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A Rhizosphere Fungus Enhances Arabidopsis Thermotolerance Through Production of an HSP90 Inhibitor¹

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The molecular chaperone Heat Shock Protein 90 (HSP90) is essential for the maturation of key regulatory proteins in eukaryotes and for the response to temperature stress. Earlier we have reported that fungi living in association with plants of the Sonoran desert produce small molecule inhibitors of mammalian HSP90. Here, we address whether elaboration of the HSP90 inhibitor monocillin I [MON] by the rhizosphere fungus *Paraphaeosphaeria quadrisepata* affects plant HSP90 and plant environmental responsiveness. We demonstrate that MON binds Arabidopsis HSP90 and can inhibit the function of HSP90 in lysates of wheat (*Triticum aestivum*) germ. MON treatment of Arabidopsis seedlings induced HSP101 and HSP70, conserved components of the stress response. Application of MON, or growth in the presence of MON, allowed Arabidopsis wild type, but not *AtHSP101* knock-out mutant seedlings to survive otherwise lethal temperature stress. Finally, co-cultivation of *P. quadrisepata* with Arabidopsis enhanced plant heat stress tolerance. These data demonstrate that HSP90-inhibitory compounds produced by fungi can influence plant growth and responses to the environment.

Environmental factors and interactions between organisms are major forces that impact the development of organisms and have helped shape the evolutionary histories of species. Recent studies have identified the highly conserved and environmentally responsive molecular chaperone HSP90 as a potential molecular link between the biotic and abiotic environment of an organism and its phenotype. HSP90 is essential for the maturation of many key regulatory proteins in eukaryotes and for the evolutionarily conserved response to temperature stress (Young et al., 2001; Picard, 2002; Pratt and Toft, 2003). In plants, reduced HSP90 function dramatically alters responses to environmental stimuli and can globally affect plant phenotype. For example, plants with reduced HSP90 function are more sensitive to microbial pathogens and also show altered responses to abiotic cues such as gravity and light (Sangster and Queitsch, 2005). Several studies have demonstrated that manipulation of HSP90 function results in expression of previously cryptic genetic and epigenetic variation, thereby dramatically altering organism phenotype in a heritable manner (Rutherford and Lindquist, 1998; Queitsch et al., 2002; Sollars et al., 2003; Yeyati et al., 2007). It has been proposed that the manifestation of such variation could occur through environmental stress which might reduce HSP90 buffering capacity (Sangster et al., 2004). Alternatively, HSP90 function could be modulated by HSP90-specific small molecule inhibitors elaborated by several fungi in natural environments (Turbyville et al., 2006). To date, however, no evidence for targeting of HSP90 in the interactions between organisms with consequences for organismic phenotypes has been reported.

In a screen of ethyl acetate extracts from over 500 Sonoran desert plant-associated endophytic and rhizosphere fungal strains, we previously identified two highly specific inhibitors of mammalian HSP90, monocillin I (MON; Fig. 1A, R = H) and radicol (RAD; Fig. 1A, R = Cl), among many other secondary metabolites (Turbyville et al., 2006). Extracts of the rhizosphere fungal strain *Paraphaeosphaeria quadrisepata*, which inhabits the Sonoran desert Christmas cactus (*Opuntia leptocaulis* DC.; Cactaceae), contained MON in surprisingly high amounts – about 30% by weight of dry extract (Wijeratne et al., 2004). Extracts of the endophytic fungal strain *Chaetomium chiversii*,

which colonizes the stem tissues of Mormon tea (*Ephedra fasciculata* A. Nels.; Ephedraceae), contained up to 10% RAD (Turbyville et al., 2006). Production of MON and RAD are not limited to these fungi; in fact, MON was first identified as a secondary metabolite elaborated by a mycoparasite of pine trees in North America (Delmotte and Delmotte-Plaquee, 1953; Omura et al., 1979; Ayer et al., 1980). Additional reports document the production of HSP90 inhibitors by bacteria and fungi in diverse ecosystems. Furthermore, *P. quadrisepata* is not restricted to the desert, but has also been isolated as the major fungal inhabitant of the rhizosphere of young maize plants in Brazil, indicating that this plant-fungus association is not limited to members of the cactus family (Gomes et al., 2003). Therefore, the extent of plant associations with fungi or other microorganisms which produce HSP90 inhibitors is most likely vastly underappreciated.

While extensive studies have been directed toward understanding the mechanisms and activities of small molecule HSP90 inhibitors in mammalian systems, especially for possible treatment of cancers (Whitesell and Lindquist, 2005), there has been virtually no investigation of the impact of production of HSP90 inhibitors on plant-microorganism associations. As an essential step in beginning to define the impact of such inhibitors on plants in the environment, we demonstrate that MON binds and inhibits plant HSP90 function and can induce components of the plant heat shock response. In addition, application of MON or co-cultivation with *P. quadrisepata* enhances plant heat tolerance. To fully understand the importance of HSP90 in plant biology, these findings make it clear that consideration must be given to HSP90 as a prominent target in mediating plant-microorganism interactions.

