Arabidopsis ROF1 (FKBP62) modulates thermotolerance by interacting with HSP90.1 and affecting the accumulation of HsfA2-regulated sHSPs

David Meiri and Adina Breiman*
Department of Plant Sciences, Tel Aviv University, Tel Aviv 69978, Israel

Received 25 November 2008; revised 15 February 2009; accepted 10 March 2009; published online 7 May 2009.
*For correspondence (fax +97 236 405958; e-mail adina@post.tau.ac.il).

SUMMARY

Arabidopsis ROF1 (AtFKBP62) is a peptidyl prolyl cis/trans isomerase and a member of the FKBP (FK506 binding protein) family. ROF1 expression is induced by heat stress and developmentally regulated. In this study, we show that ROF1 binds heat shock proteins HSP90.1 via its tetratricopeptide repeat domain, and localizes in the cytoplasm under normal conditions. Exposure to heat stress induces nuclear localization of the ROF1–HSP90.1 complex, which is dependent upon the presence of the transcription factor HsfA2, which interacts with HSP90.1 but not with ROF1. Nuclear localization of ROF1 was not detected in Arabidopsis HSP90.1 and HsfA2 knockout mutants. The rof1 knockout plants exhibited collapse when 24–48 h passed between acclimation at 37°C and exposure to 45°C. Transgenic ROF1 over-expressors showed better survival in response to exposure to 45°C than wild-type plants did. In rof1 knockout mutants, the level of expression of small HSPs regulated by HsfA2 was dramatically reduced after exposure to 37°C and recovery for 24–48 h, and correlates well with the mutant phenotype. We suggest a role for ROF1 in prolongation of thermotolerance by sustaining the levels of small HSPs that are essential for survival at high temperatures.

Keywords: ROF1, AtFKBP62, immunophilins, HSP90, HsfA2, thermotolerance.

INTRODUCTION

Peptidyl prolyl isomerases (PPIases) are ubiquitously expressed in prokaryotic and eukaryotic cells. Their primary catalytic function is to facilitate the cis/trans isomerization of peptide bonds N-terminal to proline residues within polypeptide chains. The FK506-binding proteins (FKBPs) are PPIases, and their catalytic activity is inhibited upon binding of the immunosuppressive drug FK506. The complexes formed by FKBPs and their ligands are functional modules for immunosuppression, and therefore the FKBPs are also named immunophilins. The multiple FKBPs vary with respect to molecular weight, which ranges from 12 to >77 kDa (Standaert et al., 1990; Kurek et al., 1999; Galat, 2003, 2004). Human FKBP12, which comprises a single FKBPs domain, is the gene that provides the common denominator for the family. Multi-domain FKBPs are structurally characterized by additional protein modules, typically a tripartite tetratricopeptide repeat (TPR) domain, which is involved in protein–protein interactions (Goebel and Yanagida, 1991), and a calmodulin-binding domain (CaMBD) (He et al., 2004). Mammalian FKBP51 and FKBP52 associate with HSP90 via the TPR domain in the native steroid receptor complex, and are considered the major immunophilins of this complex (Smith et al., 1993).

Sequencing of the Arabidopsis genome revealed 23 FKB family members, seven of which contain multiple domains (He et al., 2004; Romano et al., 2005), and two of which have been functionally analyzed. The PASTICCINO1 (pas1, AtFKBP72) protein is a member of the FKB family, and mutants in the coding region display severe developmental abnormalities, altered response to cytokinins and down-regulation of auxin-responsive genes (Vittorioso et al., 1998; Harrar et al., 2003). The product of pas1 has been shown to interact with the transcription factor FAN (FKBP-associated NAC) (Smyczynski et al., 2006). Characterization of the twisted dwarf1 (twd1) (Geisler et al., 2003, 2004) and ultra-curveda2 (UCU2) (Perez-Perez et al., 2004) mutants, both knockouts of AtFKBP42, revealed common pleiotropic effects leading to dwarfism, distorted roots and stems, and helical rotation of organs. Recently, the crystal structure of the AtFKBP42 was solved. These data shed light on the
The heat stress response (Baniwal et al., 2005; Weiergraber et al., 2006). The large Arabidopsis FKBP’s, AtFKBP62 (ROF1) and AtFKBP65 (ROF2), have similar domain structures and high sequence identity. Both proteins contain one domain that possesses peptidyl prolyl cis/trans isomerase activity and FK506 binding, two additional domains that have maintained only partial identity to the functional domain, a TPR region responsible for HSP90 binding, and a calmodulin-binding domain (Blecher et al., 1996; Reddy et al., 1998; Kurek et al., 1999). RNA and protein analyses of the expression profiles of ROF1 and ROF2 show that expression is regulated by age and biotic stresses. ROF1 and ROF2 are expressed in vascular tissues, seedlings and flowers, and their expression is elevated by heat stress. We have demonstrated that ROF1 binds heat shock protein HSP90, and that this interaction occurs via the TPR domain (Aviezer-Hagai et al., 2007). In contrast to the developmental phenotypes of the twd1 and pas1 knockout mutants, the Arabidopsis rof1 and rof2 mutants did not show such phenotypes under normal growth conditions (unpublished data).

