In Silico Identification of Carboxylate Clamp Type Tetratricopeptide Repeat Proteins in Arabidopsis and Rice As Putative Co-Chaperones of Hsp90/Hsp70

Bishun D. Prasad*, Shilpi Goel*, Priti Krishna*
Department of Biology, The University of Western Ontario, London, Ontario, Canada

Abstract
The essential eukaryotic molecular chaperone Hsp90 operates with the help of different co-chaperones, which regulate its ATPase activity and serve as adaptors to recruit client proteins and other molecular chaperones, such as Hsp70, to the Hsp90 complex. Several Hsp90 and Hsp70 co-chaperones contain the tetratricopeptide repeat (TPR) domain, which interacts with the highly conserved EEVD motif at the C-terminal ends of Hsp90 and Hsp70. The acidic side chains in EEDV interact with a subset of basic residues in the TPR binding pocket called a ‘carboxylate clamp’. Since the carboxylate clamp residues are conserved in the TPR domains of known Hsp90/Hsp70 co-chaperones, we carried out an in silico search for TPR proteins in Arabidopsis and rice comprising of at least one three-motif TPR domain with conserved amino acid residues required for Hsp90/Hsp70 binding. This approach identified in Arabidopsis a total of 36 carboxylate clamp (CC)-TPR proteins, including 24 novel proteins, with potential to interact with Hsp90/Hsp70. The newly identified CC-TPR proteins in Arabidopsis and rice contain additional protein domains such as ankyrin, SET, oocicosapetide/Phox/Bem1p (Phox/PB1), DnaL-like, thioredoxin, FBD and F-box, and protein kinase and U-box, indicating varied functions for these proteins. To provide proof-of-concept of the newly identified CC-TPR proteins for interaction with Hsp90, we demonstrated interaction of AtTPR1 and AtTPR2 with AtHsp90 in yeast two-hybrid and in vitro pull down assays. These findings indicate that the in silico approach used here successfully identified in a genome-wide context CC-TPR proteins with potential to interact with Hsp90/Hsp70, and further suggest that the Hsp90/Hsp70 system relies on TPR co-chaperones more than it was previously realized.

Introduction
The eukaryotic Hsp90 performs key roles in signal transduction by regulating maturation, localization, stability and protein interactions of a large number of signaling proteins [1,2]. Due to its role in chaperoning oncogenic protein kinases, Hsp90 has recently been surfaced as a drug target for inhibiting cancer progression in humans [3]. Additional roles of Hsp90 lie in chromatin remodeling, epigenetic regulation, and morphological evolution [2]. Eukaryotic Hsp90 functions as a homodimer with each monomer comprising of an N-terminal ATP-binding domain that is responsible for the ATPase activity of Hsp90, a middle domain that harbors the client protein binding site and can also regulate the ATPase activity of the N-terminal domain, and a C-terminal dimerization domain [4]. Hsp90-specific inhibitors such as geldanamycin (GA) bind to the N-terminal domain of Hsp90 and inhibit in vivo functions [3]. The C-terminus of cytosolic Hsp90 contains a conserved pentapeptide MEEVD, which is responsible for binding with the tetratricopeptide repeat (TPR) domain present in several co-chaperones of Hsp90 [1,5].

Hsp90 has an ATP-driven chaperone cycle [2]. The open V-shaped conformation of dimeric Hsp90 allows for loading of the client protein. Following binding of ATP, a conformational change in the middle domain leads to a closed conformation in which the monomers are twisted around each other and the two N-terminal domains are dimerized. With the coordinated assistance of Hsp70 and a range of co-chaperones in dynamic protein heterocomplexes, the final maturation of the client protein takes place. Upon ATP hydrolysis, the client protein and the heterocomplex dissociate and Hsp90 enters a new chaperone cycle [4].

The various co-chaperones assist Hsp90 by influencing its ATPase activity and by linking it to other proteins and providing some measure of specificity to the client protein-Hsp90 interaction [6]. On a structural basis the Hsp90 co-chaperones can be broadly divided into two categories: TPR domain proteins and non-TPR proteins. The TPR domain is a protein-protein interaction domain, comprised of a loosely conserved 34 amino acid sequence that is repeated several times [7]. Several Hsp90 co-chaperones, such as Hsp70-Hsp90 organizing protein (Hop), high molecular weight immunophilins [cyclophilin 40 (Cyp40) and FK506-binding proteins 51 and 52 (FKBP51 and FKBP52)], protein phosphatase 5 (PP5) and the carboxyl terminus of Hsc70 interacting protein (CHIP) contain a three-motif TPR domain with conserved carboxylic clamp residues [5]. Within a TPR motif, eight amino
acids at positions 4 (W/L/F), 7 (L/I/M), 8 (G/A/S), 11 (Y/L/F), 20 (A/S/E), 24 (F/Y/L), 27 (A/S/I), and 32 (P/K/E) have a higher frequency of conservation and these are important in maintaining the $\alpha$-helical structures within a motif. Clustering of several $\alpha$-helices within a tandem array of TPR motifs generates an amphipathic channel with a large amount of surface area, which allows the TPR domain to recognize its target protein [7].

A good understanding of the interaction of the TPR domain with molecular chaperones Hsp90 and Hsp70 came from cocrystallization study of TPR domains in Hop with pentapeptide MEEVD and octapeptide GPTIEEVD, which correspond to the C-termini of Hsp90 and Hsp70, respectively [8]. The side chains of several basic amino acids in the TPR groove were seen to establish interactions with the acidic side chains of the EEVD motif. These basic amino acids are referred to as ‘carboxylate clamp’ residues. With reference to the positions of the clamp residues in a three-motif TPR domain, the consensus is Lys$_2$ and Asn$_9$; Asn$_6$; Lys$_2$ and Arg$_6$. These amino acids are conserved and functionally important in most three-motif TPR containing co-chaperones of Hsp90 and Hsp70. Additional contacts between EEVD and the TPR groove involve hydrophobic interactions [9].

