Hsp90 as a capacitor of phenotypic variation

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Heat-shock protein 90 (Hsp90) chaperones the maturation of many regulatory proteins and, in the fruitfly *Drosophila melanogaster*, buffers genetic variation in morphogenetic pathways. Levels and patterns of genetic variation differ greatly between obligatorily outbreeding species such as fruitflies and self-fertilizing species such as the plant *Arabidopsis thaliana*. Also, plant development is more plastic, being coupled to environmental cues. Here we report that, in *Arabidopsis* accessions and recombinant inbred lines, reducing Hsp90 function produces an array of morphological phenotypes, which are dependent on underlying genetic variation. The strength and breadth of Hsp90's effects on the buffering and release of genetic variation suggests it may have an impact on evolutionary processes. We also show that Hsp90 influences morphogenetic responses to environmental cues and buffers normal development from destabilizing effects of stochastic processes. Manipulating Hsp90's buffering capacity offers a tool for harnessing cryptic genetic variation and for elucidating the interplay between genotypes, environments and stochastic events in the determination of phenotype.

During evolution the remodelling of shapes and functions draws on genetic differences between individuals and populations. However, species phenotypes must generally be robust to genetic variation and environmental change, requiring buffering systems to ensure normal development ('canalization')[1,2]. Environmental stress can reveal genetic variants, presumably because it compromises buffering systems. If selected for, these uncovered phenotypes can lead to heritable changes in plants and animals (assimilation)[3–5]. Recent work provided evidence that the chaperone Hsp90 can serve as such a buffer in *Drosophila melanogaster*. Remarkably, it can do so in a multitude of different morphological pathways[6].

In all eukaryotes tested, Hsp90 is essential, abundant at normal temperatures, and induced by stress. Under physiological conditions, Hsp90 dynamically interacts with a diverse but highly select set of inherently unstable 'client' proteins (for example, kinases and transcription factors). Typically, it keeps these metastable proteins poised for activation until they are stabilized by conformational changes, such as those associated with signal transduction. The requirement of many principal regulatory proteins for Hsp90 renders entire pathways sensitive to decreases in its function[7–9].

In *Drosophila*, challenging Hsp90 function by mutation, pharmacological inhibition or environmental stress can produce a profusion of morphological changes affecting virtually every structure of the fly. Notably, the particular change observed in an individual fly depends on previously silent genetic variation. In the two cases tested, multiple polymorphisms affecting specific developmental pathways could be enriched by selection so that the traits were expressed even after Hsp90 function was restored. Thus, it appears that Hsp90 allows the storage and release of genetic variation in *Drosophila*[6] as a consequence of its essential function in chaperoning regulators of growth and development. If so, this effect might be conserved in other organisms, potentially influencing the pace and nature of evolution.

The sessile, photosynthetic plant *Arabidopsis thaliana* has a vastly different lifestyle from the vagile, heterotrophic *Drosophila* and is evolutionarily distant. Moreover, the pattern and level of genetic variation differ profoundly between this inbreeding species and obligatorily outcrossing species such as the fly. Although polymorphisms are present within *Arabidopsis* populations and outcrossing does occur (estimated frequency ~0.3%; ref. 10), heterozygosity per individual is extremely low[10,11]. Because heterozygosity is such an important means of maintaining genetic variability and phenotypic robustness, it is difficult to predict whether a buffering system like Hsp90 would act in the accumulation and release of variation in such organisms.

Additional features of *Arabidopsis* provide a singular opportunity to ask whether Hsp90 influences other global aspects in the manifestation of phenotype. The *Arabidopsis* research community has gathered specimens (accessions) from diverse regions of the globe, representing virtually homozygous, divergent genomes. These accessions can be crossed to produce large numbers of virtually identical, highly heterozygous F_1 progeny, as well as recombinant inbred lines representing homozygous genetic mosaics of parental genomes. Furthermore, in *Arabidopsis* (as in plants generally), development is highly plastic: morphologies vary dramatically with even moderate changes in environment (for example, light intensity)[12–15]. These circumstances provide an opportunity to address two important questions not yet addressed in any system. First, does Hsp90 have a role in linking environmental change to the developmental dynamics that govern morphogenesis in an individual organism's lifetime? And, if so, can we assess how the strength of this linkage varies with genotype? Second, does Hsp90 influence the potentially positive and negative effects of heterozygosity on developmental stability?

Pharmacological inhibition of Hsp90 in seedlings

The *Arabidopsis* genome contains seven Hsp90 genes[16,17]. Therefore, we used pharmacological inhibition to reduce Hsp90 function. The drugs geldanamycin (GDA) and radicicol are structurally unrelated; however, each inhibits Hsp90 function through multiple, distinct contacts with residues in its highly unusual, evolutionarily conserved nucleotide-binding pocket[18,19]. Drug-binding residues are poorly conserved in the other proteins with this unconventional ATP-binding fold. An additional advantage is that both drugs are inactivated by prolonged exposure to light, thereby allowing the recovery and propagation of plants with early abnormal phenotypes caused by Hsp90 inhibition.

In the two accessions (ecotypes) initially examined, Columbia (Col) and Landsberg *erecta* (L*er*), each drug produced dosage-

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dependent phenotypes at a concentration that correlated with its affinity for Hsp90. For radicicol (dissociation constant K_d for binding Hsp90 is 19 nM), phenotypes appeared between 2 and 50 nM; for GDA ($K_d = 1.2\,\mu M$)[18], phenotypes appeared between 0.15 and 5 μM. Above the K_d, both drugs caused multiple phenotypes and reduced viability. At slightly lower concentrations, most plants remained healthy and unaffected but some exhibited strongly altered phenotypes. For example, at 1.0 μM GDA, 5–8% of seedlings showed strong morphological abnormalities, affecting shape, colour and expansion of cotyledons; shape, colour and presence of true leaves; shape and length of hypocotyls; root morphology; and the orientation of rosettes, roots or whole seedlings (Fig. 1 and data not shown). In most cases, phenotypes were distinct and not accompanied by a general failure to thrive. Without the drug, only 1–2% of plants showed far more subtle variant morphologies. Most importantly, the two chemically unrelated drugs, GDA (a benzoquinone ansamycin) and radicicol (a macrolactone), produced the same spectrum of phenotypes.