RESULTS

MON Binds and Inhibits Plant Hsp90

We first sought to establish that the fungus-derived inhibitors of mammalian HSP90, MON and RAD, can bind and inhibit plant HSP90. Although HSP90 is a conserved molecular chaperone, small molecule inhibitors can show species specificity. For example, the prototypic HSP90 inhibitor geldanamycin (GDA) fails to bind and inhibit *Caenorhabditis elegans* HSP90 (David et al., 2003), despite the fact that this protein is 83% similar (73% identical) to its human ortholog which is readily inhibited by the compound. Given that the protein sequences of plant Hsp90 are only 63-71% identical to human and yeast Hsp90 (although they are 88-93% identical between species) (Krishna and Gloor, 2001) we first tested whether fungus-derived MON and RAD could bind plant Hsp90. Using a previously reported solid phase competition approach (Whitesell et al., 1994), an amine derivative of GDA was immobilized on agarose beads and then incubated with Arabidopsis seedling lysates which had been supplemented with various concentrations of MON or RAD as soluble competitors. After washing under moderately stringent conditions, bound proteins were eluted and analyzed by SDS-PAGE and staining with Sypro Ruby. Several non-specific bands of comparable size were identified in the eluates of both control beads without attached GDA and beads with GDA. Importantly, a prominent band in the 90kD range was present only in eluate from beads with covalently bound GDA (data not shown). To establish the identity of this band, we immunoblotted eluates with an antibody to plant HSP90. Immunodetection of a specific ~90kDa band confirmed that both MON and RAD efficiently competed with immobilized GDA for binding to Arabidopsis HSP90 in a concentration-dependent manner (Fig. 1B). These data suggest that both inhibitors bind to the nucleotide-binding site in the amino terminus of HSP90, which is known to be the binding site of GDA and RAD in other organisms (Roe et al., 1999; Schulte et al., 1999).

Because ATP hydrolysis is required for its chaperoning activity, GDA binding to the nucleotide binding site of Hsp90 markedly alters its function (Roe et al., 1999). To confirm that MON and RAD could affect plant HSP90 function, we performed standard assays for chaperone activity based on the ATP-dependent refolding of heat-denatured firefly luciferase in wheat germ lysate (Kolb et al., 1994). Indeed, MON and RAD

inhibited luciferase refolding consistent with MON- or RAD-mediated inhibition of plant HSP90 function (Fig. 1C). Control experiments confirmed that neither MON nor RAD had any direct effect on the enzyme activity of native luciferase (data not shown).

MON Induces Expression of Heat Stress Responsive Genes

Having established that HSP90 from wheat and Arabidopsis were bound and inhibited by both MON and RAD, we next evaluated whether exposure to MON could alter the environmental responsiveness of plants. We first examined the impact of MON on the transcriptional activation of specific heat-stress-responsive genes. Under physiological conditions, HSP90-containing chaperone complexes are thought to sequester heat stress transcription factor (HSF) monomers in the cytoplasm. In the most widely accepted current model, HSP90 inhibitors and stressors such as heat that lead to accumulation of misfolded proteins are proposed to titrate HSP90 and other chaperones, away from HSF resulting in its release and thereby allowing the trimerization and phosphorylation that are required for its activation of HSP gene transcription (Zou et al., 1998; Guo et al., 2001; Voellmy and Boellmann, 2007). To assess potential HSF activation, mRNA levels of *AtHSP101* were measured in Arabidopsis seedlings after MON exposure using semi-quantitative RT-PCR. Exposure of seedlings to MON induced a rapid increase in *AtHSP101* mRNA level at 90 min post MON application, as is also observed during heat stress. *AtHSP101* mRNAs then declined substantially 210 min after MON exposure (Fig. 2A). A similar response was observed for *AtHSP70*, another HSP that relies on HSF for induction (data not shown). Heat stress at 38° C for 90 min. was used as a positive control for these experiments, and it markedly increased *AtHSP101* mRNA levels as expected. To determine whether MON-induced changes in mRNA levels would result in increased protein amounts, lysates of MON-treated seedlings were examined for HSP101 protein levels. A concentration-dependent increase in HSP101 level was observed in response to overnight treatment with a solution of MON in DMSO as compared to the level found in control plants which had been exposed to DMSO alone (Fig. 2B). As a positive control, heat-stressed seedlings were analyzed, and increased HSP101 levels were detected (Fig. 2B). From these results, we conclude that MON-mediated modulation of the heat shock response in plants is rapid, tunable and reversible.

MON Enhances HSP101-Dependent Heat Tolerance

In several plant species and in *Saccharomyces cerevisiae*, the HSF-dependent induction of HSP101 is crucial for acquired thermotolerance. Therefore, we hypothesized that MON-mediated induction of HSP101 might enhance thermotolerance in treated seedlings. To test this hypothesis, a quantitative assay of acquired thermotolerance based on hypocotyl elongation of dark-grown seedlings was used (Hong and Vierling, 2000; Queitsch et al., 2000). Hypocotyl elongation in the dark is slowed or completely arrested after exposure to temperature stress depending upon its severity. Prior induction of HSP101 through a pre-conditioning heat exposure, however, allows continued hypocotyl elongation even after severe stress, which can be readily measured in large numbers of replicate seedlings, permitting statistical analysis. Conversely, *AtHSP101* mutant seedlings do not acquire thermotolerance following heat pre-treatment and hypocotyl elongation remains arrested in response to severe stress (Hong and Vierling, 2000; Queitsch et al., 2000).

In the absence of heat stress (control conditions), topical MON application to dark-grown seedlings on plates did not alter hypocotyl elongation (Fig. 3A). MON application prior to heat stress at 45°C for 50 or 75 min, however, resulted in significant preservation of hypocotyl elongation consistent with enhanced thermotolerance. The 10 µM treatment appeared to cause some cytotoxicity to the heat stressed plants as measured by decreased hypocotyl elongation (Fig. 3A). This could be due to non-specific effects of the small molecule, or the loss of other essential HSP90 functions related to growth. Most strikingly, when the same plates were subsequently transferred to a growth chamber under day/night light and temperature cycling it was apparent that prior MON treatment resulted in a dramatic rescue of the seedlings from an otherwise lethal heat stress (Fig. 3B). After two weeks, the heat stressed MON-treated seedlings showed dramatic preservation of seedling growth and greening compared to the vehicle-treated seedlings.