HSP90 plays a major role in many cellular signaling systems (Picard, 2002; Pratt et al., 2004), and has widespread effects on the genesis of phenotypic diversity (Queitsch et al., 2002; Sangster et al., 2007). The interaction of mammalian HSP90 with the glucocorticoid receptor serves as a paradigm for its molecular function (Pratt et al., 2006). The glucocorticoid receptor complex includes mammalian FKBP52 and FKBP51, which are the homologous of ROF1 and ROF2 (Pratt et al., 2004; Aviezer-Hagai et al., 2007). Arabidopsis contains seven loci encoding HSP90, four of which encode cytosolic proteins (Krishna and Gloor, 2001). Cytosolic HSP90.1 is stress-inducible, and the other three cytosolic HSPs are constitutively expressed (Haralampidis et al., 2002). The Arabidopsis HSP90 isoforms are highly related (>85% identical), and the AthHSP90.2/3/4 genes are situated beside each other on Arabidopsis chromosome 5 and encode proteins that are 97% identical, suggesting that they are the result of recent gene duplication (Krishna and Gloor, 2001). The function of cytosolic HSP90 is not yet understood; however, specific roles for the independent isoforms have emerged. HSP90.1 was shown to be associated with resistance to pathogens by binding to the R proteins RAR1 and SGT1 (Hubert et al., 2003; Takahashi et al., 2003; Liu et al., 2004; Boter et al., 2007). HSP90.2 was shown to interact with the constitutively expressed transcription factor HsfA1d (Yamada et al., 2007). Similar to metazoan cells, it was proposed, that, in Arabidopsis, HSP90 regulates heat-inducible genes by suppressing HSF activity, and HSP90 is transiently inactivated upon heat shock, leading to activation of heat shock factors (Yamada et al., 2007).

The accumulation of HSPs and HSFs plays a central role in the heat stress response (Baniwal et al., 2004; Kotak et al., 2007; Larkindale and Vierling, 2008; von Koskull-Doring et al., 2007; Nishizawa et al., 2006). The family of plants HSFs shows striking multiplicity, with over 20 members in Arabidopsis. Studies of the tomato HsfA2, one of the major HSFs, have shown that its synthesis is heat-dependent and its activity is controlled by a network of proteins (HsfA1a, Hsp17-CII, Hsp17-CI) that influence its solubility, intracellular localization and activator function (Scharf et al., 1998; Baniwal et al., 2004; Port et al., 2004). Recent evidence has suggested that regulation of HsfA2 in Arabidopsis differs from that of the tomato. In tomato, HsfA1 is the master regulator and its knockdown led to a dramatic reduction of HsfA2 (Mishra et al., 2002; von Koskull-Doring et al., 2007), whereas in Arabidopsis, double knockdown of HsfA1a/1b did not affect expression of HsfA2 (Busch et al., 2005). HsfA2 has been suggested to have a protective role under conditions of repeated cycles of heat stress (Baniwal et al., 2004; Schramm et al., 2006; Nishizawa et al., 2006; Ogawa et al., 2007). The phenotype of HsfA2 knockout mutant plants was recently demonstrated; seedlings of the mutant collapse only when a prolonged recovery time from the original heat exposure treatment is used (Chang et al., 2007).

Acquired thermotolerance (AT) is the ability of plants to cope with repeated heat stress. AT is conferred by non-lethal heat treatment, and decays over time, with a simultaneous decrease in the accumulation of HSPs. An essential component of AT is the induction and synthesis of molecular chaperones and HSPs (Vierling and Kimpel, 1992; Larkindale et al., 2005; Larkindale and Vierling, 2008; von Koskull-Doring et al., 2007; Kotak et al., 2007; Nishizawa et al., 2006). Detailed characterization of AT provided evidence that HsfA2 is directly involved in the extent of duration of the AT (Chang et al., 2007).

In order to understand the function of ROF1, we investigated rof1 knockout plants and plants over-expressing ROF1. As ROF1 is heat stress-induced, we analyzed the phenotypes of the rof1 knockout and over-expressor plants under heat stress conditions. We have determined the subcellular localization of the ROF1 protein and its interactors, and propose an hypothesis by which ROF1 affects thermotolerance.

RESULTS

ROF1 interacts with HSP90.1 in the yeast two-hybrid system

ROF1 has been shown to interact with heterologous wheat (Triticum aestivum) HSP90 (Aviezer-Hagai et al., 2007). In order to identify the cytosolic Arabidopsis HSP90 isoform that interacts with ROF1, we employed a yeast two-hybrid system using AthHSP90.1 and HSP90.2 as prey and ROF1 as bait. Although AthHSP90.1 and AthHSP90.2 share 88% homology, only AthHSP90.1 was found to interact with ROF1 (Figure 1a). To identify which domain of ROF1 was responsible for the interaction with HSP90.1, yeast two-hybrid
vectors were generated to express ROF1 recombinant proteins lacking the N-terminal domain (ROF1-DN), the C-terminal domain (ROF1-DC), or a full recombinant protein with a modified TPR domain containing five mutations: K404A, N408D, N454D, N484A and R488A (ROF1_5M). The truncated protein lacking the C-terminal (ROF1-DC) and recombinant ROF1 with five mutated amino acids (ROF1_5M) were not able to recognize HSP90.1, but the truncated ROF1 without the N-terminus (which includes the PPIase domain) (ROF1-DN) did interact with HSP90.1 (Figure 1b). These results indicate that the PPIase domain is not involved in recognition of HSP90.1, and that the amino acids K404, N408, N454, K484 and R488 are essential for ROF1 interaction with HSP90.1. ROF2, the homolog of ROF1 that is detected only after heat stress (Aviezer-Hagai et al., 2007), interacts with barley (Hordeum vulgare) HvHSP90 but not with AtHSP90.1 (Figure 1c). This is evidence for ROF selectivity in terms of recognition of HSP90, and therefore we suggest a mechanism of selectivity among Arabidopsis HSP90 isoforms in their interaction with ROF proteins.

