The Protein Chaperone HSP90 Can Facilitate the Divergence of Gene Duplicates

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ABSTRACT The heat-shock protein 90 (HSP90) acts as a chaperone by ensuring proper maturation and folding of its client proteins. The HSP90 capacitor hypothesis holds that interactions with HSP90 allow proteins to accumulate mutations while maintaining function. Following this logic, HSP90 clients would be predicted to show relaxed selection compared with nonclients. In this study, we identify a new HSP90 client in the plant steroid hormone pathway: the transcription factor BES1. Its closest paralog, BZR1, is not an HSP90 client. This difference in HSP90 client status in two highly similar proteins enabled a direct test of the capacitor hypothesis. We find that *BES1* shows relaxed selection compared to *BZR1*, hallmarks of neo- and subfunctionalization, and dynamic HSP90 client status across independent evolutionary paths. These results suggested that HSP90's influence on gene evolution may be detectable if we compare gene duplicates because duplicates share most other properties influencing evolutionary rate that might otherwise conceal the chaperone's effect. We test this hypothesis using systematically identified HSP90 clients in yeast and observe a significant trend of HSP90 clients evolving faster than their nonclient paralogs. This trend was not detected when yeast clients and nonclients were compared without considering paralog status. Our data provide evidence that HSP90 influences selection on genes encoding its clients and facilitates divergence between gene duplicates.

THE phenotypic capacitor heat-shock protein 90 (HSP90) is thought to influence evolutionary processes through its ability to both conceal and release genetic variation (Rutherford and Lindquist 1998; Queitsch *et al.* 2002; Yeyati *et al.* 2007; Jarosz and Lindquist 2010). Perturbation of this conserved and essential chaperone reveals cryptic genetic and epigenetic variation in flies, plants, fish, and yeast (Rutherford and Lindquist 1998; Queitsch *et al.* 2002; Sollars *et al.* 2003; Yeyati *et al.* 2007; Jarosz and Lindquist 2010). In worms, HSP90 affects the penetrance of partial loss-of-function mutations (Burga *et al.* 2011). As expected under the capacitor hypothesis, worms with naturally lower HSP90 levels show significantly higher mutation penetrance (Casanueva *et al.* 2012). HSP90-dependent variation can be revealed by moderate environmental stress alone, providing a plausible release mechanism for this concealed variation in nature (Rutherford and Lindquist 1998; Queitsch *et al.* 2002; Jarosz and Lindquist 2010). We showed previously that HSP90-dependent variation is common in natural plant populations, implicating the chaperone as an important player in shaping phenotype and evolutionary trajectories (Sangster *et al.* 2008a,b). Together, these findings have prompted a longstanding debate about the importance of HSP90 in evolutionary processes and the magnitude of its effect (Meiklejohn and Hartl 2002; Bergman and Siegal 2003; Rando and Verstrepen 2007).

It is well-established that HSP90 recognizes metastable proteins and facilitates their folding and stability (Taipale *et al.* 2010). Recent studies demonstrate that protein stability is a major constraint on protein evolution (Bloom *et al.* 2006; Pena *et al.* 2010). Stable proteins tend to evolve faster as they can explore greater sequence space without losing function (Bloom *et al.* 2006). In prokaryotes, overexpression of the chaperonin GroEL/ES allows the evolution of a far greater number of highly active enzyme variants by compensating for their reduced stability (Tokuriki and Tawfik 2009). We

doi: 10.1534/genetics.112.148098
Manuscript received November 27, 2012; accepted for publication January 31, 2013
Supporting information is available online at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.112.148098/-/DC1.
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hypothesized that in eukaryotes HSP90 may facilitate gene divergence by similarly relaxing constraints on protein stability. If so, HSP90 clients should show greater tolerance to mutations and evolve faster than nonclients facing similar evolutionary pressures. To address HSP90's role in gene evolution in this context, we focused on recent gene duplicates, which initially face similar selection pressures and encode proteins of similar stability. We used the brassinosteroid (BR) pathway in *Arabidopsis thaliana* as an experimental model because every step in BR signaling is encoded by gene families (Kim and Wang 2010) and previous studies suggested that the BR pathway may require HSP90, although no HSP90 client has been identified (Sangster and Queitsch 2005; Sangster *et al.* 2007).

We demonstrate here that only one of two paralogous transcription factors in the BR pathway is an HSP90 client and that its encoding gene shows relaxed purifying selection compared to its nonclient paralog. Gene duplicates diverge through sub- and neofunctionalization. Consistent with subfunctionalization, only the HSP90 client is temperature sensitive; consistent with neofunctionalization, the gene encoding the HSP90 client contains a novel exon and nonsynonymous polymorphisms in divergent *A. thaliana* strains. HSP90-facilitated divergence of gene duplicates is widespread because, in the yeast *Saccharomyces cerevisiae*, genes encoding HSP90 clients tend to evolve faster than those encoding their nonclient paralogs. Together, our data provide strong evidence for HSP90-faciliated evolution in extant genomes and hence strong support for the capacitor hypothesis.

Materials and Methods

Plant growth conditions and treatments

Columbia-0 (Col-0) was used as wild type. *bes1-D*, *bzr1-D*, *DWF4*-ox, *bin2-1*, *BRI1*-ox, and *bes1-2* (WiscDsLox246D02) (Swarbreck *et al.* 2008) were in the Col-0 background. The *bes1-D* mutant is a recapitulation line using a transgene to constitutively express the mutant form of *bes1-D* in a Col-0 background. Seedlings were grown for 7 days on media with DMSO (mock) or geldanamycin, brassinolide, and brassinazole dissolved in DMSO. Statistical significance of response of hypocotyl length of seedlings from two to four replicates of 10–60 seedlings was determined using standard least-square linear regression.

Biochemistry

For western blot, 7-day-old seedlings, grown under red light emitting diodes, were ground in liquid nitrogen. Buffer (0.15 M Tris, pH 6.8) was added, and extracted protein was quantified using Bradford's assay. Proteins were resolved using SDS-PAGE, transferred to nitrocellulose, and probed with anti-BES1 antibody. For co-immunoprecipitation, ground rosette tissue was used. Extracted protein was incubated with Protein L Agarose, which was pre-incubated with anti-HSP90 3G3 antibody. Beads were pelleted and washed in buffer. Anti-BES1 antibody was used to detect BES1 in the input and pellet.

Phylogenetic tree and rate of nonsynonymous substitutions to the rate of synonymous substitutions analysis

Sequences for *BZR/BEH* family members in available sequenced plants were acquired from http://phytozome.net v5.0 from a BLAST search for gene families with similarity to *BES1*. MUSCLE 3.7 was used for amino acid alignment of the identified sequences, and Gblocks was used to remove regions with poor conservation. The remaining 86 sequences were realigned and neighbor-joining was used to create a distance tree. For the *BZR/BEH* tree, the outgroup was identified as a *BZR/BEH* family member that was closely related, but an outgroup to all *A. thaliana BZR/BEH* family members. Sequences were aligned in MUSCLE 3.7, and PhyML was used for the maximum-likelihood tree (Guindon and Gascuel 2003). For rate of nonsynonymous substitutions to the rate of synonymous substitutions (dN/dS) analysis, codeml from PAMLv4.4b was run using models 0, 1, and 2 (Bielawski and Yang 2003).

Yeast data analyses

Published HSP90 interactors were used (Zhao *et al.* 2005). The branch length of HSP90 interactors in three-member and two-member families was obtained from Ensembl Compara (release 61). See Supporting Information, File S1 for more detailed materials and methods.

Results

BES1 is an HSP90 client

Inhibition of HSP90 yields a wide variety of morphological phenotypes in *A. thaliana* plants (Whitesell *et al.* 1994; Queitsch *et al.* 2002; McLellan *et al.* 2007; Sangster *et al.* 2007). Among these phenotypes, we previously noted severely dwarfed plants that closely resembled known BR mutants. To directly test whether the BR pathway requires HSP90 function, we grew seedlings in the presence of exogenous BR (brassinolide, the most biologically active BR) with and without the highly specific HSP90 inhibitor geldanamycin (GdA) (Queitsch *et al.* 2002). Inhibition of HSP90 function significantly interfered with response to BRs (Figure S1A, $R^2 = 0.68$, $P < 0.0001$, linear regression model, standard least square fit). Consistent with this finding, GdA also reduced seedling response to brassinazole, an inhibitor of BR biosynthesis (Asami *et al.* 2000) (Figure S1B, red light, $R^2 = 0.76$, $P < 0.0001$; Figure S1C, dark $R^2 = 0.81$, $P < 0.0001$).

We next addressed which step in the BR-signaling pathway was most responsive to a loss of HSP90 function. The best-characterized HSP90 clients are the mammalian steroid hormone receptors and kinases (Picard *et al.* 1990; Whitesell *et al.* 1994; Taipale *et al.* 2010). The most common clients are transcription factors (Taipale *et al.* 2010). In *A. thaliana*, only a few clients are known, none of which function in the BR pathway (Ishiguro *et al.* 2002; Hubert *et al.* 2003; Takahashi *et al.* 2003; Iki *et al.* 2010). As HSP90 clients do not share

a common sequence or structural motif, client status is typically determined by a combination of genetic and biochemical analyses. Here, we took advantage of several well-characterized mutants in the BR pathway to test their response to HSP90 inhibition. We focused on the most likely clients: the steroid hormone receptor kinase BR INSENSITIVE1 (BRI1) and the transcription factors BES1 and BZR1 (Wang *et al.* 2001). To distinguish between HSP90 effects on BR signaling *vs.* BR synthesis, we included a mutant in DWARF4 (DWF4), an enzyme that catalyzes a rate-limiting step of BR biosynthesis. Well-characterized gain-of-function mutants were used to bypass the extensive redundancy in the BR pathway. As had been shown previously, each mutant significantly increased hypocotyl length (He *et al.* 2005) (Figure 1, A and B). Upon inhibition of HSP90 with 0.5 μM GdA, *BRI1-ox*, *DWF4-ox*, and *bzr1-D* seedlings responded like wild-type seedlings (*BRI1-ox*, $P = 0.22$; *DWF4-ox*, $P = 0.99$; *bzr1-D*, $P = 0.6273$), whereas *bes1-D* seedlings showed significant hypersensitivity to HSP90 inhibition ($P < 0.0001$, Table S1, Figure 1, A–C). *bes1-D* hypersensitivity to GdA suggests that BES1 may be an HSP90 client, while the wild-type response of *bzr1-D* mutants to GdA argues against an HSP90 client status for BZR1. Notably, the dominant *bes1-D* and *bzr1-D* mutants carry the identical amino acid change from proline to leucine (Wang *et al.* 2002; Yin *et al.* 2002; Albrecht *et al.* 2008; Guo *et al.* 2009; Li *et al.* 2009; Tang *et al.* 2011). The *bes1-D* mutant used here is expressed from a constitutive 35S CaMV promoter, providing an alternative explanation for the increased GdA sensitivity of *bes1-D*. Further increases in GdA levels yielded a significant response in *BRI1-ox*, *DWF4-ox* (*BRI1-ox*, $P < 0.0001$; *DWF4-ox*, $P = 0.0003$), but not in *bzr1-D* seedlings ($P = 0.5918$, Table S1), suggesting that there may be additional HSP90 targets upstream or alongside the transcription factors tested here (Figure 1, A and B). Similar results were obtained in the dark (Figure S2, A and B). Together, these data suggest that BES1, but not BZR1, is an HSP90 client.

