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

**Molecular mechanisms governing differential robustness of development  
and environmental responses in plants**

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• **Background** Robustness to genetic and environmental perturbation is a salient feature of multicellular organisms. Loss of developmental robustness can lead to severe phenotypic defects and fitness loss. However, perfect robustness, i.e. no variation at all, is evolutionarily unfit as organisms must be able to change phenotype to properly respond to changing environments and biotic challenges. Plasticity is the ability to adjust phenotypes predictably in response to specific environmental stimuli, which can be considered a transient shift allowing an organism to move from one robust phenotypic state to another. Plants, as sessile organisms that undergo continuous development, are particularly dependent on an exquisite fine-tuning of the processes that balance robustness and plasticity to maximize fitness.

• **Scope and Conclusions** This paper reviews recently identified mechanisms, both systems-level and molecular, that modulate robustness, and discusses their implications for the optimization of plant fitness. Robustness in living systems arises from the structure of genetic networks, the specific molecular functions of the underlying genes, and their interactions. This very same network responsible for the robustness of specific developmental states also has to be built such that it enables plastic yet robust shifts in response to environmental changes. In plants, the interactions and functions of signal transduction pathways activated by phytohormones and the tendency for plants to tolerate whole-genome duplications, tandem gene duplication and hybridization are emerging as major regulators of robustness in development. Despite their obvious implications for plant evolution and plant breeding, the mechanistic underpinnings by which plants modulate precise levels of robustness, plasticity and evolvability in networks controlling different phenotypes are under-studied.

**Key words:** Developmental robustness, species diversity, plasticity, capacitor, canalization, noise, network hubs, network motifs, redundancy, Hsp90, chromatin, rDNA, hormones.

## INTRODUCTION

Organisms constantly face changing environmental conditions, some of which are predictable and others are not. To cope and thrive in the face of changing environments, organisms have developed systems that enable phenotypic change or prevent it. At one extreme, there are some traits that exhibit little or no phenotypic change despite an environmental challenge; these traits display robustness or canalization (Waddington, 1961; Lempe *et al.*, 2012). At the other extreme, there are traits that display a large degree of plasticity – programmed shifts in phenotype in response to different environments (Pigliucci, 2001), which are important for fitness. A population of genetically identical individuals exhibits little variation in a robust trait in the face of environmental change. In contrast, a plastic trait will exhibit a mean shift across genetically identical individuals in response to environmental change. Within each organism, traits range from robust to plastic, as determined by the underlying genetic network, to provide optimal fitness within changing environments (Waddington, 1961).

Across the broad array of traits required for fitness, organisms will differentially deploy robustness and plasticity to control phenotype. The specific selection pressures on an individual trait influence the degree to which this trait will display robustness or plasticity. When stabilizing selection acts on a trait, robustness is adaptive and plasticity is costly (Wagner *et al.*, 1997; but see also Kawecki, 2000). One example is floral morphology, which displays strong stabilizing selection across angiosperms to maintain interactions with pollinators. In contrast, plasticity will be adaptive in environments with predictable changes in temperature, light or precipitation (Gomez-Mestre and Jovani, 2013). Finally, unpredictably fluctuating environments create selective regimes that favour stochastic plasticity, in which the phenotype unpredictably changes across or even within individuals (Clauss and Venable, 2000). Thus, environmental responses of all traits are likely tuned from robustness to programmed or stochastic plasticity depending upon the selective pressures and environments that influence that trait.

Although robustness and plasticity are mechanistically related, complete robustness in a trait precludes plasticity and vice

versa. This absolute relationship, however, can be altered in a temporal fashion, such as in traits that display programmed plasticity, wherein they shift from one robust state to another (Fig. 1). For example, the transition to flowering in the annual plant *Arabidopsis thaliana* demonstrates a temporal shift from robust vegetative growth via a plastic transition period to robust reproductive growth (Swiezewski *et al.*, 2009; Csorba *et al.*, 2014). Once the transition to flowering has occurred, the trait is robust and, at least in *A. thaliana*, irreversible. Other traits, however, display reversible plasticity between alternating robust states. In plants, the shade avoidance response shows reversible plasticity wherein plants can alter growth in response to varying light exposures or shade (Devlin *et al.*, 1999). Reversible plasticity is also displayed by the repeated seasonal transitions between vegetative and reproductive growth in perennial plants. As demonstrated with these examples, traits are shaped by a blend of robustness and plasticity acting at different times.

Where a trait lies on the robustness-to-plasticity spectrum is determined by the underlying genetics that controls the trait and how this genetic network responds to changing environments to optimize fitness. Within genetic networks, there are at least two components that influence a trait's position on the robust-to-plastic spectrum: redundancy among genes and topology of gene interactions. Redundancy influences both phenotypic robustness and plasticity. For example, gene duplication can provide robustness through redundant function and can promote plasticity through sub-functionalization of gene copies. Similarly, network topology also influences both phenotypic robustness and plasticity. Network topology can be expressed as gene connectivity and the pattern of wiring among genes. Both the connectivity of a given gene and its position within the wiring of the genetic network will influence a gene's role in robustness and plasticity. In most of the existing literature, the

modulation of robustness and plasticity has been traced to specific molecular mechanisms and genes, some of which we briefly review here, and we suggest further candidates. Here, we argue that the effects of these genes and mechanisms are fundamentally acting through their effects on the underlying genetic (and molecular) networks.

The fact that whole-organism traits, such as flowering, undergo transition from robust to plastic states implies that this transition must be wired within existing genetic (and molecular) networks. In other words, the properties of the underlying network controlling growth must be able to maintain robust vegetative growth but simultaneously permit the plastic transition to flowering. Here, we review how recent research is illuminating the mechanistic underpinnings of the complex relationship between robustness and plasticity.

First, we consider the influence of morphological and genetic redundancy on plasticity and robustness, particularly considering whole-genome and single-gene duplications. Second, we discuss how features of gene regulatory networks, such as connectivity and specific network motifs affect, phenotypic plasticity and robustness. In addition to such system-level features, specific molecular mechanisms have been associated with tuning environmental responses. We discuss the role of the protein chaperone Hsp90, chromatin-modifying enzymes and ribosomal DNA (rDNA) copy number variation in influencing phenotypic plasticity and robustness.

#### REDUNDANCY AS A SYSTEMS-LEVEL FEATURE GOVERNING ROBUSTNESS IN PLANTS

One systems-level feature through which robustness arises is redundancy at both the morphological and the genome level.

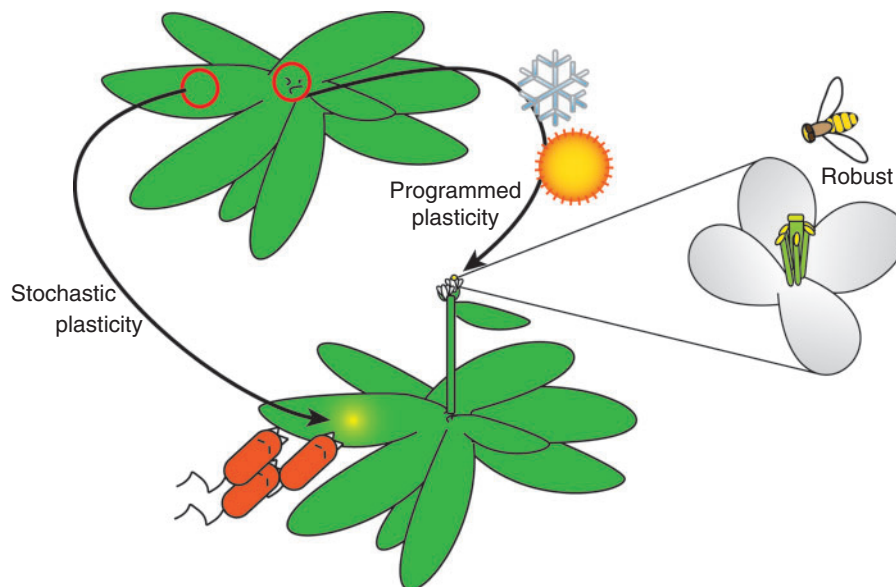


Fig. 1. Changing environments induce phenotype-specific responses. Phenotypic responses to changing environments range from plastic to robust. Environmental changes that occur predictably lead to programmed responses. Shown here is the transition between vegetative and reproductive growth induced by seasonal exposures to low and then high temperatures. Other environmental challenges are unpredictable, such as pathogen attacks (red bacteria), which trigger immune responses in plants, including the hypersensitive response (yellowed leaf). In contrast, some traits are largely robust to changing environments. For example, most floral morphs are invariant to both predictable and unpredictable environmental changes. Invariant floral forms are important for reproductive success.

