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LINKING HSP90 FUNCTION TO MICRO-ENVIRONMENTAL AND STOCHASTIC VARIATION IN FLORAL ORGANS OF *IRIS PUMILA* L.

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Abstract — Hsp90 is an environmentally responsive molecular chaperone that was found to play a key role in buffering against genetic and non-genetic perturbations in the model organisms *Arabidopsis* and *Drosophila*. Here we analyzed the buffering capacity of Hsp90 against two kinds of non-genetic factors – stochastic noise and micro-environmental variation of floral organ traits in naturally growing *Iris pumila* plants. We found no statistical association between the endogenous level of Hsp90 and the floral organ radial symmetry produced by stochastic developmental noise. Conversely, floral organ plasticity in response to micro-environmental variation tended to be greater with decrease in Hsp90b isoform expression.

Key words: Hsp90, *Iris pumila*, environmental variation, floral organs, plasticity, radial asymmetry, stochastic noise

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INTRODUCTION

Heat shock protein 90 (Hsp90) is an abundant and ubiquitous molecular chaperone that interacts dynamically with a diverse but specific group of inherently unstable substrates (client proteins), many of which are involved in signal transduction (Richter and Buchner, 2001; Nollen and Morimoto, 2002). Although the molecular interaction network of the Hsp90 chaperone system is still poorly understood (Zhao and Houry, 2007), Hsp90 has been proved to be a very useful folding machine required to switch these proteins on or off (Richter and Buchner, 2001). By promoting the activity of numerous signaling proteins in many different developmental pathways, Hsp90 plays a key role in regulating multiple cellular and developmental processes, including cell cycle and transcription control, chromatin remodeling, organism development, and responses to environmental stresses (Young et al., 2001; Terasawa et al., 2005; Rutherford et al., 2007).

Although essential for the proper folding of its clients under physiological conditions, Hsp90 can also participate in the refolding of other proteins under conditions of denaturing stress (Nathan et al., 1997; Young et al., 2001). Because of its dual role in signal transduction and stress response, Hsp90 is hypothesized to "link developmental program to environmental contingency" (Rutherford and Lindquist, 1998). Specifically, Hsp90 during stress becomes redirected from its usual substrates to other stress-damaged proteins, thereby reducing the signaling activity of its substrates through numerous target pathways. Depending upon the stress intensity and the availability of free chaperones, modulation of the chaperone and target function of Hsp90 can lead to either buffering or expression of the cryptic genetic variation that is accumulated in genomes under normal environmental conditions (Rutherford, 2003).

The role of the Hsp90 chaperone system in developmental buffering against genetic and non-genetic perturbations has recently become a topic of increased interest (Rutherford and Lindquist, 1998; Queitsch et al., 2002; Milton et al., 2003, 2006; Debat et al., 2006; Manitašević

et al., 2007; Sangster et al., 2007). However, there is still no full concensus about what component of total phenotypic variance of a given trait (genetic, stochastic, or environmental) should be buffered in particular. Contrary to theoretical predictions that genetic and non-genetic buffering should share a common mechanism(s) (Wagner et al., 1997; Meiklejohn and Hartl, 2002; Klingenberg, 2005), it has been documented in the fruit fly *Drosophila* that non-genetic sources of variation in the phenotype (measured as differences between the left and right sides of several bilaterally symmetrical bristle and wing traits) were unaffected in outbred individuals treated with an Hsp90 inhibitor or across a series of the Hsp90 mutant heterozygotes (*Hsp*83/+) (Milton et al., 2003). Conversely, Hsp90 did protect against the expression of extreme morphogenic phenotypes specific to particular genetic backgrounds or environments, suggesting that genetic and non-genetic buffering by Hsp90 are separable (Milton et al., 2003). It has been recently revealed that Hsp90 effects are remarkably specific to the most invariable and highly discrete quantitative traits, e.g., traits such as the number of scutellar (SC) and thoracic (TH) bristles in *Drosophila* (Milton et al., 2006). For these two particular traits, Hsp90 simultaneously buffered quantitative variation specific to genetic backgrounds, as well as variation from stochastic developmental and environmental effects. Since in other, normally-variable wing and bristle traits non-genetic variation remained relatively unaffected by Hsp90, this indicates that robustness against genetic and environmental influences is not a property governed by any single „canalizing" gene, but evolves or emerges „from the topology of connections between interacting genes", independently and specifically for each organismal trait (Milton et al., 2006).