**HSP90.1 and ROF1 interact in the plant cytoplasm**

To confirm the interaction between ROF1 and AtHSP90.1 in planta and to reveal its subcellular localization, we used a BiFC assay utilizing yellow fluorescent protein (YFP) split into two non-overlapping N-terminal (YN) and C-terminal (YC) fragments. Each fragment is cloned in-frame to genes of interest, enabling expression of non-fluorescent fusion proteins, which generate a positive fluorescent signal upon interaction (Bracha-Drori et al., 2004). When ROF1 and AtHSP90.1 were fused to the N- and C-terminal YFP fragments, respectively, and co-expressed in Arabidopsis leaves, a strong BiFC signal was detected in the cytoplasm (Figure 2a). Co-expression of ROF1_5M with AtHSP90.1 in Arabidopsis showed no positive signals (Figure 2a). Images of Arabidopsis cells expressing ROF1–YN and AtHSP90.1–YC after plasmolysis indicate that the interaction is located in the cytoplasm (Figure 2b). Cytoplasmic localization was confirmed by Western blot analysis of separated soluble fractions of cell proteins after ultracentrifugation (Figure 2c). ROF1 and HSP90.1 appear in the nucleus after exposure to heat stress

As expression of both HSP90.1 and ROF1 is heat-induced, we tested the interaction of HSP90.1 and ROF1 by the BiFC method after exposing the plants to heat stress (3 h at 37°C). The HSP90.1 and ROF1 interaction was detected in the nucleus as well as the cytoplasm (Figure 3a). The presence of HSP90 and ROF1 in the nucleus was confirmed by detection of the individual proteins in isolated nuclei (Figure 3b). ROF1 was detected in the nucleus after heat stress only, whereas the levels of both ROF1 and HSP90 increased in the cytoplasm (Figure 3b). The possibility that the increase in abundance of ROF1 is a mixture of ROF1 and ROF2 was ruled out by using specific anti-ROF2 antibody (data not shown). The identity of the HSP90 isoform could not be determined, as the HSP90 antibodies cannot discriminate between the isoforms. The appearance of ROF1 and HSP90 in the nucleus only after heat stress led to the speculation that they may function as a putative protein
complex with additional partners, in which ROF1 may function as a chaperone. As HSP90s and FKBP5s are known to interact with various proteins, we tested the hypothesis that HSP90 or ROF1 binds an additional protein and the putative tri-partner complex moves to the nucleus.

HsfA2 interacts with AtHSP90.1 and determines the subcellular localization of the ROF1–HSP90.1 complex

Based on the function of HsfA2 as a dominant HSF in thermotolerance in tomato and Arabidopsis (Schramm et al., 2006; von Koskull-Doring et al., 2007) and its interaction with proteins such as tomato HsfA1 and Hsp17.4-CII (Scharf et al., 1998; Port et al., 2004), we analyzed HsfA2 as a putative binding partner of ROF1–HSP90.1. We first assessed whether HsfA2 is co-expressed with ROF1 and HSP90.1. Using the Gene Investigator data mining tool (https://www.genevestigator.ethz.ch), the expression profiles of ROF1, HSP90.1 and HsfA2 were compared, indicating a high correlation coefficient between HsfA2 and both ROF1 \( r = 0.72 \) and HSP90.1 \( r = 0.80 \). Analysis using the BiFC system demonstrated an interaction between HSP90.1 and HsfA2, but no interaction between ROF1 and HsfA2 was observed (Figure 4a,b), suggesting that HsfA2 physically interacts only with HSP90.1. To determine whether this interaction occurs within the proposed trimeric complex, the BiFC assay between ROF1 and HSP90.1 was repeated, and untagged HsfA2 (HsfA2pBIN) was co-expressed, resulting in a positive BiFC signal for ROF1–HSP90.1 in the nucleus under normal conditions without heat shock (Figure 4c); in contrast, expression of the control empty vector pBin did not have this effect (Figure 4d).

Co-expression of HsfA2 increased the number of nuclei positive for ROF1–HSP90.1 interaction (68%) compared to the interaction in heat-stressed plants (24%) expressing only endogenous HsfA2 (Figure 5). When the experiment was performed in HsfA2 knockout plants injected with ROF1–YC and HSP90.1–YN, no interaction was detected in the nuclei, either before or after heat stress (Figure 5).

To determine whether the endogenous proteins are mutually dependent for nuclear localization following heat stress, we utilized rof1, HSP90.1 and HsfA2 knockout plants to test for localization of ROF1 and HsfA2 after heat stress. Nuclear and cytosolic extracts were separated by SDS–PAGE and probed with anti-ROF1 or anti-HsfA2 antibodies. Compared to wild-type cells that showed nuclear and cytoplasmic ROF1, ROF1 was present only in the cytosol in the HsfA2 and HSP90.1 knockout mutants, indicating that their presence is essential for ROF1 nuclear localization (Figure 6). HsfA2 localization in both the cytosol and the nuclei was unaffected in the rof1 and HSP90.1 knockout mutants, indicating that its localization is independent of both proteins (Figure 6).