Next, we tested several geographically and environmentally diverse *Arabidopsis* accessions (see Methods). Again, most plants bearing abnormal phenotypes on GDA had an otherwise healthy appearance. Particular phenotypes were not restricted to particular accessions, but the frequencies of their appearance varied reproducibly. For example, rare plants of all accessions exhibited abnormalities in true leaves, including radially symmetrical leaves, deformed shapes, and missing leaves, but in the Shadara accession about 30% of seedlings had these phenotypes (Fig. 1a). Shadara also frequently produced distorted rosettes with juxtaposed cotyledons (Fig. 1b). Dwarfed plants with cotyledons that accumulated purple pigment were most common in Col (Fig. 1c). Curled hypocotyls were most frequent in L*er* (Fig. 1d). For comparison, a L*er* seedling grown without GDA is shown in Fig. 1e.

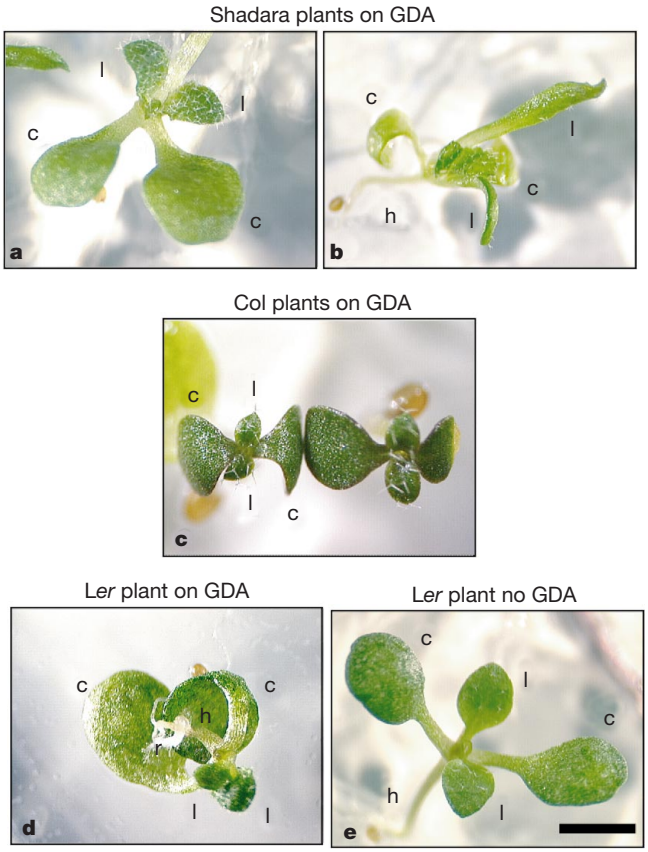

Figure 1 Common GDA-dependent phenotypes in Shadara, Col and L*er* accessions. **a**–**d**, Seedlings grown with 1 μM GDA. **e**, L*er* seedling grown without GDA. Note typical symmetry of *Arabidopsis* rosette and oval-shaped, horizontally oriented, flat leaves. **a**, Shadara, juxtaposed cotyledons. **b**, Shadara, deformed and radially symmetrical true leaves. **c**, Col, dwarfed with dark green, epinastic cotyledons and true leaves. **d**, L*er*, extremely curled hypocotyl with roots partially extended in the air. c, cotyledons; l, true leaves; h, hypocotyl; r, root. Scale bar, 2 mm.

Phenotypic variation specific to recombinant inbred lines

The predisposition of different accessions to different phenotypes suggested a genetic contribution to the phenotypic variation buffered by Hsp90. To test this, we examined recombinant inbred lines (RI lines) originating from crosses between two accessions followed by single-seed self-propagation for eight generations. Each line should be homozygous at almost all loci, with different lines representing various mosaics of the parental genomes[20]. If a decrease in Hsp90 function uncovers new phenotypes due to genetic variation, GDA-induced phenotypes should vary between RI lines but tend to be shared within them. Here, we present work with 50 RI lines derived from a cross between Cape Verde Island (Cvi) and L*er* (Cvi/L*er*, CS22477 base set, Arabidopsis Biological Resource Center, ABRC) accessions. Col/L*er* RI lines behaved similarly, although phenotypic variation was not as dramatic (data not shown), probably because these accessions are more closely related[20,21].