Another criterion for HSP90 client status is proof of physical interaction (Taipale *et al.* 2010). Using a co-immunoprecipitation assay with an HSP90-specific antibody, we found that BES1 physically interacts with HSP90 in plants (Figure 1D). In the absence of BRs, BES1 is negatively regulated by BIN2 and several related kinases (Vert and Chory 2006). Cellular perception of BRs triggers inhibition of BIN2 and activates BES1. Hypophosphorylated, active BES1 can be detected as a fast mobility band on Western blots (Yin *et al.* 2002) (Figure 1E). In this form, BES1 interacts with other transcription factors, binds DNA, and promotes plant growth (Yin *et al.* 2005). In our studies, BES1 can interact with HSP90 independently of phosphorylation, as we were able to pull-down faster and slower mobility BES1 bands (Figure 1D).

While BRs and GdA have opposite effects on plant growth, and likely on BES1 function, inhibition of HSP90 produced a similar shift in BES1 mobility as BR treatment (Figure 1E). Like other well-established clients, such as the *Drosophila* Argonaute Piwi and the human transcription factor HSF, BES1 was not degraded upon HSP90 inhibition (Figure 1E) (Zou *et al.* 1998;

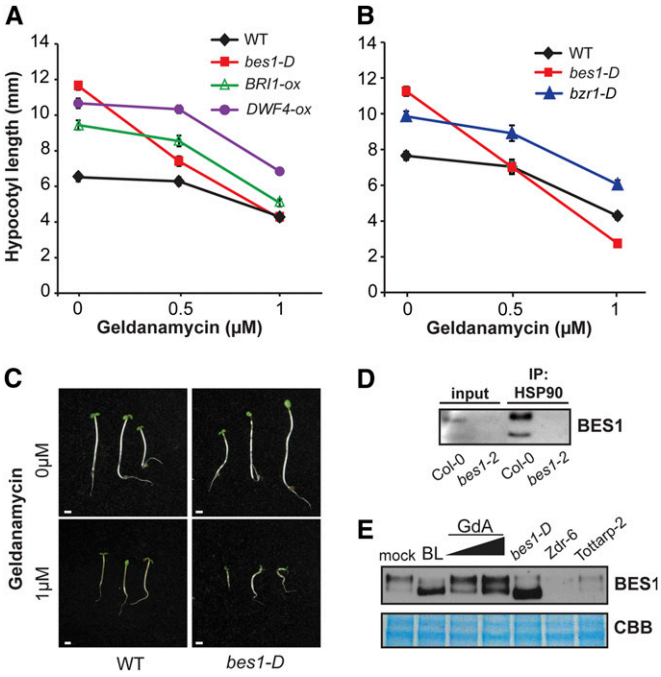

Figure 1 BES1 is an HSP90 client. (A) Seedlings with increased BR signaling through overexpression of DWF4 (*DWF4-ox*) or overexpression of BRI1 (*BRI1-ox*) showed similar sensitivity to HSP90 inhibition by GdA compared to wild type (WT). In contrast, seedlings with constitutive activation of BES1 (*bes1-D*) were significantly more sensitive than WT in red light. Standard error is shown. (B) In contrast to the dramatic GdA hypersensitivity of *bes1-D* mutants, *bzr1-D* mutants respond like WT in red light. (C) Representative WT and *bes1-D* seedling phenotypes in red light. (D) BES1 interacts physically with HSP90. BES1 was immunoprecipitated (IP) with an HSP90 antibody in WT, but not in loss-of-function *bes1-2* mutants, confirming that this antibody is specific to BES1. Input and IP are shown for both. (E) GdA treatment caused a shift of BES1 mobility. Unlike a similar shift caused by brassinolide (BL) treatment, the GdA-induced mobility shift was associated with decreased hypocotyl length. Zdr-6 and Tottarp-2 show reduced levels of BES1; detected protein is presumably due to the presence of other splice forms. Coomassie Brilliant Blue (CBB) is shown as a loading control.

Gangaraju *et al.* 2011). It appears that, while the hypophosphorylated form of BES1 accumulated upon HSP90 inhibition, it is nonfunctional (Figure 1, A–D). Our data suggest that BES1 may require HSP90 for its activation, perhaps by facilitating BES1 dimerization, promoting nuclear translocation, or interfering with phosphorylation by BIN2. If GdA indeed reduced the functional pool of BES1, we predicted that any increase in BIN2 function would sensitize plants to inhibition of HSP90. *bin2-1* mutants are semidominant hypermorphs with increased levels of phosphorylated BES1 leading to strongly reduced BES1 activity and repressed BR signaling (Kim and Wang 2010). As predicted, loss of HSP90 activity sensitized seedlings to a gain of BIN2 activity, as evidenced by a dramatically increased proportion of severely dwarfed seedlings in a segregating population of *bin2-1* mutant seedlings (Figure S2C). These results also suggest that BIN2 does not require HSP90 activity for its function; such a scenario would lead to suppression, not enhancement, of the *bin2-1* phenotype.

HSP90 facilitates the divergence of gene duplicates

If HSP90 indeed allows its clients to explore a wider range of sequence space, the *BES1* gene would be predicted to show evidence of relaxed selection compared to the *BZR1* gene. We created a phylogenetic tree of the *A. thaliana BZR/BEH* gene family using a gene from *Aquilegia coerulea* as an outgroup (Figure 2A and Figure S3). *BES1* and *BZR1* are the most recently diverged paralogs among the six *A. thaliana BZR/BEH* family members, with 88% amino acid identity (Wang *et al.* 2002). We determined the ratio of the dN/dS for *BES1* and *BZR1* under a model allowing all branches of the *A. thaliana BZR/BEH* tree to evolve at different rates. Consistent with our prediction, the gene encoding the HSP90 client BES1 (dN/dS = 0.09) shows relaxed purifying selection compared to the gene encoding the nonclient BZR1 (dN/dS = 0.04) (Figure 2A). As *BES1* and *BZR1* diverged recently and differ only in a small number of amino acids, their difference in dN/dS was not significant. However, we took advantage of the entire *BEH* gene family to test whether *BES1* shows a different evolutionary rate compared to other family members. Using a maximum-likelihood approach (Bielawski and Yang 2003), we showed that *BES1* indeed exhibits a significantly different evolutionary rate if all other branches are assumed to evolve at the same rate ($2\delta = 5.232$, d.f. = 1, $P = 0.0222$). In contrast, no significant difference was found for the evolutionary rate of *BZR1* under the same assumptions ($2\delta = 3.244$, d.f. = 1, $P = 0.0717$).

To address whether HSP90 clients generally evolve faster, we analyzed a data set of systematically identified *Saccharomyces cerevisiae* HSP90 clients (Zhao *et al.* 2005). Proteins that physically interact with HSP90 by tandem affinity purification-tagged (TAP) mass spectrometry (Zhao *et al.* 2005) were considered as likely HSP90 clients. Probable HSP90 cochaperones, identified by the TPR domain (Wegele *et al.* 2004), were removed from the analysis. Unlike clients, cochaperones interact with HSP90 through the TPR domain and modulate HSP90 activity. As expected, the evolutionary rates of HSP90 clients did not differ significantly from all other yeast genes (Figure S4A). Factors such as differences in selection pressure, protein stability, or codon bias, among others, likely obscure any impact on evolutionary rate by HSP90. To compare genes well matched for these factors, we identified gene duplicates in which one paralog encoded a likely HSP90 client. Consistent with our results for *BES1*, genes encoding yeast HSP90 clients showed significantly longer branch length than their respective closest paralog (Figure 2B, Table S2, $n = 13$, 95% confidence interval 1.02–1.64, $P = 0.002$, one-sample Wilcoxon test, testing the deviation from the expected ratio of client/nonclient branch length of 1). Likely nonclients, identified by synthetic genetic interaction (SGI) with an *hsp90* mutation, did not show this trend (Figure S4B, $n = 27$, $P = 0.97$). We then tested a more stringent situation. In cases in which yeast HSP90 interaction status was the derived state (*i.e.*, not present in a common ancestor), three of four HSP90 clients showed longer branch length than their respective closest paralog (Figure 2B, three-member families). Next, we addressed whether these differences in evolutionary rate were due to expression differences between clients and their respective nonclient paralogs. Genes that evolve faster tend to be expressed at a lower level (Drummond *et al.* 2005). In contrast, many clients are significantly more highly expressed than their nonclient paralogs across nearly 200 environmental conditions (Gasch *et al.* 2000) (Table S2 and Figure S5). We observed no correlation of expression levels and branch lengths between clients and their respective paralogs (Figure S4C, Figure S5, and Table S2). Taken together, our data suggest that HSP90 can facilitate the divergence of gene duplicates in yeast and plants.