Taken together, these results indicate that HsfA2 is responsible for the nuclear localization of the ROF1–HSP90.1 complex under conditions of over-expression, which probably mimic the natural conditions of heat stress.
ROF1 is a major player in long-term acquired thermotolerance

To study the role of ROF1 in thermotolerance, seedlings of rof1 knockout and ROF1 over-expressors were exposed to heat stress at 37°C followed by short and long recovery times before exposure to higher stress at 45°C (Figure 7). All seedlings exposed to 45°C without acclimation treatment collapsed and bleached (Figure 7a), whereas all seedlings exposed to acclimation followed by a short recovery time of 2 h were resistant (Figure 7b). Seedlings exposed to acclimation followed by a long recovery time of 2 days showed various responses (Figure 7c,d). HsfA2 and rof1 knockout seedlings collapsed and could not cope with the severe heat stress exposure at 45°C. The rof1 knockout seedlings rescued by expression of transgenic ROF1 (rof1::ROF1) coped with the severe heat stress as well as the wild-type did, indicating that loss of ROF1 was the reason for sensitivity to heat stress. Transgenic plants over-expressing ROF1 exhibited resistance to the severe heat stress treatment, indicating that ROF1 contributes to long-term acquired thermotolerance.

Steady-state levels of shSPs are dependent upon the presence of ROF1

HsfA2 is a major transcription factor for heat stress-induced proteins such as the small HSPs (shSPs) Hsp18.1-CI, Hsp25.3-P and Hsa32 (Charng et al., 2006, 2007; Schramm et al., 2006). As shSPs have been reported to play an important role in the heat stress response (Larkindale and Huang, 2004; Larkindale et al., 2005; von Koskull-Doring et al., 2007), the transcription profile of several shSPs was determined in the rof1 and HsfA2 knockout mutants (Figure 8). Hsp17.6-CII, 18.1-CI, 25.3-P and Hsa32 were detectable in the knockout rof1 and HsfA2 mutants after heat stress, but their steady-state transcript levels decreased dramatically after a recovery of 24 and 48 h. The most sensitive was Hsp25.3-P, which was undetectable in the rof1 and HsfA2 knockout mutants after 24 h of recovery.
expression of HsfA2 in transgenic ROF1 over-expressor plants (Figure 9). ROF1 over-expression by itself was insufficient to increase the expression levels of the sHSPs (Figure 9a), and expression of HsfA2 was essential for the increase in sHSP transcript levels (Figure 9b). The ROF1 over-expressor plants injected with HsfA2 expressed Hsp25.3-P, Hsa32 and Hsp18.1-CI at much higher levels than wild-type plants, whereas the level of Hsp17.6-CII transcripts was less affected by the presence of high levels of ROF1 (Figure 9). These results are even more convincing as the expression level of HsfA2 is slightly higher in wild-type plants.

**DISCUSSION**

In this study, we have demonstrated a phenotype for the rof1 knockout mutant: the rof1 knockouts collapse when 24–48 h pass between acclimation at 37°C and exposure to 45°C (Figure 7). This phenotype was abrogated by expression of recombinant ROF1 in complemented knockout mutants. Moreover, seedlings of ROF1 over-expressors were more resistant than the wild-type when challenged in the same bioassay (Figure 7). Previous efforts to identify a phenotype for the rof1 mutants by classical heat stress bioassays such as determination of hypocotyl elongation or seedling survival were not successful (Larkindale et al., 2005; and DM and AB, unpublished data). The major difference between previous assays and the one used in this study is the time interval between the first exposure to 37°C and the challenge at 45°C. Whereas in the classical assays, the delay time between exposure to 37°C and 45°C was 90 min, a recovery period of 24–48 h was implemented between the first and second exposure in this study. The phenotype observed for the rof1 knockout mutants resembled the phenotype of HsfA2 knockout plants (Chargn et al., 2007), and indicated that ROF1 is essential for extending the duration of acquired thermotolerance but not for its induction, similar to the HsfA2 gene. The distinct phenotype of the rof1 knockout was in good agreement with the RT-PCR and immunoblot results. According to these results, disruption of ROF1 did not significantly affect the transcript levels of shSP genes under normal conditions or after a 3 h treatment at 37°C. However, in the rof1 knockout mutant, the mRNA levels of selected shSPs declined faster than in the wild-type plants during the recovery period (Figure 8a). The protein levels of sHSP-CI in both rof1 and HsfA2 knockout plants were consistent with the transcript abundance but declined more slowly, presumably due to their stability (Figure 8c). The time course of the changes in protein levels correlates well with development of the mutant phenotype (Figure 7), and is probably the major factor responsible for this phenotype.

In our previous work, we showed that ROF1 interacts with heterologous wheat HSP90 (Aviezer-Hagai et al., 2007). The Arabidopsis genome contains four genes for cytosolic HSP90 (HSP90.1–4), and there is little evidence for a
specialized function of these genes (Krishna and Gloor, 2001). In this paper, we demonstrate that ROF1 interacts specifically with HSP90.1. The specificity of interaction between ROF1 and HSP90.1 is high, as ROF1 does not interact with HSP90.2 despite its 88% identity to HSP90.1 and the presence of the MEEVD motif that interacts with the ROF1 TPR. The specificity of interaction between ROF1 and HSP90.1 is even more conclusive, given that ROF2 (85% homology to ROF1) does not interact with HSP90.1 despite its ability to bind HvHSP90 (Figure 1c).

We detected the interaction between HSP90.1 and ROF1 using the BIFC method (Bracha-Drori et al., 2004), and this report shows this technique to be an extremely versatile tool. In addition to the established system in *Nicotiana benthamiana* leaves, here we successfully used the BIFC method in physiological studies such as heat stress pre-treatment and analysis of trimeric protein complex interactions (ROF1–HSP90.1–HsfA2) in Arabidopsis mutant plants (Figures 3–5). We found that, in plants grown at 24°C, the HSP90.1–ROF1 interaction was localized to the cytoplasm (Figure 2). After exposure of Arabidopsis plants to heat stress, the HSP90.1–ROF1 complex appeared in the nuclei as well as in the cytoplasm (Figure 3). Most likely, the heat stress induces HsfA2 and additional factors that contribute to nuclear translocation of ROF1–HSP90.1.

