SHOC1 and PTD form an XPF–ERCC1-like complex that is required for formation of class I crossovers

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Summary

Two distinct pathways for meiotic crossover formation coexist in most eukaryotes. The *Arabidopsis* SHOC1 protein is required for class I crossovers and shows sequence similarity with the XPF endonuclease family. Active XPF endonucleases form a heterodimer with ERCC1 proteins. Here, we show that PTD, an ERCC1-like protein, is required for class-I-interfering crossovers along with SHOC1, MSH4, MSH5, MER3 and MLH3. SHOC1 interacts with PTD in a two-hybrid assay, through its XPF-like nuclease–$(HhH)^2$ domain. We propose that a XPF–ERCC1-like heterodimer, represented by SHOC1 and PTD in *Arabidopsis*, involving Zip2 in *Saccharomyces cerevisiae* and C9orf84 in human, is required for formation of class I crossovers.

Key words: Crossover, Meiosis, Recombination, XPF, ERCC1

Introduction

Meiotic crossovers (COs) not only generate diversity in offspring, but in most eukaryotes they are also essential for the completion of meiosis (Gerton and Hawley, 2005). Two alternative CO formation pathways, resulting in class I and class II COs, coexist in most eukaryotes. These two pathways involve a distinct set of proteins. The major pathway, the class I pathway, relies on a series of proteins called the ZMMs (for Zip1, Zip2, Zip3 and Zip4, Mer3 and Msh4–Msh5) and on the Mlh1–Mlh3 heteroduplex, first identified in *Saccharomyces cerevisiae* and conserved in a large range of eukaryotes (Borner et al., 2004; Lynn et al., 2007; Youds and Boulton, 2011). In *Arabidopsis*, the ZMM proteins and MLH1–MLH3 are also required for the formation of class I COs, which represents ~85% of the total number of meiotic COs (Chelysheva et al., 2007; Dion et al., 2007; Higgins et al., 2004; Higgins et al., 2008a; Jackson et al., 2006; Mercier et al., 2005).

Class II COs, rely on the heteroduplex Mus81–Eme1 (De Los Santos et al., 2003; Hollingsworth and Brill, 2004; Whitby, 2005) and represent 5–10% of COs in *Arabidopsis* (Berchowitz et al., 2007; Higgins et al., 2008b). In addition to different genetic requirements, Class I and Class II COs have different distributions. Class I COs are sensitive to genetic interference; they have a tendency to form at a distance from each other, and consequently they are more evenly distributed than expected by chance. By contrast, distribution of class II COs is not controlled by interference (Berchowitz and Copenhaver, 2010; Mezard et al., 2007).

We previously identified the ZMM protein SHOC1 (shortage in chiasma 1) and showed that it is essential for class I CO formation in *Arabidopsis thaliana*. However, its involvement in the implementation of interference remains unclear. SHOC1 homologs are detected in a large range of eukaryotes, and include the human C9orf84 gene (Macaisne et al., 2008). SHOC1 shows similarity to the yeast ZMM protein Zip2, which is involved in the formation of class I COs in *S. cerevisiae* (Chua and Roeder, 1998). All SHOC1 homologs share a common structure, with highest conservation in their nuclease–helix–hairpin–helix $(HhH)^2$ region, which is similar to the nuclease–$(HhH)^2$ domain combination at the C-terminus of XPF family proteins. However, the consensus active site motif of XPF does not seem to be conserved in SHOC1 (Macaisne et al., 2008).

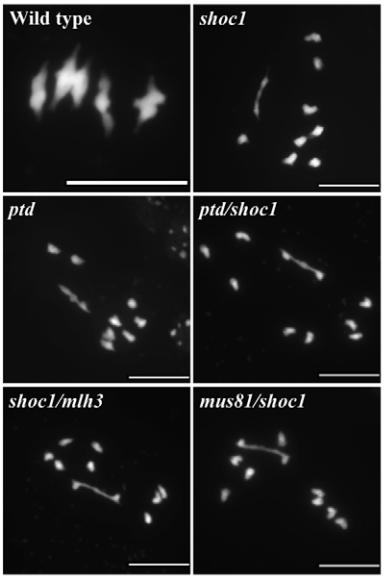
XPF-related proteins are highly conserved endonucleases that are involved in branched-DNA structure recognition and processing. Active XPF proteins form a heterodimer with a non-catalytic subunit belonging to the ERCC1 protein family. In most eukaryotes, two XPF–ERCC1-related complexes have a role in somatic DNA repair: the canonical XPF–ERCC1 complex, also known as RAD1–RAD10 in plants and fungi, and the MUS81–EME1 heterodimer, which is also involved in class II meiotic CO formation (Ciccia et al., 2008; Gaillard et al., 2003; Nishino et al., 2006; Osman et al., 2003). Interestingly, an *Arabidopsis* protein, PTD (ParTing Dancer), has sequence similarity with the ERCC1 protein family and is involved in meiotic CO formation (Wijeratne et al., 2006). Thus, an attractive model would be that SHOC1 and PTD form an XPF–ERCC1-like complex that recognizes specific DNA structures to promote class I CO formation. However, from past reports, the exact role of PTD in CO formation was unclear. In particular, it was unknown whether PTD was specifically involved in class I CO, or required for a less-specific step further upstream in the recombination pathway, as is the case for the protein ASY1 for instance (Sanchez-Moran et al., 2008). In addition, CO levels in *ptd* mutants were previously reported to be substantially higher than in the *shoc1* mutants (Macaisne et al., 2008; Wijeratne et al., 2006), arguing against a simple XPF–ERCC1-like complex.

