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Molecular mechanisms of robustness in plants

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Abstract

Robustness, the ability of organisms to buffer phenotypes against perturbations, has drawn renewed interest among developmental biologists and geneticists. A growing body of research supports an important role of robustness in the genotype to phenotype translation, with far-reaching implications for evolutionary processes and disease susceptibility. Like for animals and fungi, plant robustness is a function of genetic network architecture. Most perturbations are buffered; however, perturbation of network hubs destabilizes many traits. Here, we review recent advances in identifying molecular robustness mechanisms in plants that have been enabled by a combination of classical genetics and population genetics with genome-scale data.

Introduction

Phenotypic robustness is a measure of an organism's ability to buffer phenotype against genetic and environmental perturbations during development [2–4] (Box 1). Robustness is commonly attributed to features of the underlying genetic networks, such as connectivity, redundancy, feedback, and oscillators, as well as to non-genetic mechanisms [4,6,7]. Targeted perturbation of these features decreases phenotypic robustness and releases cryptic genetic or epigenetic variation. The release of accumulated variation has been invoked as an important factor in evolutionary processes [9] and in disease susceptibility in humans [5].

Box 1

The term phenotypic robustness is often conflated with other terms, some of which have slightly different meanings or denote entirely different phenomena. In the following, we attempt to clarify our view and usage of these terms:

Developmental stability – is equivalent to robustness as defined here, describes “the ability of organisms to withstand genetic and environmental during development, so as to produce a predetermined phenotype” [1].

Canalization – describes the notion that genetic systems evolve to a robust optimum through stabilizing selection. This robust optimum is thought to arise through elimination

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of deleterious alleles and reduction of additive genetic effects. Canalization pertains to populations with most individuals clustering around an optimal phenotype [3,5].

Cryptic genetic variation – is genetic variation that is phenotypically silent until revealed by environmental, genetic, or epigenetic perturbations [8].

Developmental noise – was used originally by Waddington to refer to differences among homologous replicated parts within a single individual and to describe the absence of developmental stability [2]. The term is currently often used as “noise” to describe stochastic variation in traits such gene expression, caused by both intrinsic errors and extrinsic micro-environmental fluctuations [10,11]. Noise is thought to play an important role in fate determination and circadian clock function [12–14].

Fluctuating asymmetry – describes an organism’s deviation from bilateral symmetry for the whole organism or particular morphological features such as fly wings or bristles. FA is an individual-based measure of robustness. Low FA is thought to correlate with high fitness [15].

Variable mutation penetrance – describes the phenomenon that certain mutations show different expressivity (i.e. severity of phenotypic effect) among isogenic individuals. We attribute these expressivity differences to differences in robustness among these individuals. Less robust individuals are expected to show higher mutation penetrance. Variable mutation penetrance among genetically divergent individuals arises from individual-specific genetic and non-genetic modifiers.

Phenotypic plasticity – is the ability of an organism to alter its physiology, morphology, and development in response to changes in its environment [2]. In our view, phenotypic plasticity describes changes in phenotype that are pre-determined in existing genetic networks, rather than consequences of stochastic errors in development (that may be ultimately due to extrinsic micro-environmental differences rather intrinsic errors).

Epistasis – is the nonreciprocal interaction of nonallelic genes, in which one gene masks the effects of another. More recently also used to describe interactions of variants with a gene or regulatory region.

Pleiotropy – describes the phenomenon in which a single gene is responsible for several distinct and seemingly unrelated phenotypic effects.

Robustness master regulator – is used here to denote genes that strongly affect robustness. We use this term interchangeably with the terms network hub and fragile node. In yeast, genes that strongly affect robustness are network hubs [28]. Studies in plants [30] and worms [32] have identified a small number of fragile nodes that affect the penetrance of mutants and natural variants in many other genes. Another frequently used term is ‘capacitor’ which refers to genes that keep genetic variation phenotypically silent when fully functional and release genetic variation when perturbed [22,23].