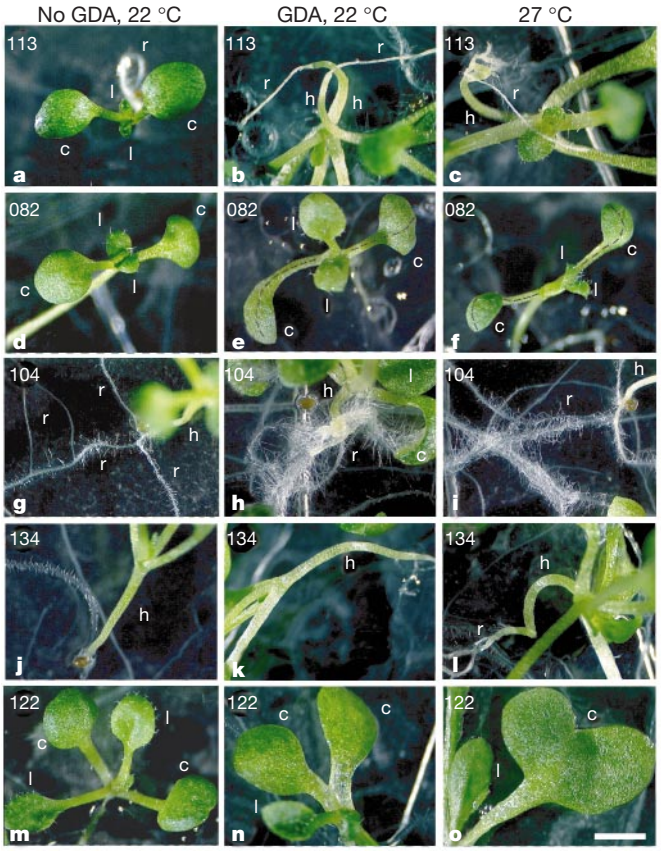

Figure 2 The same RI line-specific phenotypes are uncovered when buffering capacity is challenged by GDA (middle) and by growth at 27 °C (right). **a**, **d**, **g**, **j**, **m**; RI line seedlings grown without GDA. **b**, **c**, Line 113 seedlings, extreme hypocotyl curls and roots partially extended into air. **e**, **f**, Line 082 seedlings, S-shaped rosettes with vertically oriented leaf blades. **h**, **l**, Line 104 seedlings, abundant root hair growth. **k**, **l**, Line 134 seedlings, bent hypocotyls with rosette touching the medium surface. **n**, **o**, Line 122 seedlings, juxtaposed cotyledons (similar to those in Shadara) fused to varying degrees. In extreme cases, seedlings appeared to have only one very large, malformed cotyledon and one apparently healthy true leaf. Because GDA activity is lost after prolonged light exposure (see Methods), this phenotype (and other leaf phenotypes) did not appear in later-developing leaves. Plant organs are indicated, as defined in Fig. 1. Scale bar: 2 mm for **a**–**f**, **j**–**m**; 1 mm for **g**–**f**, **n**, **o**.

The most striking Hsp90-buffered phenotypes in Cvi/L*er* RI lines were distinct and restricted to particular lines (Fig. 2, middle; Table 1). For example, seedlings of line CS22113 (113) developed normally for about 8 days. Thereafter, their hypocotyls started to curl, lifting the root above the medium surface (Fig. 2b). By day 10, about 30% of the seedlings showed extreme hypocotyl curls and unusually large, round cotyledons. For seedlings of line 082, leaf blades of cotyledons were positioned vertically instead of horizontally, twisting the seedling rosette in an S shape (Fig. 2e). Seedlings of lines 104 (Fig. 2h) and 159 (data not shown) displayed a profuse increase in root-hair growth. Some line 104 seedlings also accumulated purple pigment in cotyledons and emerging adult leaves (data not shown). For line 134 seedlings, arched hypocotyls were observed (Fig. 2k).

Other RI lines exhibited phenotypes that were variable but appeared related: fused cotyledons and distorted rosettes (line 122, Fig. 2n), radially symmetrical leaves and missing true leaves (lines 119 and 120, data not shown), and other leaf deformities (line 119, data not shown). Lines 119 and 120 also showed occasional trichomes on the abaxial side (underside) of the first true leaf (data not shown).

Notably, a few seedlings in most lines exhibited altered phenotypes without GDA. Owing to potential pitfalls of subjective analysis, all seedlings with detectable phenotypes were scored in Table 1. As with accessions (Fig. 1), these phenotypes were not only less frequent without GDA, but they were also generally more subtle in character (data not shown). They were, however, specific to individual lines and related to the stronger phenotypes observed with GDA. That is, although seedlings of line 113 sometimes exhibited slightly curled hypocotyls without GDA, none exhibited S-shaped rosettes, malformed true leaves, or abnormally abundant root-hair growth. Thus, GDA phenotypes were based on uncovering the genetic predispositions of different RI lines to produce different traits (Table 1), rather than to spurious drug effects. Some of the traits (for example, juxtaposed cotyledons) observed in RI lines were never observed in their parents, presumably because the lines represent new combinations of the original genotypes. These combinations of parental genomes produce the potential for a wide variety of genetically determined traits, which are expressed when Hsp90 chaperone capacity falls below a certain level.

The diversity and specificity of the phenotypes observed indicates that the level of Hsp90 inhibition required to uncover them is relatively modest. If inhibition had been strong enough to interfere with developmental pathways that have an absolute dependence on Hsp90 in this species, all plants would exhibit similar phenotypes. Moreover, the robust health of most seedlings displaying strong phenotypes suggested that although inhibition was sufficient to uncover certain genetic predispositions, it was not so severe as to interfere with the general 'housekeeping' functions of Hsp90. To test whether our conditions induced a general stress response, eight randomly selected lines, including two that produced the strongest phenotypes (113 and 119), were examined for expression of Hsp101 and Hsp70 by western blotting. All exhibited normal Hsp inductions with a brief 38 °C heat shock[22]. None showed detectable inductions with GDA at their normal growth temperature (22 °C; data not shown). Certainly, phenotypes might be influenced by other Hsps, but the level of Hsp90 inhibition required to uncover the specific polymorphisms that can give rise to strong phenotypes was generally lower than that required to produce a detectable stress response.

Phenotypes uncovered by environmental change

If Hsp90- buffered variation acts in the storage and release of genetic variation, then moderate environmental changes might be sufficient to cross the threshold to express particular traits in plants carrying particular polymorphisms. To test this, we grew Cvi/L*er* RI line seedlings without GDA, but at 27 °C instead of 22 °C. (As with GDA, this moderate temperature increase did not detectably induce other Hsps; data not shown.) All but one of the lines that exhibited strong phenotypes with GDA showed the same phenotypes at 27 °C (compare Fig. 2b with c, e with f, h with i, k with l, and n with o; Table 1). Even in the exceptional case, line 104, one of its two GDA-induced phenotypes (abundant root-hair growth) was expressed (Fig. 2i, Table 1). Furthermore, the ability of environmental change to reveal previously hidden genetic variation was precise: among the seven lines presented in Table 1, the phenotypes were distinct, whereas in the other 42 lines examined, some seedlings exhibited altered phenotypes at 27 °C, but rarely these. Other environmental conditions, such as the nature of the growth substratum, also had the capacity to uncover Hsp90-buffered traits, and these could function in a combinatorial fashion (see Supplementary Information).