In order to establish the contribution of HsfA2 to nuclear re-localization of the ROF1–HSP90.1 complex, ROF1 and HSP90.1 were injected into leaves of Arabidopsis HsfA2 knockout mutants (Figure 5). In the HsfA2 knockout plants, heat stress did not lead to the expected nuclear localization; however, translocation was detected (in 88% of the cells) only after the addition of unlabeled HsfA2 (Figures 5 and 6).

The ability of HsfA2 to shuttle between the cytoplasm and nucleus has been demonstrated for tomato HsfA2 (Heerklotz et al., 2001). The tomato HsfA2 was found to serve as a shuttling protein that is predominantly localized in the cytoplasm. It contains both a nuclear localization signal (NLS) and a C-terminal leucine-rich motif that functions as a nuclear export signal (NES) (Heerklotz et al., 2001). Translocation of tomato HsfA2 to the nucleus was found to be dependent on HsfA1, whereas HsfA2 is regulated in a different manner in Arabidopsis (Scharf et al., 1998; Port et al., 2004; Schramm et al., 2006). In Arabidopsis, there is no or very little accumulation of the HsfA2 protein without heat stress; however, it is the most strongly expressed member of the Hsf family after heat stress (Port et al., 2004; Schramm et al., 2006). Moreover, an *HsfA1b/A1a* double knockout does not show altered expression of HsfA2 or its putative target genes (Busch et al., 2005). Here we show that, after heat stress, the HsfA2 protein localizes to both the nucleus and the cytoplasm, with clear preference to the nucleus (Figure 6). We suggest that HsfA2, which does not interact directly with ROF1 but strongly interacts with HSP90.1 (Figure 4b), is the factor responsible for ROF1–HSP90.1 translocation to the nucleus. We show both by BIFC and in isolated nuclei that ROF1 is present exclusively in the cytosol in HsfA2 and HSP90.1 knockout mutants, indicating that their presence is essential for its localization (Figure 6). In contrast, the absence of ROF1 or HSP90.1 proteins in knockout mutants did not affect HsfA2 localization or expression, indicating that its localization and expression are independent of both proteins (Figure 6). It is worth mentioning that some FKBP5s are known to play a key role in translocation. The mammalian FKBP52 (ROF1 homolog) is responsible for translocation of the glucocorticoid receptor complex to the nucleus by linking the receptor to the dynein motor protein, which interacts with the PPlase domain in FKBP52 (Davies et al., 2005). Wheat FKBP73 has been shown to interact in a similar manner with dynein, and experiments were performed to replace mammalian FKBP52 in forming an active steroid receptor complex (Harrell et al., 2002; Wochnik et al., 2005).
Arabidopsis ROF1 interacts with HSP90.1, and the translocation to the nucleus is induced by heat stress and depends on the presence of HsfA2. Therefore, the effect of ROF1 on sHSP expression is not facilitated by nuclear transport as described for its mammalian homologs (Banerjee et al., 2008).

In mammalian cells, the general concept is that the transcriptional activity of HSF1 is repressed by the HSP90–p23–FKBP52 complex by repressing trimerization of HSF1 (Voellmy, 2004). FKBP52 (the homolog of ROF1) could be crossed-linked in situ to HSF1 in heat-treated cells (Guo et al., 2001), and it has been shown that Hsf1 forms complexes with HSP90 and FKBP52 (Nair et al., 1996).

HSP90 is a major factor in regulation of thermotolerance, and sHSPs were not able to promote thermotolerance when HSP90 activity was repressed (Duncan, 2005). The regulation of the heat stress response is very complex, and additional co-factors have been identified that may compete with binding of the Hsp90 complex to trimeric HSF1 (Boellman et al., 2004).

We propose that ROF1 participates in thermotolerance, and there are several ways for interpretation of its role. One possibility is that HsfA2 activity requires ROF1 as its direct or...
indirect chaperone due to ROF1 activity as PPIase and/or chaperone activity similar to the wheat FKBP73 (Blecher et al., 1996; Kurek et al., 2002). A second possibility is that ROF1, together with HSP90.1, associates to form a complex that contributes to the ability of HsfA2 to recognize and interact with other nuclear candidates such as histones. The results of our studies are compatible with the following hypothesis. Under normal growth conditions, ROF1 interacts with HSP90.1 in the cytoplasm. After heat stress, among the proteins synthesized are sHSPs and HsfA2. HsfA2 interacts with HSP90.1 and is responsible for translocation of the ROF1–HSP90.1–HsfA2 complex to the nucleus. The interaction between ROF1–HSP90.1 and HsfA2 stabilizes HsfA2, and this complex is responsible for the continuity of sHSP synthesis during the recovery period, which enables the plants to cope with repeated heat stress. Interestingly, our recent studies indicate that the ROF1 homolog ROF2 has the opposite effect and negatively regulates thermotolerance (DM and AB, unpublished results).

Further evidence for the role of ROF1 will be obtained by analysis of the protein complexes with HSP90.1 and HsfA2 and their function in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Yeast two-hybrid analysis

The coding region of the ROF1 gene (codons 1–552) was amplified by PCR (forward primer 5'-CGCCCCGGGATGGATGCTAATTTCGAGATGCCT-3'; reverse primer 5'-CGCCCATGGTTATTCCTTACTAGTTTCGCAAAC-3'), and cloned in-frame into pLexA (Clontech, http://www.clontech.com/) via XmaI and NcoI restriction sites. The specific fragments for deletion constructs were ROF1-Dc (amino acid residues 1–389 of AtROF1) and ROF1-Dn (amino acid residues 390–552 of AtROF1). ROF1_5M was constructed as described previously (Aviezer-Hagai et al., 2007).