Robustness is a quantitative trait. Traditionally, robustness of individuals has been measured as the degree of symmetry in morphological features [15]. Another robustness measure is the degree of accuracy with which a genotype produces a phenotype across many isogenic siblings. Robustness thus measured is trait-specific and may not be predictive of robustness in other traits [15]. Like any quantitative trait, robustness shows a distribution among genetically divergent individuals of a species and can be mapped to distinct genetic loci [16–18]. Non-genetic mechanisms also affect robustness, as mutation penetrance can vary among isogenic individuals [4,6,7]. Plants are excellent models to probe the molecular underpinnings of robustness. Due to their sessile life-style and continuous development, plants have likely optimized molecular mechanisms that buffer phenotype in the face of

ever-changing environmental conditions. Here, we review some advances in identifying molecular mechanisms that contribute to robustness in plants and discuss future directions and challenges.

‘Master regulators of robustness’ affect connectivity of genetic networks

One of the best characterized ‘master regulators of robustness’ is the molecular chaperone HSP90 [6,18–27] (Box 1). HSP90 assists the folding of key developmental proteins, a function that is of even greater importance under stresses that compromise protein folding [29]. HSP90 inhibition decreases robustness in plants, flies, yeast, and fish and releases previously cryptic genetic and epigenetic variation [18,21–24,27] (Fig. 1a, b, Fig. 2a, b). In worms, low HSP90 levels correlate with high mutation penetrance [6]. HSP90’s capacity to buffer many developmental phenotypes has been attributed to its high connectivity in genetic networks [31]. Perturbing HSP90 function impairs its numerous substrates, which is thought to reduce network connectivity and lead to decreased robustness and release of variation. In genetically divergent *A. thaliana* strains, every tested quantitative trait is affected by at least one HSP90-dependent polymorphism; most traits are affected by several [18,24].

The circadian regulator *ELF4* is another gene that reduces robustness when perturbed [33]. Circadian clocks are endogenous oscillators with remarkably robust periods, which persist in the absence of light cues and under increased temperature [14]. The robustness of plant clocks is thought to arise from multiple interconnected feedback loops [14]. In reporter assays, *elf4* mutants show highly variable periods before turning arrhythmic (without periods) [33]. It is unclear whether the initial, variable periods translate into increased variation of developmental traits or released cryptic variation; both seem likely given the importance of the circadian clock in orchestrating growth and development. In fact, HSP90’s effect on robustness may arise in part from disrupted clock function: ZTL, a circadian regulator, is chaperoned by HSP90 [34].

Of course, HSP90 and ELF4 are not the only robustness master regulators in plants. However, unlike in yeast, in which a systematic mutant analysis identified 300 robustness master regulators, all highly connected ‘network hubs’ [28], in plants a similar analysis has not been conducted; the sheer number of genes and the lack of high-throughput robustness assays have so far made such analysis unfeasible. In our hands, most tested plant mutants, some of them affecting key developmental genes, do not affect robustness of quantitative seedling traits.

Fine-tuning of gene expression stabilizes developmental traits

The origins and consequences of gene expression noise have been extensively studied in single celled organisms [10–12], but less so in multicellular organisms [35], including plants [13]. In 2006, Hornstein and Shomron [36] hypothesized that microRNAs (miRNAs) may reduce gene expression noise and sharpen developmental transitions. In particular, feed-forward loops, in which a transcription factor regulates both a target and its miRNA with opposing effects on target protein levels, were predicted to buffer stochastic expression fluctuations [36]. As plant miRNAs tend to target key transcription factor and F-box genes, they modulate developmental transitions, variation in leaf morphogenesis, reproductive development, and root architecture [37]. miRNAs have recently been shown to facilitate robustness. For example, *miRNA164* miRNAs control plant development by dampening transcript accumulation of their targets *CUC1* and *CUC2*, wherever expression of miRNAs and targets overlap. *miRNA164* miRNAs define boundaries for target mRNA accumulation in addition to reducing target expression levels [38].

In plants, small RNA-dependent regulation of gene expression is not limited to miRNAs – in fact, there are many plant-specific small interfering RNAs (siRNAs), some of which are mobile and facilitate robust pattern formation. Chitwood and co-authors [39] demonstrated that a subset of trans-acting siRNAs (tasiRNAs), the low-abundant and conserved tasiR-ARFs, move intercellularly from the upper leaf side (adaxial), where they originate, to the lower leaf side (abaxial), generating a small RNA gradient that defines the expression boundaries of the abaxial determinant ARF3. tasiR-ARF biogenesis requires both miRNA activity (*miR390*) and siRNA pathway components, including the specialized Argonaute AGO7. Although *miR390* accumulates in a seemingly non-specific pattern throughout the developing leaf, tasiR-ARF biogenesis is restricted to the most adaxial leaf cell layers by the localized expression of *AGO7* [39]. Consistent with the notion that the tasiR-ARF gradient mediates robust adaxial-abaxial fate decision, *ago7* mutants show significantly increased variance in adaxial leaf width (Fig. 2d–g).