Hsp90-dependent phenotypic plasticity

Next, we asked whether developmental plasticity was dependent on Hsp90 function, employing traits that could be scored objectively. The dark response is a classic example of developmental plasticity: in contrast to light-grown *Arabidopsis* seedlings, dark-grown seedlings have elongated hypocotyls; small, yellow and non-expanded cotyledons; and short roots[23,24]. In Fig. 3, RI lines and their parents (red labels) are sorted by their degree of hypocotyl elongation when grown in the dark without GDA. Notably, the breadth of RI line responses was greater than those of parental lines (transgression). Thus, genetic variants affecting elongation were contributed by both parents and segregated in the original cross.

Table 1 Phenotypes specific to RI lines

RI line	Without GDA, 22 °C		With GDA, 22 °C		Without GDA, 27 °C	
	Phenotype*	Frequency†	Phenotype	Frequency	Phenotype	Frequency
CS22113	1	3.5% (1/28)	1	32% (9/28)	1	32% (9/28)
CS22082	2	1.7% (2/112)	2	32% (64/201)	2	95% (53/56)
CS22104	3	0% (0/75)	3	34% (29/84)	3	0% (0/47)
	4	16% (12/75)	4	52.4% (44/84)	4	38% (18/47)
CS22159	4	6.3% (8/127)	4	50.5% (53/105)	4	28% (18/63)
CS22134	5	0% (0/28)	5	87.5% (49/56)	5	46% (19/41)
CS22119	3	0% (0/28)	3	7% (2/28)	3	0% (0/28)
	6	7% (2/28)	6	68% (19/28)	6 or 7	90% (25/28)
	7	0% (0/28)	7	7% (2/28)		
CS22120	6	7% (2/28)	6	64% (18/29)	6	82% (23/28)
CS22122	6	0% (0/110)	6	2.5% (4/158)	6	0% (0/130)
	7	15% (17/110)	7	28% (44/158)	7	31.5% (41/130)

Phenotypes specific to RI lines appeared when buffering capacity was reduced by GDA or by a moderate temperature increase. These phenotypes were distinct and restricted to particular lines. In the absence of GDA at 22 °C, rare seedlings of some lines (113, 082, 119 and 120) exhibited phenotypes that were similar to those observed in the presence of GDA, but these were much more subtle. For lines 122, 104 and 159, phenotypes in the absence of GDA ranged from subtle to as strong as those observed with GDA.

*Phenotypes: 1, extreme hypocotyl curl and roots partially extended into air; 2, S-shaped rosettes with vertically oriented leaf blades; 3, accumulation of purple pigment; 4, abundant root hairs; 5, bent hypocotyls with rosette touching the medium surface; 6, malformed true leaves; 7, juxtaposed cotyledons, malformed cotyledons.

†Per cent penetrance, followed by affected plants/total number of plants scored (in parentheses).

The effects of GDA on hypocotyl elongation were uniform within lines but differed markedly between lines. In some (for example, lines 077 and 129; Fig. 3, thin arrows), the drug had little effect. In others (for example, lines 086 and 116; Fig. 3, thick arrows), it reduced elongation to nearly the values obtained in white light. GDA did not simply intensify the variation observed without the drug. Lines with naturally long or short hypocotyls (Fig. 3, grey bars) were found both in the group that was strongly affected by Hsp90 inhibition and in the group that was little affected. Both parental lines showed moderate reductions in hypocotyl length. Thus, the degree to which the dark response was affected segregated in RI lines, producing lines with extreme Hsp90 dependence and others with much lower dependence.

We examined the Hsp90 dependence of several other developmental plasticity traits: root growth in the dark promoted by sucrose in the medium; germination in the dark; the ability of cotyledons to become green after growth in the dark; and the ability of roots to respond to a change in the direction of gravity (Fig. 3, more details are provided in Supplementary Information). If Hsp90 buffers polymorphisms in many different environmental response pathways, we would expect the Hsp90 dependence of different traits to vary both within a given RI line and between RI lines. Indeed, in the same seedlings, measured on the same day, GDA affected different traits in different ways. For example, consider lines 132 and 079 (Fig. 3, blue labels): hypocotyl elongation and 'greening' were more strongly affected in 132; root elongation and germination were more affected in 079. Gravitropism responses were assessed in a separate experiment by rotating plants grown on vertical plates by 90°. This trait also showed different sensitivities to GDA in different lines, including one (129) in which roots turned more efficiently with GDA than without (Wilcoxon two-sample test, $P \leq 0.018$). Most notably, GDA's effects on specific plasticity responses seemed largely independent of each other (Spearman's rank tests, turning versus root elongation, $P \leq 0.7$; versus hypocotyl elongation, $P \leq 0.3$; versus germination rate, $P \leq 0.3$; germination rate versus root elongation, $P \leq 0.6$; versus hypocotyl elongation, $P \leq 0.5$).

Thus, the Hsp90-dependent variation was due neither to random nor to line-specific differences in drug uptake or toxicity. Rather, it resulted from genetic variation in different environmental response pathways that rendered them more or less dependent on full Hsp90 function. By QTL mapping, the uncovered phenotypic variation in hypocotyl elongation did not map to the cytoplasmic Hsp90 gene cluster (C.Q., J. Borevitz, J. Maloof, D. Weigel, J. Chory and S.L., unpublished observations). This confirms that, for this trait, the phenotypic variation uncovered by Hsp90 inhibition is not due to polymorphisms in Hsp90 itself but to a wide variety of polymorphisms elsewhere in the genome.