The coding region of the ROF2 cDNA gene (codons 1–578) was amplified by PCR (forward primer 5'-CCGGAATTCATGGAAGAGCGATTTCGAC-3'; reverse primer 5'-CGCGGATCCATGCCTTGGTGTCAATAC-3'), and cloned in-frame into pLexA (Clontech) via EcoRI and BamHI restriction sites.

The coding region of the HSP90.1 gene (codons 1–706) was amplified by PCR (forward primer 5'-GGGATCTGAGGGATTATGGCGGACCTGAAACATCG-3'; reverse primer 5'-GGCCCATGGTTATTCCTTACTTAGTTTCGCAAAC-3'), and cloned in-frame into pLexA (Clontech) via EcoRI and BamHI restriction sites.

The coding region of the HSP90.2 gene (codons 1–706) was amplified by PCR (forward primer 5'-GGGATCTGAGGGATTATGGCGGACCTGAAACATCG-3'; reverse primer 5'-GGCCCATGGTTATTCCTTACTTAGTTTCGCAAAC-3'), and cloned in-frame into pLexA (Clontech) via EcoRI and BamHI restriction sites.

Figure 9. The steady-state level of sHSPs is increased by ROF1. Semi-quantitative RT-PCR analysis of mRNA levels for ROF1, HsfA2, Hsp17.6-cII, Hsp18.1-cI, Hsp25.3-p and Hsa32 before (a) and after (b) transient expression of HsfA2 on leaves from 5-week-old wild-type (wt) and ROF1-over-expressing plants (ROF1 o.e.). Plants were injected with the HsfA2 pBIN plasmid and harvested 48 h after the injection. The control samples were wild-type leaves injected with empty pBIN plasmid. The RT-PCR products are shown as a loading control. The numbers of PCR cycles for ROF1, HsfA2, Hsp17.6-cII, Hsp18.1-cI, Hsp25.3-p, Hsa32 and ribosomal 40S protein were 30, 30, 32, 32, 32 and 24, respectively. The expected sizes of the PCR products are indicated on the right. The experiments were repeated three times.

(c) For semi-quantitative analysis, the intensity of the bands from (b) and another two independent experiments were determined by densitometry using IMAGE MASTER ID PRIME software, dividing the observed signal of each gene by that for ribosomal 40S protein. The mean intensities of the mRNA levels are presented, with error bars indicating the standard deviation of three independent replicates.
AGTCGACTCTCTCATCTTGCCT-3'). A silent point mutation (A → C) was made at position 1108, which disrupted an XhoI restriction site, and the mutant HSP90.2 was then cloned in-frame into pB42AD (Clontech) via XhoI restriction sites. HvHSP90 pB42AD (Takahashi et al., 2003) was kindly provided by K. Shirasu. The selected plantlets were then transferred to soil and plant were germinated on MS agar plates containing 60 mg/ml hygromycin. The selected plantlets were then transferred to soil and then grown to yield T2 seeds. Three homozygous T3 lines of wild-type were co-transformed by injection into the WS ecotype, the ecotype represented in the Col-0 line in the Col-0 ecotype were kindly provided by K. Shirasu (Department of Cell Biology, Goethe University von Koskull-Doring, Germany). The Arabidopsis line (Col-0) containing a T-DNA insertion in the AtHsfA2 gene (SALK_008978) (Schramm et al., 2006) was obtained from Pascal von Koskull-Doring (Department of Cell Biology, Goethe University Frankfurt, Germany). The Arabidopsis AtHSP90.1 T-DNA insertion line in the Col-0 ecotype was kindly provided by K. Shirasu (Takahashi et al., 2003). The rol1 knockout mutant in the Arabidopsis WS ecotype has been described previously (Aviezer-Hagai et al., 2007).

To generate ROF1 over-expressor plants and rol1–::ROF1 plants, the coding region of the ROF1 gene (codons 1–552) was amplified by PCR (forward primer 5'-CCGGATCCATGTGGTATATGTTTCGCAAAC-3' and reverse primer 5'-CTCCTGCCATATGGTCTTTTAGTTTCGCAAAC-3') and cloned in-frame into the pLB19 binary vector via XhoI restriction sites. pLB19-ROF1 was digested with SacI and XbaI, and the resulting fragment (ROF1 + 35S PRO/TERM cDNA) was cloned into a SacI/XbaI-digested pCAMBIA 1300 vector. The construct was sequenced to confirm there were no errors generated by PCR, and then introduced into Agrobacterium tumefaciens strain GV3101 (Bechtold and Pelletier, 1998). Arabidopsis wild-type ecotype Wassilewskija (WS) and rol1 knockout plants were transformed with the constructs by the floral dip method (Clough and Bent, 1998).

Approximately 100 sterilized T1 seeds from each T0 transformed plant were germinated on MS agar plates containing 60 mg/ml hygromycin. The selected plantlets were then transferred to soil and grown to yield T2 seeds. Three homozygous T2 lines of wild-type and rol1 knockout plants containing ROF1::35S (ROF1 o.e. #6, ROF1 o.e. #8, ROF1 o.e. #12; rol1–::ROF1 #1, rol1–::ROF1 #3, rol1–::ROF1 #6) were selected for further analysis by PCR and Western blot.