Hsp90 has been studied widely in animal and yeast model systems, but little is known by comparison about plant Hsp90. The Hsp90 family in the model plant Arabidopsis thaliana comprises of seven members: four closely related isoforms are cytosolic (AtHsp90-1 to AtHsp90-4), one is chloroplastic (AtHsp90-5), one mitochondrial (AtHsp90-6), and one is localized to the endoplasmic reticulum (ER) (AtHsp90-7) [10]. The occurrence of multiple Hsp90 isoforms that display both developmental and stress-responsive gene expression, suggests a range of specific functions for these proteins. The recent demonstration of the involvement of Hsp90 and co-chaperones RAR1 (required for M1a12 resistance) and SGT1 (suppressor of G2 allele of skp1), in plant disease resistance [11,12] has provided impetus for further investigation of the roles of Hsp90 in plant signaling pathways. Apart from RAR1 and SGT1, Hsp70 [13], high molecular weight immunophilins [14], Hop-like protein [15], PP5 [16], and p23-like proteins [17] have been found to associate with plant Hsp70. Plant orthologs of the previously characterized Hsp90/Hsp70 co-chaperones with TPR domains include Hop [15], PP5 [16], immunophilins like Cyp40 (SQUINT) [18], Rotamase AtFKBP62 (AtROF1) and AtFKBP65 (AtROF2) [19], PASTICCINO1 (AtPASt1) [20], AtFKBP12 (AtTWD1) [21,22], Translocon of the outer envelope of chloroplast (TOC64) [23] and AtCHIP [24]. Given the conservation and preponderance of the TPR domain in established and potential co-chaperones of the plant Hsp90/Hsp70 system, we set out to uncover all carboxylate clamp (CC) type TPR proteins (CC-TPR proteins) in Arabidopsis as an in silico approach and following the residues conserved for interaction with the EEVD motif of cytosolic Hsp90/ Hsp70. Here we report the identification of 24 new CC-TPR proteins in Arabidopsis, and similar as well as distinct CC-TPR proteins in rice (Oryza sativa), as putative Hsp90/Hsp70 interactors. Some of the newly uncovered proteins contain additional protein domains such as ankyrin, SET, octocaspase/Phox/Bem1p (Phox/Phb1), DnaJ, thioredoxin, FBD and F-box, and protein kinase and U-box, indicating novel functions that may add new dimensions to the Hsp90/Hsp70 chaperone complex in plants.

Methods

Identification of CC-TPR proteins in Arabidopsis

The term ‘TPR’ submitted as query at the InterPro home page: http://www.ebi.ac.uk/interpro/ [25] retrieved 31 entries of which IPR013026 was found to best conform to TPR structure and function. The ‘Taxonomic coverage’ of IPR013026 revealed 235 TPR proteins in the Arabidopsis proteome (taxon ID 3702). The sequences of all proteins and the Arabidopsis Genome Initiative Identifier (AGI ID) were recorded and confirmed against the database of National Centre for Biotechnology Information (NCBI) and each sequence was analyzed for the presence of TPR domain using InterProScan (http://www.ebi.ac.uk/InterProScan/) [26]. Since the known co-chaperones that interact with MEEVD of Hsp90 consist of three-motif TPR domain, proteins with one or more TPR domains comprising of three motifs were short-listed. Subsequently, these motifs were analyzed for the presence of the conserved residues (K$_5$N$_9$-N$_6$-K$_2$R$_6$). A second round of search was carried out where protein sequences with one or two TPR motifs were manually mapped for the presence of the second and/or third motif and the conserved residues (K$_5$N$_9$-N$_6$-K$_2$R$_6$). Following selection of the CC-TPR proteins, these proteins were searched for additional known domains and localization signal sequences. Multiple sequence alignment for each of the three motifs of the CC-TPR proteins identified in Arabidopsis (above-described search) and human Hop TPR2a as the reference sequence was used to build a statistical model of the corresponding motif using the software package HMMER 3.0 (http://hmmer.org/). Each of these three models was queried on the basis of HMM profile against TAIR9 - pep_20090619 (Arabidopsis annotation from TAIR) and Rice Genome Annotation Project (ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/O_sativa/annotation_dbs/pseudomolecules/version_6.0). The HMMER-selected proteins (E-value <10) were scanned for conserved residues (K$_5$N$_9$-N$_6$-K$_2$R$_6$), and BLAST and manual editing were used to remove redundancy.

Phylogenetic tree construction

Full-length sequences of CC-TPR proteins of Arabidopsis and rice were aligned using Clustal X 2.0.10. The phylogenetic tree was derived from the sequence comparisons using the neighboring method in Clustal X.

Protein localization prediction

The electronic Fluorescent Pictograph (eFP) Browser [27] from the Bio-Array Resource (BAR) (http://bar.utoronto.ca/) was used to predict the subcellular localization (SL) of the CC-TPR proteins. The information retrieved was based on computational prediction and/or experimental documentation compiled in SUBA; the Arabidopsis SUBcellular localization database [29]. If experimental documentation for SL was available for a protein, it was considered as significant. However, for proteins lacking experimental documentation we inferred data available from several prediction algorithms such as iPSORT (http://hc.imis.athena-innovation.gr/iPSORT/), LOCtree (http://cubic.bioc.columbia.edu/cgi-bin/vari/nair/loc/tree/query), MitoPred (http://bioapps.rit.albany.edu/MITOPRED/), MitoProt II (http://bioinfak.sbc.waseda.jp/iPSORT/), MitoPred (http://www.lifl.fr/cellbio/mtopred.html), MitoP (http://www.cbs.dtu.dk/services/MitoP/), PeroxP, Predotar (http://urgi.versailles.inra.fr/predotar/predotar.html), SubLoc (http://www.bioinfo.tsinghua.edu.cn/SubLoc/), Target P (http://www.ebs.dtu.dk/services/TargetP/) and WOLFPSORT (http://wolfpsort.org/). This data was cross-checked with the actual eFP Browser data that was generated using heuristic prediction algorithms [27].

Expression analyses using AtGenExpress Visualization Tool

Affymetrix microarray data provided by Weigel World (http://www.weigelworld.org/) was accessed using AtGenExpress Visual-
frozen. 24-epibrassinolide (EBR) or 0.01% ethanol (solvent for EBR) for 3 h and 12 h. After the treatment, the plant material was quick-frozen. Total RNA was prepared using SV Total RNA Isolation System (Promega). First strand cDNA was synthesized from 1 μg of total RNA using QuantTect Reverse Transcription Kit (Qiagen) and used as a template for quantitative RT-PCR (qRT-PCR). qRT-PCR was performed in 200 μl tubes with a Rotor-Gene RG-3000 real-time thermal cycling system (Corbett Research) using SYBR green to monitor double-strand DNA synthesis. Three independent biological samples were used with gene-specific primers (Table S1). Data were analyzed using Rotor-Gene 6.0.16 software (Corbett Research). Values were normalized using ubiquitin as the internal reference, and fold change in the expression level was calculated.

Yeast two-hybrid assay
To determine interaction of the two newly identified proteins, AtTPR1 and AtTPR2, with AtHsp90-2 by the yeast two-hybrid approach, AtHsp90-2 and AtTPR1/AtTPR2 coding sequences were cloned into bait and prey vector, respectively. AtHsp90-2 was amplified by PCR using the Arabidopsis Biological Resource Center (ABRC) cDNA clone C105037 and primers attB1-Hsp90-2F 5’-GGGG ACA AGT TTG TAC AAA AAA GCA GGC TCC ATG GCG GAC GCT GAA ACC TTT GCT TTC-3’ and attB2-Hsp90-2R 5’-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC GTC CAT CTC CAT CTT GAC TTC-3’. The PCR product was cloned into pDONR221 by in vitro BP recombination (Invitrogen) to generate pDONRHsp90-2, which was used in LR reaction with pDestDB (bait vector) to generate pDBHsp90-2. AtTPR1 and AtTPR2 cDNAs were synthesized from total RNA from Arabidopsis leaf using gene-specific gateway primers (attB1-TPR1F 5’-GGGG ACA AGT TTG TAC AAA AAA GCA GGC TCC ATG GAG AG-3’ and attB2-TPR1R 5’-GGGG ACA AGC TTC GTA CAA GAA AGC TGG GTC GTC TA TGG CTC TTC CAC TAA ACC CG-3’) as described for AtHsp90-2, into pDestAD (prey vector) to generate pDBAtTPR1 and pDBAtTPR2. PCR products were cloned, as described, into pDEST4 (prey vector) to generate pADTPR1 and pADTPR2. Following sequence verification, pDBHsp90-2 and pADTPR1/pADTPR2 were transformed into Y8930 and Y8800 yeast strains, respectively. Transformants were selected on synthetic complete (SC) media lacking either leucine (bait vector) or tryptophan (prey vector). Bait and prey transformants were then mated and selected on SC media lacking leucine and tryptophan. The interaction of pDBHsp90-2 with pADTPR1/pADTPR2 was assayed based on the ability of cells to grow on SC-Leu-Trp-His plus 3 mM 3-aminotriazole (3AT).