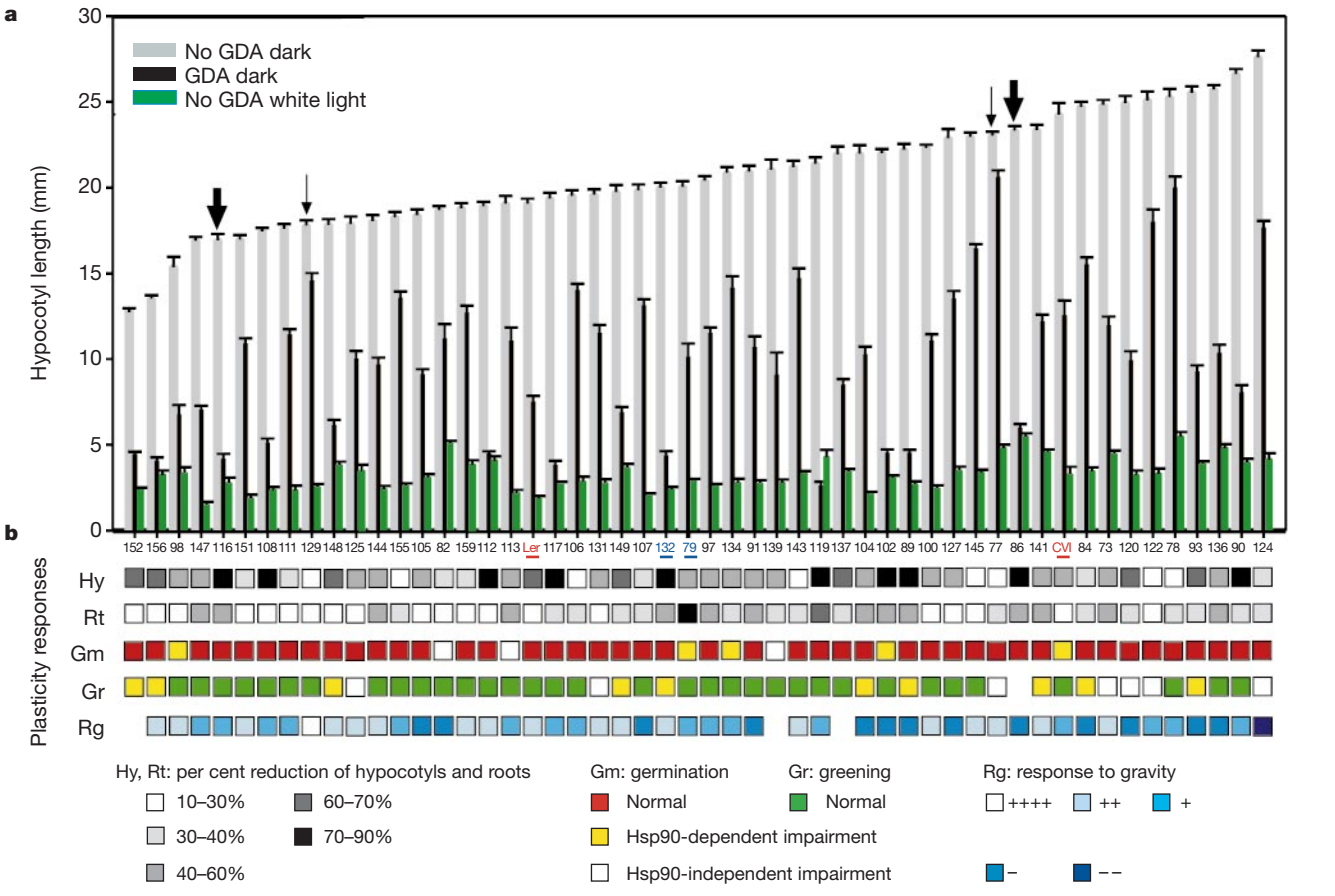

Figure 3 The effect of GDA on developmental plasticity responses differs between 50 Cvi/L*er* RI lines and their parents. **a**, Response of hypocotyls to growth in the dark without GDA (grey bars), with GDA (black bars) and in white light (green bars). Error bars represent s.e.m. Red labels indicate parental accessions; blue labels indicate examples of RI lines for which GDA's effects on developmental responses differed. Thick arrows, examples of lines in which GDA reduced hypocotyl elongation to values close to those of seedlings grown in white light; thin arrows, lines for which hypocotyl length was little affected by GDA. **b**, Hy and Rt, degree of hypocotyl and root reduction: white, least affected lines; black, most affected lines. Gm, germination scored by radicle emergence: red, nearly complete germination; yellow, poor germination only on GDA; white, poor germination without GDA and further reduction with GDA. Gr, greening: green, complete greening; yellow, greening partially or completely impaired only on GDA; white, greening impaired with or without GDA. Rg, response to gravity: light blue, most seedlings in category 1 or 2; intermediate blue, most in category 2 or 3; dark blue, most in category 3 or 4; deep blue, most in category 4; white, more seedlings scored in category 1 with GDA than without GDA (see Methods). Missing boxes, not tested.

Three conclusions follow: Hsp90 has an important role in many aspects of developmental plasticity; these roles vary in different genetic backgrounds; and the dependencies of different pathways on Hsp90 segregate independently of each other.