**Plant material and heat stress conditions**

**Generation of transgenic plants.** The Arabidopsis line (Col-0 ecotype) containing a T-DNA insertion in the HsfA2 gene has been described previously (Aviezer-Hagai et al., 2007). To generate ROF1 over-expressor plants and rol1–::ROF1 plants, the coding region of the ROF1 gene (codons 1–552) was amplified by PCR (forward primer 5'-CCGGATCCATGTGGTATATGTTTCGCAAAC-3' and reverse primer 5'-CTCCTGCCATATGGTCTTTTAGTTTCGCAAAC-3') and cloned in-frame into the pLB19 binary vector via XhoI restriction sites. pLB19-ROF1 was digested with SacI and XbaI, and the resulting fragment (ROF1 + 35S PRO/TERM cDNA) was cloned into a SacI/XbaI-digested pCAMBIA 1300 vector. The construct was sequenced to confirm there were no errors generated by PCR, and then introduced into Agrobacterium tumefaciens strain GV3101 (Bechtold and Pelletier, 1998). Arabidopsis wild-type ecotype Wassilewskija (WS) and rol1 knockout plants were transformed with the constructs by the floral dip method (Clough and Bent, 1998).

Approximately 100 sterilized T1 seeds from each T0 transformed plant were germinated on MS agar plates containing 60 mg/ml hygromycin. The selected plantlets were then transferred to soil and grown to yield T2 seeds. Three homozygous T2 lines of wild-type and rol1 knockout plants containing ROF1::35S (ROF1 o.e. #6, ROF1 o.e. #8, ROF1 o.e. #12; rol1–::ROF1 #1, rol1–::ROF1 #3, rol1–::ROF1 #6) were selected for further analysis by PCR and Western blot.

**Data analysis**

To estimate the relative levels of ROF1, HSP90.1 and HsfA2 mRNA in various tissues and in response to stress treatments, we used the Gene Correlator tool from the Genevestigator Arabidopsis thaliana microarray database (https://www.genevestigator.ethz.ch) (Zimmermann et al., 2004, 2005), which shows how two genes are co-expressed over selected chips in the database. Pearson’s correlation coefficient ($r^2$) is given as an indication of the quality of least-squares fitting to the data.

**Bimolecular fluorescence complementation analysis**

To generate ROF1 over-expressor plants and rol1–::ROF1 plants, the coding region of the ROF1 gene (codons 1–552) was amplified by PCR (forward primer 5'-CCGGATCCATGTGGTATATGTTTCGCAAAC-3' and reverse primer 5'-CTCCTGCCATATGGTCTTTTAGTTTCGCAAAC-3') and cloned in-frame into the pLB19 binary vector via XhoI restriction sites. pLB19-ROF1 was digested with SacI and XbaI, and the resulting fragment (ROF1 + 35S PRO/TERM cDNA) was cloned into a SacI/XbaI-digested pCAMBIA 1300 vector. The construct was sequenced to confirm there were no errors generated by PCR, and then introduced into Agrobacterium tumefaciens strain GV3101 (Bechtold and Pelletier, 1998). Arabidopsis wild-type ecotype Wassilewskija (WS) and rol1 knockout plants were transformed with the constructs by the floral dip method (Clough and Bent, 1998).

Approximately 100 sterilized T1 seeds from each T0 transformed plant were germinated on MS agar plates containing 60 mg/ml hygromycin. The selected plantlets were then transferred to soil and grown to yield T2 seeds. Three homozygous T2 lines of wild-type and rol1 knockout plants containing ROF1::35S (ROF1 o.e. #6, ROF1 o.e. #8, ROF1 o.e. #12; rol1–::ROF1 #1, rol1–::ROF1 #3, rol1–::ROF1 #6) were selected for further analysis by PCR and Western blot.

**Plant materials and growth conditions.** In all experiments, we used wild-type controls of both ecotypes Col-0 and WS. As both ecotypes exhibited a similar pattern, and rol1 knockout and ROF1 over-expressors were in the WS ecotype, the ecotype represented in all figures is WS.

For leaf injection (BiFC method and HsfA2 expression), Arabidopsis thaliana plants were grown on soil for approximately 5 weeks and irrigated from below. Plants were grown in an environmental growth chamber under short-day conditions (10 h light/14 h dark cycles) at 22°C.

For RNA preparation, protein extraction and heat stress bioassays, Arabidopsis thaliana seeds were germinated on plates containing MS medium (Murashige and Skoog medium including vitamins, Duchefa, comprising 0.2% w/v MS, 2.5 mM MES, 1% sucrose and 0.5% plant agar (Duchefa, http://www.duchefa.com) for 5–14 days. After 2–3 days at 4°C in the dark for stratification, seeds were grown on plates at 22°C under long-day conditions (16 h light, 8 h dark) unless otherwise mentioned.

**Heat-stress conditions**

For BiFC, 2 days after injection, the whole plant was incubated for 3 h at 37°C in an incubator with high humidity to avoid drought stress. For RNA preparation and protein extraction, 5-day-old seedlings were incubated for 3 h at 37°C in an incubator, and then returned to recovery conditions of 22°C under long days (16 h light, 8 h dark) for various durations (see figure legends). For thermotolerance bioassays, the plates were sealed with parafilm (Pechiney Plastic Packaging Company, Chicago, IL) after recovery, and then incubated for 60 min in a 45°C water bath. After the 45°C treatment, the plates were incubated at 22°C for recovery under long-day conditions (16 h light, 8 h dark) for another 8 days.