The role of Hsp90 in buffering genetic variation in morphogenetic pathways appears to be strictly conserved among biological kingdoms. Several recent studies have emphasized the importance of this molecular chaperone for plant development and responsiveness to environmental cues (Queitsch et al., 2002; Sangster and Queitsch, 2005; Sangster et al., 2007; Manitašević et al., 2007). Partial inhibition of the Hsp90 function in accessions and recombinant lines of *Arabidopsis thaliana* produced a number of morphological phenotypes that were found to be specific to underlying genetic backgrounds (Queitsch et al., 2002). The robust health of most seedlings exhibiting strong phenotypes indicates that the level of impairment of Hsp90 required to uncover specific polymorphisms was lower than that necessary to interfere with its „housekeeping" functions (Queitsch et al., 2002). Phenotypic analyses of an *A. thaliana* line with constitutively reduced Hsp90 expression by RNAi targeting revealed that Hsp90 reduction affects an array of quantitative life-history traits, including flowering time and total seed set, and decreases the developmental stability of repeated traits (Sangster et al., 2007). Recent reports indicating altered environmental sensitivity in Hsp90-reduced *Arabidopsis* lines (revealed by genome-wide expression analyses) have widened the functions of Hsp90 to include the „genesis and maintenance of plastic responses" as well (Sangster et al., 2007).

The purpose of this study was to investigate the effect of the molecular chaperone Hsp90 on two sources of non-genetic variation in the same set of floral organ traits from a sample of distinct *Iris pumila* genotypes naturally growing in the wild. Within-flower variation, i.e., deviations from radial symmetry of identical floral organ parts, results from stochastic perturbations (developmental noise) in repeated floral organ parts as they develop within a single floral whorl of the same flower and environment. Environmental variation, i.e., variation in trait expressions among different flowers within the same clonal individual, arises due to heterogeneity of the micro environments experienced by each flower of a given clone.

The main questions addressed were: (1) Is there covariation of floral organ radial asymmetry (RA) in *I. pumila*? (2) Do the micro-environmental sensitivities of different floral organ traits covariate among themselves? (3) Does the endogenous level of Hsp90 affect plasticity of floral organ traits in response to micro-environmental variation? and (4) Does Hsp90 buffer flower development against stochastic noise?

MATERIAL AND METHODS

The studied species

Iris pumila L. is a rhizomatous perennial herb widely distributed in the Deliblato Sands, a sandy area situated about 50 km northeast of Belgrade (44º 47' 39" N/ 21º 20' 00" E to 45º 13' 10" N/ 28º 26' 08" E). In its natural habitats, the species forms round shaped clones, markedly polymorphic for flower color. Because the flower color distinctiveness of *I. pumila* clones is specified by segregation at several gene loci, each of the flower color variants commonly found in the population can be regarded as a unique genotype (Tucić et al., 1988).

Like other congeneric taxa, *Iris pumila* has flowers that display a highly specialized morphology. The perianth (the outer, sterile whorls of a flower) is not differentiated into the calyx (the outermost perianth whorl) and the corolla (the inner perianth whorl). Instead, all of the perianth elements are similar in appearance (color, shape) and hence are collectively termed the tepals. The tepal whorls are radially symmetrical and united towards the base, forming a floral tube. The interior, fertile whorls of the flower consist of the male sex organs or stamens (anthers plus filaments), which are sheltered beneath the overarching style arms (part of the female sex organ) below the stigmatic lip. In *I. pumila*, the upper surface of the falls is equipped with a beard consisting of short fine hairs. Because all of the tepals from the same whorl are essentially identical in size and shape, such flower morphology is said to be actinomorphic (meaning "ray-formed") or regular.