Redundancy is the presence of nearly identical duplicate parts wherein each duplicate suffices to replace the function of the other copy. Thus, redundancy allows a system to function fully even when a duplicate is lost. At the morphological level, redundancy manifests through continuous development, in which multiple copies of organs, such as branches and leaves, are produced as defined by a plant's developmental programme and as enabled by environmental conditions.

At the genome level, plants show an unusual capacity to tolerate whole-genome duplication and hybridization – and thus redundant gene copies – presumably due to RNA-directed DNA methylation (RdDM), a plant-specific mechanism of gene silencing that prevents complications due to increased gene dosage (Ha *et al.*, 2009b). The functional redundancy created by these events likely contributes to plant robustness. In this section, we discuss recent evidence that plants indeed utilize redundancy at the morphological and genetic levels to increase robustness.

#### *Morphological redundancy in plant development promotes trait robustness*

Plants are composed of repeating units, such as individual leaves, flowers, branches and roots. Unlike most animals, plants replace damaged units with new ones through continuous development, creating morphological robustness. Others have described plants as 'populations of organ modules' and suggested that the extreme morphological redundancy observed in plants compensates for their lack of mobility (Harper, 1980).

The trait leaf venation is a key example of robustness arising from morphological redundancy. Veins provide mechanical support to leaves as well as structures for the transfer of water and solutes (Roth-Nebelsick *et al.*, 2001). Major or primary veins branch into minor or secondary veins and so forth, creating a branching network of veins. In angiosperms, these veins form a reticulated network of criss-crossing lines (Roth-Nebelsick *et al.*, 2001). This reticulated network creates robustness by providing multiple alternative pathways by which solutes can move if veins are damaged by herbivore feeding or strong winds (Roth-Nebelsick *et al.*, 2001). Does the generation of reticulate networks with high redundancy and robustness come at a cost? In other words, can an argument be made that the robustness of the vein network is selected? Price and Weitz (2014) modelled the costs of increased redundancy in reticulated vein patterns across 324 angiosperm species. On average, the length of the network only increased by 6.3 % when reticulated networks were compared with branched ones. Although this length increase may be an evolutionarily important cost, it is also correlated with increased photosynthetic rate (Price and Weitz, 2014). Thus, the formation of these highly redundant networks is likely cost-neutral or driven by increased photosynthetic rate. The presence of reticulated venation networks has been suggested to be a driver of the success of angiosperms (Brodrribb and Feild, 2010), possibly due to both increased photosynthetic rate and increased robustness.

The reticulated nature of leaf venation is robust, not plastic. In contrast, components that underlie leaf venation, such as vein diameter and vein density, are plastic (Sack and Scoffoni, 2013). For example, the minor veins in leaves under high irradiance show a greater diameter than those in shaded leaves; the

greater diameter of minor veins is also observed in leaves exposed to drought (Dorantes and Sánchez, 2006; Brodrribb and Jordan, 2011). Vein density is also plastic; sun-exposed leaves tend to show higher density of minor veins compared with shaded leaves within trees (Sack and Frole, 2006). Thus, in order to maximize solute flow and photosynthesis, sun-exposed leaves develop additional redundant minor veins. It is unknown whether the genes generating the vein network control both its reticulated venation pattern *and* its plasticity or whether the two are controlled by separate molecular entities. As increased vein density increases network robustness, we speculate that the same genes may govern both.

#### *Genome duplication/redundancy generates robustness while simultaneously generating the raw material for wiring networks for trait plasticity*

Whole-genome duplication (WGD) and hybridization within plants are well-studied mechanisms by which plants can simultaneously generate robustness as well as increase the potential for fine-tuning development and environmental responsiveness and evolving new traits (Rieseberg and Carney, 1998; Fawcett *et al.*, 2009).

After WGD or hybridization of closely related species, all genes are present in duplicate. Over time, many copies are lost and revert to their prior copy number. In some cases, the presence of a paralogue relieves the selection pressure acting on the original gene copy. This relief of selective pressure can result in the emergence of a novel function in one of the paralogues (neo-functionalization). In other cases, both gene copies become specialized in different aspects of the original function (sub-functionalization) (Conant and Wolfe, 2008). Both WGD and hybridization escape the challenge of dosage compensation that individual gene duplication events face. At their core, WGD and hybridization enable the building of the intricate genetic networks that allow both robustness and plasticity.

Many genes that are maintained in duplicate post-WGD continue to share some function. This redundancy in function is a well-documented source of robustness in all organisms, including plants. Indeed, in plants and many other organisms, single-gene deletions of duplicated genes have smaller effects on morphology than deletions of singletons (Hanada *et al.*, 2009). The same tendency was observed for metabolite levels (Hanada *et al.*, 2011). Thus, duplicated genes provide trait robustness in the presence of mutations. However, it should be noted that the extent of the functional redundancy provided by gene duplicates is rarely studied in much detail. Members of a closely related gene family may compensate for the loss of one gene under laboratory conditions or even most conditions, yet defects may arise in the context of a particular environment or a particular genomic background. Consequently, the concept of redundancy depends critically on the level of detail studied or the precision with which the phenotype was measured.

As paralogues diverge from one another in function, the robustness they provide for each other's loss is reduced. For example, the well-studied BES1 and BZR1 transcription factors (TFs) of the brassinosteroid signalling pathway are frequently assumed to function redundantly (Krizek, 2009; Ye *et al.*, 2012). This assumption arises from their high homology

(88% amino acid identity) and the fact that these TFs have been typically studied with individual dominant mutants. These mutations prevent degradation and convert the affected protein, either BES1 or BZR1, into the major effector of brassinosteroid signalling, obscuring subtle differences between the TFs. Indeed, close examination of BES1 and BZR1 reveals that the two TFs have different protein interaction partners, different splice forms and different patterns of expression, as well as different mutant phenotypes (Wang *et al.*, 2002; Yin *et al.*, 2002; Lachowiec *et al.*, 2013; Jiang *et al.*, 2015).

Paralogues are known to diverge in expression patterns in different subsets of tissues, developmental stages or environments (Diss *et al.*, 2014). We argue that plasticity in gene expression patterns emerges because the presence of duplicated genes enables expression patterns to be specifically associated with each copy. The diversity in gene expression across closely related species is greater among duplicated genes than singletons (Gu *et al.*, 2004; Ha *et al.*, 2009a), and genes with more paralogues diverge in expression to an even greater degree (Ha *et al.*, 2009a). For example, in cotton over 99% of gene duplicates have diverged in tissue-specific expression patterns over the last 60 My (Renny-Byfield *et al.*, 2014). These gene duplicates often complement one another's expression pattern, suggesting that gene expression divergence contributed to the maintenance of these duplicated genes (Renny-Byfield *et al.*, 2014).