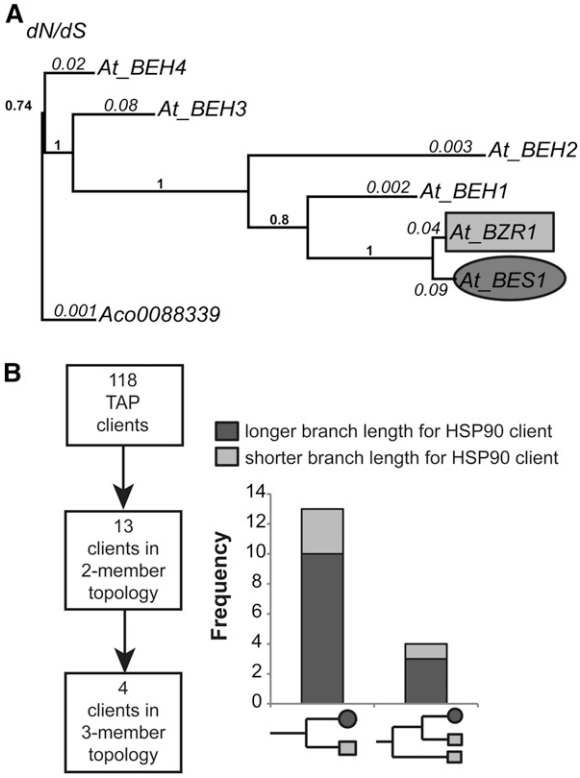

Figure 2 HSP90 clients show relaxed selection compared to their paralogs. (A) The branch leading to *BES1* has a larger dN/dS ratio than the branch leading to *BZR1*. dN/dS ratios are in italics. Branch lengths represent the difference in the number of amino acid substitutions among family members from an unrooted tree. The fraction of 100 bootstraps supporting each branch are shown in boldface type. (B) *S. cerevisiae* HSP90 clients tend to evolve faster than their paralogs in two-member and three-member families. Clients are dark-gray circles; nonclients are light-gray squares. Significance was determined by using a one-sample Wilcoxon test, which tests the deviation from the expected ratio of client/nonclient branch length of 1, $n = 13$, 95% confidence interval is 1.02–1.64, and $P = 0.002$.

HSP90 client BES1 shows hallmarks of sub- and neofunctionalization

Evolutionary theory holds that, after gene duplication, one copy dies off quickly or changes function (Conant and Wolfe

2008). A surviving gene copy can retain part of the ancestral gene function, such as expression in fewer tissues or under certain environmental conditions (subfunctionalization), and/or acquire a novel beneficial function (neofunctionalization) (Conant and Wolfe 2008). An obvious subfunctionalization path for an HSP90 client is loss of function under environmental conditions that challenge HSP90 chaperone activity, such as increased temperature. HSP90 clients are typically less stable than other proteins and hence lose function at increased temperature despite induced HSP90 expression (Taipale *et al.* 2010). We grew seedlings at 27°, a temperature known to challenge HSP90 function but not induce heat stress in *A. thaliana* (Queitsch *et al.* 2002). The temperature response of *BRI1-ox*, *DWF4-ox*, and *bzr1-D* mutants closely resembled the response of wild-type seedlings (Figure 3D). In contrast, not one of >100 *bes1-D* seeds in multiple independent experiments germinated at 27° (Figure 3, D and E). This germination phenotype was completely suppressed at standard growth conditions (22°) (Figure 3, D and E, and Figure S2D). The loss of BES1 function at moderately elevated temperature, likely a direct result of challenged HSP90 function, is a strong support for subfunctionalization.

If genes encoding HSP90 clients evolve faster than their nonclient paralogs, neofunctionalization may be facilitated. Although there appears to be extensive redundancy between the HSP90 client BES1 and the nonclient BZR1 (Yin *et al.* 2005), we found evidence of BES1 neofunctionalization. First, the major *BES1* splice variant At1g19350.3 encodes a novel exon not found in *BZR1* or in any of the other *BEH/BZR* family genes (Figure 3A). This exon shares significant homology with intergenic regions on chromosomes 3 and 5 (Figure 3B) and, together with an adjacent intron sequence, has additional matches to the 5′ UTR *BZR1* sequence and to another intergenic region (Figure 3C). Gene chimeras are a hallmark of neofunctionalization (Hahn 2009), and these findings suggest that distant genomic regions may have contributed to the novel *BES1* exon.

Second, we found that *BES1* polymorphisms across divergent *A. thaliana* accessions were significantly associated with the phenotypic variation these strains showed in response to HSP90 inhibition. Wild *A. thaliana* accessions harbor considerable genetic variation, yet due to *A. thaliana*'s inbreeding lifestyle, individual accessions are nearly isogenic. Sensitivity to HSP90 inhibition varied dramatically among accessions (Figure 4A). Some accessions grouped with the hypersensitive *bes1-D* mutant (Figure 4A, red bar), whereas others responded very little. Accessions that grouped with the hypersensitive *bes1-D* mutant contained three intronic polymorphisms, but this association was not significant (Figure 4, A and B, light blue). In contrast, we found significant associations between decreased sensitivity to HSP90 inhibition and two other *BES1* polymorphisms (Figure 4B and Figure S6A). The first polymorphism is a frameshift mutation in the strains Zdr-6 and Tottarp-2 that results in an early stop codon (Figure 4A, black bars, and Figure 1E). Consistent with a severely truncated BES1 protein,

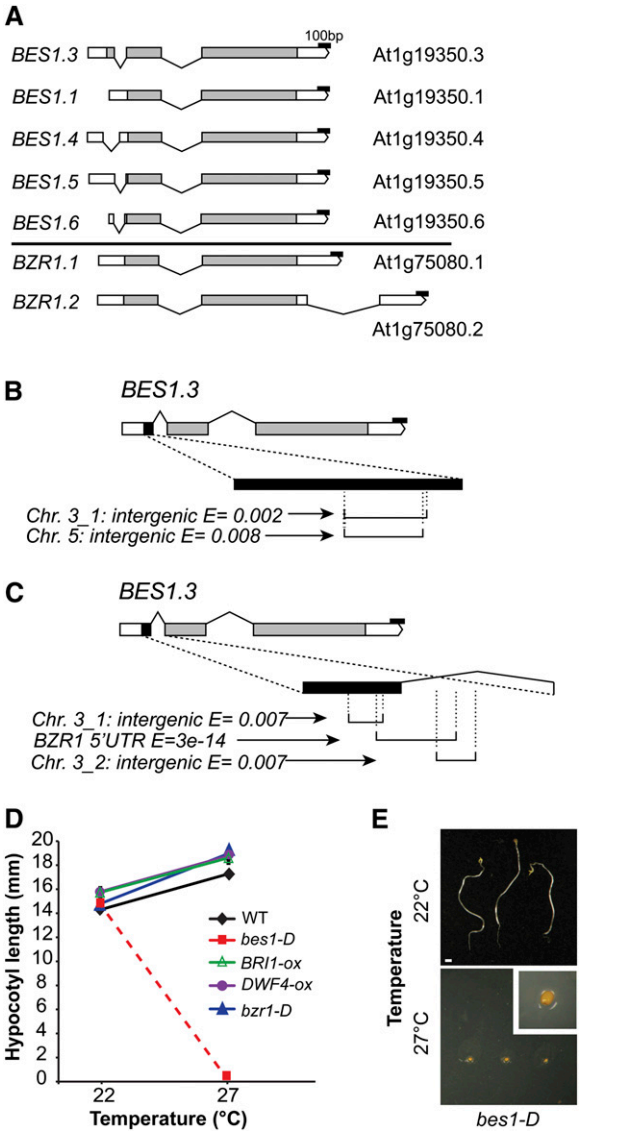

Figure 3 BES1 shows evidence of neo- and subfunctionalization. (A) The major *BES1* splice variant, *BES1.3*, encodes a novel exon. Shaded boxes are exons, solid lines are introns, and open boxes are UTRs. (B) A BLASTn search for regions with homology to the novel *BES1.3* exon identifies intergenic loci. (C) A BLASTn search for regions with homology to the novel exon and first intron of *BES1.3* identifies *BZR1* 5′ UTR sequence in addition to intergenic loci. (D) At 27°, the *bes1-D* mutant failed to germinate, while all other mutants showed a wild-type response. (E) Representative *bes1-D* seedlings and seeds at 22° and 27°, respectively. Enlarged image of *bes1-D* (inset) shows a seed that failed to germinate at 27°.

these strains responded little to HSP90 inhibition. The second polymorphism was found in four different strains (Figure 4A, green bars). This synonymous polymorphism in the 5′ end of *BES1* alters the preferred codon for alanine to a rarely used codon (Wright *et al.* 2004). Changes in codon usage can alter translation efficiency and protein-folding kinetics, potentially making stabilization by HSP90 superfluous. A similar change in codon usage has been observed for evolutions of viral proteins under conditions of reduced HSP90 in mammalian cells (Vaughan *et al.* 2010). Thus, in