Robust flower development through combinatorial gene interaction

In core eudicots, flower organs – sepals, petals, stamens and carpels – are organized in four concentric whorls, giving rise to a highly reproducible pattern that attracts pollinators and human admirers. First proposed for *A. thaliana* and *Antirrhinum majus*, the ABC model describes how three classes (A, B, and C) of homeotic transcription factors pattern flowers through antagonistic and combinatorial interactions [40] (Fig. 1c–f). The ABC model is conserved in flowering plants, with different flower phenotypes arising from fading expression boundaries and gene duplication or loss of the A, B, and C class transcription factor genes [41]. In the original model, A and C activities are mutually exclusive, establishing the boundary between the sterile outer whorls (perianth, A function) and the reproductive inner whorls (C function) [40]. Curiously, the A gene *AP2* is uniformly expressed throughout young floral primordia. Wollmann and co-authors [42] reconciled this paradox with the discovery that *miR172* and *AP2* expression overlap transiently, which restricts *AP2* activity and reinforces the robust boundary between perianth and reproductive organs (Fig. 1c). Robust boundaries may also be facilitated by the oligomerization dynamics of A, B, C, and E class proteins. The floral quartet hypothesis [43] proposes the existence of tetrameric complexes of various ABCE proteins (floral quartets). The increased cooperativity and the higher local concentrations of specific A, B, C, and E class proteins involved in tetramer formation are predicted to sharpen organ identity boundaries.

Population genetics and large-scale phenotypic data demonstrate the role of genetic architecture in robustness

Hall and co-authors [16] mapped the first quantitative trait loci (QTL) for trait robustness rather than trait mean in two recombinant inbred populations (RILs), estimating within-genotype robustness with Levene's statistic. They identified 22 robustness QTL across five developmental traits in two conditions. Of these, only three QTL affected exclusively trait robustness, whereas all the others coincided with mean QTL. This strong correlation of robustness and mean QTL agrees with Waddington's view that decreased robustness is associated with decreased function [16]. Nearly half of the robustness QTL were linked to *ERECTA*, for which a mutant allele segregated in both RILs. *ERECTA* controls aerial organogenesis, and *erecta* mutants show strong pleiotropic phenotypes [44,45]. Using the same approach, Sangster and colleagues [18] mapped robustness QTL for two seedling traits in control and HSP90-reduced conditions, including a third RIL population without a segregating *erecta*-allele. Most robustness QTL did not coincide with mean QTL under control conditions. In contrast, under HSP90-reduced conditions, mean QTL strongly correlated with robustness QTL, consistent with Waddington's notion that newly released variants are poorly buffered [3]. As in the previous study, heritability of robustness QTL was significantly lower than for mean QTL. Both studies provided empirical evidence for

network elements that stabilize particular traits, which was subsequently also shown in maize [46].

Moving from developmental traits to large-scale molecular traits, Jimenez-Gomez and colleagues [17] mapped robustness QTL in Bay x Sha RILs for defense metabolite levels and genome-wide expression. For both datasets, the authors were limited to about four replicates per line for estimating within-genotype robustness with the coefficient of trait variation (CV, standard deviation/mean). CV of small samples is an unreliable robustness estimate (Fig. 2c). As CV is strongly mean-driven, the identified robustness QTL may be largely driven by mean differences. Countering this concern, the authors point out that not all mean QTL coincided with robustness QTL. However, all but one robustness QTL coincided with mean QTL. The authors addressed the sample size problem by mapping line-specific CV averages for all 22,746 transcripts, identifying loci that affect global gene expression CV. The major effect QTL contained *ELF3*, an important circadian and flowering time regulator. In a reference background, *ELF3*-Bay and -Sha alleles differentially affected robustness for some traits but not others, possibly due to buffering of the significant global CV expression differences. *ELF3*-Bay and -Sha alleles produce significant mean differences in circadian and developmental phenotypes in reference backgrounds [47–49]. In contrast to *ELF4* [33], *elf3* loss-of-function mutants do not show decreased robustness in circadian or developmental phenotypes [47–49], although the *ELF4* and *ELF3* proteins are known to interact [50].