Hsp90 buffers developmental stability

Given the near homozygosity of inbred lines and accessions, the partial penetrance of certain phenotypes was surprising. Although some morphogenetic traits were highly penetrant (for example, S-shaped rosettes in line 082 at 27 °C), most were not. Similarly, responses of individual seedlings for some developmentally plastic traits (for example, germination and gravitropism) varied within a line. One explanation for this phenotypic variability is the segregation of genetic variation. Low levels of heterozygosity are present in RI lines (~1 site per 5,000 base pairs) and might also be present in accessions. Indeed, in one case tested, segregating genetic variation did affect penetrance. About 30% of Shadara seedlings exhibited deformed, missing or radially symmetrical true leaves with GDA. Without the drug, subtle abnormalities of true leaf development appeared more frequently in Shadara (~3–6%) than in other accessions (<1%). When plants displaying subtle abnormalities without GDA were self-bred, 50% of progeny exhibited the phenotype without GDA, and 75% did with GDA. Thus, genetic variants contributing to abnormal leaf development were still segregating in the Shadara seeds we sampled and could be enriched by selective breeding, increasing phenotypic penetrance. In contrast, in an RI line displaying partial penetrance of one altered phenotype (line CS1941, radially symmetrical and missing true leaves), self- and cross-breeding the affected progeny did not increase penetrance in the next generation (data not shown). Here, partial penetrance was not due to segregating genetic variation, suggesting that stochastic processes influenced the propensity of genomes to produce particular traits.

To investigate the interplay of genetic variation, stochastic processes, and their dependence on the Hsp90 buffering system, we tested crosses of different, nearly homozygous accessions (Col × *Ler* and *Ler* × Cvi) that are believed to have been recently evolving independently. If stochastic events contribute to phenotypes predisposed by genotype, then mixing genomes might disrupt developmental stability in F_1 progeny. Further, if Hsp90 buffers developmental stability against such events, reducing Hsp90 function in nearly identical F_1 progeny should increase phenotypic variance.

Indeed, F_1 progeny exhibited higher frequencies of subtle altered phenotypes than their parental accessions even without GDA (frequency 5–7%). In the presence of GDA, not only did the frequency of abnormal phenotypes increase dramatically (up to 25%), but the severity and complexity of the phenotypes also

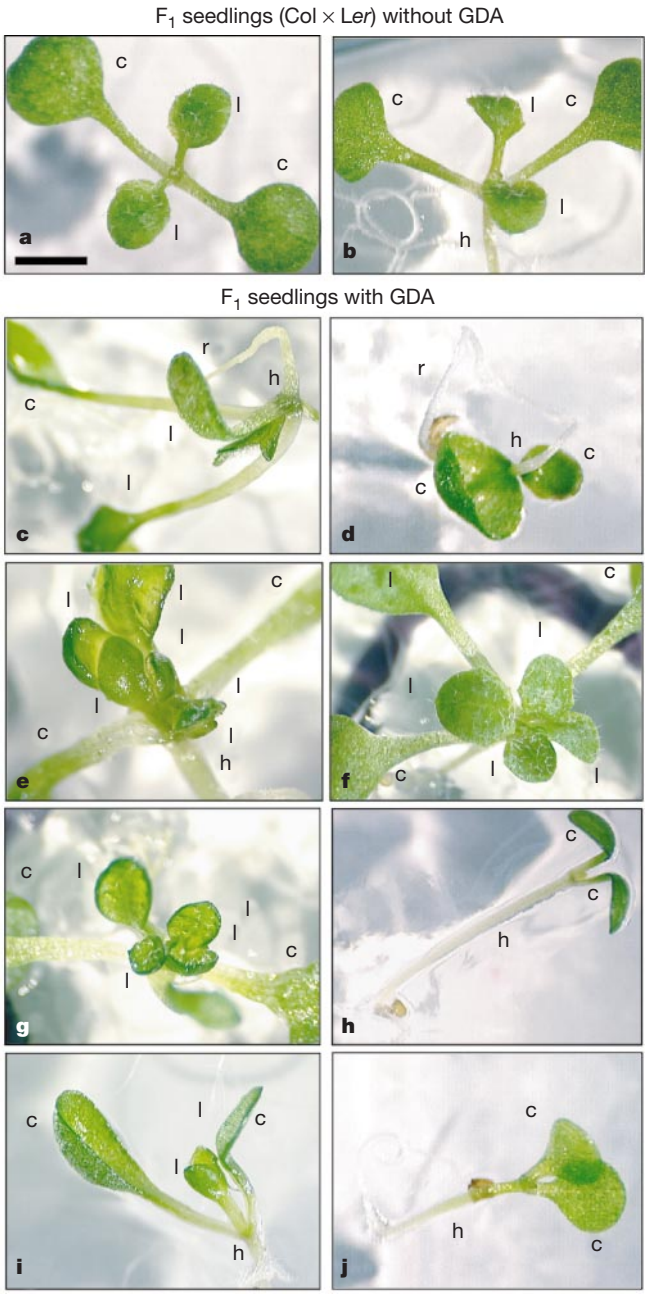

Figure 4 F_1 progeny of accession crosses exhibit higher diversity and greater phenotype complexity than parental accessions, especially with GDA. **a**, **b**, F_1 seedlings of Col × *Ler* cross without GDA. **c**–**j**, Seedlings with GDA. **c**, **d**, seedlings with hypocotyl curls and different cotyledon shapes. **e-g**, seedlings with multiple true leaves emerging apparently simultaneously: **e**, at different angles; **f**, with a leaf in first whorl missing; **g**, as seemingly separated rosettes. **d**, **h**–**j**, seedlings with diverse hypocotyl and cotyledon phenotypes: **d**, short curled hypocotyl, inverted orientation, epinastic cotyledons; **h**, long hypocotyl, small, dark-green cotyledons; **i**, large spoon-shaped cotyledons, extremely short hypocotyls; **j**, hypocotyl of intermediate size, light, non-expanded round cotyledons. Plant organs indicated, as defined in Fig. 1. Scale bar: 2 mm for **a**–**d**, **h**–**j**; 1 mm for **e**–**g**.