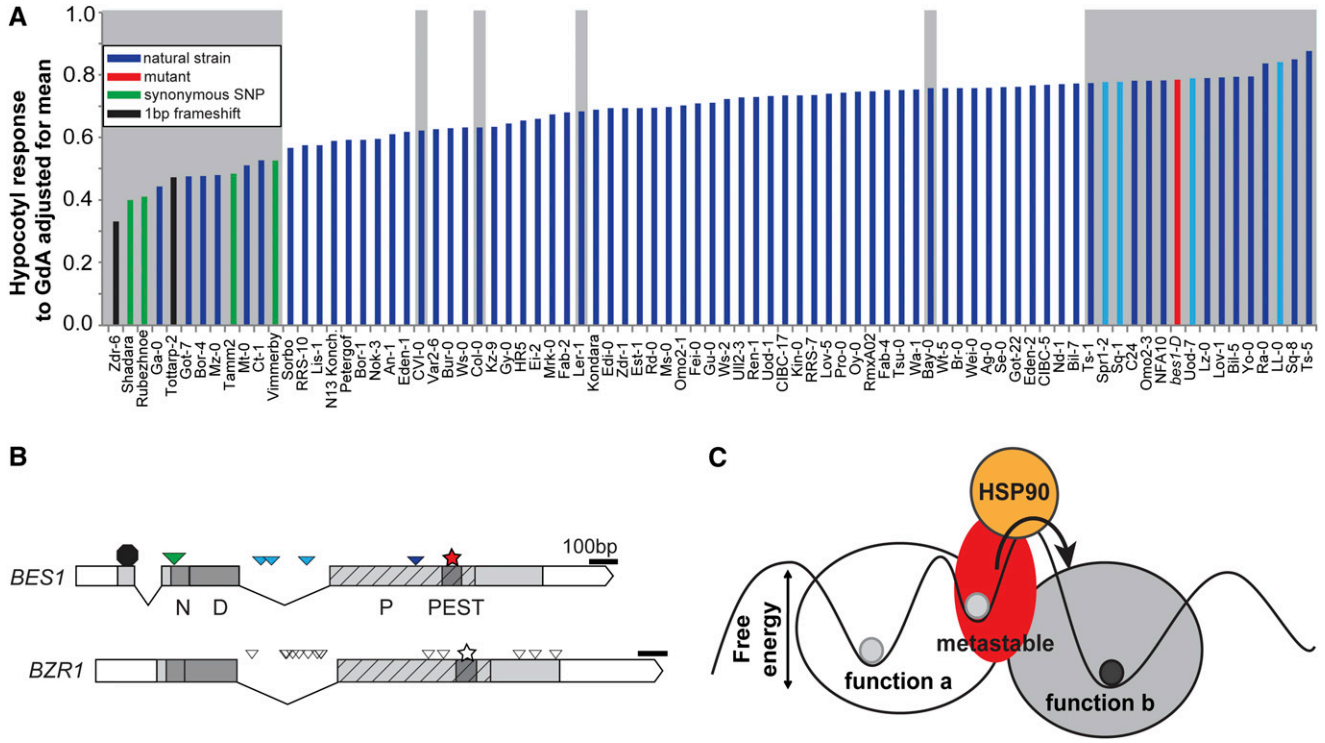

Figure 4 BES1 HSP90 client status is dynamic. (A) Hypocotyl length GdA sensitivity of divergent *A. thaliana* strains and *bes1-D* (red). *BES1* and *BZR1* were sequenced in strains highlighted in gray. Strains with frameshift mutations are in black; strains with *BES1*-synonymous polymorphisms are green. Both polymorphisms are significantly associated with GdA sensitivity. Strains with *BES1* intronic polymorphisms are light blue. (B) *BES1.3* and *BZR1.1* polymorphisms. Gray boxes are exons, black lines are introns, and white boxes are UTRs. The hexagon marks the frameshift mutation, the green triangle marks the synonymous SNP, the dark-blue triangle marks the nonsynonymous polymorphism, white triangles mark noncoding or synonymous polymorphisms, light-blue triangles mark intronic polymorphisms in *BES1*, and stars mark the *bzr1-D* and *bes1-D* dominant mutations. The domains are N-nuclear localization signal, D-DNA binding domain, P-phosphorylation domain, and PEST-PP2A interaction domain. (C) A protein that exists in a free-energy minimum ("function a") acquires mutations that render it metastable and thus recognized by HSP90. As an HSP90 client, the protein can visit a greater sequence space, increasing the chance of reaching another free-energy minimum associated with a novel function ("function b") and loss of client status.

strong support of the capacitor hypothesis, HSP90 inhibition revealed phenotypic differences among divergent *A. thaliana* strains that are associated with *BES1* polymorphisms. Moreover, HSP90 client status appears to be highly dynamic with BES1 losing HSP90 dependence in some strains.

Another nonsynonymous polymorphism in *BES1* did not correlate with response to HSP90 inhibition (Figure 4B). Consistent with HSP90 facilitating *BES1* evolution, this polymorphism may represent a step toward a novel phenotype through acquisition of a second mutation, with which it interacts epistatically (Figure 4B) (Salverda *et al.* 2011). Neutral nonsynonymous mutations, such as mutations that increase protein stability without affecting function, can facilitate ascent to a new fitness optimum (Tokuriki and Tawfik 2009). In contrast, all 20 *BZR1* polymorphisms were synonymous (Figure 4B), consistent with *BZR1*'s lower evolutionary rate compared to *BES1*. None of these were associated with sensitivity to HSP90 inhibition, supporting BZR1's nonclient status (Figure 4B and Figure S6B). Eight *BZR1* polymorphisms were unique to the strain Uod-7, which carries a large intronic insertion in addition to other intronic polymorphisms (Figure 4B). These polymorphisms may lead to mis-regulation of *BZR1* (Le Hir *et al.* 2003), thereby increasing the need for BES1. This scenario is consistent with Uod-7's hypersensitivity to GdA (Figure 4A). Taken together, our data suggest that HSP90 facilitates divergence of gene duplicates by promoting subfunctionalization through temperature sensitivity of its clients and neofunctionalization through their increased tolerance of mutations.

Discussion

With BES1, we have identified a novel HSP90 client in a crucial plant growth pathway (Figure 1). By necessity, plant growth must be finely tuned to the environment. The temperature sensitivity of BES1 tightly links BR pathway function to the ambient environment. We and others showed previously that HSP90 plays an important role in defenses against herbivores and microbial pathogens (Hubert *et al.* 2003; Takahashi *et al.* 2003; Sangster and Queitsch 2005; Sangster *et al.* 2007), as well as in the timing of flowering (Sangster *et al.* 2007). Our findings add an important layer of complexity to known hormone–environment interactions (Nemhauser

2008; Robert-Seilaniantz *et al.* 2011), especially in light of HSP90's known role in resource allocation among defense and growth pathways. Our data also add to the emerging evidence of functional divergence between BES1 and BZR1—the critical downstream targets of BR signaling. For example, recent genome-wide chromatin immunoprecipitation experiments show only partial overlap of gene targets in BES1 and BZR1 (Sun *et al.* 2010; Yu *et al.* 2011). This partial overlap highlighted key unresolved questions about BR transcriptional responses. Specifically, what distinguishes BES1- or BZR1-specific targets from targets regulated by both proteins? And what determines whether BES1 and BZR1 act as repressors or activators? As BES1, but not BZR1, is an HSP90 client, we speculate that interaction with the chaperone may facilitate association of BES1 with specific partner proteins, resulting in BES1-specific functions (Yin *et al.* 2005).

In addition to identifying a novel plant HSP90 client, our study provides support for the capacitor hypothesis by garnering evidence of HSP90-facilitated evolution in extant genomes. When we compared paralogs, we found a significant trend that genes encoding diverse HSP90 clients evolved faster than nonclients (Figure 2). In contrast, when we compared diverse yeast HSP90 clients and nonclients as aggregate groups, we could not detect any significant difference in evolution rate (Figure S4B). This result is consistent with a similar analysis comparing prokaryotic genes encoding diverse clients of the bacterial chaperonin GroEL/ES and nonclients (Williams and Fares 2010). On the contrary, within the superfamily of mammalian kinases, strong HSP90 clients carry more nonsynonymous mutations than nonclients (Taipale *et al.* 2012). These different results are not surprising because many other factors or gene properties influence evolutionary rate, most of which will differ for a diverse set of genes. Combined, these factors can outweigh and conceal effects of HSP90 and GroEL/ES client status on evolutionary rate. By focusing on gene duplicates of diverse HSP90 clients, which share many properties influencing evolutionary rate, we found support for HSP90's previously hypothesized effect on gene evolution. As overexpression of GroEL/ES also increases the evolutionary rates of its clients (Tokuriki and Tawfik 2009), our study supports an ancient and conserved role for protein chaperones in gene evolution.

Our findings also help resolve an apparent paradox about the fate of gene duplicates. Gene duplicates are functionally redundant immediately after duplication, rendering one copy superfluous or even harmful (Lynch and Conery 2000; Papp *et al.* 2003). Evolutionary theory predicts nonfunctionalization—one gene copy is silenced and subsequently lost—as the fate of most duplicated genes. Recent studies have challenged this view by revealing that gene duplicates with partially redundant function are maintained much longer than expected (Lynch and Conery 2000; Maere *et al.* 2005; Conant and Wolfe 2008; Dean *et al.* 2008; DeLuna *et al.* 2008). Moreover, in yeast, plants, insects, and humans, genes that can be maintained in duplicate show strong functional bias, with transcription factors and kinases significantly overrepresented (Maere *et al.* 2005; Aury *et al.* 2006; Guan *et al.* 2007; Wapinski *et al.* 2007; Conant and Wolfe 2008). Both observations raise questions about the molecular mechanism(s) that aid the initial preservation, continued maintenance, and eventual divergence of a specific subset of gene duplicates. Our data are consistent with a model in which acquisition of HSP90 client status is a molecular mechanism aiding each of these steps. Acquisition of HSP90 client status can occur through a single mutational step (Citri *et al.* 2006; Taipale *et al.* 2010). A new HSP90 client will be subject to immediate environmental subfunctionalization. As we observed for BES1, HSP90 clients are exquisitely sensitive to environmental conditions affecting protein folding (Nathan *et al.* 1997). Immediate and efficient subfunctionalization will counteract deleterious dosage effects and foster long-term maintenance of gene duplicates and hence provide opportunity for their functional divergence. In fact, *bes1-D* mutants were more sensitive to a temperature increase than to the HSP90 inhibition, which is consistent with the fact that temperature change is a more complex perturbation compared to the specific inhibition of a single chaperone. In addition to providing evidence for HSP90-faciliated subfunctionalization, we show that HSP90 client status correlates with hallmarks of neofunctionalization and increased evolutionary rates of the genes encoding them. HSP90 recognizes metastable signal transduction proteins, most of which are transcription factors and kinases (Taipale *et al.* 2010). We speculate that HSP90's specificity for these substrates contributes to the observed functional bias among gene duplicates. Additional support for HSP90's role in gene duplicate divergence comes from our observation that HSP90 client status is dynamic in the BZR/BEH family and in yeast gene families, with frequent gains and losses (Figure 4, A and B). Dynamic client status is also observed in the mammalian kinome (Taipale *et al.* 2012). In fact, even across wild *A. thaliana* accessions BES1 HSP90 client status itself appears to be dynamic.

The presence of HSP90 clients and nonclients in gene families with partially redundant function has important implications for another aspect of HSP90-mediated capacitance. Inhibition of HSP90 increases phenotypic variation even in the absence of genetic variation (Queitsch *et al.* 2002; Sangster *et al.* 2008a,b). This increase of phenotypic variation in isogenic lines has been attributed to an increased frequency of stochastic events in development or to a greater sensitivity to microenvironments. As recently shown, significant phenotypic variation arises in isogenic worms due to the loss of functional redundancy between paralogs (Burga *et al.* 2011; Casanueva *et al.* 2012). Under conditions that challenge HSP90 function, HSP90-dependent paralogs will become inactive, thereby reducing functional redundancy and increasing phenotypic variation. We suggest a general role for HSP90 in maintaining a reservoir of phenotypic variation through facilitating conditional functionality of gene duplicates.

Acknowledgments

We thank members of the Queitsch and Nemhauser groups for useful discussions; Orrin Stone for experimental assistance; Yanhai Yin for anti-BES1 antibody; Elhanan Borenstein, Maitreya Dunham, Stanley Fields, Jesse Bloom, and Harmit Malik for helpful comments; Magnus Nordborg and Scott Hodges for use of unpublished *Aquilegia* sequences; and Neeraj Salathia for manual curation of TPR domain-containing proteins. This work was supported by grants from the National Human Genome Research Institute Interdisciplinary Training in Genomic Sciences (T32 HG00035) (J.L.), the National Science Foundation Graduation Research Fellowship (DGE-0718124) (J.L.), National Institute of General Medical Sciences Academic Research Enhancement Award R15-GM086822 (P.J.M.M.), and National Science Foundation grant IOS-0919021 (J.L.N.).