Plasmid expressing AtHsp90-2 with a cleavable N-terminal glutathione-S-transferase (GST) tag in pGEX-6p-1 (Hsp90-GST) was kindly provided by Dr. David Hubert, University of North Carolina. Plasmid expressing Hsp90AMEEV-GST was generated by PCR amplification and cloning of the PCR product into pGEX-6p-1 (GE Healthcare). The chitin-binding domain (CBD) affinity tag was fused to the C-terminus of TPR1 by cloning TPR1 cDNA in frame into pITYB2 (New England Biolabs) to prepare TPR1-CBD. Similarly, the GST tag was fused to the N-terminus of TPR2 by cloning TPR2 cDNA in frame into pGEX-6p-1 to prepare TPR2-GST fusion protein.

In vitro protein-binding assay
Plasmid expressing AtHsp90-2 with a cleavable N-terminal glutathione-S-transferase (GST) tag in pGEX-6p-1 (Hsp90-GST) was kindly provided by Dr. David Hubert, University of North Carolina. Plasmid expressing Hsp90AMEEV-GST was generated by PCR amplification and cloning of the PCR product into pGEX-6p-1 (GE Healthcare). The chitin-binding domain (CBD) affinity tag was fused to the C-terminus of TPR1 by cloning TPR1 cDNA in frame into pITYB2 (New England Biolabs) to prepare TPR1-CBD. Similarly, the GST tag was fused to the N-terminus of TPR2 by cloning TPR2 cDNA in frame into pGEX-6p-1 to prepare TPR2-GST fusion protein.

Hsp90-GST and Hsp90AMEEV-GST were expressed in BL21 cells. Expressed proteins were purified on Glutathione-Sepharose 4B beads as per the protocol provided by GE Healthcare. The beads were washed once with PBS buffer containing 500 mM NaCl, five times with PBS buffer, and once with cleavage buffer (50 mM Tris, pH 7.0, 150 mM NaCl, 1 mM EDTA and 1 mM DTT). The beads were incubated at 4°C overnight with cleavage buffer containing PreScission Protease to cleave the GST tag. The cleaved protein was eluted with the cleavage buffer. Fresh Glutathione-Sepharose 4B beads were
added to the eluate (cleaved Hsp90 and Hsp90ΔMEEVD) to remove the PreScission Protease. Purified Hsp90 and Hsp90ΔMEEVD were concentrated, dialyzed against 10 mM HEPES, pH 7.5, and 50 mM NaCl, and stored at −80°C until further use.

TPR1-CBD and TPR2-GST proteins were produced in E. coli strains ER2566 and BL21 cells and immobilized on chitin beads and Glutathione-Sepharose 4B beads, respectively. For in vitro binding assays, 50 μl aliquot of beads with immobilized proteins were incubated for 60 min on ice with 1 μg of purified Hsp90 or Hsp90ΔMEEVD in reaction buffer (20 mM HEPES, pH 7.5, 20 mM KCl, 1 mM MgCl₂, 0.01% NP-40) containing either no nucleotide, 5 mM ADP or 5 mM ATP. After incubation the supernatant was removed and the beads were washed thrice with the reaction buffer containing either no nucleotide, 5 mM ADP or 5 mM ATP. Proteins retained on the beads were extracted into SDS-sample buffer, separated by SDS-PAGE and immunoblotted with the R2 anti-Hsp90 antibody [33].

Results

Identification of CC-TPR proteins in Arabidopsis

Orthologs of most of the known CC-TPR co-chaperones of animal and yeast Hsp90 have also been identified in plants. To understand the extensiveness of CC-TPR proteins in the model plant Arabidopsis, the InterPro database was first searched for TPR proteins. The initial big list of 235 TPR proteins was narrowed down to 52 proteins on the criterion of proteins possessing a Hop TPR2a-like three-motif domain. The start and end sites of each of the three motifs in the 52 proteins were identified and the motifs were then searched for the conserved residues (K₅N₉-N₆-K₂R₆) responsible for interacting with the MEEVD motif of cytosolic Hsp90. This led to the identification of 24 proteins, including SQUINT, which appeared in the database as a two-motif TPR domain. Following from this example, proteins showing one or two motifs of TPR were manually searched for the conserved residues, leading to another 12 proteins being identified as CC-TPR proteins with potential to interact with Hsp90/Hsp70. Thus, a total of 36 CC-TPR proteins were identified in Arabidopsis (Table 1). Analyses of these genes in TAIR, which provides structural and functional annotation as well as links to different databases containing information of specific gene, transcript, and/or protein, led us to infer that 24 of the 36 genes in the list were novel, while 12 genes had been characterized previously in Arabidopsis or another plant species (Table 1).

As an additional search of TPR proteins in Arabidopsis, we used the HMMER program, which aims to detect remote homologs on the basis of mathematical models, to generate a list of proteins against each motif of the CC-TPR proteins. The number of proteins generated against each motif varied and each list of proteins (data not shown) could be divided into four categories: 1) All 36 proteins described in Table 1 were among the top hits identified for all three motifs. An additional 2-6 proteins were detected in this list of proteins, which on analysis fell in the second category of proteins; 2) True TPR domain proteins (entry no. IPRO13026), but lacking a three-motif TPR domain and/or the conserved carboxylate clamp residues. These proteins had been analysed earlier and rejected; 3) TPR-like domain containing proteins (InterPro entry no. IPRO11990). The motif structure of this domain varied in the number of amino acids in different proteins and there was no conservation of the carboxylate clamp residues; 4) Proteins with no TPR domain. In conclusion, the HMMER search identified the same 36 CC-TPR proteins, lending further support to the results shown in Table 1.

Previously known CC-TPR proteins, such as Hop (AtHop1, AtHop2 and AtHop3), immunophilins (AtROF1, AtROF2, PAS1, AtTWD1 and SQUINT), AtCHIP, TOC64 (AtTOC64-III, and AtTOC64-V) and AtPP5 were all present in the list represented in Table 1. Hop, immunophilins, CHIP and PP5 are known co-chaperones of Hsp90, while soybean Hop [15] and tomato PP5 [16] have been shown to act as co-chaperones of Hsp90. Of the newly identified proteins, TTL1 has been associated with salt sensitivity and altered abscisic acid (ABA) responses [35], but no links have been made of this protein to the Hsp90/Hsp70 chaperone machinery. The closest protein to TTLs in other organisms is Tpr2, a relatively new co-chaperone of mammalian Hsp90 that contains a DnaJ-like domain instead of the thioleoidin domain [35-37].