Divergence in expression patterns among duplicate genes likely promotes plasticity in gene expression in response to changing environments. In *A. thaliana*, gene duplicates tend to be upregulated when exposed to biotic and abiotic stresses, unlike singletons (Ha *et al.*, 2007). Divergence in gene expression between duplicates was higher in response to abiotic and biotic stresses when compared with expression divergence in different tissues (Ha *et al.*, 2007). In one specific example, the two copies of the alcohol dehydrogenase gene (*Adh*) in cotton diverged in their expression responses to cold, dark and submersion (Liu and Adams, 2007). Thus, we suggest that the transient robustness provided by redundant gene duplicates increases regulatory precision in response to specific environmental stimuli and enables appropriate plastic responses.

It is undisputed that WGD and hybridization of closely related species provide raw material for evolution, specifically the emergence of novelty. Plant defence metabolism or secondary metabolism is a key model system in which the role of WGD in generating trait plasticity and novelty is being studied (Chae *et al.*, 2014). The continuous battle between pests and plants leads to continuous evolutionary pressure to evolve novel defence metabolites (Ehrlich and Raven, 1964). This Red Queen evolutionary scenario has led to the production of over 200 000 secondary plant compounds across angiosperms (Hartmann, 2007). Studies of the reference plant *A. thaliana* generated evidence supporting the role of both WGD and gene duplications in creating new glucosinolates, which are common defence compounds against herbivores (Kliebenstein, 2008; Hofberger *et al.*, 2013). Duplicate genes for enzymes within the glucosinolate pathway show a higher level of retention in comparison with genes involved in primary metabolism (Kliebenstein, 2008; Hofberger *et al.*, 2013). The redundancy produced by duplication has led to a number of instances in which one paralogue has evolved a brand-new enzymatic

function or even a novel TF function leading to the generation of a new defence compound (Kliebenstein *et al.*, 2001; Kroymann *et al.*, 2003; Hansen *et al.*, 2007, 2008; Li *et al.*, 2008; Sønderby *et al.*, 2010). Thus, WGD facilitates plasticity, here as the programmed response to herbivores, while also contributing to robustness.

Like WGD, hybridization of related species doubles genome content. Unlike WGD, hybridization combines two different genotypes, which permits the emergence of transgressive phenotypes. As a result, hybrids may be more robust to novel environments than progenitor lines. For example, the hybrid sunflower *Helianthus paradoxus* is significantly more salt-tolerant in laboratory experiments than either of its progenitor lines, *H. annuus* and *H. petiolaris*. The increased capacity of *H. paradoxus* hybrids to tolerate high-salt environments allows them to occupy salt marsh habitats (Welch and Rieseberg, 2002). Thus, in this case, increased robustness to environmental stress allows hybrids to colonize a new environment.

## NETWORK WIRING AND ROBUSTNESS AND PLASTICITY

In addition to redundancy, the topology and connectivity of a network can dramatically influence robustness and plasticity. Connectivity describes the number of nodes with which a given node interacts. In terms of genetic networks, this concept is often applied to TF networks, by enumerating the input edges and output edges (targets) of TFs. In addition to simply counting the number of interactions per node, as we discuss in this section, the specific pattern of the connections or network wiring is critical for robustness and plasticity. For example, some TFs, like HY5, the master regulatory of photomorphogenesis, show many input and output edges despite changing environments. In contrast, some TFs show dramatic changes in both input and output edges, and yet others only show change in either input or output edges in response to stimuli (Sullivan *et al.*, 2014). These patterns give clues as to the importance of a particular TF in a studied process, as well as to its possible role in robustness or plasticity. Intuitively, a factor that vastly changes connectivity in response to a given stimulus is of great importance for the ensuing plastic response. An example would be HSFA2, the known 'amplifier' of the heat-shock response, which gets more connected upon heat shock (Sullivan *et al.*, 2014). In contrast, TFs that drastically change in the number of input edges while output edges do not change, are implicated as possible noise buffers. In fact, such detailed analyses of network topology can detect changes in regulatory feedback loops, including autoregulatory ones, along a time course. Feedback loops are associated with robust molecular phenotypes (Hornstein and Shomron, 2006).

### Connectivity in gene networks is associated with robustness

Connectivity in gene networks follows an exponential distribution, with most genes having few interactors and few genes – the so-called network hubs – having many. This pattern is associated with robustness because the likelihood of a disrupting a hub is small, and disruptions elsewhere are buffered through the modular structure of genetic networks (Albert *et al.*, 2000;

Lehner *et al.*, 2006; Levy and Siegal, 2008; Fu *et al.*, 2009). However, high connectivity is not the only possible definition of a hub. We prefer the more specific definition of hubs as crucial nodes for information flow in which disruption produces significant phenotypic consequences and loss of robustness (Table 1). Such genes/molecules must not necessarily be highly connected within a network module; rather they could touch many modules across the entire network (Whitacre, 2012).

How are hubs identified? Traditionally, network hubs have been identified through genetic analysis as those genes that show outsize epistasis with many other genes. Examples include the chaperone Hsp90 in yeast, plants, flies, nematodes and fish (Rutherford and Lindquist, 1998; Queitsch *et al.*, 2002; Yeyati *et al.*, 2007; Jarosz and Lindquist, 2010; Casanueva *et al.*, 2012), in addition to chromatin remodelling proteins in nematodes (Lehner *et al.*, 2006), developmental regulators such as ERECTA in plants (Hall *et al.*, 2007) and key molecules in microRNA pathways in both plants and animals (Cassidy *et al.*, 2013; Schmiedel *et al.*, 2015). Notably, Hsp90 is an example of a hub that is not part of a specific network module or pathway, but rather peripherally but essentially affects many, if not most, processes in living cells (Whitacre, 2012). Chromatin remodelling fits into this category too as it globally modulates gene expression.

Similar to identifying hubs in genetic networks, hubs can be defined as proteins that engage in many more functionally relevant protein interactions than others (Arabidopsis Interactome Mapping Consortium, 2011). Surely, most components of the protein degradation machinery would cause large phenotypic change when disrupted. Hsp90, a well-known player in trait robustness and plasticity (Sangster and Queitsch, 2005; Lempe *et al.*, 2012), acts at the level of protein interactions together with its army of co-chaperones that define substrate specificity. Another example is the protein ELF3, which appears to serve as a scaffold for potentially many other proteins (Nusinow *et al.*, 2011). Through its various interactions, ELF3 contributes

to circadian regulation (Nusinow *et al.*, 2011; Anwer *et al.*, 2014), regulation of growth and development (Zagotta *et al.*, 1996; Liu *et al.*, 2001) and temperature response (Box *et al.*, 2014; Mizuno *et al.*, 2014; Nieto *et al.*, 2015). By mapping gene expression noise, ELF3 was also identified as a major player in the robustness of gene expression and whole-organism traits (Jimenez-Gomez *et al.*, 2011).

The importance of protein complexes and their changing connectivity for robustness is also illustrated by recent work on floral homeotic genes. The identity of floral organs is determined by the concentration of homeotic TF proteins and their complexes as described in the classic ABC model (Bowman *et al.*, 1991) and its descendant, the ABCDE model (Theißen, 2001). The B-genes DEF and GLO (also known as PISTILLATA and APETALA 3 in other species) are primarily expressed in the second and third floral whorls, defining petal and stamen identity. Unlike the other MADS-box homeotic TFs, DEF and GLO exclusively heterodimerize with one another in derived angiosperms; their connectivity is reduced in comparison with other related TFs. Disrupting either gene suffices for B loss-of-function phenotypes. However, in basal angiosperms they can also homodimerize, thereby increasing DEF and GLO connectivity. Early angiosperms tend to have floral organs with weaker identity boundaries. Melzer and co-authors (2014) argue that the defined floral forms and sharp organ identity boundaries of derived angiosperms may be due to the obligate heterodimerization of the B-class proteins. Here, it appears that reduced rather than increased connectivity among DEF and GLO TF proteins results in more robust floral organ formation. It remains to be seen whether these same genes play a role in the plastic shifts of floral shape as seen in the plant species with environmentally determined monoecious flower formation.