Literature Cited

Albrecht, C., E. Russinova, B. Kemmerling, M. Kwaaitaal, and S. C. de Vries, 2008 Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE proteins serve brassinosteroid-dependent and -independent signaling pathways. Plant Physiol. 148: 611–619.

Asami, T., Y. K. Min, N. Nagata, K. Yamagishi, S. Takatsuto *et al.*, 2000 Characterization of brassinazole, a triazole-type brassinosteroid biosynthesis inhibitor. Plant Physiol. 123: 93–100.

Aury, J. M., O. Jaillon, L. Duret, B. Noel, C. Jubin *et al.*, 2006 Global trends of whole-genome duplications revealed by the ciliate Paramecium tetraurelia. Nature 444: 171–178.

Bergman, A., and M. L. Siegal, 2003 Evolutionary capacitance as a general feature of complex gene networks. Nature 424: 549–552.

Bielawski, J. P., and Z. Yang, 2003 Maximum likelihood methods for detecting adaptive evolution after gene duplication. J. Struct. Funct. Genomics 3: 201–212.

Bloom, J. D., S. T. Labthavikul, C. R. Otey, and F. H. Arnold, 2006 Protein stability promotes evolvability. Proc. Natl. Acad. Sci. USA 103: 5869–5874.

Burga, A., M. O. Casanueva, and B. Lehner, 2011 Predicting mutation outcome from early stochastic variation in genetic interaction partners. Nature 480: 250–253.

Casanueva, M. O., A. Burga, and B. Lehner, 2012 Fitness tradeoffs and environmentally induced mutation buffering in isogenic C. elegans. Science 335: 82–85.

Citri, A., D. Harari, G. Shohat, P. Ramakrishnan, J. Gan *et al.*, 2006 Hsp90 recognizes a common surface on client kinases. J. Biol. Chem. 281: 14361–14369.

Conant, G. C., and K. H. Wolfe, 2008 Turning a hobby into a job: how duplicated genes find new functions. Nat. Rev. Genet. 9: 938–950.

Dean, E. J., J. C. Davis, R. W. Davis, and D. A. Petrov, 2008 Pervasive and persistent redundancy among duplicated genes in yeast. PLoS Genet. 4: e1000113.

DeLuna, A., K. Vetsigian, N. Shoresh, M. Hegreness, M. Colon-Gonzalez *et al.*, 2008 Exposing the fitness contribution of duplicated genes. Nat. Genet. 40: 676–681.

Drummond, D. A., J. D. Bloom, C. Adami, C. O. Wilke, and F. H. Arnold, 2005 Why highly expressed proteins evolve slowly. Proc. Natl. Acad. Sci. USA 102: 14338–14343.

Gangaraju, V. K., H. Yin, M. M. Weiner, J. Wang, X. A. Huang *et al.*, 2011 Drosophila Piwi functions in Hsp90-mediated suppression of phenotypic variation. Nat. Genet. 43: 153–158.

Gasch, A. P., P. T. Spellman, C. M. Kao, O. Carmel-Harel, M. B. Eisen *et al.*, 2000 Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell 11: 4241–4257.

Guan, Y., M. J. Dunham, and O. G. Troyanskaya, 2007 Functional analysis of gene duplications in *Saccharomyces cerevisiae*. Genetics 175: 933–943.

Guindon, S., and O. Gascuel, 2003 A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52: 696–704.

Guo, H., L. Li, H. Ye, X. Yu, A. Algreen *et al.*, 2009 Three related receptor-like kinases are required for optimal cell elongation in Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA 106: 7648–7653.

Hahn, M. W., 2009 Distinguishing among evolutionary models for the maintenance of gene duplicates. J. Hered. 100: 605–617.

He, J. X., J. M. Gendron, Y. Sun, S. S. Gampala, N. Gendron *et al.*, 2005 BZR1 is a transcriptional repressor with dual roles in brassinosteroid homeostasis and growth responses. Science 307: 1634–1638.

Hubert, D. A., P. Tornero, Y. Belkhadir, P. Krishna, A. Takahashi *et al.*, 2003 Cytosolic HSP90 associates with and modulates the Arabidopsis RPM1 disease resistance protein. EMBO J. 22: 5679–5689.

Iki, T., M. Yoshikawa, M. Nishikiori, M. C. Jaudal, E. Matsumoto-Yokoyama *et al.*, 2010 In vitro assembly of plant RNA-induced silencing complexes facilitated by molecular chaperone HSP90. Mol. Cell 39: 282–291.

Ishiguro, S., Y. Watanabe, N. Ito, H. Nonanka, N. Takeda *et al.*, 2002 SHEPARD is the Arabidopsis GRP94 responsible for the formation of functional CLAVATA proteins. EMBO J. 21: 898–908.

Jarosz, D. F., and S. Lindquist, 2010 Hsp90 and environmental stress transform the adaptive value of natural genetic variation. Science 330: 1820–1824.

Kim, T.-W., and Z.-Y. Wang, 2010 Brassinosteroid signal transduction from receptor kinases to transcription factors. Annu. Rev. Plant Biol. 61: 681–704.

Le Hir, H., A. Nott, and M. J. Moore, 2003 How introns influence and enhance eukaryotic gene expression. Trends Biochem. Sci. 28: 215–220.

Li, L., X. Yu, A. Thompson, M. Guo, S. Yoshida *et al.*, 2009 Arabidopsis MYB30 is a direct target of BES1 and cooperates with BES1 to regulate brassinosteroid-induced gene expression. Plant J. 58: 275–286.

Lynch, M., and J. S. Conery, 2000 The evolutionary fate and consequences of duplicate genes. Science 290: 1151–1155.

Maere, S., S. De Bodt, J. Raes, T. Casneuf, M. Van Montagu *et al.*, 2005 Modeling gene and genome duplications in eukaryotes. Proc. Natl. Acad. Sci. USA 102: 5454–5459.

McLellan, C. A., T. J. Turbyville, E. M. K. Wijeratne, A. Kerschen, E. Vierling *et al.*, 2007 A Rhizosphere fungus enhances Arabidopsis thermotolerance through production of an HSP90 inhibitor. Plant Physiol. 145: 174–182.

Meiklejohn, C. D., and D. L. Hartl, 2002 A single mode of canalization. Trends Ecol. Evol. 17: 468–473.

Nathan, D. F., M. H. Vos, and S. Lindquist, 1997 In vivo functions of the Saccharomyces cerevisiae Hsp90 chaperone. Proc. Natl. Acad. Sci. USA 94: 12949–12956.

Nemhauser, J. L., 2008 Dawning of a new era: photomorphogenesis as an integrated molecular network. Curr. Opin. Plant Biol. 11: 4–8.

Papp, B., C. Pal, and L. D. Hurst, 2003 Dosage sensitivity and the evolution of gene families in yeast. Nature 424: 194–197.

Pena, M. I., M. Davlieva, M. R. Bennett, J. S. Olson, and Y. Shamoo, 2010 Evolutionary fates within a microbial population highlight

an essential role for protein folding during natural selection. Mol. Syst. Biol. 6: 387.
Picard, D., B. Khursheed, M. J. Garabedian, M. G. Fortin, S. Lindquist *et al.*, 1990 Reduced levels of hsp90 compromise steroid receptor action in vivo. Nature 348: 166–168.
Queitsch, C., T. A. Sangster, and S. Lindquist, 2002 Hsp90 as a capacitor of phenotypic variation. Nature 417: 618–624.
Rando, O. J., and K. J. Verstrepen, 2007 Timescales of genetic and epigenetic inheritance. Cell 128: 655–668.
Robert-Seilaniantz, A., M. Grant, and J. D. Jones, 2011 Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. Annu. Rev. Phytopathol. 49: 317–343.
Rutherford, S. L., and S. Lindquist, 1998 Hsp90 as a capacitor for morphological evolution. Nature 396: 336–342.
Salverda, M. L. M., E. Dellus, F. A. Gorter, A. J. M. Debets, J. van der Oost *et al.*, 2011 Initial mutations direct alternative pathways of protein evolution. PLoS Genet. 7: e1001321.
Sangster, T. A., and C. Queitsch, 2005 The HSP90 chaperone complex, an emerging force in plant development and phenotypic plasticity. Curr. Opin. Plant Biol. 8: 86–92.
Sangster, T. A., A. Bahrami, A. Wilczek, E. Watanabe, K. Schellenberg *et al.*, 2007 Phenotypic diversity and altered environmental plasticity in Arabidopsis thaliana with reduced Hsp90 levels. PLoS ONE 2: e648.
Sangster, T. A., N. Salathia, H. N. Lee, E. Watanabe, K. Schellenberg *et al.*, 2008a HSP90-buffered genetic variation is common in Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA 105: 2969–2974.
Sangster, T. A., N. Salathia, S. Undurraga, R. Milo, K. Schellenberg *et al.*, 2008b HSP90 affects the expression of genetic variation and developmental stability in quantitative traits. Proc. Natl. Acad. Sci. USA 105: 2963–2968.
Sollars, V., X. Lu, L. Xiao, X. Wang, M. D. Garfinkel *et al.*, 2003 Evidence for an epigenetic mechanism by which Hsp90 acts as a capacitor for morphological evolution. Nat. Genet. 33: 70–74.
Sun, Y., X.-Y. Fan, D.-M. Cao, W. Tang, K. He *et al.*, 2010 Integration of brassinosteroid signal transduction with the transcription network for plant growth regulation in Arabidopsis. Dev. Cell 19: 765–777.
Swarbreck, D., C. Wilks, P. Lamesch, T. Z. Berardini, M. Garcia-Hernandez *et al.*, 2008 The Arabidopsis Information Resource (TAIR): gene structure and function annotation. Nucleic Acids Res. 36: D1009–D1014.
Taipale, M., D. F. Jarosz, and S. Lindquist, 2010 HSP90 at the hub of protein homeostasis: emerging mechanistic insights. Nat. Rev. Mol. Cell Biol. 11: 515–528.
Taipale, M., I. Krykbaeva, M. Koeva, C. Kayatekin, K. D. Westover *et al.*, 2012 Quantitative analysis of HSP90-client interactions reveals principles of substrate recognition. Cell 150: 987–1001.
Takahashi, A., C. Casais, K. Ichimura, and K. Shirasu, 2003 HSP90 interacts with RAR1 and SGT1 and is essential for RPS2-mediated disease resistance in Arabidopsis. Proc. Natl. Acad. Sci. USA 100: 11777–11782.
Tang, W., M. Yuan, R. Wang, Y. Yang, C. Wang *et al.*, 2011 PP2A activates brassinosteroid-responsive gene expression and plant growth by dephosphorylating BZR1. Nat. Cell Biol. 13: 124–131.
Tokuriki, N., and D. S. Tawfik, 2009 Chaperonin overexpression promotes genetic variation and enzyme evolution. Nature 459: 668–673.
Vaughan, C. K., L. Neckers, and P. W. Piper, 2010 Understanding of the Hsp90 molecular chaperone reaches new heights. Nat. Struct. Mol. Biol. 17: 1400–1404.
Vert, G., and J. Chory, 2006 Downstream nuclear events in brassinosteroid signalling. Nature 441: 96–100.
Wang, Z. Y., H. Seto, S. Fujioka, S. Yoshida, and J. Chory, 2001 BRI1 is a critical component of a plasma-membrane receptor for plant steroids. Nature 410: 380–383.
Wang, Z. Y., T. Nakano, J. Gendron, J. He, M. Chen *et al.*, 2002 Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. Dev. Cell 2: 505–513.
Wapinski, I., A. Pfeffer, N. Friedman, and A. Regev, 2007 Natural history and evolutionary principles of gene duplication in fungi. Nature 449: 54–61.
Wegele, H., L. Muller, and J. Buchner, 2004 Hsp70 and Hsp90: a relay team for protein folding. Rev. Physiol. Biochem. Pharmacol. 151: 1–44.
Whitesell, L., E. G. Mimnaugh, B. De Costa, C. E. Myers, and L. M. Neckers, 1994 Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc. Natl. Acad. Sci. USA 91: 8324–8328.
Williams, T. A., and M. A. Fares, 2010 The effect of chaperonin buffering on protein evolution. Genome Biol. Evol. 2: 609–619.
Wright, S. I., C. B. Yau, M. Looseley, and B. C. Meyers, 2004 Effects of gene expression on molecular evolution in Arabidopsis thaliana and Arabidopsis lyrata. Mol. Biol. Evol. 21: 1719–1726.
Yeyati, P. L., R. M. Bancewicz, J. Maule, and V. van Heyningen, 2007 Hsp90 selectively modulates phenotype in vertebrate development. PLoS Genet. 3: e43.
Yin, Y., Z. Y. Wang, S. Mora-Garcia, J. Li, S. Yoshida *et al.*, 2002 BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. Cell 109: 181–191.
Yin, Y., D. Vafeados, Y. Tao, S. Yoshida, T. Asami *et al.*, 2005 A new class of transcription factors mediates brassinosteroid-regulated gene expression in Arabidopsis. Cell 120: 249–259.
Yu, X., L. Li, J. Zola, M. Aluru, H. Ye *et al.*, 2011 A brassinosteroid transcriptional network revealed by genome-wide identification of BESI target genes in Arabidopsis thaliana. Plant J. 65: 634–646.
Zhao, R., M. Davey, Y. C. Hsu, P. Kaplanek, A. Tong *et al.*, 2005 Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the hsp90 chaperone. Cell 120: 715–727.
Zou, J., Y. Guo, T. Guettouche, D. F. Smith, and R. Voellmy, 1998 Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. Cell 94: 471–480.