In addition to identifying members of a gene family, transcript data from NCBI also provides information on the number of splice variants for each gene family member. It is noted in Table 1 that there are two splice variants for PAS1, AtROF1, AtPP5, AtHop3, AtTPR8, AtTPR10 and AtTPR15, and three splice variants for AtTPR1. The DnaJ-domain containing AtTPR15 has two CC-TPR domains both of which are present in the first variant, but the second variant appears to be missing the first motif of the second TPR domain. With the exception of AtPP5 that has been shown to contain two splice variants localized in the cytoplasm/nucleus and ER, respectively [16], the splice variants of all other genes remain to be verified experimentally.

Multiple sequence alignments of excised TPR motifs of proteins listed in Table 1 against human Hop TPR2a as reference sequence showed high degree of conservation of the consensus residues K₅N₉-N₆-K₂R₆ (Table 2). In the case of proteins with more than one TPR domain (AtTTLs, AtTPR12-16), the domain with highest conservation was used for alignment. It can be seen in Table 2 that with one exception, the substitutions for K₅ and K₂ in the first and third motif, respectively, are mostly conservative, whereby Lys (K) is replaced with Arg (R). Since this substitution occurs in confirmed Hsp90 interactors such as Hop, it is concluded that this substitution does not interfere with the binding of the TPR domain to Hsp90/Hsp70. The substitution of Asn (N) with Gln (Q) in the first motif is also conservative. All others replacements are radical according to classification of amino acids by volume and polarity, with the K₅ to Gln (Q) replacement in AtTPR4 being most noteworthy. With the exception of AtPhox1 and AtPhox4, the consensus residue N₆ of the second motif is highly conserved in Arabidopsis proteins. It should be noted that Ala (A) and Met (M) substitute R₆ in the third motif of known co-chaperones AtHop1-3 and AtCHIP, respectively, indicating that some radical changes are accommodated in the TPR-Hsp90/ Hsp70 interaction. How such substitutions affect interaction with Hsp90/Hsp70 remains to be seen.

A notable feature of AtTPR5 is that the conserved N is detected in the fifth position of the second motif as opposed to the sixth position. If, however, the second motif is initiated at residue 117 instead of 118 in the protein sequence, then the N residue falls in the right place. The perfect match of residues in the first and third TPR motifs of AtTPR5 to the consensus K₅N₉ and K₂R₆, respectively, as well as the presence of a relatively conserved R residue after N in the second motif, suggest that AtTPR5 qualifies as a CC-TPR protein.
In silico characterization of the newly identified CC-TPR proteins in Arabidopsis

To obtain clues about the functions of the newly identified CC-TPR proteins, InterProScan against InterPro database was used to identify additional functional domains as well as subcellular localization signal sequences within these proteins. The known functional domains were highlighted on the output display page for each input FASTA sequence. Using this information the schematics of domain FASTA architecture of the new proteins were prepared (Figure 1). Eight of the proteins contained a single TPR domain, seven contained a single TPR domain plus another functional domain, three contained two TPR domains, and six contained two TPR domains plus another functional domain. The second functional domains in these proteins include SET, ankyrin, Table 1.

### Table 1. Properties of the Arabidopsis CC-TPR proteins.

| AGI ID  | Name            | Number of amino acids | Additional functional domains | Subcellular localization | mRNA species |
|---------|-----------------|-----------------------|-------------------------------|--------------------------|--------------|
| AT4G30480 | AtTPR1         | 161/208/277           |                               | C/N                      | 3            |
| AT1G04130 | AtTPR2         | 360                   |                               | C/N                      | 1            |
| AT1G04190 | AtTPR3         | 328                   |                               | C/N/M                    | 1            |
| AT1G04530 | AtTPR4         | 310                   |                               | C                        | 1            |
| AT1G56440 | AtTPR5         | 476                   |                               | N/P                      | 1            |
| AT1G58450 | AtTPR6         | 164                   |                               | M/C                      | 1            |
| AT5G21990 | AtTPR7         | 554                   |                               | M/N                      | 1            |
| AT4G08320 | AtTPR8         | 426/427               |                               | C/M/N/P                  | 2            |
| AT1G33400 | AtTPR9         | 798                   |                               | SET                      | 1            |
| AT3G04710 | AtTPR10        | 455/456               |                               | Ankyrin                  | 1            |
| AT2G25290 | AtPhox1*       | 697                   |                               | Phox/PB1                 | 2            |
| AT1G62390 | AtPhox2*       | 751                   |                               | Phox/PB1                 | 1            |
| AT5G20360 | AtPhox3*       | 809                   |                               | Phox/PB1                 | 1            |
| AT4G32070 | AtPhox4*       | 811                   |                               | Phox/PB1                 | 1            |
| AT4G22670 | AtTPR11        | 441                   |                               | Heat shock chaperonin-binding | V    | 1 |
| AT2G15790 | AtSquint       | 361                   |                               | Cyclophilin              | 1            |
| AT3G54010 | AtAPAS1        | 635/545               |                               | Peptidyld-prolyl-cis-trans isomerase | C/N | 2 |
| AT3G25230 | AtROF1         | 551/562               |                               | Peptidyld-prolyl-cis-trans isomerase | C  | 2 |
| AT5G45870 | AtROF2         | 578                   |                               | Peptidyld-prolyl-cis-trans isomerase | C/Pe | 1 |
| AT3G21640 | AtTWD1         | 365                   |                               | Peptidyld-prolyl-cis-trans isomerase | PM | 1 |
| AT3G07370 | AtCHIP         | 278                   |                               | U-box                    | 1            |
| AT2G42810 | AtPP5          | 464/538               |                               | PPS                      | 2            |
| AT3G17970 | AtToc64-III    | 589                   |                               | Amidase                  | 1            |
| AT5G09420 | AtToc64-V      | 603                   |                               | Amidase                  | 1            |
| AT1G78120 | AtTPR12        | 530                   |                               | M                        | 1            |
| AT5G10090 | AtTPR13        | 594                   |                               | M/N/P                    | 1            |
| AT5G65160 | AtTPR14        | 593                   |                               | M/N                      | 1            |
| AT2G41520 | AtTPR15        | 1077/1108             |                               | DnaJ                     | P/N          | 2 |
| AT5G12430 | AtTPR16        | 1165                  |                               | DnaJ                     | P/N          | 1 |
| AT1G53300 | AtTTL1         | 699                   |                               | Thioredoxin              | N/M/C        | 1 |
| AT3G14950 | AtTTL2         | 721                   |                               | Thioredoxin              | M/N/P        | 1 |
| AT2G42580 | AtTTL3         | 691                   |                               | Thioredoxin              | M/N/P        | 1 |
| AT3G58620 | AtTTL4         | 682                   |                               | Thioredoxin              | P/M/N        | 1 |
| AT1G12270 | Athop1         | 572                   |                               | Heat shock chaperonin-binding | C/N | 1 |
| AT1G62740 | Athop2         | 571                   |                               | Heat shock chaperonin-binding | P/M/N | 1 |
| AT4G12400 | Athop3         | 530/558               |                               | Heat shock chaperonin-binding | M/P/N | 2 |

The 24 new CC-TPR proteins are referred to as AtTPR1-16, AtPhox1-4 and AtTTL1-4, while the known CC-TPR proteins are referred to by their names. *represents new names of the proteins. For subcellular localization the bolded represent experimental documentation, while the italicized represent the most significant according to computational predictions. C, cytoplasm; ER, endoplasmic reticulum; M, mitochondria; N, nucleus; P, plastid; Pe, peroxisome, PM, plasma membrane; V, vacuole.