To address whether root exposure to MON could enhance thermotolerance, comparable to topical treatment, seedlings were germinated on media supplemented with varying concentrations of MON. An Arabidopsis HSP101 null mutant, *hot1-3* (Hong and Vierling, 2000), was included in these experiments to determine if differences in response

to MON are linked to the observed MON-induced expression of this critical chaperone. Growth of seedlings on MON-containing plates resulted in modest stunting of overall hypocotyl growth when compared to controls, similar to what has been reported for the HSP90 inhibitor GDA. However, growth on MON-supplemented plates protected dark-grown seedlings from the adverse effects of severe temperature stress as demonstrated by their normal light-induced greening response in contrast to untreated seedlings (Fig. 4). Most importantly, the HSP101 null mutant *hot1-3* failed to green and develop normally, confirming that induction of HSP101 is required for the enhanced thermotolerance observed after MON treatment (Fig. 4).

Co-cultivation of Arabidopsis and *P. quadrisepata* Enhances Heat Tolerance

To test whether the presence of MON-producing fungus in the rhizosphere could alter plant responsiveness to thermal stress, we investigated the consequences of co-cultivation of *P. quadrisepata* with Arabidopsis as a model for this type of interaction. When Arabidopsis seedlings were co-cultured with *P. quadrisepata* on plates, hypocotyl elongation under non-heat shock conditions was reduced, most likely due to the presence of MON. Importantly, despite overall reduced growth, the hypocotyls of seedlings co-cultivated with *P. quadrisepata* continued to elongate after heat stress, resulting in significantly longer hypocotyls than seen in seedlings heat-stressed in the absence of fungus (Fig. 5A and B, $p < 0.0001$ for the effect of fungus, effect of heat treatment and the interaction of the two variables). Although the heat treatment appears to have decreased fungal growth, extraction of fungus recovered from the plates and of the agar substrate showed that the fungus produced MON under these culture conditions and that MON had diffused into the growth medium (Fig. 6).

Co-cultivation with *P. quadrisepata* also improved the survival of soil-grown plants after severe heat stress (Fig. 7). In the presence of the fungus, surviving soil-grown plants recovered readily from heat stress, appearing indistinguishable from non-stressed controls with no significant difference in leaf number or rosette diameter observed after 12 days of growth (data not shown).

DISCUSSION

We have demonstrated that the fungal secondary metabolites MON and RAD can bind and inhibit plant HSP90. Application of MON leads to expression of major components of the heat stress response, HSP101 and HSP70, and can promote heat tolerance of *Arabidopsis* seedlings in an HSP101-dependent manner. In addition, co-cultivation of the MON-producing fungus, *P. quadrisepitata*, with *Arabidopsis* leads to enhanced heat tolerance of *Arabidopsis*. Taken together, these data demonstrate that the presence of an HSP90 inhibitor-producing fungus can dramatically alter plant responsiveness to environmental stresses such as heat. Based on extensive evidence in other systems that HSF activation of HSP gene expression is stimulated by inhibition of HSP90, we conclude that the enhanced *Arabidopsis* heat tolerance observed in our studies is the result of MON inhibition of HSP90. The elaboration of HSP90 inhibitors by plant-associated microorganisms implicates HSP90 as a direct target in organismic interactions, possibly among microbial communities competing in the plant rhizosphere. In light of the multi-faceted role that plant HSP90 plays in response to biotic and abiotic stresses (Sangster and Queitsch, 2005), in normal plant development (Krishna and Gloor, 2001), and in the buffering of genetic variation (Queitsch et al., 2002), HSP90-based plant-fungus interactions could impact plant phenotypes in numerous ways.

Induced responses to environmental stimuli such as heat and drought and defense responses against microbial pathogens are typically tightly regulated. Constitutive activation of these normally inducible environmental response pathways can result in slower growth and abnormal pleiotropic phenotypes (Kasuga et al., 1999; Noutoshi et al., 2005). At this point, it is not known whether fungal production of MON or RAD might be regulated by abiotic conditions or by plant signals, either in the Sonoran desert, where our isolates were obtained, or in other environments where these fungi occur. In laboratory monoculture, however, elaboration of these secondary metabolites can be markedly altered by growth conditions (Gunatilaka, 2006). Even if such compounds are continually produced by fungi in association with plants, it remains possible that stress pre-conditioning as a result of plant exposure to fungal HSP90 inhibitors could be advantageous in certain environments such as the desert, where plants must survive rapid, drastic temperature changes. Indeed, extreme environments may favor constitutive upregulation of HSPs as demonstrated for larvae, but not adults, of the polar insect

Belgica antarctica. The larvae of this flightless midge are exposed to dramatic temperature differences, being encased in ice during the lengthy Antarctic winter and then emerging to complete their life cycle during the austral summer (Rinehart et al., 2006). In contrast, adults show the typical stress-inducible expression of HSPs.

Previous studies of grass-endophyte associations have also shown that continual presence of an endophyte can improve the tolerance of infected plants to high temperatures (Marquez et al., 2007). In these studies, however, molecular mechanisms responsible for induced thermotolerance including the potential elaboration of heat shock-active secondary metabolites were not investigated. Much more research on the occurrence, concentration and environmental stability of HSP90 inhibitors, as well as the control of their production by plant-associated microorganisms is clearly warranted. Ecological and physiological studies will also be advanced by development of genetically tractable microorganisms, in which production of MON can be controlled, an approach which is not currently feasible in *P. quadriseptata*.