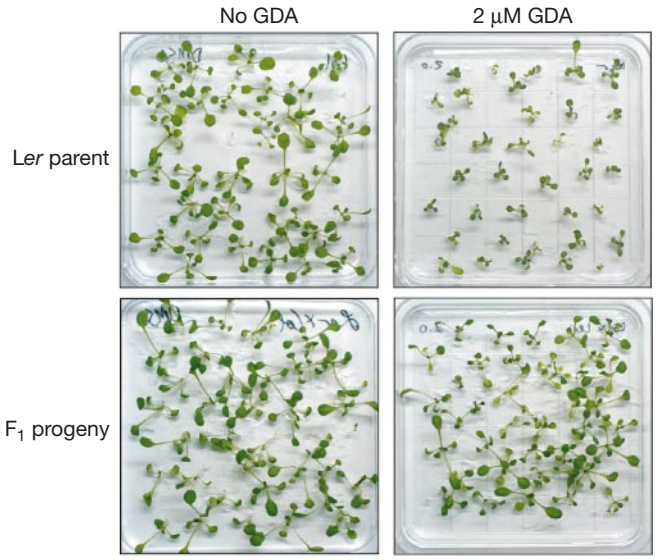

Figure 5 F_1 progeny grow more robustly in the presence of 2 μM GDA than do parental accessions. Left, without GDA; right, with GDA. Top, parental accession *Ler*; bottom, F_1 progeny of Col × *Ler* cross. Similar numbers of seeds (~40) were plated for all.

increased (Fig. 4). Phenotypes were stronger and more frequent in seedlings with a maternal contribution from L*er* (~20–25% affected seedlings) than with maternal contributions from Col or Cvi (~10–15%).

Notably, the developmental diversity of F_1 seedlings with GDA was not due to reduced viability. When the concentration of GDA was doubled (to 2 μM), Col × L*er* F_1 seedlings exhibited an even greater abundance of phenotypes, but most were more vigorous and developmentally advanced than those of either parental accession at the same GDA concentration (Fig. 5). Significantly, when buffering functions were challenged by a higher growth temperature (27 °C) instead of by GDA, phenotypes of Col × L*er* F_1 seedlings were also more diverse than those of their parental accessions. Again, the F_1 seedlings were generally larger and healthier (data not shown). Preliminary analysis of other cross-bred seedlings suggests that the effects of heterozygosity on developmental stability and stress tolerance vary, depending on the genetic divergence of the accessions and the nature of the stress, offering a rich subject for further analysis.

Discussion

Our work establishes three aspects of Hsp90 function in organismal biology. First, its role in buffering the expression of genetic variation[6] is conserved across plant and animal kingdoms. Although it is difficult to predict what might prove adaptive in evolution, it is noteworthy that the phenotypes revealed in plants by challenging Hsp90 buffering capacity are not 'monstrous' in character (as they might be described in fruitflies). Indeed, some would seem plausibly advantageous under particular conditions: for example, altered leaf shapes, purple pigment accumulation, and different degrees of hypocotyl extension. Notably, most Hsp90-buffered traits we tested were also uncovered by moderate changes in growth conditions (that is, a mild temperature increase). This establishes that the Hsp90 buffer has global effects on the storage of cryptic polymorphisms and their release in response to shifting environments, converting neutral to non-neutral variation.

Second, Hsp90 profoundly affects developmental plasticity. Plasticity allows morphogenetic variation in response to circumstances and environments and is particularly salient in plants. Our observations suggest that Hsp90 functions at the interface between genotypes and environments, a unique vantage point from which to influence the dynamic nature of developmental processes. Most remarkably, the degree to which the plasticity of individual traits depends on Hsp90 varied greatly between RI lines, and different traits exhibited different levels of Hsp90 dependence within each RI line. Apparently, the genetic networks that affect both the likelihood that a particular trait might appear, as well as the extent to which it may respond to changes in the environment, are commonly polymorphic with respect to the influence of Hsp90. We suggest that, in the face of changing selective pressures, Hsp90 buffering may provide an avenue by which populations can evolve different genotypic states—from those that produce a particular trait, to those in which the trait dynamically responds to the environment, to those in which a different developmental endpoint has become a fixed characteristic.

Third, Hsp90 buffers developmental stability against stochastic processes. Individual seedlings in nearly isogenic RI lines and accessions produced strong phenotypes when Hsp90 function was reduced, but these were only partially penetrant. Also, the nearly genetically identical F_1 progeny of crosses between Col and L*er* parents produced an unprecedented diversity of phenotypes when Hsp90 function was challenged. These phenotypes were not due to a general loss of vigour, because F_1 progeny from the same cross were generally more robust than either parent under conditions of increased stress. Thus, Hsp90 normally acts to reduce the likelihood that stochastic events will alter the deterministic unfolding of a multitude of developmental programmes.

These seemingly diverse effects of Hsp90 are readily encompassed by a single, simple molecular framework. The biochemical function of Hsp90 is to chaperone metastable proteins, including diverse regulators of growth and development. In doing so, Hsp90 stabilizes its 'client' proteins in conformations that would otherwise be prone to misfolding and potentiates their capacity to be activated in the proper time and place, by associations with partner proteins, ligand binding, post-translational modifications and correct localization[7–9]. Under stressful conditions, Hsp90 is induced in response to problems in protein folding. Polymorphisms with the potential to alter a particular trait may be so enriched in individual genomes that Hsp90's ability to maintain the functional pathway is exceeded. These polymorphisms might occur not only in client proteins themselves but also in upstream or downstream components of the pathway in which they function. Indeed, even in the absence of stress, some populations may be so close to the trait expression threshold that stochastic events in development will produce a few individuals displaying the altered trait. Once the pathway is diverted, the expression of a new trait may become robust through the influence of auto-regulatory feedback loops, self-perpetuating protein conformations, and developmental windows. Thus, Hsp90 contributes to phenotypic variance by buffering the functional state (folding, accumulation and local concentration) of gene products that contribute to altered traits through the effects of chance, genotypes and environments.