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Table 2. Multiple sequence alignment of excised TPR motifs of Arabidopsis proteins.

| Name | Helix 1A Hel: 1b | Helix 2 Hel: 2b | Helix 3A Hel: 3b |
|------|-----------------|-----------------|-----------------|
| KKN | 259 | MTYIT | NAYA |
| AtTPR1 | AT4G30480 | 105 | ANEA |
| KKN | 180 | NALV | RAEAHLEFKLEHFEDAVTDLKKILELDPSN |
| AtTPR2 | AT4G30480 | 105 | ANEA |
| KKN | 140 | VNLHNLGLLKESLRDCHRALRIDPYY |
| AtTPR3 | AT5G21990 | 103 | AQML |
| KKN | 146 | LACSL |
| AtTPR4 | AT4G08320 | 175 | AETL |
| KKN | 209 | AVFYC |
| AtTPR5 | AT1G56440 | 84 | SSSE |
| KKN | 118 | VTYAN |
| AtTPR6 | AT1G58450 | 10 | ANRK |
| KKN | 92 | VSCFL |
| AtTPR7 | AT5G21990 | 103 | AQML |
| KKN | 146 | LACSL |
| AtTPR8 | AT4G08320 | 175 | AETL |
| KKN | 209 | AVFYC |
| AtTPR9 | AT1G33400 | 63 | SLDL |
| KKN | 140 | A |
| AtTPR10 | AT3G04710 | 327 | AAEA |
| KKN | 126 | S |
| AtPhox3 | AT5G20360 | 126 | AQGL |
| KKN | 164 | SHVRA |
| AtPhox4 | AT4G32070 | 51 | ALEL |
| KKN | 89 | AYLRT |
| AtTPR11 | AT4G22670 | 123 | AQEA |
| KKN | 126 | S |
| AtSquint | AT2G15790 | 212 | VDFV |
| KKN | 200 | N |
| AtPAS1 | AT3G54010 | 310 | ADKI |
| KKN | 324 | AVYWA |
| AtROF1 | AT3G25230 | 400 | ASKK |
| KKN | 410 | AGKK |
| AtROF2 | AT5G48570 | 410 | AGKK |
| KKN | 410 | AGKK |
| AtTWD1 | AT3G21640 | 179 | ADRR |
| KKN | 230 | NPCHL |
| AtCHIP | AT3G07370 | 10 | AERL |
| KKN | 44 | PAYWT |
| AtPP5 | AT2G42810 | 13 | AEEF |
| KKN | 47 | AVYWA |
| AtTPR12 | AT1G78120 | 159 | PETL |
| KKN | 193 | PTYWP |
| AtTTL2 | AT3G14950 | 258 | PEEV |
| KKN | 258 | PEEV |
| AtTTL3 | AT2G42580 | 458 | VVRA |
| KKN | 492 | SVLYC |
| AtTTL4 | AT3G58620 | 449 | VAKA |
| KKN | 499 | AILYC |

The conserved residues are indicated in bold, and the substitutions in bold-italics. The numbers on the left of each motif refer to the amino acid positions in the sequences of proteins.
octicosapeptide/Phox/Bem1p (PB1), DnaJ, and thioredoxin (Figure 1). With the exception of DnaJ, these domains have not previously been linked with CC-TPR proteins.

The ankyrin repeat is a protein-protein interaction motif comprised of a tandemly repeated 33 amino acid sequence. Each repeat folds into a helix-loop-helix structure [38]. Ankyrin repeats are present in functionally diverse proteins involved in transcription initiation, cell-cycle regulation, ion transportation and signal transduction. This domain was identified in AtTPR10, which is predicted to localize to the plastid (Table 1).

As a conserved sequence motif made up of 130–140 amino acids, the SET domain occurs as a part of multidomain proteins involved in histone methylation, which regulates chromatin structure and gene transcription [39]. These enzymes use S-adenosylmethionine (AdoMet) as a donor substrate and add methyl groups to lysine residues of histone H3. SET domain proteins can also methylate non-histone proteins [40]. Recently it was shown that a human protein called SMYD2 interacts with Hsp90α, and that this interaction enhances the histone methyltransferase activity of SMYD2 [41]. The presence of the SET domain in AtTPR9 and its predicted localization to the nucleus (Table 1) suggest that this protein may be involved in methylating histone or non-histone proteins.

AtPhox1 to AtPhox4 constitute a gene family (Figure S1). In addition to the TPR domain, the AtPhox proteins contain the Phox/PB1 domain (Figure 1), which is known to mediate protein-protein interactions in cell processes such as cell polarity, pheromone signaling, cytoskeletal organization, osteoclastogenesis,
angiogenesis and early cardiovascular development [42]. Hetero-
dimerization of two PB1 domains is important in the formation of
cellular signaling complexes. PB1 domains can also
interact with other protein domains. The presence of Ser (S) in
the context of the TPR domain contributes to the formation of
the TPR domain.

The heat shock chaperonin-binding motif found in Hop is for
binding heat shock proteins. This domain is found singly or
interactions and various chaperone functions [44].

The heat shock chaperonin-binding motif found in Hop is for
binding heat shock proteins. This domain is found singly or
duplicated in proteins [45]. The newly identified protein AtTPR11
contains this domain. As would be expected, transcripts of proteins
with this domain are induced by high temperature.

Identification of CC-TPR proteins in rice

The HMM profile created with Arabidopsis sequences was used
to search for CC-TPR proteins in rice. The top hits for each of the
motifs were searched for the conserved carboxylate clamp
residues. Sequence alignments of each of the three motifs of the
top scoring CC-TPR proteins in rice are shown in Table 3. In case
of proteins with more than one three-motif TPR domain, the most
conserved domain was used for alignment. Proteins with both
conservative and non-conservative amino acid substitutions in the
carboxylate clamp residues were identified. The protein sequences
of CC-TPR proteins identified in Arabidopsis (Table 1) and rice
(Table 3) were used to generate a phylogenetic tree (Figure 2). The
various additional domains detected in Arabidopsis CC-TPR
proteins were also found in rice CC-TPR proteins. In addition,
we found that unlike in Arabidopsis, rice contains numerous ankyrin
containing CC-TPR proteins (Figure 2, highlighted in red), one
CC-TPR protein with FBD and F-box (highlighted in green), and
one CC-TPR protein with protein kinase (STYKc) and U-box
(highlighted in blue). The latter two proteins in rice likely expand
the ability of the Hsp90/Hsp70 system to link cellular proteins to
the ubiquitin proteasome system.