In addition to genetic and protein interaction networks, much effort has been devoted to building expression-based networks (Ma *et al.*, 2013, 2015) or networks based on TF occupancy at

TABLE 1. Definition of some terms

Robustness	The ability of an organism to maintain a specific phenotypic value or state in the face of environmental and genetic perturbations (Lempe <i>et al.</i> , 2012). Traditionally, robustness of individuals has been measured as the degree of symmetry in morphological features (Auffray <i>et al.</i> , 1999). Another robustness measure is the degree of accuracy with which a genotype produces a quantitative phenotype across many isogenic siblings. Thus, robustness measurements are frequently trait-specific and may not be predictive of robustness in other traits (Lempe <i>et al.</i> , 2012).
Canalization	This assumes that genetic systems evolve to an optimum phenotype via stabilizing selection and this phenotype is robust. The robust optimum arises through elimination of deleterious alleles and reduction of additive genetic effects (Waddington, 1942; Gibson, 2009).
Plasticity	The ability of organisms to alter their physiology, morphology and development in response to environmental changes (Debat and David, 2001; Pigliucci, 2001). Here, we distinguish between programmed plasticity and stochastic plasticity. Programmed plasticity refers to <i>predictable</i> changes in phenotype in response to a particular environmental change. In contrast, stochastic plasticity refers to <i>unpredictable</i> phenotypic changes that may or may not be in response to a stimulus. Both types of plasticity are enabled by the features of the underlying genetic networks. The former is adaptive in predictable environments; the latter is associated with unpredictable, fluctuating environments.
Network hubs	Network nodes that are crucial for information flow and in which disruption causes severe phenotypic consequences, including loss of robustness (Albert <i>et al.</i> , 2000). More recently this term has been applied to highly connected nodes in different networks based on genetic interaction networks, protein interactions, gene expression data or transcription factor occupancy.
Phenotypic capacitor	This refers to genes that keep genetic variation phenotypically silent when fully functional and release genetic variation when perturbed. The term was initially coined to describe the role of the chaperone Hsp90 in maintaining phenotypic robustness (Rutherford and Lindquist, 1998). We view phenotypic capacitance as an epistasis phenomenon, in which a network node epistatically interacts with many other loci and hence acts as a strong genetic modifier. This epistasis phenomenon differs critically from the traditional pairwise ‘gene × gene’ interaction and could be thought of as a ‘gene × genome’ interaction. Network nodes that keep genetic variation phenotypically silent typically also maintain phenotypic robustness in the face of environmental perturbation and vice versa, although exceptions exist.

promoters. Using the latter approach, Sullivan *et al.* (2014) studied the dynamics of TF networks in *A. thaliana* seedlings transitioning from dark to light growth conditions and seedlings exposed to heat shock (Sullivan *et al.*, 2014). Although many of their findings are validated by prior classic studies, the functional relevance of the many novel connections that are described has yet to be explored. As discussed above, when connectivity is divided between input and output edges for each TF, stark differences arise for TFs with comparable overall connectivity across conditions. These detailed patterns hold information about potential roles of particular TFs in robustness. For example, some TFs, such as EIN3 and MYC2, drastically change the number of input edges across conditions while their output edges remain more or less stable. This pattern suggests that such factors may act as environmental noise buffers across conditions because, despite vastly increased inputs, the outputs of these TFs remain stable.

However, it is non-trivial to predict the influence of TF hubs based on their connectivity. Due to the hierarchical nature of regulatory networks, regulatory effects of a TF can be multiplied or dampened across the regulatory network (Taylor-Teeples *et al.*, 2014). For example, let us assume a TF directly interacts with only a few other TFs; however, these secondary TFs have a large number of targets. In this scenario, disruption of the upstream, less connected TFs may produce more severe phenotypic consequences than disrupting one of the secondary, highly connected TFs. The regulatory hierarchy may be particularly important in the regulation of metabolism (Taylor-Teeples *et al.*, 2014). Support for the idea that direct connectivity is not highly predictive of phenotypic consequence was recently found by examining TF connectivity to genes in the biosynthetic pathway for the glucosinolate defensive metabolites in *A. thaliana* (Sønderby *et al.*, 2010). The authors observed no relationship between the connectivity of a TF and the likelihood or magnitude of a corresponding TF mutation having a phenotypic effect on glucosinolate accumulation. It remains to be tested whether this conclusion holds when secondary connections are included in the network analysis.

Hub genes are known to be key targets of biotic factors, which may promote effective programmed plasticity responses. For example, plant-infecting viruses encode a small number of proteins but elicit broad transcriptional responses throughout a plant. A meta-analysis of transcriptional studies examining the effect of viral infection suggests that, across viral infections of *A. thaliana*, the most consistently responsive genes are hub genes (Rodrigo *et al.*, 2012). Similar patterns were found for bacterial infections, which also preferentially affect hub genes (Mukhtar *et al.*, 2011). A systematic test of *A. thaliana* proteins interacting with pathogen effector proteins from two bacteria that diverged 2 billion years ago discovered that a limited set of hub proteins interacts with both pathogen effectors (Mukhtar *et al.*, 2011). Together, these studies suggest that diverse pathogens display convergent evolution such that they recurrently obtain the capacity to target hub genes within plants. Thus, in the case of biotic attackers, it appears that hubs are targeted and elicit broad, robust responses. These results create a centrally important chicken–egg question. Do the biotic attackers target hub genes to overcome plant defence? Or do plants utilize these hubs as trip-wires to detect biotic attackers and elicit a strong and robust defence? In the larger context of this review, are

hubs generally maintaining robustness? Are hubs enabling both short-term plastic responses to environmental change and long-term evolutionary adaptation? The available evidence suggests that both are true (Lehner *et al.*, 2006; Fu *et al.*, 2009).

#### Network motifs associated with robustness

In addition to connectivity, the topology of connections among genes determines the robustness of gene networks. Networks are composed of simple patterns or motifs of connections among nodes. Probably the best known motifs associated with robustness are feed-forward and feed-back loops (Alon, 2003; Hornstein and Shomron, 2006; Lempe *et al.*, 2012; Payne and Wagner, 2014; Schmiedel *et al.*, 2015). Feed-forward loops come in two flavours: coherent and incoherent.

*Coherent feed-forward loops* typically consist of a TF that regulates another TF or miRNA and they jointly regulate a target gene either positively or negatively (Fig. 2A). This network motif is often found in cell fate determination (Hornstein and Shomron, 2006) because its redundancy reinforces fate decisions. Examples have been described in mammalian cells (Xie and Cvekl, 2011) and flies (Cassidy *et al.*, 2013). In plants, coherent feed-forward loops also contribute to the robustness of secondary cell wall synthesis. Prior studies attributed the robustness of secondary cell wall synthesis to the extensive functional redundancy among the contributing TFs (Carlsbecker *et al.*, 2010). An in-depth survey of the TF network directing secondary cell wall synthesis demonstrates that TF redundancy arises from 96 feed-forward loops regulating the activity of TFs and enzymes driving this process (Taylor-Teeples *et al.*, 2014).