While the responses we have measured are most likely the result of the interactions of HSP90 with plant HSFs, HSP90 inhibitors can also be expected to disrupt processes mediated by other substrates that HSP90 chaperones, its so called client proteins. The only endogenous HSP90 clients characterized so far in plants are R-proteins, whose rapid degradation in HSP90-deficient mutant plants translates into greater sensitivity to microbial pathogens (Belkhadir et al., 2004; Schulze-Lefert, 2004). Because the entry of symbiotic organisms into the root system of host plants is accompanied by a local defense response similar to that observed in response to pathogens, manipulation of host HSP90 by secondary metabolites might facilitate the establishment and maintenance of fungal symbionts. MON and other HSP90 inhibitors could also be toxic to certain plant pathogens (Ayer et al., 1980), suggesting another potentially beneficial aspect of MON production by fungi for their associated plant communities. As the clients of HSP90 in plants are further elucidated, the range of possible phenotypes impacted by HSP90-producing microorganisms will no doubt continue to expand.

HSP90 clearly plays an important role in eukaryotic development and environmental responsiveness. The elaboration of HSP90 inhibitors by microorganisms

in both natural and agricultural ecosystems and the myriad ways in which such small molecules could affect organismic interactions and phenotypes open up many exciting new avenues for investigation.

MATERIAL AND METHODS

Solid Phase Competition Assay

MON binding to Arabidopsis HSP90 was assessed using a solid phase competition assay (Whitesell et al., 1994). Plant tissue was prepared by grinding aerial tissue from 14-day old Arabidopsis in a liquid nitrogen-cooled mortar and pestle. The tissue was extracted with assay buffer containing 20 mM HEPES pH 7.3, 50 mM KCl, 5 mM MgCl₂, 20 mM Na₂MoO₄ and 0.01% NP-40 by rocking for 2 h at 4°C. The lysate was clarified by centrifugation for 30 min at 4°C, followed by supplementation with 1 mM fresh DTT, and frozen at -80°C. Binding assays were performed using 0.5 ml aliquots (1.2 mg of total protein) of plant lysate supplemented with the indicated concentrations of soluble drug as competitor or an equal volume of DMSO vehicle. After addition of agarose beads on which geldanamycin (GDA) had been previously immobilized as described, the reaction tubes were incubated for 1 h with gentle agitation at 4°C. Beads were then washed extensively and bound proteins eluted into Laemmli sample buffer followed by SDS-PAGE and western blotting with an Arabidopsis-specific anti-HSP90 antibody (Santa Cruz Biotechnology, Inc.).

Luciferase Renaturation Assay

Inhibition of chaperone-mediated re-folding of heat-denatured firefly luciferase was analyzed using an adaptation of previously published methods (Kolb et al., 1994). Recombinant firefly luciferase [Promega, 3 µg/ml in sample buffer (SB) (Thulasiraman and Matts, 1996) was denatured at 40°C for 5 min. Native or denatured luciferase was then diluted 1:10 into a 50 % solution of wheat germ lysate in H₂O (Promega) that had been previously supplemented with the indicated compounds or an equal volume of DMSO vehicle. After incubation at 25°C for the indicated time intervals, 5 µl aliquots of lysate mixture were removed, added to 50 µl of luciferase assay reagent (Promega) and the light intensity measured using a microplate luminometer. Each time point was

assayed in triplicate and the results presented depict the mean +/- SD of pooled data from two independent experiments.

Measurement of *AtHSP101* mRNA and Protein

Surface sterilized Arabidopsis seeds (Col-O accession) were germinated on nutrient agar plates and allowed to grow for 10 d at 24°C. Seedlings were then sprayed with aqueous solutions of MON at various concentrations. RNA was extracted from pools of 12 seedlings at 90 and 210 min post MON application while for protein lysates, seedlings were harvested approximately 18 h after being sprayed. For semi-quantitative measurement of *AtHSP101* mRNA, 5 µg of total RNA from each sample was reverse transcribed and a 648 bp *AtHSP101* fragment amplified using forward (CTGCTCAGCTGTCTGCTCG) and reverse (GCCCTTGACCTTAGAATTGCC) primers derived from Arabidopsis locus At1g74310. The *AtHSP101* fragment was co-amplified in the same PCR reactions with primers for a control 542 bp fragment of glyceraldehyde-3-phosphate dehydrogenase C subunit (*GAPC*) cDNA (Arabidopsis locus At3g04120) and 20% *GAPC* amplification inhibitors as described previously (Kerschen et al., 2004). After agarose gel electrophoresis, relative PCR product amounts were measured by UV image optical density (Labworks Analysis Software, UVP, Inc., Upland, CA). The reduced intensity of *GAPC* control bands in heat-shocked samples is due to competition for amplification by elevated *AtHSP101* transcript levels. The ratio of target to control band intensity in the untreated sample was assigned a value of 1.0 and the ratios determined for all other samples were normalized based on this measurement to yield values for relative *AtHSP101* transcript levels. The relative level of HSP101 protein in seedlings was measured by immunoblotting protein extracts (10 µg/lane) using a rabbit polyclonal primary antibody (AZ 561, 1:5,000), peroxidase-conjugated secondary antibody and chemiluminescent detection. As a positive control, seedlings were heat shocked at 38°C for 90 min. RNA and protein extracts were analyzed in the same manner as extracts prepared from MON-exposed seedlings.