Expression patterns of CC-TPR proteins in Arabidopsis

A collaborative microarray project (based on Affymetrix ATH1
arrays), dubbed AtGenExpress, has utilized 79 different Arabidopsis
samples in triplicate to generate expression data [29,30]. We
rationalized that the expression patterns of Hsp90 co-chaperones
must overlap to some degree with the expression patterns of Hsp90
family members. Data to this end could help formulate functional
hypotheses for these proteins in the context of the Hsp90/Hsp70
chaperone machinery. We first performed e-Northern analysis
with the Expression Browser at BAR using the AtGenExpress
extended tissue and stress series data sets for the AtHsp90 gene
family. The Arabidopsis Hsp90 gene family members are referred to as
AtHsp90-1 (AT3G32840), AtHsp90-2 (AT3G35630), AtHsp90-3
(At3G356010), AtHsp90-4 (AT3G35600), AtHsp90-5 (AT2G4030),
AtHsp90-6 (AT3G07770), and AtHsp90-7 (AT4G24190). AtHsp90-1
to AtHsp90-4 are cytoplasmic, AtHsp90-5 is plastidial, AtHsp90-6
and AtHsp90-7 are localized to the mitochondria and ER, respectively [10]. Of these, AtHsp90-3 was not present on the ATH1 array and is therefore missing from our analysis.

When absolute expression values retrieved by AVT were used
to compare the expression levels of AtHsp90 gene family members,
AtHsp90-4 was found to have the highest level of constitutive expression, and AtHsp90-1 to be most responsive to HS (data not
shown). For e-Northern data from BAR depicting tissue
expression, the median level of expression across all samples
displayed for a particular gene is used as control value for
calculating the relative level. Data in Figure 3A shows that with
the exception of AtHsp90-1, which is highly expressed in seeds,
most other members have higher expression in the shoot apex,
followed by roots and flowers. Further dissection of the flower into
sepalas, petals, stamens and carpels revealed that in general carpels
and petals have higher levels of AtHsp90 transcripts as compared to
the other two floral organs (Figure 3B). AtHsp90 genes are induced
by various abiotic stresses, such as cold, salt, UV-B, etc., although
maximum induction is in response to HS (Figure 3C). These data
are in accordance with our earlier observations in Brassica napus of
the relatively high expression of Hsp90 in apex, flowers and seeds,
as well as of its responsiveness to cold temperatures [46].

To compare the expression levels of CC-TPR genes (AtTPR7 and
AtPhox1 were not present on the ATH1 array) in different plant
parts, the absolute expression values retrieved by AVT were plotted
(Figure 4). This was done because the low level expression of some TPR genes led to artifacts in the output of the Expression
Browser. Several conclusions can be drawn from the data
represented in Figure 4: 1) most genes are expressed in different
plant parts, albeit at much lower levels than the highly expressed
AtHsp90-4; 2) of the newly identified genes, AtTPR11 and AtPhox2
are expressed at relatively high levels in most tissues (Figure 4A),
suggesting that the encoded proteins may be general co-
chaperones of Hsp90/Hsp70; 3) of the known co-chaperones,
immunophilins (AtPAS1, AtROF1 and 2) and Hop (AtHop1, 2 and
3), are expressed at relatively high levels (Figure 4B) and, like their
animal counterparts, are abundant co-chaperones of plant Hsp90.
To the near exclusive expression of AtTPR13 and AtTPR14 in
stamens, and the relatively high expression of AtPhox genes,
AtTPR16, AtTTLL2, AtTPR6 and AtTPR18 in stamens as compared to
other floral organs (Figure 4C) is noteworthy. The immunophone
d molecules and Hop, similar to AtHsp90, have predominant
expression in petals and carpels (Figure 4D). Together, these data
dicate that the Hsp90/Hsp70 chaperone machinery components
are expressed in all plant tissues, albeit at different levels. It is
possible that some components may function as general co-
chaperones, while others may have tissue-specific functions within
the context of the Hsp90/Hsp70 chaperone machinery. The
expression profiles of the newly identified CC-TPR proteins
provide an initial working hypothesis for delineating their
functions.

B. Northern analysis of CC-TPR genes by BAR and AVT using
the stress series data set indicated that heat is the most prominent
signal for induction of transcript abundance. AtTPR2, AtTPR5 (by
AVT), AtTPR8, AtTPR10, AtPhox2 and AtTPR11 are heat-
responsive in both root and shoot (Figure 5A, B). The fold-change
values for AtTTLL2, AtTPR13 and AtTPR18 in the output of
Figure 5 are unreliable due to their low expression; these genes are
therefore not considered as heat-responsive. AtROF1, AtROF2,
AtHop2, AtHop3 and AtSquint are also heat-induced both in root and
shoot (Figure 5C, D), an attribute that has been noted before
[15,19]. The heat-induced expression patterns of CC-TPR genes
are similar to that of AtHsp90-1. This observation supports a co-
chaperone role for the encoded proteins during HS. The co-

PLOS ONE | www.plosone.org 8 September 2010 | Volume 5 | Issue 9 | e12761
| Locus ID | Motif 1 | Motif 2 | Motif 3 |
|----------|---------|---------|---------|
| Os04g59350 | 105 | 64 | AINQGKGERLYVEQIYYGKDEATEAQPY |
| Os09g13340 | 14 | 329 | RSLCWHHMGNGGKALLDAYECRKLRPDW |
| Os02g29150 | 46 | 353 | ATEL |
| Os03g50010 | 470 | 14 | ATEL |
| Os05g31062 | 98 | 12 | LLSH |
| Os06g06760 | 154 | 15 | ADHH |

**Carboxylate Clamp-TPR Proteins**

The observed residues are indicated in bold, and the substitutions in italics. The numbers on the left of each motif refer to the amino acid positions in the sequence of proteins.
expression of this subset of genes with AtHsp90-1 was confirmed using Expression Angler. AtHsp90-1 was used as query in AtGenExpress stress series, and the data generated by Expression Angler was searched for the presence of CC-TPR genes during HS. AtROF1, AtROF2, AtHop2, AtHop3, AtTPR2, AtTPR10 and AtTPR11 were within the top 156 proteins that are HS-induced and co-expressed with AtHsp90-1 (data not shown). As a final confirmation, the HS response of AtTPR2, AtTPR3, AtTPR8, AtPhox2, AtTPR10 and AtTPR11 in leaf tissue was checked by qRT-PCR. All of these genes showed induction of expression in response to heat (Figure 6A).