*Incoherent feed-forward loops* consist of a TF regulating another TF or miRNA. Together, they regulate a target in opposite ways, e.g. the first TF activates the target gene and the second TF or miRNA represses it (Fig. 2B). This motif has been implicated in dampening noise in gene and protein expression levels as it exists in regulation of both transcription and translation (Hornstein and Shomron, 2006; Schmiedel *et al.*, 2015). In plants, incoherent feed-forward loops play a role in defence against biotic attacks. For example, an incoherent feed-forward loop exists in pattern-triggered immunity (PTI), which is driven by at least four signalling pathways: the jasmonate, ethylene, phytoalexin-deficient 4 and salicylic acid pathways (Tsuda *et al.*, 2009; Kim *et al.*, 2014). Using a multiple regression model, Kim and co-authors (2014) attributed robustness in PTI to an incoherent feed-forward loop in which biotic attacks induce both ethylene and jasmonate signalling, but the latter is repressed by ethylene signalling. Their argument is supported by a recent study demonstrating robust transcriptional responses to diverse *Botrytis cinerea* strains in wild-type *A. thaliana* plants compared with highly variable responses in jasmonate mutants (Rowe *et al.*, 2008). In addition to providing robust responses to diverse biotic attacks, the jasmonate/ethylene incoherent feed-forward loop also prevents ectopic activation of PTI by damping noise (Kim *et al.*, 2014). Both coherent and incoherent feed-forward loops containing miRNA were hypothesized in 2006 to provide robustness in development and reduce noise in gene expression and protein levels (Hornstein and Shomron, 2006). Recent studies have provided several specific examples in different organisms, although systematic analyses are

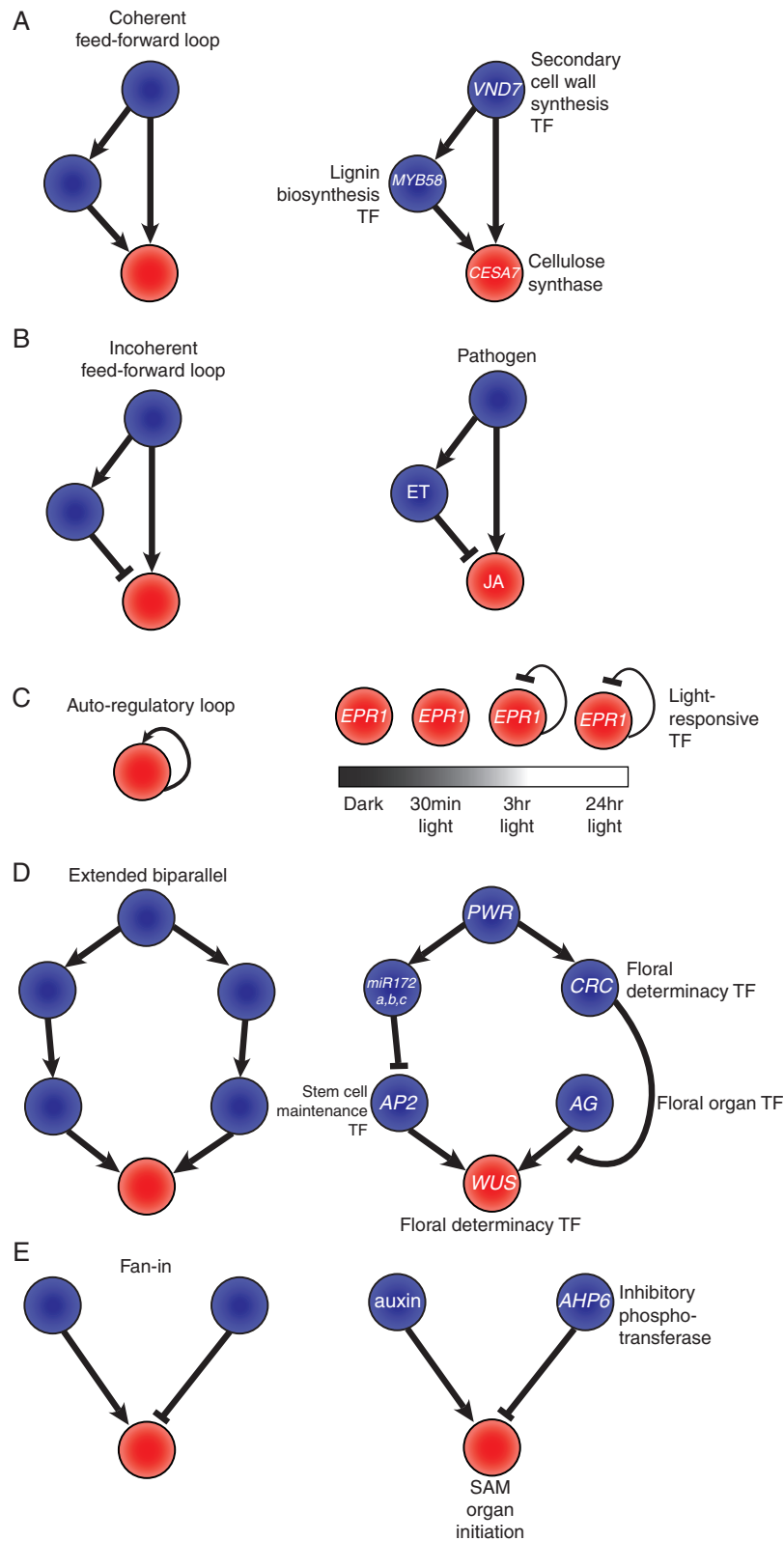


FIG. 2. Network motifs associated with robustness and plasticity. Several network motifs are associated with robust phenotypes. (A) Coherent feed-forward loops (left), such as those acting in secondary cell wall synthesis (right), maintain robust development (TF, transcription factors). (B) An incoherent feed-forward loop (left) triggered by pathogens induces plant immunity (right), a plastic response. (C) An autoregulatory loop (left) in which *EPR1* negatively regulates itself is important for the plastic developmental process of photomorphogenesis (right). The *EPR1* autoregulatory loop is not present in the dark or upon initial light exposure but emerges with increased exposure to light. (D) A variant of an extended biparallel motif (left) is important for robust floral and shoot determinacy (right). (E) Phyllotaxy relies on a fan-in motif (left) for robust patterning of leaf emergence (right).



lacking. Such analyses may be forthcoming, especially in the light of detailed occupancy-based TF networks in plants and other organisms and improved predictions for miRNA target sites. Like feed-forward loops, feedback loops have been known for decades to provide robustness in regulatory networks. This motif describes TFs, which regulate their own expression in autoregulatory feedback loops or their respective activator or repressor (Fig. 2C). Autoregulatory feedback loops play an important role in reinforcing cell fate or robust responses to the environment.

Another type of motif that promotes phenotypic robustness through redundancy is the biparallel motif. Biparallel motifs contain four nodes, in which one node regulates two others, and these two downstream factors jointly regulate a single target node (Fig. 2D). A variant of the biparallel motif regulates the termination of floral stem cells, a process critical for plant reproduction. *POWERDRESS*, a newly identified gene, regulates floral determinacy by promoting the transcription or PolII occupancy at *miR172* and *CRC*, both of which regulate the floral determinacy gene *WUSCHEL*. Notably, *miR172* and *CRC* regulate floral determinacy at different times, with *miR172* acting in early floral development and *CRC* acting later (Yumul et al., 2013). Using two functionally redundant pathways to control *WUSCHEL* expression likely contributes to robustness in floral and shoot determinacy (Laux et al., 1996).