Flower harvesting and measurement

In April of 2004, at the peak of blooming phase, 10 large clones differing in flower color (distinct genotypes) were randomly chosen over an exposed dune site and marked with plastic pegs. Thereafter, two or three just opened flowers (in the early male stage) were randomly harvested from each of these clones and placed individually in a 0.5-l-bottle of 70% ethanol for storage until dissection. Later, in the laboratory, each flower was cut up at the base of the perianth, and its upper parts, the falls and the standards, were flattened over a transparent glass plate covered with 50% glycerol. Digital images of the floral organs were recorded using an optical scan-

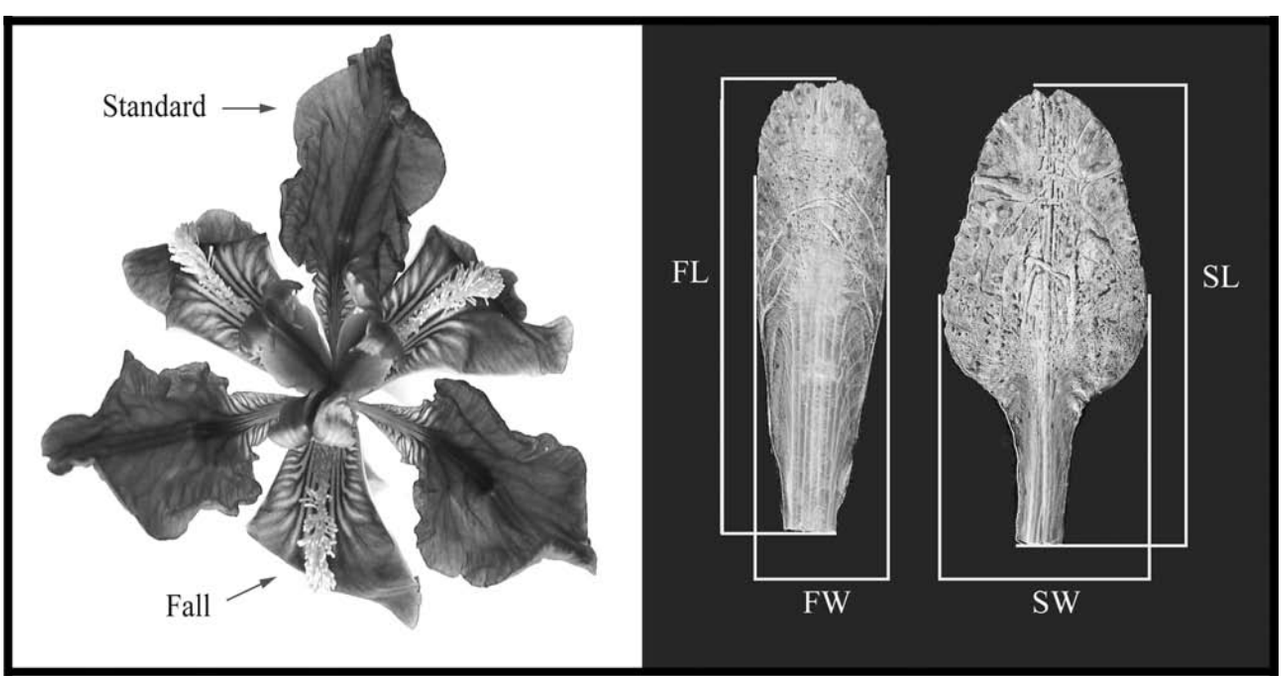

Fig. 1. *Iris pumila* flower and measured floral organ traits. FL, fall length; FW, fall width; SL, standard length; SW, standard width

Fig. 2. Representative immunoblot of Hsp90 isoforms in *Iris pumila*.

ner (HP ScanJet 3800; 600 dpi resolution). Analyses of digital images were conducted using IT software (UTHSCSA Image Tool, Version 3.0, San Antonio, Texas). The floral organ traits analyzed in this study were fall length (FL), fall width (FW), standard length (SL), and standard width (SW), all expressed in mm (Fig. 1). All measurements were performed blind with respect to clone genotype. To reduce measurement error, the size of each floral organ was taken two times from the same digital pictures by the same person (AV). Repeated measurement of the whole sample was conducted after the first measurement was completed.

Most floral traits are quantitative in nature, which means they are influenced by multiple gene loci with small additive effects, as well as by various sources of environmental heterogeneity, including random developmental noise (Conner, 2002). Environmental heterogeneity originates from fluctuations in external environmental conditions, while developmental noise results from the stochastic nature of cellular processes regularly occurring even in a homogeneous environment. In this study, sensitivity to stochastic noise (developmental instability) was measured by radial asymmetry (RA), defined as random deviations away from radial symmetry and expressed in terms of within-flower standard deviation of the repeated floral organ parts. Plasticity (PL) of a floral trait was estimated as the absolute difference between the means of repeated floral parts measured on different flowers from the same clone. This measure is an estimate of the effects of micro-environmental variation on floral trait expressions within a given clone, where environmental robustness decreases as PL increases.