Communicating editor: J. Borevitz

GENETICS

Supporting Information
http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.112.148098/-/DC1

The Protein Chaperone HSP90 Can Facilitate the Divergence of Gene Duplicates

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DOI: 10.1534/genetics.112.148098

File S1

Supplementary Materials and Methods

Plant growth conditions and treatments

Columbia-0 (Col-0) was used as wild type. *bzr1-D*, *DWF4*-ox, *bin2-1*, *BRI1*-ox, and *bes1-2* (WiscDsLox246D02) were in the Columbia-0 background. The recessive, loss-of-function mutant *bes1-2* was isolated in this study, using a T-DNA insertion line (WiscDsLox246D02) . The *bes1-D* mutant is a recapitulation line using a transgene to constitutively express the mutant form of *bes1-D* in Col-0. Seeds were stratified for three nights at 4°C and grown vertically in the dark or red LED light for seven days on 0.5x LS 0.08% bactoagar growth media at 22°C. Geldanamycin (Sigma #G3381), brassinolide (Chemiclones #101), and brassinazole (Chemiclones #117) were dissolved in DMSO. Drugs and mock (DMSO only) treatments were added to growth media. For growth at 27°C, seedlings were placed in 22°C for 24 hours and then transferred to 27°C. For response to GdA in accessions, 15 seeds from 96 accessions were grown in the dark for seven days on medium containing DMSO or 1µM GdA. Accessions in which less than seven seeds germinated were removed from further analysis. Difference between hypocotyl length in mock and treatment was taken and adjusted by the mock mean per genotype.

Statistical analyses

Analyses of data were performed with JMP7 (SAS Institute). To measure the effect of drug treatments and interactions on hypocotyl length, standard least square linear regression was used. Each drug, drug by drug interaction (all fixed effects), and replicate (random effect) was modeled (2-4 replicates for each experiment, 10-60 seedlings each). When comparing the effect of genotype on response to drug treatment, standard least square linear regression was used. Genotype, drug, genotype by drug interaction (all fixed effects), and replicate (random effect) was modeled (2-4 replicates of each experiment, 10-60 seedlings each). To associate *BES1* and *BZR1* SNPs with response to GdA, the GdA response was normalized using the mock treatment mean for each strain. Each SNP in the 30 strains was tested for association with GdA response using ANOVA (One-way).

Western blot

Seedlings were grown for seven days in red LED light and then ground in liquid nitrogen. Buffer (0.15M Tris pH 6.8) was then added, and extracted protein was quantified using Bradford's assay (Pierce #1856210). Approximately 15ug of protein was loaded per lane. Gel Code Blue Stain Reagent (Pierce #24590) was used to determine loading. Proteins were transferred to nitrocellulose and probed with anti-BES1 antibody (gift of Yanhai Yin).

Co-immunoprecipitation

Col-0 (WT) and *bes1-2* rosette leaves grown in long day conditions were harvested, frozen in liquid nitrogen, ground, and resuspended in HEM buffer (10mM HEPES, 1mM EDTA, 20mM sodium molybdate, 1mM PMSF (Fluka 78830) and protease inhibitor (Roche 11-836-153-001)). The solution was ultracentrifuged for 100,000 x g for 30 minutes at 4°C. The resulting supernatant was incubated for three hours with Protein L Agarose (Pierce 20510), which was pre-incubated with anti-HSP90 3G3 antibody (Enzo Lifescences ALX-804-079-R400). Beads were pelleted and washed with HEM buffer. Anti-BES1 antibody (gift of Yanhai Yin) was used to detect BES1 in the pellet. The newly isolated *bes-2* mutant (recessive, loss-of-function) was used to confirm that this antibody is specific to BES1.

Phylogenetic tree and dN/dS analysis

One hundred eighteen sequences for *BZR/BEH* family members in all sequenced plants were acquired from http://phytozome.net v5.0 from a BLAST search for gene families with similarity to *BES1* at the node for Viridiplantae. Sequences from genomes that were not publically available were removed manually. MUSCLE 3.7 was used for amino acid alignment of the identified sequences, and Gblocks was used to remove regions with poor conservation. Sequences with low similarity in the high conservation region identified with Gblocks were removed manually. The remaining 86 sequences were re-aligned and neighbor-joining was used to create a distance tree. For the *BZR/BEH* tree, the outgroup was identified as a *BZR/BEH* family member that was closely related, but an outgroup to all *A. thaliana BZR/BEH* family members. MUSCLE 3.7 was used for amino acid alignment of outgroup and *A. thaliana* family members using default parameters and manually examined for errors. PhyML was used for maximum likelihood tree (GUINDON and GASCUEL 2003). For dN/dS analysis, PAL2NALv13 was used to convert amino acid alignment to codon alignment. The codeml program from PAMLv4.4b was run with gaps removed using models 0, 1, and 2 (BIELAWSKI and YANG 2003). The branch leading to *BES1* or *BZR1* was allowed to vary in model 2. The dN/dS values for the *BZR/BEH* family came from model 1.

Yeast data analyses

For this study, published HSP90 interactors were used (ZHAO *et al.* 2005). TPR-domain containing proteins were curated from available literature and sequence information and excluded from further analysis. We used the Ensembl Compara (release 61) database to obtain the branch length of HSP90 interactors in three-member and two-member families. For the three-member families, we filtered out genes in which the inner or outer-paralog were also HSP90 interactors. For the two-member families we filtered out those in which both genes were HSP90 interactors. To determine whether there was a significant difference in

branch length between clients, non-clients, and their paralogs, we calculated the proportion of total branch length for each client or non-client and their paralogs. A one-sided Wilcoxon test was used to determine significance of detected differences in proportional branch length between client and paralog or non-client and paralog.

Table S1 Significance of response to HSP90 inhibition

	0.5uM GdA		1.0uM GdA	
genotype	R^2	p-value	R^2	p-value
bes1-D	0.73	<0.0001	0.89	<0.0001
bzr1-D	0.26	0.63	0.63	0.59
BRI1-ox	0.48	0.22	0.74	<0.0001
DWF4-ox	0.75	0.99	0.8	0.0003

Seedlings with increased BR signaling through overexpression of DWF4 (*DWF4-ox*) or overexpression of BRI1 (*BRI1-ox*) showed WT-like sensitivity to HSP90-inhibition at 0.5µM GdA. In contrast, seedlings with constitutive activation of BES1 (*bes1-D*) were significantly more sensitive than WT at 0.5µM GdA in red light. At 1.0 µM GdA, *BRI1-ox, DWF4-ox*, but not *bzr1-D* showed increased sensitivity compared to WT.