Robust morphological pattern formation allows plants to maintain structure and harvest resources such as light. The positioning of lateral organs, phyllotaxis, is stereotypical for most plants and arises from the periodic initiation of organ primordia at the shoot apical meristem (SAM). The robustness of phyllotaxis appears to depend on a network motif in which one regulator promotes and a second regulator represses organ formation (Fig. 2E). The accumulation of the plant hormone auxin promotes the initiation of organ primordia (Reinhardt et al., 2000), and polar transport of auxin out of cells creates an inhibitory field that precludes cells from forming organ primordia (Reinhardt et al., 2003). Deterministic models of phyllotaxis show that interaction of inhibitory fields among cells composing the SAM explains the spatial and temporal initiation of organ primordia (Mirabet et al., 2012). Mirabet and co-authors (2012) instead modelled the transport of auxin among cells at the SAM as a stochastic process. In these stochastic models, irregularities in phyllotaxis arise, such as the simultaneous initiation of two primordia or reversals in spiral handedness. The same irregularities are observed in phyllotaxis mutants, suggesting that the stochastic model misses parameters. In *Drosophila melanogaster* embryonic development, two inhibitory fields interact to increase robustness (Okabe-Oho et al., 2009). Inclusion of a second inhibitory field in the model decreased irregularities. The dual field model traced irregularities to particular mis-functions in the SAM, such as the incorrect ranking of primordium age, to cause reversals in spiral handedness.

Indeed, 2 years later, cytokinin signalling emerged as the unknown secondary inhibitory field required for robust phyllotaxis (Besnard et al., 2014). Cytokinins were already known to regulate SAM size, and cytokinin signalling mutants also alter the shape of the SAM (Leibfried et al., 2005). Expression studies implicated *AHP6*, which is a cytokinin signalling inhibitor, as a strong candidate. Strikingly, mutants in *AHP6* exhibit

spiral handedness reversals. Closer inspection of *ahp6* SAMs reveals simultaneous initiation of multiple primordia, indicating that *AHP6* directs the temporal initiation of organ formation. Like auxin, *AHP6* was present in a gradient among cells, centred on primordia. Thus, robust phyllotaxis arises through the activity of two inhibitory fields formed by auxin depletion and *AHP6* accumulation, which together tightly control the temporal and spatial initiation of organ primordia (Besnard et al., 2014).

#### Network topology and motif rewiring controls robustness across environments

As network topologies are maintained or altered in response to changing environments, the same is observed for network motifs. The wiring between genes and targets can be altered to reinforce the programmed, plastic shift to another robust phenotypic state. For example, Sullivan et al. (2014) showed several examples of gain and loss of autoregulatory loops throughout photomorphogenesis (Sullivan et al., 2014). One example is EARLY PHYTOCHROME RESPONSIVE 1 (*EPR1*, *REVILLE7*), which is one of the first genes to respond to light exposure via phytochrome A and B networks. Upon exposure to light, *EPR1* expression is induced and a negative autoregulatory loop emerges, tightly controlling expression (Kuno et al., 2003). Another distinct example is *MYC2* (*JAI1*, *JIN1* for jasmonate insensitivity), which is both an inhibitor of seedling photomorphogenesis and a key biotic defence regulator (Dombrecht et al., 2007; Shin et al., 2012; Schweizer et al., 2013). In response to light, *MYC2* loses its positive autoregulation and generally loses connectivity. Thus, network topology is readily rewired in response to changing environments.

This plastic wiring also controls robustness within the shade avoidance response, in which connections between key morphological genes are re-wired. The shade avoidance response describes the stereotypical developmental switch that occurs when plants are in competition for light (grown in the shade of other plants) compared with when they are grown in open conditions. During high-light conditions, the underlying network is wired to produce high levels of auxin that lead to robust growth even in the presence of decreased auxin sensitivity (Hersch et al., 2014). In low light, however, the network is re-wired to produce less auxin due to the upregulation of the negative regulator of auxin production *HFR1* and concomitantly increases in auxin sensitivity, possibly through increased expression of auxin co-receptors such as *AFB1*. Comparing high- and low-light networks by modelling demonstrates that the auxin response during high light is more robust (Hersch et al., 2014). In contrast, in low-light conditions plants show a more acute response to auxin, which is enabled by decreased lower robustness. Thus, plants modulate network topology and motifs to shift phenotype between robustness and plasticity in certain environments.

#### Temporal staggering of signalling produces robust responses

In response to pathogens, plants activate signalling cascades to reduce harm and overwhelm invaders (reviewed in Cui et al., 2015). As a first line of defence, plants recognize general

pathogen features through activity of cell surface proteins and mount PTI. However, many pathogens have evolved to circumvent the innate immune response. Thus, plants utilize a back-up system in which specific pathogens elicit an immune response, known as effector-triggered immunity (ETI). In ETI, the innate immune response is raised and amplified, through signalling cascades triggered by the defence hormone salicylic acid (SA), among others. The SA signalling pathway induces the hypersensitive response, which leads to localized cell death at the site of infection, as well as systemic acquired resistance in uninfected parts of the plant. Of course, pathogens have evolved mechanisms to circumvent ETI by neutralizing factors that act in the SA pathway. It is clear that robustness in these mechanisms is critical to withstanding bacterial attack. Tsuda *et al.* (2009) report that downstream responses of the SA pathway are also activated in an SA-independent fashion. They trace this SA-independent activation to the delayed expression and sustained activity of the kinase MPK3. This is one of the kinases activated by the detection of pathogens in ETI, but activation of its expression is delayed relative to other SA signalling components. Therefore, robustness in ETI, despite SA signalling circumvention, emerges from the temporal staggering of SA signalling, specifically the sustained activity of MAPK3 to form a coherent feed-forward loop. It would be interesting to explore whether the robustness of ETI signalling is primarily due to the staggered, temporal component or the mere redundancy between the SA and MAPK3 signal elicitation, or both.

#### THOUGHTS ON ESTABLISHED MOLECULAR MECHANISMS THAT PROVIDE ROBUSTNESS AND ENABLE PLASTICITY AND POSSIBLE CANDIDATE MECHANISMS

In several species, robustness across diverse traits has been traced to the function of genes that perform crucial cellular functions and are involved in cellular homeostasis (Queitsch *et al.*, 2002; Levy and Siegal, 2008; Lempe *et al.*, 2012; Bauer *et al.*, 2015). Examples include genes encoding certain protein chaperones (Rutherford and Lindquist, 1998; Queitsch *et al.*, 2002; Yeyati *et al.*, 2007), genes functioning in proper genome maintenance and genes modifying expression of many others (Lehner *et al.*, 2006; Levy and Siegal, 2008). Importantly, such genes may affect diverse specific regulatory networks and motifs without being an integral component of these networks (Whitacre, 2012). We discuss specific examples below.