Together, these studies prove that robustness is a quantitative trait with strong genetic contributions. In reference or hybrid backgrounds, natural alleles can cause different robustness levels in specific traits. In their natural backgrounds, however, these low robustness alleles may be buffered by compensatory mutations. Most *A. thaliana* robustness QTL were trait-specific, suggesting that natural robustness alleles reside in trait-specific sub-networks rather than in robustness master regulators that destabilize many traits and reduce fitness when perturbed. For example, an *HSP90* allele that subtly decreases robustness in some traits has been found in wild flies, yet this slightly deleterious allele is exceedingly rare [51,52].

In 2009, Fu and colleagues [30] addressed a different robustness angle, genetic buffering, by identifying allelic variation that affects many different traits simultaneously. The authors mapped trait means for 139 developmental traits and 40,580 molecular traits in *Ler* x *Cvi* RILs. Although the parental lines are highly divergent, the authors found only six QTL hot spots with major system-wide effects. These hotspots included well-studied candidate genes such as *ERECTA*, for which a mutant allele segregated, *CRY2*, *HUA2* or *FRL1*, all of which affect plant development pleiotropically. These results are consistent with pervasive genetic buffering that renders the majority of genetic variation phenotypically silent [30]. These data are reminiscent of data in other organisms showing that most single loss-of-function mutants are phenotypically silent, even in pair-wise combinations [28,32,53–55], whereas a small group of highly connected network ‘hubs’ show epistasis with many different genes [28,32]. Although natural alleles are not equivalent to interaction studies with loss-of-function mutants, the fundamental message is the same: eukaryotic genetic networks are mostly robust to perturbation, unless one of a small number of ‘fragile nodes’ is perturbed [28,30,32].

Most recently, Shen and colleagues [56] developed a statistical framework to associate trait variation across accessions with genetic variation (vGWAS), re-analyzing a published *A. thaliana* GWA dataset. Their analysis found loci in which allelic variation is associated with accessions that either vary little from each other in trait mean (e.g. flowering time for accessions with loss-of-function *flc* and *fri* mutants, high penetrance alleles) or vary greatly

from each other (e.g. flowering time in accessions with functional *FLC* and *FRI*, variable penetrance alleles) (Fig. 3). The authors demonstrate that accounting for these variance or penetrance differences significantly improves heritability and hence mapping of trait means. By eliminating invariant accessions (e.g. accessions with loss-of-function *flc* and *fri* mutants) and focusing on those with variable penetrance (e.g. accessions with functional *FLC* and *FRI*) one could identify background-specific trait determinants. Curiously, the study did not attempt to identify loci that affect accession-variance for many traits.

Future directions and challenges

Similar to other organisms, plants achieve robustness by tightly controlling and buffering developmental decisions in a modular fashion. Whereas the vast majority of perturbations are either phenotypically silent or affect only small sub-networks (local), perturbation of a highly connected fragile node, network hub or robustness master regulator destabilizes many traits (global) [28,30,32,53–55]. The importance and relative contribution of local and global robustness mechanisms to evolvability and adaptation to new environments remain unresolved.

In the absence of systematic deletion or double mutant analyses – thus far phenotype descriptions for 2400 single and 401 mutant combinations have been compiled [57] – what is the best way to characterize robustness mechanisms in plants going forward? It seems reasonable to focus on robustness master regulators found in other organisms. For example, yeast and worm studies strongly implicate chromatin modifiers in robustness [28,32]. Consistent with a role of chromatin in plant robustness, maize mutants in RNA-directed DNA methylation (RdDM) show stochastic developmental defects [58,59], suggesting yet another class of small RNAs as robustness agents (Fig. 1g). Further, defects in ribosome function result in highly pleiotropic developmental defects in humans [60,61] and maize [62], suggesting that genes involved in ribosome biogenesis, rRNA processing, and RNA splicing may also maintain robustness.