Table S2 Yeast HSP90 clients tend to evolve faster than their respective non-client paralogs independent of expression levels.

client			non-client paralog			comparison		
gene	expression mean	branch length	gene	expression mean	branch length	client minus paralog expression mean		client branch/non-client branch
YBR172C	-0.3258	1.0072	YPL105C	-0.1654	0.7407	-0.1603	*	1.3598
YCL024W	-0.3181	0.3717	YDR507C	-0.4492	0.2050	0.1311		1.8136
YDL025C	0.3978	0.5842	YOR267C	0.2715	0.4900	0.1263		1.1923
YDL199C	0.5594	1.7737	YFL040W	0.0277	1.7386	0.5317	*	1.0202
YDR001C	0.7171	0.2090	YBR001C	0.5592	0.1304	0.1579	***	1.6024
YFL011W	-0.1650	0.2859	YMR011W	-0.4621	0.1549	0.2971	***	1.8461
YFR024C-A	0.0450	0.3320	YHR016C	0.3144	0.2701	-0.2694		1.2291
YGL077C	-0.3462	0.4449	YNR056C	0.0736	1.0123	-0.4198		0.4395
YHR080C	0.3019	0.5174	YDR326C	-0.3168	0.2316	0.6187	***	2.2340
YKR003W	0.0862	0.2907	YHR001W	0.1561	0.2927	-0.0700	***	0.9931
YMR192W	-0.0287	1.6764	YPL249C	0.0550	0.8823	-0.0837		1.9000
YNR011C	-0.1769	1.0256	YKL078W	-1.1110	1.2136	0.9340	***	0.8451
YNR031C	0.0252	0.5748	YCR073C	-0.2379	0.5540	0.2630	***	1.0375

Mean expression was calculated from (GASCH *et al.* 2000). Differences in mean expression were calculated using a Wilcoxon paired sign rank test. Significance values are abbreviated as * p<0.05, ** p<0.01, *** p<0.001.

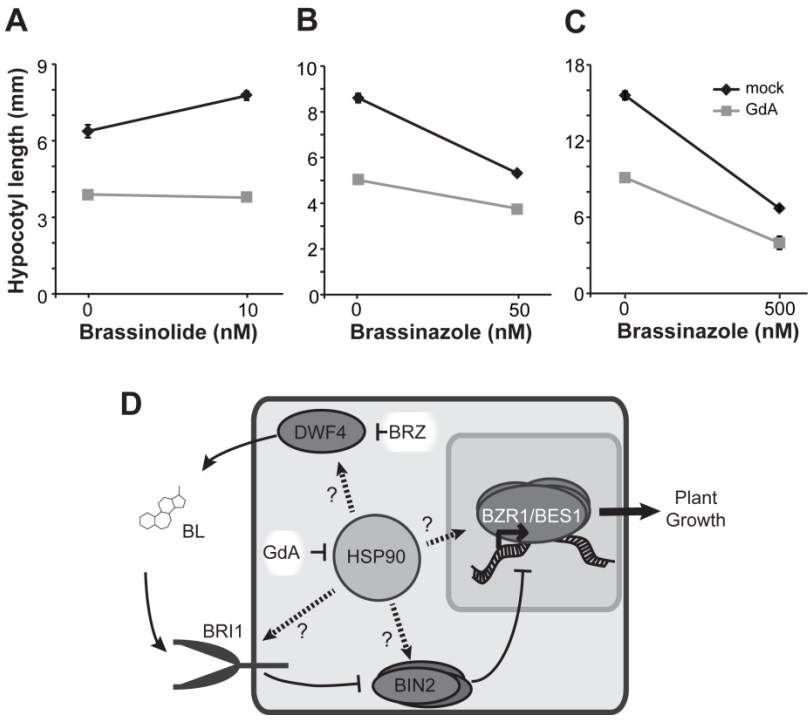

Figure S1 HSP90 is required for BR signaling.
(A) GdA reduces seedling response to brassinolide (BL), the most biologically active BR in red light. Red light was used because GdA decays rapidly in white light. Standard error is shown for all values.
(B) Treatment with brassinazole (BRZ), a BR biosynthetic inhibitor, greatly reduces the effect of GdA treatment on seedling growth in red light.
(C) Treatment with BRZ greatly reduces the effect of GdA treatment on seedling growth in the dark.
(D) The BR signaling pathway. BRs (brassinolide-BL) are synthesized through a number of enzymatic reactions, including the rate-limiting enzyme DWF4, the target of BRZ. When BR levels are low, BIN2 and related kinases inhibit the activity of a family of transcription factors, including BES1 and BZR1. As BRs accumulate, they are detected by the plasma-membrane localized receptor BRI1. Activated BRI1 triggers a series of phosphorylation and dephosphorylation events that ultimately inhibit the activity of BIN2 and its paralogs. Hypophosphorylated BES1 and BZR1 then can bind DNA and trigger changes in target gene transcription. Each of the indicated proteins is a potential HSP90 client.

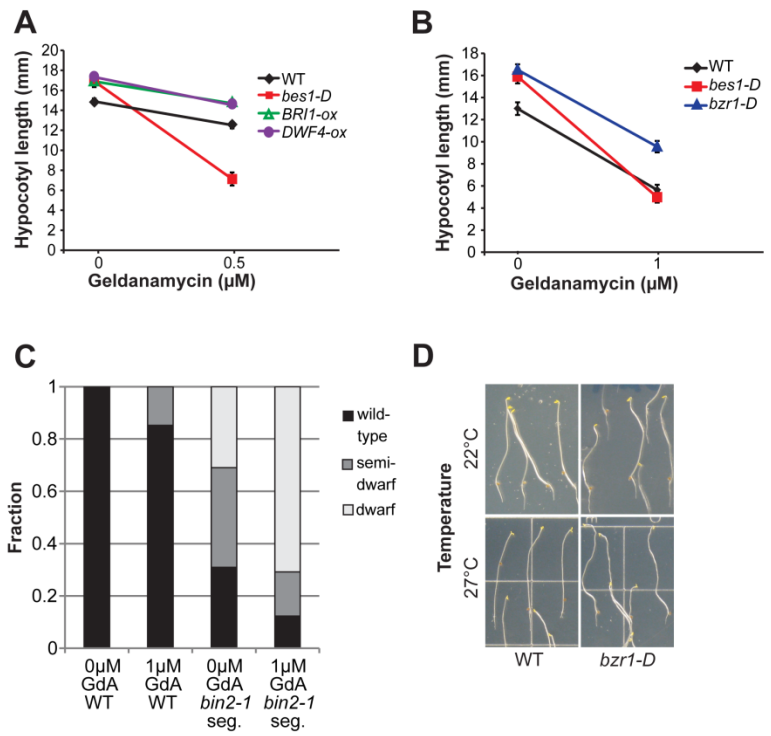

Figure S2 BES1 is an HSP90 client.
(A) Seedlings with increased BR signaling through overexpression of DWF4 (*DWF4-ox*) or overexpression of BRI1 (*BRI1-ox*) showed similar sensitivity to HSP90-inhibition by GdA compared to WT in the dark. In contrast, seedlings with constitutive activation of BES1 (*bes1-D*) were significantly more sensitive than WT the dark. Standard error is shown.
(B) In contrast to the dramatic GdA hypersensitivity of *bes1-D* mutants, *bzr1-D* mutants respond like WT in the dark.
(C) BIN2 is not a likely HSP90 client. The *bin2-1* mutation results in a dwarfed phenotype compared to WT. The phenotypes of a segregating population of *bin2-1* were enhanced rather than alleviated upon inhibition of HSP90. Plant growth phenotypes were categorized as wild-type, semi-dwarf, or dwarf and assessed for 60 seedlings per genotype and condition.
(D) Hyper-sensitivity to increased temperature is not observed in WT and *bzr1-D* seedlings grown at 22°C and 27°C.

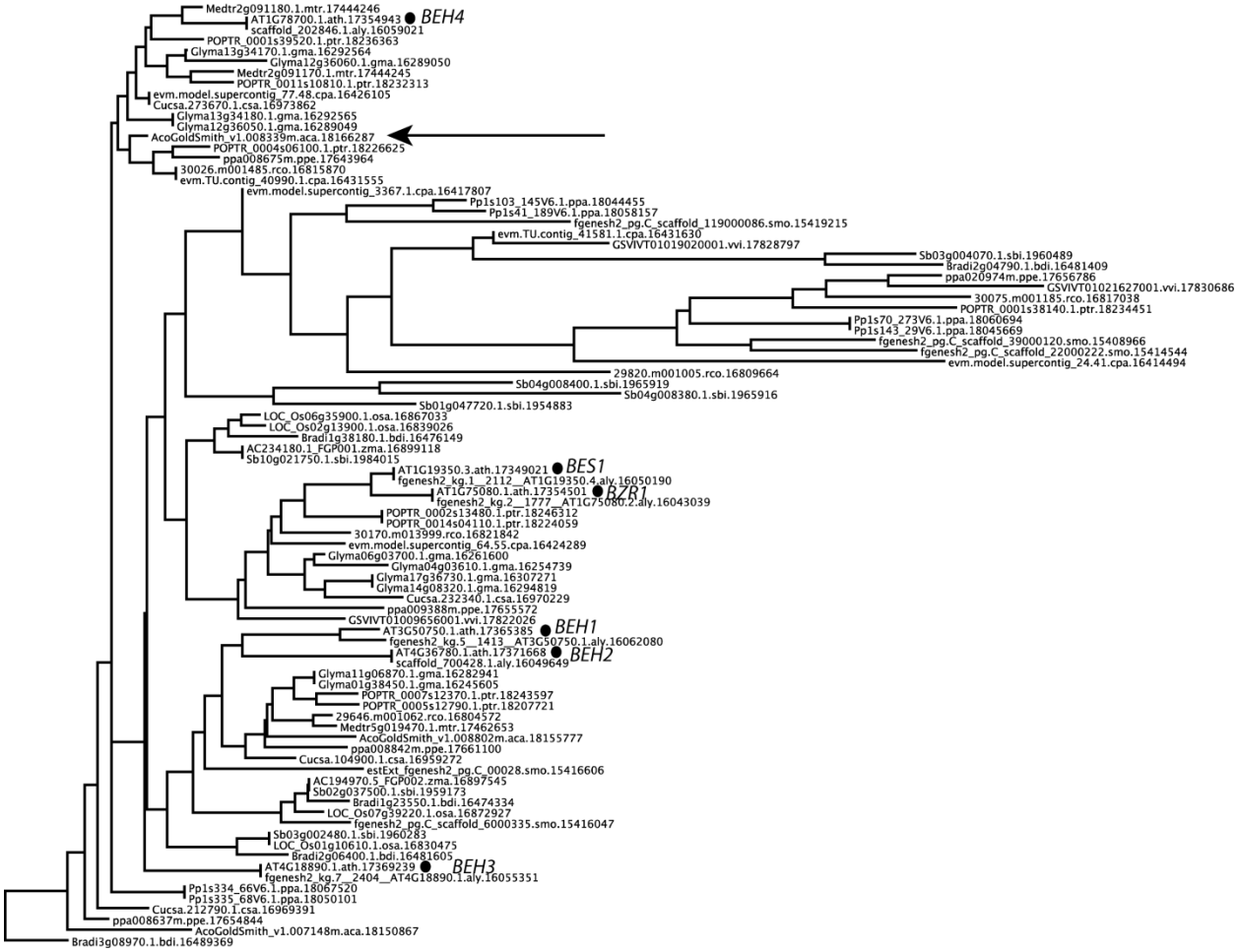

Figure S3 BZR/BEH family genes are found in many plant species. A neighbor-joining tree, using percent amino acid identity among genes identified as potential BZR/BEH family members among 16 plant species, shows that a gene from *Aquilegia coerulea* (arrow) is the most closely related outgroup to all BZR/BEH family members in *A. thaliana* (indicated by "·").