##### *Hsp90-dependent robustness emerges from the maintenance of genetic networks*

Hsp90 is a highly connected and evolutionarily conserved protein chaperone that facilitates the maturation of certain proteins (clients) and keeps them poised for activation. Hsp90 clients are often conserved hub proteins that comprise about 10–20% of the cellular proteome (Zhao *et al.*, 2005; McClellan *et al.*, 2007; Jarosz and Lindquist, 2010; Gopinath *et al.*, 2014). Hsp90 clients, among them many kinases, TFs and receptors, are conformationally flexible, which is the property that the chaperone is thought to recognize (Taipale *et al.*, 2010). We argue that the well-documented role of Hsp90 in maintaining

phenotypic robustness (Rutherford and Lindquist, 1998; Queitsch *et al.*, 2002; Sangster *et al.*, 2007; Yeyati *et al.*, 2007; Jarosz and Lindquist, 2010) is fundamentally an epistasis phenomenon on a network scale. The interaction of Hsp90 with its many clients maintains robustness, yet the chaperone's property of enabling signal transduction proteins to perceive and transduce signals is also of fundamental importance for phenotypic plasticity, in particular programmed plasticity. Environmental stresses such as increased temperature, drought, oxidative stress and altered conductivity compromise protein folding and increase demands for Hsp90 (Gerspacher *et al.*, 2009; Cid *et al.*, 2010; Park *et al.*, 2010; Pratt *et al.*, 2010; Sekimoto *et al.*, 2010; Stensløkken *et al.*, 2010). Under these conditions, Hsp90 clients will be less functional and the genetic network will be perturbed at many nodes. Hence, it is not surprising that Hsp90 function is environmentally responsive. Hsp90 levels are strongly upregulated in conditions that compromise protein folding to bolster protein homeostasis (Gerspacher *et al.*, 2009; Cid *et al.*, 2010; Park *et al.*, 2010; Pratt *et al.*, 2010; Sekimoto *et al.*, 2010; Stensløkken *et al.*, 2010).

As has been widely reported and reviewed (Rutherford and Lindquist, 1998; Queitsch *et al.*, 2002; Sangster and Queitsch, 2005; Sangster *et al.*, 2008; Jarosz and Lindquist, 2010; Jarosz *et al.*, 2010), Hsp90 also maintains phenotypic robustness in the presence of genetic variation, a phenomenon that gave rise to the term 'phenotypic capacitor' (Rutherford and Lindquist, 1998). We view this aspect of Hsp90 function as an extension of the above-described epistasis phenomenon (gene  $\times$  genome), in which the chaperone epistatically suppresses the phenotypic consequences of polymorphisms in either its clients or their direct and indirect interactors (which may include regulatory sequences) (Jarosz and Lindquist, 2010).

Because perturbing Hsp90 function impairs its numerous clients, it greatly alters network connectivity and wiring (Lempe *et al.*, 2012). Across the human proteome, 9200 of the 70 000 known protein interactions are lost across five cancer cell lines when Hsp90 function is inhibited with potent and specific inhibitors (Echeverria and Picard, 2014). Lost connections are due to aggregation and degradation of misfolded Hsp90 clients as well as the depletion of protein complexes that contain Hsp90 clients. Clearly, this loss of connectivity will lead to network fragmentation (Echeverria and Picard, 2014). A similar loss of connectivity is observed for occupancy-based TF networks upon heat shock (Sullivan *et al.*, 2014).

Perturbing Hsp90 in isogenic plant populations gives rise to a multitude of abnormal phenotypes (Queitsch *et al.*, 2002). It remains unclear whether this diversity of phenotypes arises from the chaperone's interactions with many specific clients or is due to generally increased noise in the genetic network causing random developmental errors. We speculate that the former, simpler explanation might be true; the published data are consistent with both scenarios. Most developmental decisions, many of which are governed by diverse Hsp90 clients, are thought to adhere to the two-forked checkpoint type, resulting in two possible fates. Let us assume an isogenic population that has to pass through many such Hsp90-dependent forks to complete development. To simplify matters further, all clients have an equal chance of being perturbed when Hsp90 function is inhibited, in which case the alternative fate is produced. Each individual decision at an Hsp90 fork is stochastic; yet, the

available developmental paths and spectrum of possible phenotypes is in principle predictable. This simple model would produce the observed wide array of Hsp90-dependent phenotypes among isogenic seedlings (Queitsch *et al.*, 2002), ranging from seeds failing germination to fully developed seedlings with specific organ defects. Applied to a simple quantitative trait like stem length, the significantly wider distribution of stem length in response to Hsp90 perturbation would arise as the overlap of two distributions representing a short and a long stem fate. In fact, there is increasing evidence from other systems that wide distributions can arise as the overlap of two or more sub-distributions. For example, differences in transcriptional bursts among mammalian cells have been recently linked with their cell volumes, presumably to keep transcript concentrations stable (Padovan-Merhar *et al.*, 2015). Returning to Hsp90, let us now assume an isogenic population in which a particular client is sensitized to Hsp90 perturbation by genetic variation. Due to the sensitized client, this fork decision is now strongly skewed towards a particular developmental path and phenotype upon Hsp90 perturbation. Indeed, strong genotype-specific phenotypes are observed in plants for both morphological traits and stem length (Queitsch *et al.*, 2002). In short, we propose that Hsp90 perturbation does not simply increase random noise in genetic networks, but rather that the multitude of Hsp90 clients governing different fate decisions generates this impression. An empirical test of this hypothesis is lacking, but could be accomplished by focusing on a specific fate decision or the simple stem-length trait and profiling the molecular properties of phenotypic extremes.

#### Regulation of chromatin modulates phenotypic robustness

Mechanisms of chromatin regulation have the potential to affect phenotypic robustness because of the centrality of chromatin in regulating gene expression (Tirosh *et al.*, 2010). Proteins involved in DNA methylation, chromatin remodelling and histone modifications are prime candidates for modulators of phenotypic robustness.

Chromatin modifications are proposed to be fast-changing, potentially heritable sources of phenotypic variation. For example, DNA methylation is thought to be important in fluctuating environments, because though it is heritable, it is also readily reversible, unlike DNA sequence variation. However, a recent study of genome-wide DNA methylation patterns for almost 100 generations does not show any acceleration in the accumulation of epimutations compared with mutations among plants, even among plants grown in diverse environments (Hagmann *et al.*, 2015). Despite the fact that certain epialleles do influence phenotypes (Weigel and Colot, 2012) and plasticity (Zhang *et al.*, 2013; Kooke *et al.*, 2015), there is little evidence for epimutations as a source of fast-acting heritable phenotypic change (Schmitz *et al.*, 2011; Li *et al.*, 2014a, b; Hagmann *et al.*, 2015). This result supports the older notion that changes in DNA methylation primarily reinforce tissue- and cell-type-specific expression patterns and hence the robustness of cell fate decisions.

*Chromatin remodelling genes maintain phenotypic robustness.* Ensuring that chromatin marks and nucleosomes are properly placed is crucial for trait robustness and plasticity. Like Hsp90,

regulators of nucleosome positioning, are associated with maintaining robust phenotypes and enabling plasticity. In worms (Lehner *et al.*, 2006), yeast (Levy and Siegal, 2008) and now in plants, SWI/SNF ATPase chromatin remodellers specifically have been identified as regulators of phenotypic robustness. In isogenic *A. thaliana*, over-expression of the SWI/SNF-type ATPase *CHR23* results in increased variability in root length and leaf size in long-day but not short-day conditions (Folta *et al.*, 2014). Long-day conditions typically promote more rapid growth, which may sensitize plants to robustness loss. However, *CHR23* loss-of-function mutants show wild-type variance in root length and leaf size, suggesting that *CHR23* dosage matters for phenotypic robustness. *CHR23* has a close paralogue with many overlapping, essential functions; the double mutant is lethal. We speculate that the right balance of both chromatin-remodelling proteins generates phenotypic robustness. One may imagine that the *CHR23* paralogue compensates for loss of *CHR23*; yet *CHR23* overexpression overrides this cross-regulation and perturbs robustness. Notably, the increased phenotypic variation upon *CHR23* overexpression correlates with increased variation of expression in genes associated with environmental stress (Folta *et al.*, 2014).