Thermotolerance Assays

As a quantitative indicator of thermotolerance, hypocotyl elongation following heat shock of 2.5 d old, synchronized dark grown seedlings (between 10-15 per condition) was measured as previously described (Hong and Vierling, 2000). The evening prior to heat shock, plates were sprayed with aqueous solutions of MON at various concentrations or an equivalent volume of DMSO vehicle. Plants were maintained in the dark continuously and the extent of hypocotyl growth over a 4.5 d following heat shock was measured. After measuring hypocotyl growth, plates were transferred to a growth chamber with a day/night cycle and photographed 14 days later. This same experimental design was repeated three times with similar results. Mutant *hot 1-3* seedlings (expressing no functional HSP101) and wild type (Col-0) planted on the same plates were sprayed, as above, with varying concentrations of MON or DMSO. 18 h later the plates were heat shocked at 45°C for 50 min, transferred to a growth chamber with a day/night cycle and then photographed 14 d later. To directly assess the effect of the fungus *P. quadrisepata* on Arabidopsis heat tolerance, seeds were co-cultured with fungus on plates and in soil. For these experiments seeds were sterilized and imbibed in water at 4°C for a minimum of 2 d prior to transfer to sucrose-enriched plates or autoclaved soil. Approximately 25 colony forming units (cfu) of *P. quadrisepata* were then applied on top of each seed in 10 µl of water. Cfus were determined experimentally by plating dilutions of fungal suspension on potato dextrose agarose plates followed by incubation at 28°C for 4 d. Seeds grown on plates were heat treated at 45°C 3 d after plating. Hypocotyl elongation occurring in the dark after 4 d period was measured. Soil grown plants were cultivated in 24-well tissue culture plates with drainage holes drilled into the bottom of each well to facilitate watering. Plates were incubated under 50% humidity and a physiological light/dark cycle. After 7 days of growth, the plants were photographed, counted and transferred during the light phase of their cycle to an identical incubator maintained at 45°C. After 2 h treatment at the higher temperature, the plants were returned to their original growth conditions for 5 d at which time they were photographed and recounted.

Preparation of Extracts

Samples of nutrient agar or actual fungal mass were transferred to three 50 ml centrifuge tubes, sonicated twice in methanol (30 ml each time for 30 min) and filtered. Combined filtrates from each of the three tubes were evaporated separately under reduced pressure to afford crude methanolic extracts. Each of these extracts was then partitioned between water and ethyl acetate (3 x 10 ml). Combined ethyl acetate layers were washed separately with water (3 x 10 ml), dried over anhydrous Na₂SO₄ and evaporated under reduced pressure to yield ethyl acetate extracts for LC-MS analysis.

Analysis of Extracts for the Presence of MON by LC-MS

To detect the presence of MON, ethyl acetate extracts were dissolved in methanol to a final concentration of 1.0 mg/ml and analyzed by LC-MS using a Shimadzu LCMS-QP800α equipped with LC-10AD Liquid Chromatograph, SCL-10A System controller, DGU-14A Degasser and SPD-M10A Diode Array Detector. The following conditions were used: Cromasil C-18 5 μm column (250 mm x 4.6 mm), 35 min linear gradient from 60% methanol in water (containing 0.25% HCOOH) to 100% methanol, 0.4 ml/min flow rate. APCI (Atmospheric Pressure Chemical Ionization) positive mode was used for data acquisition. Spectra were monitored and processed using Shimadzu LabSolutions LC-MS software.

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Figure Captions

Figure 1. MON binds plant HSP90 and inhibits its activity. (A) Structures of MON (R = H) and RAD (R = Cl). (B) HSP90 binding. Aliquots of drug-supplemented lysate of 14-day old aerial tissue from Arabidopsis seedlings were incubated with bead-immobilized GDA. The relative binding of HSP90 was assessed by immunoblot using an Arabidopsis HSP90-specific antibody. Control indicates a sample of resin beads without immobilized GDA. (C) Luciferase renaturation. Heat-denatured luciferase was added to wheat germ lysate supplemented with the indicated compounds and the time-dependent recovery of activity was measured. Each time point was assayed in triplicate. The mean and standard deviation of data derived from two independent experiments is depicted. Recovery of total input luciferase activity for DMSO control samples over the course of experiments was 61 % +/- 4%.

Figure 2. MON increases *HSP101* expression in Arabidopsis seedlings. (A) Semi-quantitative measurement of *AtHSP101* mRNA by RT-PCR. Seedlings were sprayed with aqueous solutions of MON or DMSO vehicle and harvested for RNA extraction at the times indicated. As a positive control, seedlings were heat shocked (Heat). Fragments of the *AtHSP101* gene (black arrow) and the control housekeeping gene *GAPC* (gray arrow) were co-amplified from the same reverse transcribed RNA samples and analyzed by gel electrophoresis. Relative levels of *AtHSP101* normalized to *GAPC* signal are indicated below the gel. (B) Measurement of relative HSP101 protein levels by immunoblotting. Seedlings were sprayed with aqueous solutions of MON at the indicated concentrations or DMSO vehicle, and total protein was extracted the following day. Lysate from heat-shocked seedlings (38°C for 90 min) served as a positive control (Heat).

Figure 3. MON induces a thermotolerant phenotype in Arabidopsis seedlings. (A) Measurement of hypocotyl elongation after heat treatment. MON was applied topically to dark grown seedlings 18 h prior to heat shock under the conditions indicated. Hypocotyl elongation occurring in the dark over a 4 d period following heat treatment was measured

as previously described (Hong and Vierling, 2000; Queitsch et al., 2000). The mean increase observed for 15 plants per condition is plotted. Error bars: SD. Star indicates that no column appears because the value was 0. (B) MON-treated seedlings continue to develop normally. Plates were transferred to a growth chamber with a day/night cycle, and photographed 14 d later. Results presented are representative of 3 independent experiments.