**Table 1 Vector combination and YFP reconstitution in BiFC analysis**

<table>
<thead>
<tr>
<th>Combination of constructs</th>
<th>YFP fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROF1–YN/pSY735–YC</td>
<td>–</td>
</tr>
<tr>
<td>ROF1–YN/HS90.1–YC</td>
<td>+</td>
</tr>
<tr>
<td>ROF1–YN/HsfA2–YC</td>
<td>–</td>
</tr>
<tr>
<td>ROF1_5M–YN/HS90.1–YC</td>
<td>–</td>
</tr>
<tr>
<td>ROF1–YN/pSY736–YN</td>
<td>–</td>
</tr>
<tr>
<td>ROF1–YC/HS90.1–YN</td>
<td>–</td>
</tr>
<tr>
<td>ROF1–YC/HsfA2–YN</td>
<td>–</td>
</tr>
<tr>
<td>ROF1_5M–YC/HS90.1–YN</td>
<td>–</td>
</tr>
<tr>
<td>HSP90.1–YN/pSY735–YC</td>
<td>–</td>
</tr>
<tr>
<td>HSP90.1–YN/HsfA2–YC</td>
<td>–</td>
</tr>
<tr>
<td>HSP90.1–YC/pSY736–YN</td>
<td>–</td>
</tr>
<tr>
<td>HSP90.1–YC/HsfA2–YN</td>
<td>+</td>
</tr>
<tr>
<td>HSP90.1–YC/pSY735–YN</td>
<td>+</td>
</tr>
<tr>
<td>HSP90.1–YC/HsfA2–YN</td>
<td>+</td>
</tr>
</tbody>
</table>
Leaf injections

Arabidopsis leaves were injected with Agrobacterium tumefaciens strains GV3101/mp90 harboring the appropriate plasmids. Cultures (10 ml) were grown overnight at 28°C in Luria–Berti liquid medium with kanamycin (30 μg/ml). The next day, 500 μl of the cultures were transferred into induction medium [50.78 mM MES, 0.5% Glc, 1.734 mM NaH₂PO₄, 0.2 mM acetylserine, and 5% 20× AB mix (373.9 mM NH₄Cl, 24.34 mM MgSO₄, 40.23 mM KCl, 1.36 mM CaCl₂ and 0.18 mM FeSO₄·7H₂O)] and grown for an additional 4 h until the OD₆₀₀ reached 0.2–0.6. Before injection, cultures were diluted with induction medium until the OD₆₀₀ reached 0.12. YFP fluorescence was observed in leaves for 24 and 48 h after injection.

4′,6-diamidino-2-phenylindole (DAPI) staining

An intact leaf was dipped in 1 M KCl and incubated in 100 μg/ml 4′,6-diamidino-2-phenylindole (Sigma, http://www.sigmaaldrich.com/) for 30 min at 4°C.

Plasmolysis

Leaves were plasmolyzed by incubation in 0.8 M NaCl. Leaf sections were mounted on microscope slides in the plasmolysis solution.

RNA isolation and RT-PCR

Total RNA from seedlings or leaves (100 mg) before and after heat stress treatment was isolated as described previously (Aviezer-Hagai et al., 2007). All RNA samples were quantified at 260 nm using a NanoDrop ND-1000 spectrophotometer (http://www.nanodrop.com/). First-strand cDNA was produced using 2 μg of total RNA, oligo(dT)₁₅ primer and M-MLV reverse transcriptase (Promega, http://www.promega.com/). The resulting cDNA was diluted 40 times, and 5 μl were used as a template in a 25 μl PCR reaction using gene-specific primers (Table S1). PCR amplification was performed as follows: 94°C for 4 min, followed by five cycles at 94°C for 45 sec, 55°C for 45 sec and 72°C for 1 min, then 19 (for ribosomal 40S) or 25 cycles (for ROF1 and all other genes) at 94°C for 30 sec, 55°C for 45 sec and 72°C for 2 min. The reaction was terminated by incubation at 72°C for 5 min. The PCR reactions were performed using a PTC-200-PCR Peltier Thermal Cycler (MJ Research, http://www.mjp.com/). For semi-quantitative analysis, the intensity of the bands was determined by densitometry using IMAGE MASTER ID PRIME software version 4.1 (Amersham Pharmacia Biotech, http://www5.amershambiosciences.com/).

Preparation of protein extracts for SDS-PAGE and Sup-100

Protein extractions were performed as previously described (Aviezer-Hagai et al., 2007). For Sup-100 (soluble fraction after centrifugation at 100 000 g) proteins extracted from 2-week-old Arabidopsis seedlings were centrifuged for 1 h at 200 000 g.

Antibodies used for Western blot analysis

ROF1 was detected using polyclonal antibodies raised against recombinant wFKBP73 (x73) (Blecher et al., 1996; Kurek et al., 1999) used at a dilution of 1:3000. Arabidopsis HSP90.1 was detected using rabbit R2 antisera against Brassica HSP90 (Krishna et al., 1997) diluted 1:2500. The antibodies against HsfA2 were provided by Pascal von Koskull-Doring (Goethe University Frankfurt, Germany) and used at a dilution of 1:1000. The antibodies against SHSPs-Cl were provided by Dr Adam (Institute of Plant Sciences and Genetics in Agriculture, Hebrew University)

替代材料（其他除丢失材料外的任何查询）应转至The Authors（其他除丢失材料外的任何查询）
REFERENCES


学霸图书馆

www.xuebalib.com

本文献由“学霸图书馆-文献云下载”收集自网络，仅供学习交流使用。

学霸图书馆（www.xuebalib.com）是一个“整合众多图书馆数据库资源，提供一站式文献检索和下载服务”的24小时在线不限IP图书馆。

图书馆致力于便利、促进学习与科研，提供最强文献下载服务。

图书馆导航：

图书馆首页 文献云下载 图书馆入口 外文数据库大全 疑难文献辅助工具