*Histone modifications promote robust plastic responses.* In plants, trimethylation of lysine 27 on histone H3 (H3K27me3) is associated with robust silencing of gene expression, which is critical for the transition between vegetative and reproductive growth. Certain *A. thaliana* strains require cold exposure to induce flowering. This cold exposure silences the expression of *FLC*, which encodes a potent flowering repressor, due to the accumulation of H3K27me3 marks. The initiation of *FLC* repression is aided by the long non-coding RNA *COOLAIR* (Swiezewski *et al.*, 2009). Polycomb-group proteins, which are enzymes with histone methylase activity, also contribute to H3K27me3 at *FLC* (Angel *et al.*, 2011). In general, Polycomb-group proteins silence gene expression and interact antagonistically with Trithorax-group proteins, which maintain active gene expression (Kennison, 1995). The non-coding RNA *COOLAIR* and Polycomb-group proteins work independently to generate a threshold of H3K27me3 levels that silences *FLC* expression and hence triggers flowering (Csorba *et al.*, 2014).

A recent study argues that *FLC* H3K27me3 silencing translates into a robust transition to flowering due to the utilization of a digital, rather than an analogue, mechanism to monitor temperature (Angel *et al.*, 2015). In the analogue (continuously varying) mechanism model, the probability of adding H3K27me3 modifications at *FLC* increases with increased cold exposure and that of removal of H3K27me3 modifications decreases. In the digital mechanism model (all or nothing), the number of cells containing enough H3K27me3 modifications to reach the threshold to silence *FLC* increases irreversibly with increased exposure to cold. The authors found that the latter model provided a better fit for available chromatin immunoprecipitation data for H3K27me3 and *FLC* expression. Under the digital model, intermittent periods of higher temperature during cold exposure should not affect the length of cold exposure necessary for transition to flowering. In contrast, the analogue model predicts that intermittent warm periods should lengthen the cold exposure necessary to initiate flowering. Testing both models experimentally, the authors found that the timing of

flowering was not lengthened by intermittent short periods of higher temperature. The plant's utilization of a digital all-or-nothing mechanism contributes to the robust transition between vegetative and reproductive growth and its irreversible nature, at least in the annual *A. thaliana* (Angel *et al.*, 2015). How precisely *COOLAIR* and Polycomb-group proteins contribute to this digital mechanism of adding H3K27me3 modifications remains to be determined, but their role in *FLC* silencing makes both likely candidates for the underlying molecular mechanism.

#### *Ribosomal DNA copy number variation as a putative source of phenotypic robustness akin to Hsp90*

Ribosomal RNA (rRNA) is encoded in large repetitive DNA arrays, which are highly mutable and prone to copy number variation. Recent studies in humans, mice, worms and plants have uncovered vast variation in estimated rDNA copy number among individuals (Long *et al.*, 2013; Thompson *et al.*, 2013; Gibbons *et al.*, 2014). Across 97 tested humans, rDNA copy number variation is associated with global gene expression changes and altered abundance of mitochondrial DNA (Gibbons *et al.*, 2014). As one may expect, increased rDNA dosage is associated with increased expression of genes related to rDNA expression and function, such as those encoding ribonucleoproteins, various nucleolar proteins and many chromatin-remodelling proteins. Deletions of rDNA arrays in *Drosophila melanogaster* also modulates global gene expression (Paredes *et al.*, 2011). It is unknown whether rDNA copy number variation affects phenotype primarily through altered rRNA expression or through replication conflicts arising from large arrays of repetitive DNA. The latter mechanism is supported by recent evidence, largely in bacteria and yeast (Merrih *et al.*, 2011, 2012; Kwan *et al.*, 2013). The former would minimize interest in rDNA as a robustness mechanism in plants because plant rDNA arrays are largely silenced by RdDM (Douet *et al.*, 2009; Benoit *et al.*, 2013). In the absence of this mechanistic knowledge, the observed vast variation in rDNA copy number and its association with global gene expression and altered chromatin remodelling raise the question of whether rDNA dosage affects phenotypic robustness.

rDNA is intricately linked with cellular physiology, stress response and senescence. The encoded rRNAs are essential structural and catalytic components of ribosomes, the protein factories of every living cell (Kobayashi, 2011, 2014). Ribosome biogenesis is by far the most energy-consuming cellular process: rRNA accounts for ~80% of total transcription, and transcription of genes encoding ribosomal proteins accounts for ~50% of total Pol II-dependent transcription (Rudra and Warner, 2004; Gibbons *et al.*, 2014). Thousands of large and small ribosome subunits are synthesized every minute in growing cells.

The rDNA arrays are organized within the nucleolus, in which ribosome assembly takes place (Olson *et al.*, 2000). In the last decade, the nucleolus has emerged as a key player in sensing cellular stress and is considered the central hub for coordinating the stress response and maintaining protein homeostasis (Boulon *et al.*, 2010). Many molecular chaperones, including Hsp90, are involved in nucleolar functions (Bański *et al.*, 2010; Boulon *et al.*, 2010). Given the extraordinary

importance of rDNA and ribosome biogenesis, it is not surprising that a host of human diseases is associated with mutations in rDNA expression, rRNA processing and ribosome biogenesis (Freed *et al.*, 2010). Several of these diseases show large differences in expressivity among carriers of the same mutation (Freed *et al.*, 2010), possibly because of underlying, but uncharacterized, differences in rDNA dosage, which results in differential robustness to these mutations or aggravating environmental perturbations (Gibbons *et al.*, 2015).

Although rDNA is traditionally under-studied in multicellular organisms, studies in yeast provide ample support for the importance of rDNA dosage in organismal fitness and lifespan (Kobayashi *et al.*, 1998; Kobayashi, 2011, 2014; Kwan *et al.*, 2013). Yeast cells with artificially increased or decreased rDNA copy number rapidly revert to wild-type rDNA copy number, suggesting strong selection (Kobayashi *et al.*, 1998). The stability and replication of rDNA affect yeast replicative lifespan (Kobayashi *et al.*, 1998; Kobayashi, 2011, 2014; Kwan *et al.*, 2013). Together, the cumulative evidence of prior studies calls for a systematic and accurate assessment of rDNA copy number and its effect on phenotypic robustness.

## CONCLUSIONS

Plants are remarkable – they accommodate environmental changes both predictable and unpredictable every day without the benefit of mobility and a centralized response system and while saddled with the task of continuous development. Although the fundamental principles governing robustness of development and environmental responses are conserved between animals and plants, important details differ, such as the well-documented ability of plants to tolerate WGD and hybridization. In the absence of systematic studies of network topology and motifs, we can only speculate whether strategies to ensure robustness and plasticity differ only quantitatively or also qualitatively. At a superficial glance, gene families involved in environmental responses, such as the heat shock TFs and various chaperones, have considerably expanded in plants compared with animals. Similar considerations apply among plants; one may expect different strategies between perennials and annuals, with the latter primarily investing in large numbers of offspring rather than continued robustness over often long periods of time. Lastly, even among annuals life history and ecological niche will influence where traits will fall on the robustness-to-plasticity spectrum.

As this review demonstrates, there are plenty of specific examples that highlight different aspects of how network architecture contributes to robustness and plasticity. Efforts to systematically probe genetic architecture in any organism are rare, and possibly even rarer in plant research with its limited resources. As a community we may want to move away from our standard strategy of throwing out outliers, as commonly done in plant pathology or circadian research. These outliers will hold essential information about altered wiring of underlying pathways and hence illuminate the network architecture maintaining trait robustness. In our quest to understand the universal principles governing trait architecture, its robustness, plasticity and ultimately evolvability, we should not lose sight of discovering mechanistic underpinnings. Outliers may hold

clues to mechanisms; advanced computational and experimental approaches such as single-cell-derived networks hold promise for their discovery. Lastly, our new-found prowess in mechanistic and systems-based experimental approaches will not yield universal principles, assuming these exist, unless we apply them in the field and include diverse species in our studies.

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