Leaf sample collection

Simultaneously with flower harvesting, a fully expanded leaf from two ramets per each clone was collected and immediately frozen in liquid nitrogen. Samples were transported to the laboratory and stored at -70 °C until preparation.

Tissue extract preparation

Tissue extracts were prepared by pulverization of frozen leaves under liquid nitrogen, followed by sonification on ice in 2 volumes (w/v) of 0.1 M Tris buffer, pH 7.6, containing 1 mM EDTA and 1 mM dithiothreitol. The samples were centrifuged at 12000 x g at 4°C for 15 min. The supernatants were re-centrifuged and analyzed for total protein content by the method of Spector (1978) with bovine serum albumin used as a standard.

SDS-PAGE and immunoblotting

Aliquots of tissue extracts containing 40 µg of proteins were mixed with an equal volume of ^{2X}SDS-sample buffer, boiled for 5 min at 100°C, and loaded onto 7.5% SDS-polyacrylamide gels (Laemmli, 1970). Proteins were separated by electrophoresis at 120 V and 4°C using a Mini-Protean II Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA). Myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were used as molecular mass references.

After electrophoresis, Western transfer of proteins from the gels to nitrocellulose membranes (Hybond-C, Amersham, UK) was carried out at 135 mA and 4°C overnight using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA) in a transfer buffer containing 25 mM Tris buffer, pH 8.3, 192 mM glycine, and 20% (v/v) methanol. The membranes were blocked for 1.5 h at room temperature in PBS (1.5 mM KH_2PO_4, 6.5

mM Na_2HPO_4, 2.7 mM KCl, and 0.14 M NaCl, pH 7.2,) containing 0.25% nonfat dry milk. Isoforms of Hsp90 were detected with the aid of mouse monoclonal antibody SPA-830 (1:2000; StressGen, Canada). Immunoreactive bands were visualized and quantified by an enhanced chemifluorescence (ECF) detection system using STORM Imager and ImageQuant software (Amersham Biosciences Limited, UK). In order to make quantitative comparisons between multiple immunoblots reliable, an internal reference sample, the leaf extract of an *I. pumila* plant growing independently of the studied populations, was run on each gel. Prior to any comparison, the intensity of each analyzed immunospecific band was normalized to the intensity of the respective internal reference band on the same blot. A representative immunoblot is presented in Fig. 2.

Statistical analyses

Floral measurements from three replicate flowers were taken individually to assess RA and PL or were averaged together within each clone to obtain genotypic means of floral organ traits. Genotypic means for each floral organ trait and the corresponding means for Hsp90 levels were used to estimate the Pearson product moment correlation coefficients.

RESULTS AND DISCUSSION

Flowers of most angiosperms consist of four types of specialized organs (sepals, petals, stamens, and carpels) that are arranged in a series of concentric rings or whorls. Development of these organs is under the control of several classes of regulatory genes (A, B, C, and E), each of which is expressed in spatially restricted positions within the floral meristem (Weigel and Meyerowitz, 1993; Bowman, 1997; Krizek and Fletcher, 2005). Apart from class E genes (which specify identity of all organ types), sepal identity is directed by class A genes, petal identity by classes A + B, stamen by classes B + C, while carpel identity is specified by the expression of class C genes. The molecular and genetic mechanisms that control flower development are to a large extent similar between eudicots and monocots, suggesting that all angiosperms employ homologous genes to specify the identity of their floral organ primordia (Krizek and Fletcher, 2005).

The flower is a highly evolved plant structure that manifests a considerable degree of robustness to perturbations from genetic and non-genetic sources. This is visible in the relatively constant floral phenotype within populations and the enormous phenotypic diversity among species and higher taxa.

Table 1 summarizes the results of correlation analysis, which mirrors the strength of statistical associations among RAs of four floral organ traits (FL, FW, SL, and SW) in *I. pumila*. Although all of these traits share the same precursor tissue - the floral meristem - and, as in other monocots, their identities are specified by the combined expression of A + B class genes (see Bowman, 1997 for details), none of the correlation coefficients between floral

Table 1. Pearson correlation coefficients (P-values in parenthesis) between radial asymmetries (RA) of floral organ traits in *Iris pumila*; N = sample size.