Figure 4. HSP101 is required for MON-induced thermotolerance. A solution of MON (2 μm) or DMSO vehicle were applied to dark grown wild type (Col) or mutant (*hot 1-3*) Arabidopsis seedlings (Col-O accession). *hot 1-3* mutant seedlings express no functional HSP101 (Hong and Vierling, 2000). 18 h after MON application, seedlings were heat shocked at 45°C for 50 min (45 X 50). Plates were then transferred to a growth chamber with a day/night cycle, and photographed 14 d later.

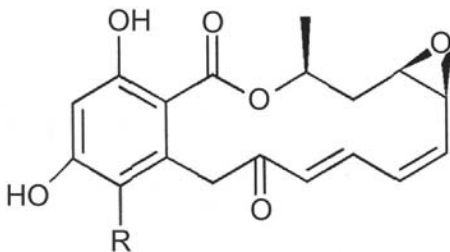
Figure 5. Co-culture with the fungus *P. quadrisepata* induces a thermotolerant phenotype in Arabidopsis seedlings. (A) Measurement of hypocotyl elongation after heat stress. Seeds were plated along with approximately 25 colony forming units (cfu) of *P. quadrisepata*. Three days later, plates were heat treated at 45°C for the indicated time intervals and hypocotyl elongation occurring in the dark over a 4 d period following heat treatment was measured. The mean increase observed for 20 to 30 plants per condition is indicated. Error bars: SEM. (B) Plants that were not heat shocked and plants heat shocked at 45°C for 75 min were photographed at the conclusion of the experiment.

Figure 6. Detection of MON in nutrient agar recovered from plates following plant-fungus co-culture. (A) Analysis of methanolic extract prepared from agar approximately 1 cm distant from the location of Arabidopsis – *P. quadrisepata* growth zone. (B) Analysis of authentic MON as a standard. The liquid chromatograms (LC) in panels A and B demonstrate a major UV absorbance peak eluting at approximately 17.5 min as indicated by the arrows. UV and mass spectrometry (MS) performed on material recovered from these peaks confirm that the peak in panel A contains MON with the expected molecular mass $[\text{M}+1]^+$ of 331 Da. The chromatogram and spectra of extract prepared directly from

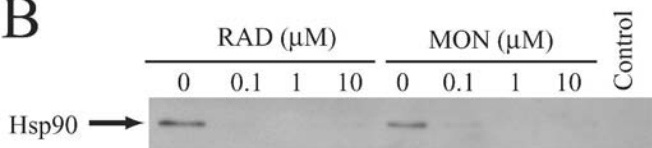
plate-grown fungus appeared essentially identical to that shown in panel A, while extract of agar from plates containing seedlings but no fungus was found to contain no detectable MON (data not shown).

Figure 7. Cultivation with the fungus *P. quadrisepitata* induces a thermotolerant phenotype in soil grown Arabidopsis. Height of each bar depicts the percent of plants surviving 5 d after a 2 h heat shock at 45°C. Results from two independent experiments are displayed in which each experimental group consisted of approximately 20 plants. In both experiments, 90-100% of plants that received no heat shock survived over the 5 d time period, irrespective of the presence or absence of fungus.

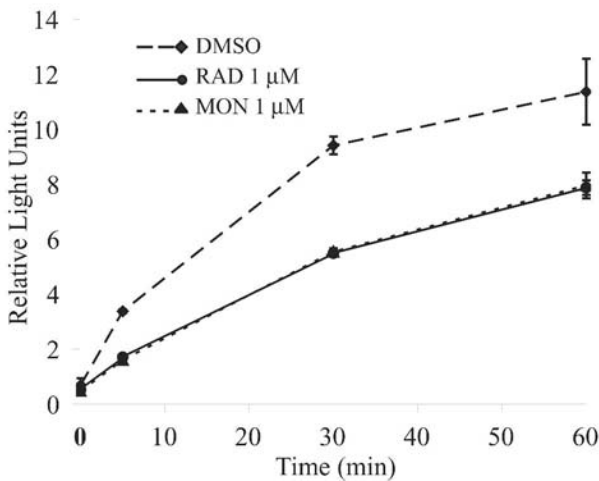
A

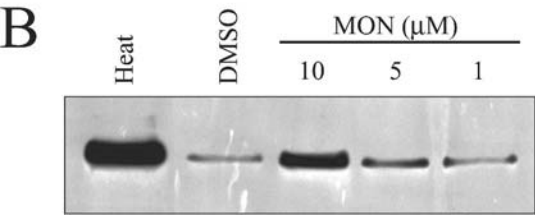
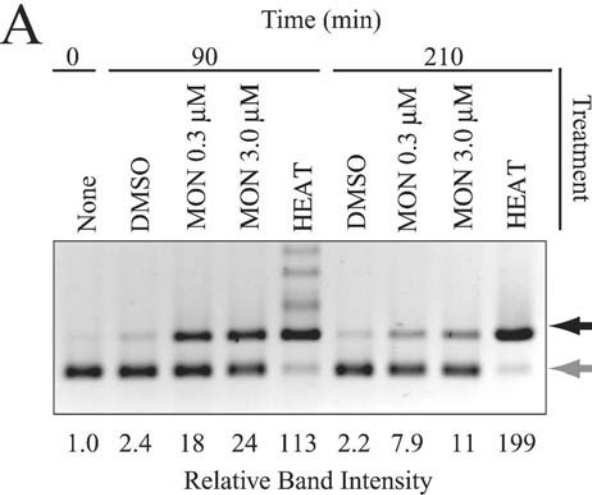