Here, we propose that a XPF–ERCC1-like heterodimer, represented by SHOC1 and PTD in *Arabidopsis*, and involving Zip2 in *S. cerevisiae* and C9orf84 in human, is required for class I CO formation.

Results and Discussion

PTD is required for class I CO formation

CO levels in *ptd* mutants were previously reported to be substantially higher than in the *shoc1* mutants, but CO counts might be sensitive to growth conditions (Francis et al., 2007) and/or counting methods (i.e. diakinesis vs metaphase I) (Armstrong



B

	Genotype	Chiasma per cell	SNK grouping
	Wild type	9.20 ±1.00 (n=55)	
single mutants	*ptd*	1 41 ±1.10 (n=100)	*a*
	shoc1	1.27 ±1.06 (n=88)	*a*
	msh5	1.41 ±1.15 (n=133)	*a*
	mer3	2.25 ±1.33 (n=311)	*b*
	mlh3	3.64 ±1.75 (n=103)	*c*
double mutants	*ptd/msh5*	1.40 ±1.01 (n=42)	*a*
	shoc1/msh5	1.28 ±0.98 (n=60)	*a*
	shoc1/ptd	1.42 ±1.20 (n=71)	*a*
	shoc1/msh4	1.19 ±1.04 (n=64)	*a*
	mer3/msh4	1.35 ±1.05 (n=204)	*a*
	shoc1/mer3	1.41 ±0.92 (n=61)	*a*
	mlh3/msh5	1.52 ±1.17 (n=133)	*a*
	shoc1/mlh3	1.69 ±1.02 (n=67)	*a*
	shoc1/mus81	0.85 ±0.78 (n=59)	*d*

Fig. 1. Chiasma frequencies. (**A**) Representative examples of metaphase I in indicated genotypes, showing five bivalents in the wild type and a mixture of bivalents and univalents for the mutants. Scale bars: 10 µm (**B**) Average number of chiasma per cell counted at metaphase I, in single and double mutants compared with the wild type. The number of cells counted is indicated in brackets. The means that are followed by different letters (a, b or c) are significantly different from each other, according to the Student–Newman–Keuls (SNK) multiple range test ($P<0.05$). Chiasma number is not significantly different in any mutant genotypes, except *mer3* and *mlh3* which have more chiasma, and *shoc1/mus81*, which has less chiasma than the other genotypes.

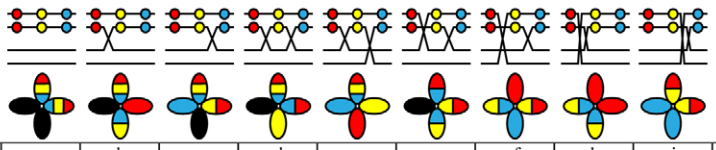
and Jones, 2003). We thus compared the number of chiasma in parallel, under exactly the same conditions, in the *ptd* and *shoc1* mutants, compared with the wild type and several known *zmm* mutants, by studying the shape of metaphase I bivalents (Sanchez Moran et al., 2001) (Fig. 1A). The chiasma frequency per cell was 9.2±1 in the wild type, 1.41±1.1 in *ptd*, 1.27±1.06 in *shoc1* and 1.41±1.15 in *msh5* mutants. Thus, under the same conditions, the level of residual chiasma in *ptd* mutants was not significantly different from that in *shoc1* and *msh5*, suggesting that they act in the same pathway (Fig. 1B).