This approach, however, may not suffice to characterize plant-specific robustness mechanisms. Plants, in particular angiosperms, readily tolerate changes in chromosome number (aneuploidy, polyploidy) and interspecific hybridization (allopolyploidy) [63,64], both of which are thought to be major drivers in angiosperm divergence. A recent analysis of *Arabidopsis* allotetraploids implicated RdDM-specific siRNAs in chromatin and genome stability, whereas miRNAs and tasiRNAs mediated gene expression diversity, possibly facilitating hybrid vigor and adaption [65]. Curiously, the emergence of RNA polymerase V and IV, key enzymes in RdDM, coincides with the rise of angiosperms [66].

One of the major draw-backs for current robustness studies is the large sample sizes required for population-based robustness measures. Thus far, individual-based robustness measures remain less amenable to high-throughput analysis. We speculate that comparing organisms perturbed in functionally distinct robustness master regulators may reveal shared molecular features such as specific changes in gene expression, methylation or nucleolar function. These shared features could be leveraged as molecular robustness markers that are applicable to individuals and large populations. Molecular robustness markers would revolutionize the study of robustness in non-model organisms, including humans, and allow us to explore the role of robustness in evolutionary processes and disease susceptibility.

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- 56••. Shen X, Pettersson M, Rönnegård L, Carlborg Ö. Inheritance Beyond Plain Heritability: Variance-Controlling Genes in *Arabidopsis thaliana*. *PLoS Genet*. 2012; 8:e1002839. This paper describes a new approach (vGWA) to improve traditional GWA by taking trait variance across individuals (here individual accessions) carrying a particular polymorphism into account when

Highlights

- Most genetic perturbations are buffered.
- A small number of network hubs disrupt robustness when mutated.
- for specific traits, natural alleles confer different robustness levels in reference-backgrounds.
- As population-based robustness measures need large samples, the discovery of individual-based molecular robustness markers is a future goal.

Fig. 1. Loss of robustness in developmental traits

a) A wild-type *A. thaliana* seedling shows normal phyllotaxy and leaf shape. b) An HSP9-reduced *A. thaliana* seedling shows distorted phyllotaxy and meristem defects. c) *A. thaliana* transgenic plant with *miR172*-resistant *AP2* under the control of the *AP3* promoter (B-gene) shows disrupted floral patterning. Transgenic plants mis-expressing wild-type *AP2* have mostly normal flowers, whereas those misexpressing *rAP2* often have petaloid stamens or as pictured here, complete conversion of stamens into petals in severe cases. Reproduced/adapted with permission [42]. d- f) A homeotic flower *Thalictrum thalictroides* mutant with disrupted floral patterning (no carpels, little or no stamens). Mutant expressivity differs for different floral meristems within the same plant. Three flowers of the same plant are shown (unpublished data. C. Snelson, V.S. diStilio). g) A *Zea mays* RNA polymerase IV mutant shows abnormal lateral outgrowths. Reproduced/adapted with permission [58].

Fig. 2. Using quantitative traits to measure robustness

a–b) The early seedling trait hypocotyl length is a sensitive robustness read-out. a) robust 7 day-old, dark-grown wild-type *A. thaliana* seedlings, b) non-robust 7 day-old, dark-grown mutant *A. thaliana* seedlings. Reproduced/adapted with permission [67]. c) Coefficient of variation (CV, standard deviation/mean) is an unreliable estimate of population robustness at small sample sizes. Here, we show the distribution of CV estimates for various sample sizes from simulated data. For each sample size N, 1000 samples of size N were drawn from a normal distribution with mean = 10 and SD = 1 (true CV = 0.1), and a CV estimate was calculated for each sample. d–e) *ago7* mutants show decreased robustness in leaf width. Representative plants of d) wild-type Col-0 and e) *ago7-1*. f) Mean width and g) CV of the longest leaf at flowering time was measured in Col-0, an HSP90-reduced line (HSP90-RNAi), and *ago6-1*, *ago7-1*, and *ago8-1*, n=15.

Fig. 3. vGWAS loci that cannot be mapped with GWAS

Traditional GWAS associates significant mean differences in quantitative traits with genetic variants (top and middle panel). In contrast, vGWAS associates significant variance differences in trait means with common genetic variants in the same individuals. This approach does not consider within-genotype robustness, rather it is a measure of penetrance of a given polymorphism. Alleles associated with higher variance may or may not affect the trait mean (middle and bottom panels respectively). Thus, taking variance effects into account may improve heritability estimates relative to traditional GWAS.