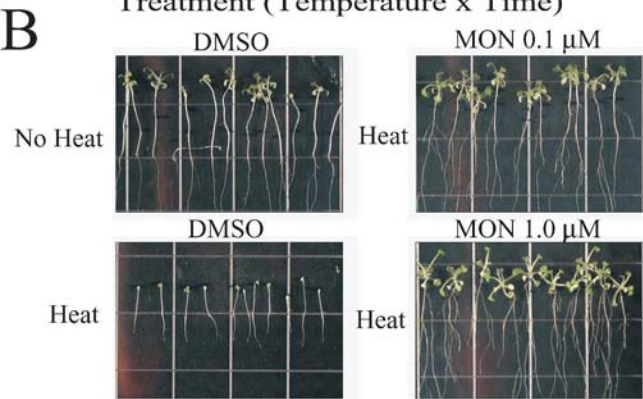
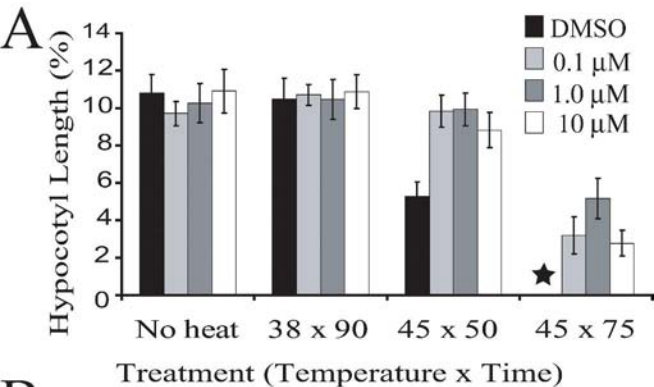
B



C



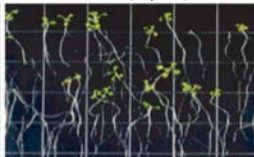
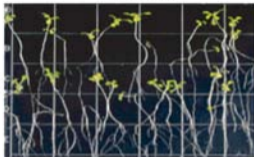




DMSO

MON (2 μ M)

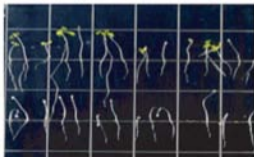
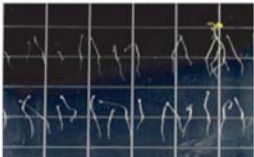
No Heat



Col

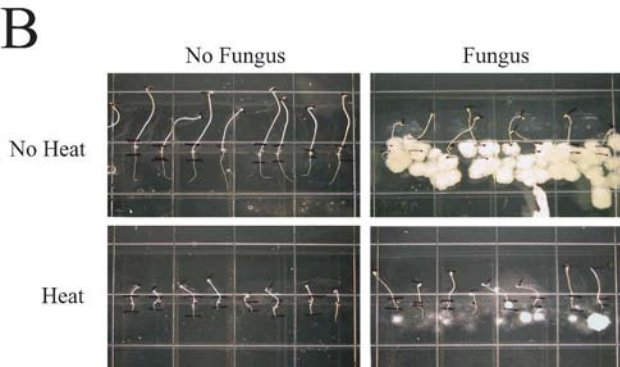
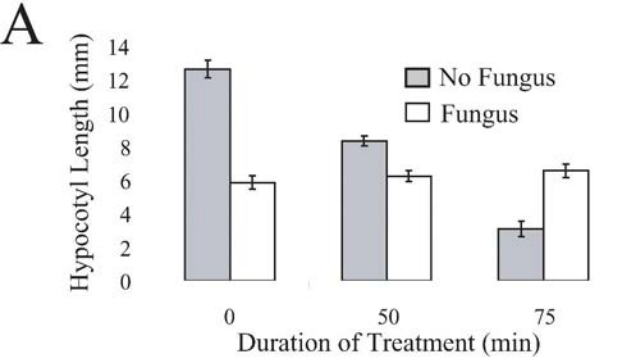
hot1-3