Trait	RA_{FW} (N = 28)	RA_{SL} (N = 28)	RA_{SW} (N = 28)
Fall length (RA_{FL})	0.170 (0.388)	0.314 (0.103)	0.021 (0.917)
Fall width (RA_{FW})		-0.168 (0.394)	0.043 (0.830)
Standard length (RA_{SL})			0.123 (0.514)

Table 2. Pearson correlation coefficients (P-values in parenthesis) between plasticities (PL) of floral organ traits in *Iris pumila*; N = sample size.

Trait	PL_{FW} (N = 10)	PL_{SL} (N = 10)	PL_{SW} (N = 10)
Fall length (PL_{FL})	0.896 (0.0005)	0.921 (0.0002)	0.867 (0.0012)
Fall width (PL_{FW})		0.758 (0.0111)	0.809 (0.0046)
Standard length (PL_{SL})			0.880 (0.0008)

Table 3 Pearson correlation coefficients (P-values in parenthesis) between radial asymmetry (RA) and plasticity (PL) of floral organ traits in *Iris pumila*; N = sample size.

Trait	PL_{FL} (N = 10)	PL_{FW} (N = 10)	PL_{SL} (N = 10)	PL_{SW} (N = 10)
Fall length (RA_{FL})	0.441 (0.202)	0.437 (0.206)	0.318 (0.370)	0.058 (0.874)
Fall width (RA_{FW})	0.138 (0.704)	0.205 (0.571)	0.333 (0.347)	0.310 (0.384)
Standard length (RA_{SL})	-0.181 (0.617)	-0.124 (0.734)	-0.351 (0.320)	-0.362 (0.303)
Standard width (RA_{SW})	0.106 (0.770)	-0.111 (0.759)	0.225 (0.532)	0.294 (0.409)

Table 4. Pearson correlation coefficients (P-values in parenthesis) between Hsp90 (Hsp90a and Hsp90b) expression and stochastic (RA, radial asymmetry) and micro-environmental (PL, plasticity) variation in floral organ traits of *Iris pumila*. N = sample size.

Trait	Hsp90a (N = 10)	Hsp90b (N = 10)
Radial asymmetry		
Fall length (RA_{FL})	-0.414 (0.235)	-0.489 (0.152)
Fall width (RA_{FW})	0.127 (0.726)	0.198 (0.583)
Standard length (RA_{SL})	-0.214 (0.553)	0.442 (0.201)
Standard width (RA_{SW})	0.730 (0.016)	0.056 (0.879)
Plasticity		
Fall length (PL_{FL})	-0.139 (0.701)	-0.577 (0.080)
Fall width (PL_{FW})	-0.305 (0.391)	-0.426 (0.219)
Standard length (PL_{SL})	-0.006 (0.987)	-0.568 (0.087)
Standard width (PL_{SW})	0.214 (0.552)	-0.375 (0.286)

RAs was statistically significant, implying that small random perturbations of cellular processes producing asymmetry of repeated floral parts exerted their effects very locally and separately.

Unlike RAs, plasticities in response to micro-environmental variation of floral traits appeared to be strongly correlated in *I. pumila*, especially between two length traits: FL and SL (Table 2). Plastic correlations are thought to originate between traits that exhibit similar sensitivity to a given environmental factor, exert the same function, and/or have a shared genetic basis (Schlichting, 1989; Juenger et al., 2005). These comparative plastic responses are

postulated to promote the maintenance of an integrated phenotype in the face of changing environmental conditions (Schlichting, 1989).

Associations between the parts of fully formed morphological structures can originate in two different ways: by direct connections between the developmental pathways that produce these structures, or by parallel variation of separate pathways that respond to the same extrinsic factors (Klingenberg, 2005). In the first case, a developmental precursor can be partitioned into two or more descendant organs or tissues, or there can be inductive signaling from one developing part to another. In either of these cases, perturbations that occur during the interaction or variation that has arisen in the common upstream part of the pathway can be shared or passed down through the partitioning step to multiple parts simultaneously (Klingenberg, 2005). The main feature of covariation caused by direct interaction of developmental pathways is that variation arising within the pathways themselves is the source of covariation between traits.