Analysis of the AtGenExpress data set revealed some interesting information. One was the stress and ABA-responsive expression of the AtPhox gene family. AtPhox2 was found induced by heat (Figure 5), AtPhox3 by cold and salt stress, and AtPhox4 by cold, osmotic and salt stress, as well as pathogen infection. In accordance with the role of ABA in stress tolerance [47], AtPhox3 and 4 were found to be ABA-responsive (data can be retrieved by AVT). The second interesting observation was the brassinosteroid (BR) responsive expression of the AtTTL gene family. Three out of four TTL genes that have wide spread tissue expression, were found to be most responsive to BR as compared to other hormones. Since BR is a relatively new hormone [48,49], the list of BR-regulated genes is still growing. To add AtTTL genes to this list we confirmed the BR-responsive expression of AtTTL1, AtTTL3 and AtTTL4 in Arabidopsis seedlings by qRT-PCR. All three genes showed 2 to 3-fold induction by BR at 12 h (Figure 6B). These results strongly suggest that AtTTL1, 3 and 4 are BR response genes.

Expression patterns of CC-TPR proteins in rice

We searched tissue-specific and stress-related libraries in the rice MPSS database (http://mpss.udel.edu/rice/) using 17 nucleotides long signatures to obtain information on the relative transcript abundance of rice CC-TPR genes. Of the 35 rice CC-TPR genes being reported here, no expression was detected for seven genes (Os02g29190, Os02g29210, Os01g07640, Os05g31056, Os05g50990, Os04g45480 and Os02g29150) in the tested conditions. Genes that were widely expressed (several tissues) at relatively high levels (Figure 7A) and most strongly induced by stress (salinity, drought and cold) (Figure 7B) are Os10g34540 (AtTPR1), Os08g41390 (AtROF1), Os05g11550 (AtTPR5), Os05g03010 (AtTPR6), Os06g00750 (AtToc64-III), Os03g50010 (AtToc64-V), Os02g18100 (AtTPR8), Os04g50010 (AtTPR11), AtTTL1, AtTTL2 and AtTTL4 in Arabidopsis seedlings by qRT-PCR. All three genes showed 2 to 3-fold induction by BR at 12 h (Figure 6B). These results strongly suggest that AtTTL1, 3 and 4 are BR response genes.
Os05g11990 and Os01g42960 (Figure 7B), together with their sequence and structural similarities with AtTTLs (Figure 2), suggest that these proteins are functional orthologs of each other. The protein with the unique combination of CC-TPR, protein kinase, and U-box domains (Os06g06760) was found expressed at low levels in immature panicle and to be induced by drought stress.

AtTPR1 and AtTPR2 interact with Hsp90 by the molecular ‘clamp’ mechanism

We applied yeast two-hybrid and in vitro binding assays as a proof-of-concept trial for analysis of the newly identified proteins for binding to Hsp90. AtHsp90-2 fused to the DNA-binding domain (pDBHsp90-2) and AtTPR1/TPR2 fused to the activation domain (pADTPR1/TPR2) were co-expressed in yeast. Growth of yeast on selection medium when co-transformed with pDBHsp90-2 and pADTPR1/TPR2 only, but no other plasmid combination (Figure 8A), indicated positive interaction between Hsp90 and AtTPR1/TPR2. This interaction was further validated in in vitro binding assays. TPR1-CBD and TPR2-GST immobilized on chitin and Glutathione Sepharose 4B beads, respectively, were incubated with purified recombinant AtHsp90-2 in the absence or presence of ATP and ADP. Both TPR1-CBD (Figure 8B, top panel) and TPR2-GST (bottom panel) bound Hsp90 in a nucleotide independent manner, but no binding was seen to CBD or GST alone. Neither TPR1-CBD nor TPR2-GST bound to Hsp90AMEEVD, confirming that the interaction requires MEEVD for carboxylate clamp formation. These results provide evidence for the validity and success of our in silico strategy of identifying TPR proteins with potential to interact with Hsp90/ Hsp70.

Figure 3. Expression profiles of AtHsp90 genes. (A) e-Northern results for expression of AtHsp90 genes in root, stem (second internode), leaf (cauline leaves), apex (shoot apex, inflorescence), flowers (stage 12), and seeds (stage 10, without siliques). (B) e-Northern results for expression of AtHsp90 genes in individual floral organs (flowers stage 12), sepals, petals, stamens and carpels. (C) e-Northern results for expression of AtHsp90 genes in response to different abiotic stresses. The control is mock treatment at each time point. The colour scale indicates the log2-level of expression above or below the median. Dark red indicates more than 4-fold above the median, while dark blue indicates 4-fold below. The clustering tree can be seen to the right of the heatmap.
doi:10.1371/journal.pone.0012761.g003
Identification of new CC-TPR proteins in Arabidopsis and rice by in silico methods

Co-chaperones are an integral part of the Hsp90/Hsp70 chaperone protein folding machinery, which has key roles in numerous cellular processes [1–5]. The known CC-TPR co-chaperones (Hop, immunophilins, PP5, CHIP, TOM70, Tpr2) bind to the MEEVD motif of Hsp90 and regulate the functions of Hsp90 [5,8]. Orthologs of these are present in plants, and mutations in some have shown striking phenotypes. Due to the significance of the CC-TPR co-chaperones in the biochemical regulation of the Hsp90/Hsp70 machinery, we set out to uncover all CC-TPR proteins encoded by the model plant Arabidopsis genome based on the information available through studies in animal and yeast systems. The confirmation of two of the 24 newly identified CC-TPR candidates to bind to the MEEVD motif of Hsp90 indicates a high probability for these proteins as bona fide interactors of either Hsp90 or Hsp70 or both. This data set provides a useful framework that will accelerate studies of the Hsp90/Hsp70 chaperone machinery in plants, as well as of the newly identified CC-TPR proteins, most of which have no assigned functions.

To predict the CC-TPR co-chaperone network in Arabidopsis, sequences with the well characterized protein interaction domain called ‘TPR’ were extracted from the InterPro database, followed by careful identification of the conserved residues involved in binding with the MEEVD motif of Hsp90 [8]. Orthologs of these are present in plants, and mutations in some have shown striking phenotypes. Due to the significance of the CC-TPR co-chaperones in the biochemical regulation of the Hsp90/Hsp70 machinery, we set out to uncover all CC-TPR proteins encoded by the model plant Arabidopsis genome based on the information available through studies in animal and yeast systems. The confirmation of two of the 24 newly identified CC-TPR candidates to bind to the MEEVD motif of Hsp90 indicates a high probability for these proteins as bona fide interactors of either Hsp90 or Hsp70 or both. This data set provides a useful framework that will accelerate studies of the Hsp90/Hsp70 chaperone machinery in plants, as well as of the newly identified CC-TPR proteins, most of which have no assigned functions.

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Figure 5. Expression profiles of *Arabidopsis* CC-TPR genes in response to HS. e-Northern results for expression of new CC-TPR genes in roots (A) and shoots (B) of seedlings exposed to HS. e-Northern results for expression of known CC-TPR co-chaperones in roots (C) and shoots (D) of seedlings exposed to HS.

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proteins in a genome-wide context with potential to interact with Hsp90/Hsp70 by the carboxylate clamp mechanism.