Heat

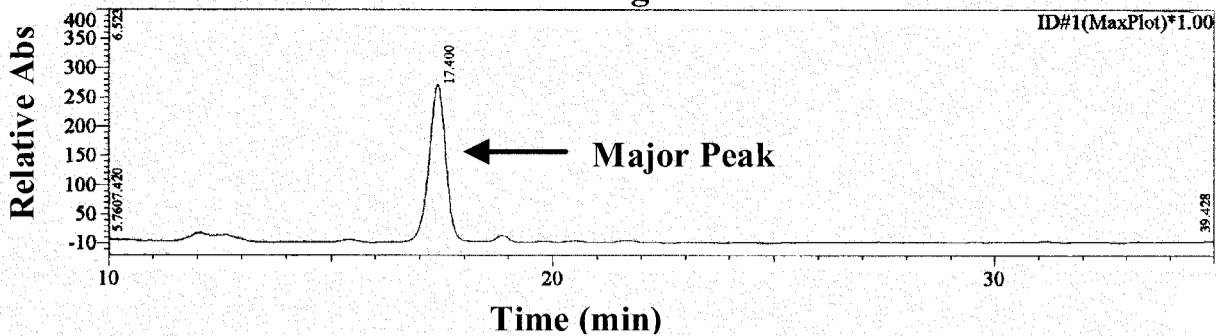


Col

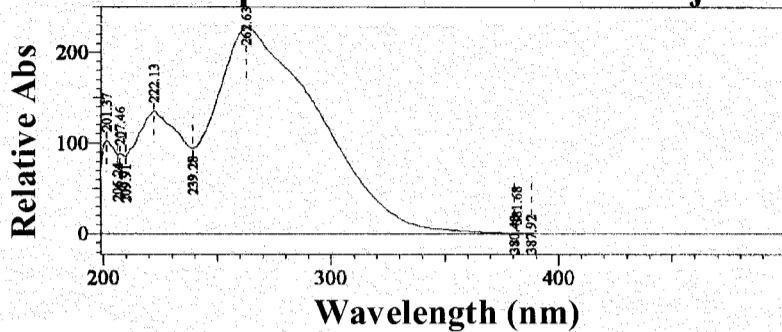
hot1-3



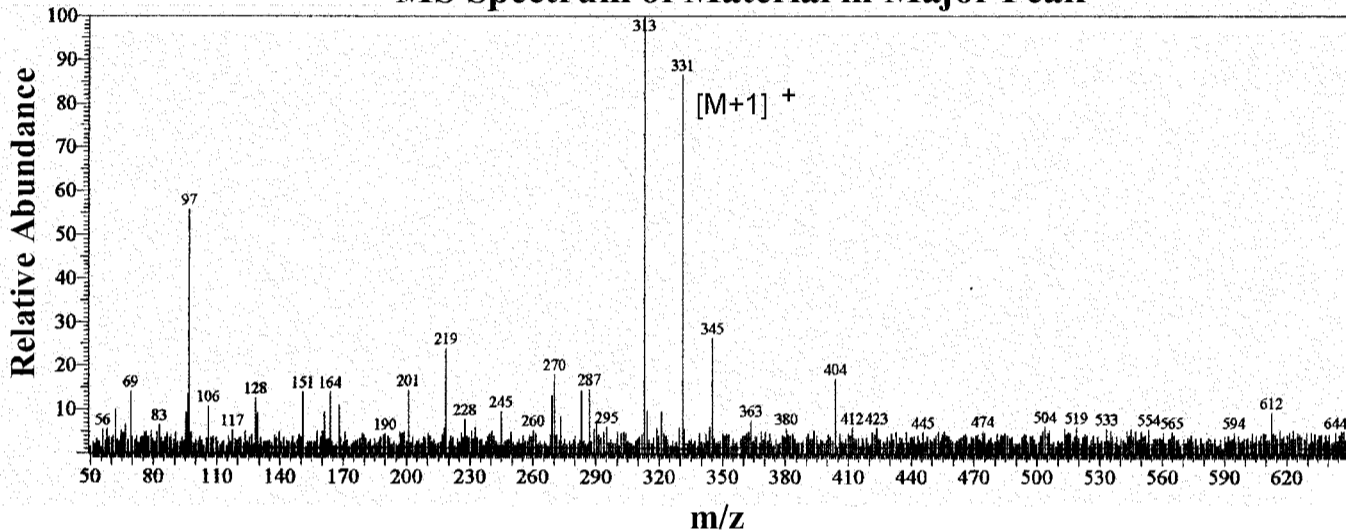
LC Chromatogram of Extract



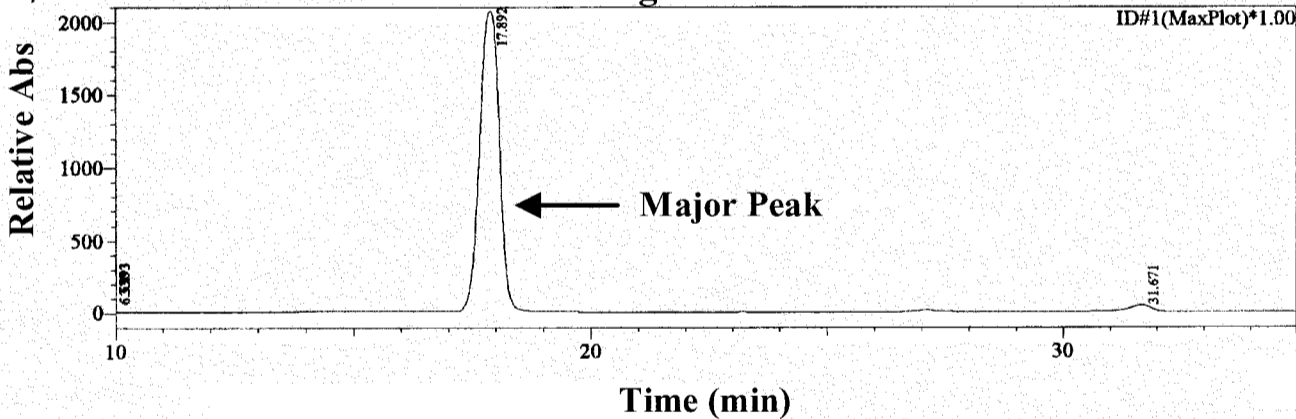
UV Spectrum of Material in Major Peak



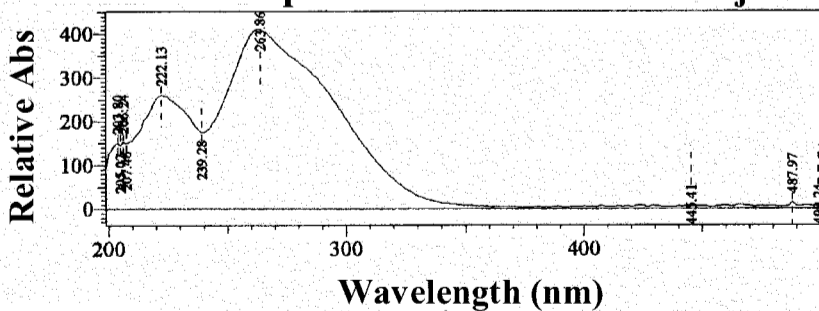
MS Spectrum of Material in Major Peak



LC Chromatogram of Authentic MON



UV Spectrum of Material in Major Peak



MS Spectrum of Material in Major Peak

