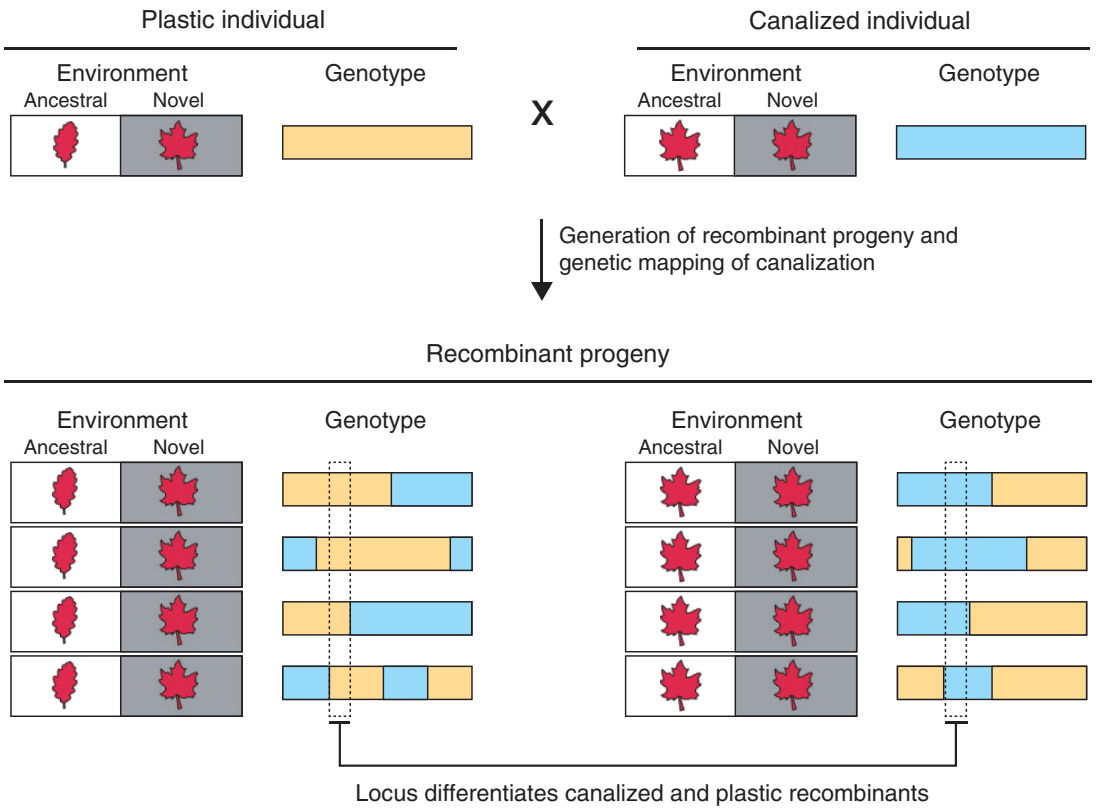

FIG. 5. Genetic mapping of loci that underlie genetic assimilation. Crosses between canalized and plastic genotypes can be used to determine the loci that underlie canalization of a novel trait.

CONCLUSIONS

Improving our understanding of genetic assimilation may provide valuable new insights into the origins of novel traits and species. Because the number of examples in which genetic assimilation has been convincingly demonstrated is small, efforts to develop more case studies of genetic assimilation, especially in natural populations, may facilitate major advances in this research area. Identifying and cloning the genes and genetic variants that underlie these examples can shed crucial new light onto the mechanisms underlying genetic assimilation. However, to fully explain the mechanisms that cause genetic assimilation, it will likely be necessary to examine how the genetic variants underlying genetic assimilation alter developmental gene regulatory programmes across genotypes and environments. Examples in which genetic assimilation has been shown and characterized at the molecular level will facilitate new insights into the processes that shape diversity in nature. Such work may also have impacts outside of evolutionary biology by expanding our basic knowledge of how to modify biological systems to produce new traits.

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