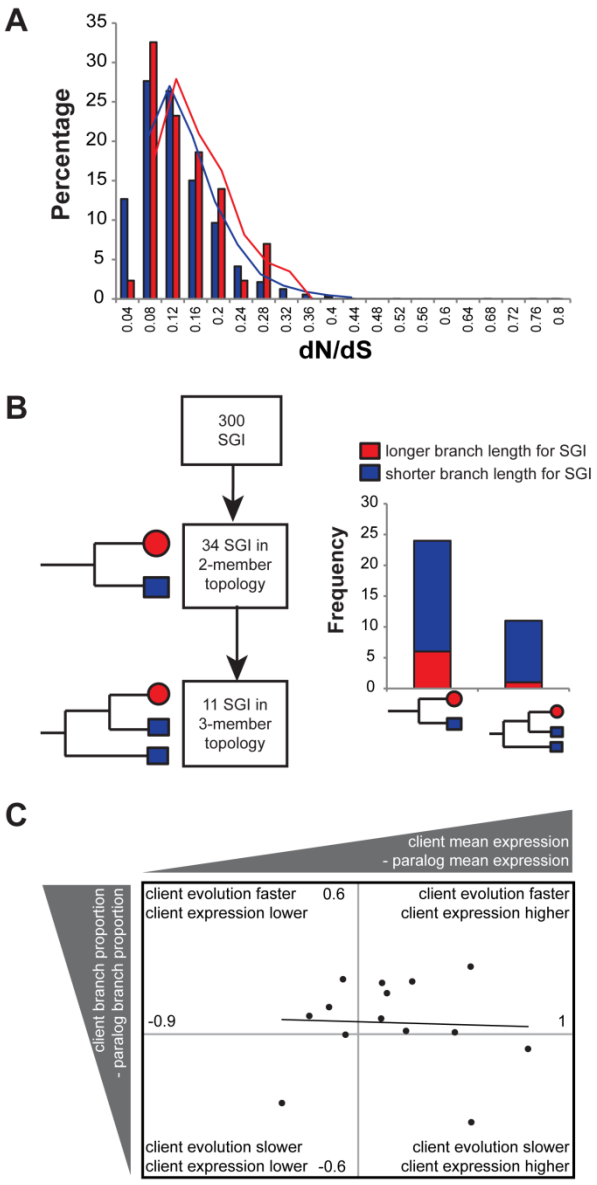

Figure S4 *S. cerevisiae* HSP90 genetic interactors do not show elevated rates of evolution.

(A) *S. cerevisiae* HSP90 TAP-identified clients do not show a significant difference from all other yeast genes in their dN/dS ratios.
(B) *S. cerevisiae* HSP90 synthetic genetic interactors (SGI) do not show longer branch lengths than their paralogs in two-member and three-member families. In fact, they tend to show significantly shorter branch lengths (p=0.03, Wilcoxon, one-sided). SGI are red circles; SGI paralogs are blue squares.
(C) Increased evolutionary rate of yeast HSP90 clients is not explained by lower expression. X-axis shows client mean expression minus non-client paralog mean expression across many environmental conditions (GASCH *et al.* 2000). Y-axis shows client evolutionary rate minus non-client paralog evolutionary rate (using proportional branch length for both). Labels in quadrants indicate relationship of client evolutionary rate and expression level. Numbers indicate scale. Solid trend line shows lack of correlation between evolutionary rate and expression levels (p=0.882, R^2=0.0019).

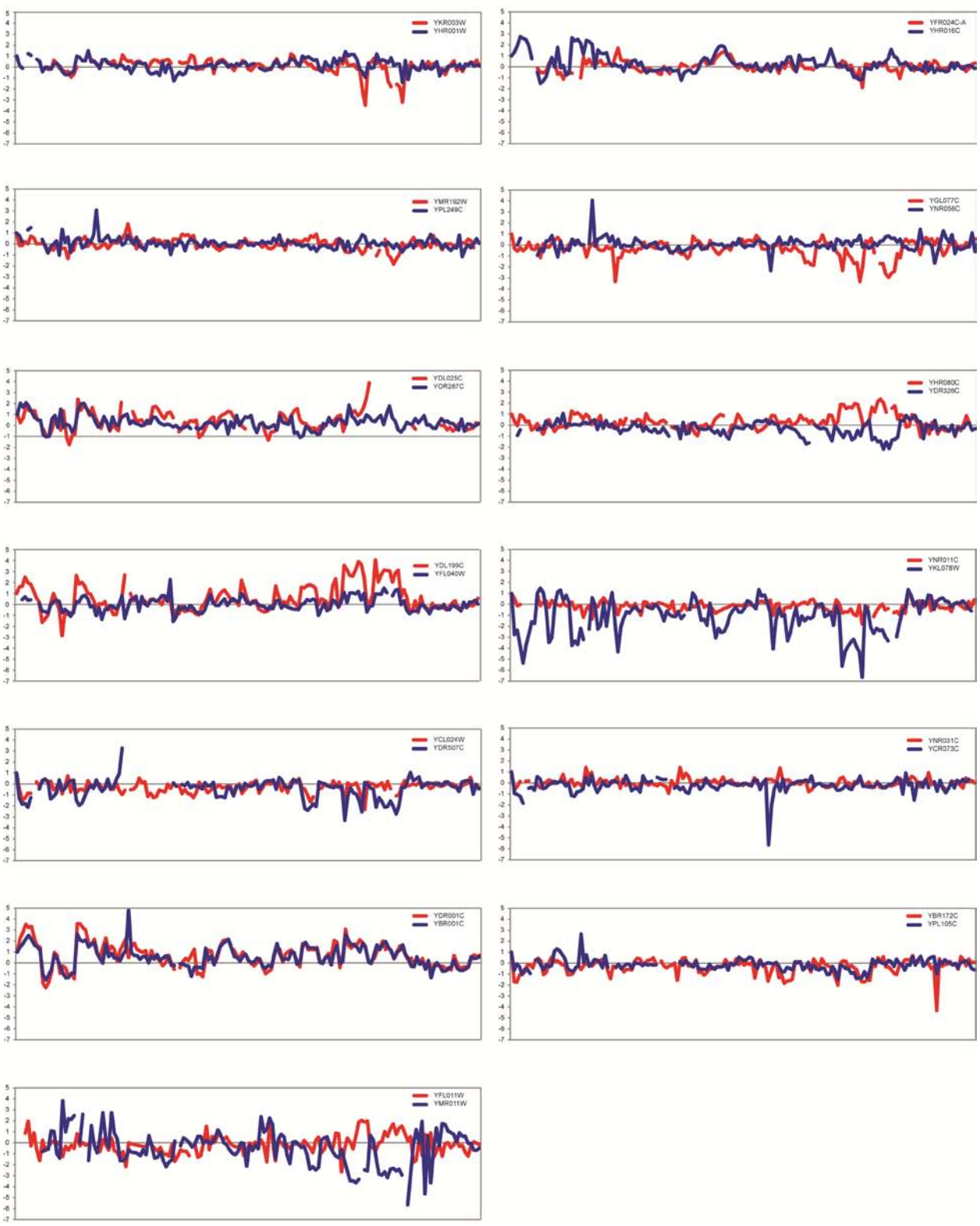

Figure S5 Expression profiles of yeast HSP90 clients and their respective non-client paralogs across 173 environmental conditions (GASCH *et al.* 2000). Client expression is in red; non-client expression is in blue. All 14 pairs are shown. Statistics for observed differences appear in Table S2.

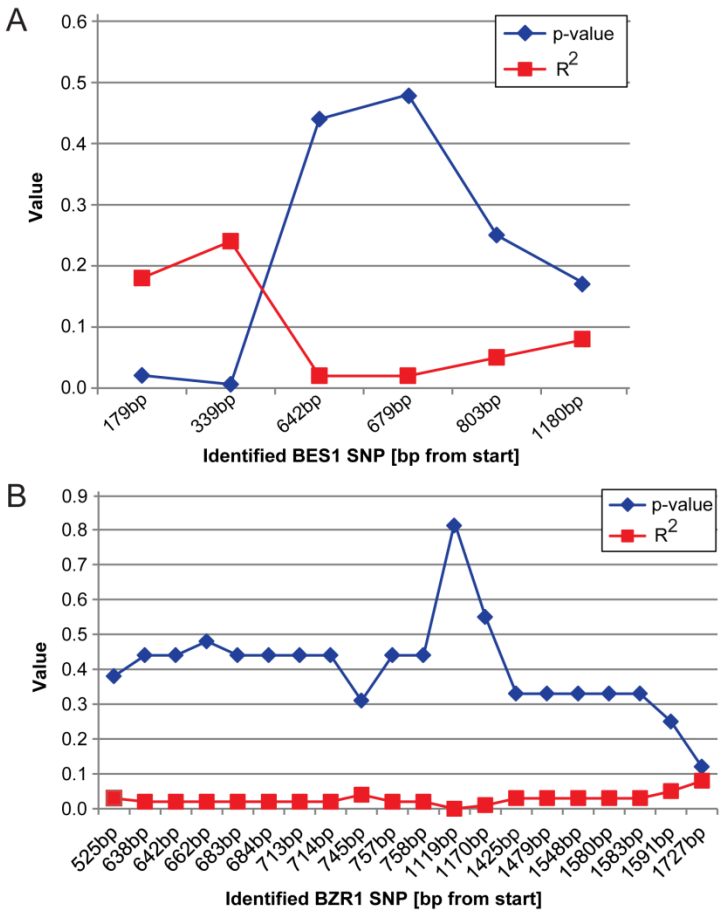

Figure S6 Significant associations were observed between SNPs and hypocotyl response to HSP90 inhibition in *BES1* (A) but not *BZR1* (B) among the 30 sequenced accessions. ANOVA was used to assess significance.