From a the viewpoint of a population geneticist, buffering systems like Hsp90, without regard to any possible adaptive value, would decrease selection on nucleotide substitutions, allowing storage of an expanded spectrum of selectively nearly neutral ones. Under stable environmental conditions, a population arrives at a local fitness optimum in an adaptive landscape. Given that natural selection can only further increase the fitness of a population, it is a perplexing evolutionary question how a population might move to a different local optimum without an intervening period of reduced fitness (adaptive valley). Previously proposed mechanisms include genetic drift in small populations, compensatory mutations and gene conversion[25–27]. As a by-product of its biochemical function, Hsp90 may allow the neutral accumulation of potentially selectable polymorphisms and synchronize their conversion to a non-neutral state. Certainly, most combinations of polymorphisms will be deleterious, and these may be periodically purged from the population through the environmental coupling of the Hsp90 buffer. However, rare combinations may produce a new, advantageous phenotype, thereby providing a molecular means by which adaptive peak shifts in large populations may occur without passing through an adaptive valley. None of the other mechanisms discussed can be modulated by environmental contingencies.

Finally, deliberate manipulation of the Hsp90 buffer may offer an opportunity to speed the identification of genetic variation for academic investigations and commercial applications. Developmental pathways might be deciphered more rapidly by using multiple, subtle polymorphisms contributing to a trait than by traditional approaches of sequentially identifying strong alleles, enhancers and suppressors. Harnessing such variation to produce valuable phenotypes would also bypass public concern about transgenic plants. Our results also suggest possible connections to medical maladies with stochastic genotypic and environmental aetiology, because they provide a global molecular explanation for why genotype does not always directly translate into phenotype. Indeed, our findings situate Hsp90 at the critical intersection of genotype, environment and development. □

Methods

Plant material and growth conditions

Accession seeds (Mr-0, Shadara, Ts-1, Tsu-1) and RI lines (CS22477, CS1899) were from the ABRC. Columbia (Col-0), L*er* and Cvi seeds were laboratory stocks propagated from seeds (D. Preuss).

Accessions were independently cross-fertilized several times to produce F_1 seeds. To minimize variation in growth conditions, surface-sterilized seeds were plated with equal spacing on germination (GM) medium[22] containing either 1 μM GDA ($C_{30}H_{42}N_2O_8$, formula weight 559, from 1 mg ml^{-1} stock in dimethyl sulphoxide, DMSO) or equivalent concentration of DMSO alone (0.056%). Plates were wrapped in foil to allow germination while avoiding degradation of light-sensitive GDA, and incubated at 22 °C after cold treatment for 24–48 h. Plates were unwrapped after 48 h at 22 °C, incubated in continuous light at ~150 μmol m^{-2} s^{-1}, and analysed after 8 and 10 days. Digital images were taken after 10–12 days (Figs 1, 2 and 4) and after 14 days (Fig. 5) (at × 10 or × 20 magnification, as indicated).

Experiments in altered environments

Incubation at 27 °C increased growth rates (by ~0.5–1 day after 8 days), and GDA decreased growth rates (by ~0.5–1 day) relative to plants grown without GDA at 22 °C. To accommodate these differences, one set of seeds (~28 per RI line) was plated with GDA on day 1; a second set (22 °C) was plated without GDA on day 2. On day 3, three sets were plated: one for growth at 27 °C and two at 22 °C, with and without GDA. To minimize variation and provide more objective comparisons, two RI lines were plated together on each plate. Sets were cold treated for 48 h. Foil-wrapped plates incubated at 22 °C were treated as described above. Because development was more rapid at 27 °C, these plates were unwrapped after 24 h. Phenotypes were scored at 8 and 10 days.

Developmental plasticity responses

Seedlings respond to subtle environmental changes that are difficult to control (for example, small variations in incubator conditions, or slight substratum changes). Values for individual lines varied somewhat between experiments, but within experiments seedlings on multiple replicate plates behaved similarly. For example, for the dark response of hypocotyls and roots, 50 seeds for each RI line were plated on vertical plates (10 seeds per plate) and intermixed with multiple RI lines in the incubator. A second screen for the dark response analysed ten seeds for each RI line with a 2-h light pulse and 48 h of cold treatment. Hypocotyl length reductions were similar to those of Fig. 3 for nearly all RI lines (hypocotyls of lines 117 and 086 were much longer than those of light-grown seedlings).

After 7 days in the dark, hypocotyl and root lengths were measured on digitized images using Scion Image (http://www.scioncorp.com). The number of germinated seeds and morphological characteristics of seedlings grown with and without GDA were documented. Germination efficiency of Cvi seeds increased (up to 100%) with a 2-h light pulse. This light pulse also increased germination efficiencies of lines indicated with white or yellow boxes in Fig. 3, except line 134.

The light-induced rate of GDA decay was assessed phenotypically. GDA-containing plates were pre-exposed to light for various periods before seeds were plated, and hypocotyl growth was measured after 7 days in the dark, as in Fig. 3. Exposures of 2–4 h had little effect on GDA potency; 48 h eliminated it.

Because GDA affects root extension, the response to changing the direction of gravity was tested at GDA concentrations of 0.75 and 1 μM, ensuring that a set of seedlings was available for each RI line with root growth comparable to controls. Forty seeds for each condition were plated, cold treated for 48 h, and incubated at 22 °C in the dark for 4 days (to exclude phototropism signals). Root growth and germination were scored, and plates were turned by 90°. Responses were scored 12, 24, 36 and 48 h after turning. Each seedling was assigned a turning category (1, complete turn; 2, incomplete turn; 3, no turn; 4, turning upward; 5, coiled growth). Line responses (blue shading, Fig. 3) were scored according to the number of seedlings in each category. Seedlings of most RI lines without GDA scored in category 1, except lines 129 and 131, which were distributed between categories 1 and 2.

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Competing interests statement

The authors declare that they have no competing financial interests.

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