In contrast to covariation due to direct connections between the developmental pathways, the mechanism of parallel variation in independent developmental pathways is based on the action of a shared variable step, such as an allelic variation of a gene that is involved in both pathways, or an environmental factor to which both pathways respond simultaneously but separately (Klingenberg, 2005). In this case, the factor responsible for the association between traits lies outside the individuals.

Table 3 shows the Pearson correlation coefficients between RA and PL of the same floral traits in *I. pumila*. Since none of these coefficients approached statistical significance, it suggests that the mechanism maintaining the stability of floral organ phenotypes against stochastic noise is not the same as that buffering environmental variation.

Molecular chaperone proteins belonging to the Hsp90 family are reported to be components of a buffering mechanism against genetic and environmental perturbations in the model organisms *Arabidopsis* and *Drosophila* (Rutherford and Lindquist, 1998; Queitsch et al., 2002; Milton et al., 2003, 2006; Sangster et al., 2007). In *I. pumila*, Hsp90 was found to buffer cryptic genetic variation that can be uncovered during environmental stress (Manitašević et al., 2007).

In spite of significant progress achieved during the last years in elucidating the functions of Hsp90s across biological kingdoms, the relationship between processes buffering against genetic, environmental, and stochastic variation are mostly unidentified, especially in wild plants. To determine whether Hsp90 controls robustness of *I. pumila* flowers under physiological conditions, we contrasted natural variation in the level of two Hsp90 isoforms with the strength of two kinds of non-genetic perturbations, developmental noise and micro-environmental variation within a wild population of this species. Table 4 presents the magnitude of correlations between the level of expression of Hsp90 isoforms designated Hsp90a and Hsp90b (Manitašević et al., 2007), and stochastic and micro-environmental variation in four floral organ traits of *I. pumila*. Our study provides evidence that with the exception of a highly significant positive correlation between RA_{SW} and Hsp90a ($r = 0.73$; $P = 0.02$), Hsp90 did not affect the asymmetry of any floral trait, suggesting that it cannot be a general buffer against random perturbations in *I. pumila*. In contrast to RA, PL of floral organs in *I. pumila* tended to be negatively related with the endogenous level of Hsp90b, which was previously found to be a strong capacitor of leaf variation in *I. pumila* (Manitašević et al., 2007). The impact of Hsp90b on floral plasticity appeared to be stronger in organ lengths than organ widths ($r = -0.58$ and -0.57 vs. $r = -0.43$ and -0.37, respectively; Table 4), indicating that Hsp90-dependence of environmental responsiveness in *I. pumila* is trait-specific. Analyses of *Arabidopsis* plants with genetically and/or pharmacologically reduced Hsp90 levels established that Hsp90 is essential not only for maintaining the wild-type phenotype against developmental noise and environmental variation, but also for the origin and maintenance of plastic responses to environmental cues (Queitsch et al., 2002; Sangster

et al., 2007). Previous reports on the participation of Hsp90 in standard plastic responses such as the response to light and gravitropism, together with recent genome-wide expression analyses, clearly indicate that "Hsp90's effects on plant plasticity are generalizable to many different stimuli and that Hsp90 plays a global role in the environmental responsiveness of plants" (Sangster et al., 2007).

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ПОВЕЗАНОСТ ФУНКЦИЈЕ HSP90 СА МИКРО-СРЕДИНСКИМ И СЛУЧАЈНИМ ВАРИРАЊЕМ ЦВЕТНИХ ОРГАНА *IRIS PUMILA* L.

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Молекуларни шаперон Hsp90 има кључну улогу у неутралисању генетичких и негенетичких поремећаја код модел организама *Arabidopsis* и *Drosophila*. У овом раду анализирали смо заштитни капацитет Hsp90 у односу на две врсте негенетичких фактора – стохастичко и микро-срединско варирање особина цвета у природној популацији *Iris pumila*. Утврдили смо да нема статистички значајне корелације између ендогеног нивоа протеина Hsp90 и радијалне асиметрије условљене случајним променама у току развића анализираних цветних органа. Насупрот томе, пластичност цветних органа у односу на микро-срединско варирање повећавала се са смањењем експресије изоформе Hsp90b.