CC-TPR proteins can bind to both Hsp90 and Hsp70

The interaction of Hsp90 and Hsp70 with TPR proteins occurs via the conserved C-terminal EEVD motif in both Hsp90 and Hsp70 and the conserved carboxylate clamp residues (K$_5$N$_9$N$_{13}$-K$_2$R$_6$) in the TPR domain [8]. Additional contacts involving charged and hydrophobic amino acids determine the specificity of interaction. In case of Hop, Hsp70 and Hsp90 interact with two separate domains, i.e. TPR1 and TPR2a, respectively [45], but in the case of CHIP a single TPR binds either chaperone protein [50]. This is accomplished by accommodating either the methionine of Hsp90 (DDTSRMEEV) or the isoleucine of Hsp70 (GSQPTEEEV) into a hydrophobic pocket of CHIP, which is not present in Hop, resulting in the peptide being twisted into a conformation so that no further specific contacts are required [50]. Additional examples of TPR proteins binding to both Hsp90 and Hsp70 include the yeast CNS1 [51], and the human Tpr2 [36]. CNS1 binds to both Hsp90 and Hsp70 with comparable affinities and while it exerts no influence on the ATPase activity of Hsp90, it activates the ATPase activity of Hsp70 up to 30-fold. Tpr2 binds Hsp90 with slightly lower affinity as compared to Hsp70, but requires ATP for binding Hsp70 in the presence of Hsp90. The DnaJ homologus J domain in Tpr2 stimulates ATP hydrolysis and polypeptide binding by Hsp70 [36]. These data bring to light the importance of TPR co-chaperones and of Hsp70 regulation in the context of the Hsp90 chaperone cycle, and raise the possibility that the TPR proteins identified herein may interact with either Hsp90 or Hsp70 or both. Experimental analyses of interaction with both Hsp90 and Hsp70 are required for each of these proteins to understand their mode of action and its implications within the context of the Hsp90/Hsp70 chaperone machinery.

Figure 6. Transcript expression analysis by qRT-PCR in response to HS and BR treatment. (A) For HS treatment, 10 day-old Arabidopsis seedlings were exposed to 38°C for 1 and 3 h at which time the plant tissue above the medium was collected and quick-frozen for RNA isolation. (B) BR treatment was given to 21 day-old seedlings for 3 and 12 h and the tissue was collected as in A. Transcripts were analyzed by qRT-PCR. Bars indicate mean ± SD. doi:10.1371/journal.pone.0012761.g006
AtTPR1 and AtTPR2 bind Hsp90 through its MEEVD motif

AtTPR1 and AtTPR2 were verified for binding to Hsp90 as proof-of-concept of interaction of newly identified CC-TPR proteins with Hsp90 by the ‘carboxylate clamp’ mechanism. Both proteins bound Hsp90 in yeast two-hybrid and in vitro binding assays. Neither protein bound to Hsp90 lacking MEEVD, indicating that interaction occurs by the ‘carboxylate clamp’ mechanism [52,53]. During the course of this study, a tomato TPR protein (SlTPR1) with highest similarity to AtTPR1 and Os10g34540 was demonstrated to interact with ethylene receptors, and its overexpression in tomato and Arabidopsis caused a range of developmental phenotypes [54]. Although no connections were made with Hsp90/Hsp70, we checked and found the presence of the consensus carboxylate clamp residues in the tomato protein, suggesting that it is an interactor of Hsp90/Hsp70. Based on the above information, it can be expected that important cellular functions will be unveiled in the future for the newly CC-TPR identified proteins.

Putative functions of the CC-TPR proteins

The different subcellular localization possibilities and the different combinations of protein domains (Table 1, Figures 1 and 2), suggest a diverse range of functions for the CC-TPR proteins, which likely add to and/or regulate the functional capacity of the Hsp90/Hsp70 chaperone system. Proteins that have a single TPR domain and no additional known domains may function as co-chaperones by modulating the ATPase activity of Hsp90/Hsp70. It is possible that their functional specificity is derived, at least in part, through specific temporal and spatial expression patterns. Proteins with more than one TPR domain or...
other domains like DnaJ, ankyrin, or Phox/PBI may function additionally through recruitment of other proteins. The presence of the SET domain in AtTPR9 and Os10g36250, together with the high probability of AtTPR9 protein to be localized in the nucleus (Table 1) suggests that it cooperates with the Hsp90/Hsp70 machinery in the process of chromatin remodelling [2]. Recent identification of the targets of thioredoxins in plants suggests that these proteins could influence nearly every major cellular process [55]. The involvement of AtTTL1 in ABA signaling pathway and stress responses [35] has set the stage for further investigation of the functions of TTLs along these lines. Subcellular localization of a protein can provide clues to its functions. AtTPR10 with one TPR domain and ankyrin repeats has a high probability of being localized to the plastid. Since the plastidial Hsp90 and Hsp70 lack the EEVD motif at their C-terminus, we speculate that the interaction of the TPR domain is limited to cytosolic Hsp90/Hsp70, which chaperones the trafficking of precursor proteins to the chloroplast [23]. Whether the ankyrin domain recruits a client for the cytosolic Hsp90/Hsp70 system or a plastidial partner for AtTPR10 itself, are questions that need to be answered in the future. Experimental documentation of the plastidial localization of AtTPR10 is a prerequisite for addressing the functions of this protein. The identification of a protein in rice (Os06g06760) that contains both protein kinase and U-box domains is intriguing. To date, no such combination of domains has been noted in a CC-TPR protein. As a unique CC-TPR protein, functional analysis of this protein should be a priority.

In conclusion, the present study has uncovered a number of new potential interactors of Hsp90/Hsp70: future investigations of these will provide a better understanding of the Hsp90/Hsp70 chaperone machinery, its functions and its mode of action in plant cells. Due to the critical requirement of the Hsp90/Hsp70 system for cell viability and normal functioning of the cell, which has ramifications in human health, knowledge of this system is a high priority in any model organism.

Supporting Information

Figure S1  Amino acid sequence alignment of AtPhox1-4. The numbers on the side indicate the amino acid positions in the proteins. Alignment was performed using MEGA4 software. Black, grey and light grey shading indicates 100%, 75% and 50% conservation of amino acids, respectively.

Found at: doi:10.1371/journal.pone.0012761.s001 (3.51 MB TIF)

Figure S2  Amino acid sequence alignment of of AtTTL1-4. The numbers on the side indicate the amino acid positions in the proteins. Alignment was performed using MEGA4 software. Black, grey and light grey shading indicates 100%, 75% and 50% conservation of amino acids, respectively.

Found at: doi:10.1371/journal.pone.0012761.s002 (3.41 MB TIF)
Table S1 Sequences of primers used in quantitative RT-PCR (qPCR) analysis. Found at: doi:10.1371/journal.pone.0012761.s003 (0.03 MB DOC)

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Author Contributions
Conceived and designed the experiments: BDP SG PK. Performed the experiments: BDP SG PK. Analyzed the data: BDP SG PK. Contributed reagents/materials/analysis tools: PK. Wrote the paper: BDP SG PK.

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