It was previously unknown whether PTD belonged to the ZMM epistasis group. A previous report showed that the number of residual chiasma in *ptd* mutants per cell and per chromosome is randomly distributed (Wijeratne et al., 2006), but these data do not provide information on chiasma distribution within chromosomes, and thus do not imply that interference is impaired in this mutant. It was therefore unclear whether PTD was specifically involved in class I CO formation or required for a less-specific step further upstream in the recombination pathway. To test whether *PTD* acts in class I pathway, similarly to *MSH5* and *SHOC1*, we performed epistasis tests. There was no significant difference in chiasma counts between the two double mutants (*ptd/msh5* and *ptd/shoc1*) or the corresponding single mutants, with 1.40±1.01 in the *ptd/msh5* double mutant and 1.42±1.20 in *ptd/shoc1* (Fig. 1B). Thus, *PTD* belongs to the same epistasis group as *MSH5* and *SHOC1* and is thus required for class I CO formation.

Residual COs in *shoc1* do not display interference

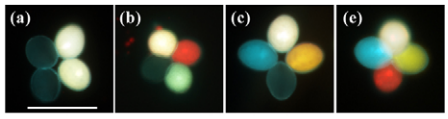
Because only class I COs are sensitive to interference, the distribution of residual CO in a *zmm* mutant is not expected to be controlled by interference. We quantified interference in the *shoc1* mutant compared with the wild type using the fluorescent tetrad lines (FTL) tool developed previously (Berchowitz and Copenhaver, 2008). The FTL system is a visual assay based on fluorescent proteins produced in the pollen grains of the quartet mutant (*qrt1*), in which the pollen grains remain attached as tetrads (Fig. 2A,B). We generated *shoc1-1 qrt1-2* plants that were heterozygous for three genetically linked reporter transgenes defining two adjacent

A



Tetrad class	a	b	c	d	e	g	f	h	i	Total
Wild type	9460	1446	1474	9	13	3	16	7	9	12437
shoc1	2918	205	185	3	2	2	3	0	0	3318

B



C

	Wild type	*shoc1*
d I1 (cM)	6.15 ±0.16	3.20 ±0.21
d I2 (cM)	6.31 ±0.16	2.94 ±0.20
d I1 with CO in I2	1.35 ±0.21	2.56 ±0.79
d I1 without CO in I2	6.82 ±0.18	3.28 ±0.22
IR	0.1973 ±0.0308	0.7812 ±0.2463

Fig. 2. Interference assay in the wild type and *shoc1* mutant. (**A**) The number of tetrads observed in wild type and *shoc1*, according to the nomenclature of Berchowitz and Copenhaver (Berchowitz and Copenhaver, 2008). A schematic representation of the corresponding CO events is shown above each class of tetrad. (**B**) Examples of images of pollen grain tetrads. The class of tetrad is indicated in brackets (YFP, CFP and DsRed images were merged). Scale bar: 50 µm. (**C**) d I1 and d I2 are map distances of intervals I1 and I2, respectively. d I1 with COs in I2 and d I1 without COs in I2 are map distances in I1 when a CO occurred in I2 and when no COs occurred in I2, respectively. Interference ratio (IR) is the ratio of d I1 with COs in I2 to d I1 without COs in I2 (Berchowitz and Copenhaver, 2008).

intervals on chromosome 5 (referred to as I1 and I2) (Fig. 2C). The I1 and I2 map distances in *shoc1* were reduced by half compared with the wild type. Thus recombination is reduced in *shoc1* (χ^2, $P<10^{-18}$ for both intervals). The map distances appear to be less reduced than the chiasma number (50% vs 85%). This could be a consequence of selecting for viable four-pollen tetrads or due to variation of the relative frequency of class I or class II COs along the genome (Shinohara et al., 2008). To measure interference, we used the interference ratio, which is defined as the ratio of the I1 map distance when a CO occurred in I2, to the genetic distance of I1 when no COs occurred in I2. In the wild type, the interference ratio was 0.2, which is indicative of strong interference as the map distance of I1 is five times shorter when a CO occurred in I2 compared with when no COs occurred in I2 (χ^2, $P<10^{-30}$). By contrast, the interference ratio was 0.78 in *shoc1*, which is not significantly different from the absence of interference (i.e. the recombination status of I2 having no effect on the I1 map distance) (Z test, P=0.4). The interference ratio in *shoc1* was significantly different from the interference ratio in the wild type (Z test, P=0.02; Stahl Lab Online Tools). In conclusion, whereas our data cannot rule out a small amount of residual interference in *shoc1* in the studied pair of intervals, interference is strongly reduced in *shoc1* mutants compared with the level in the wild type.

SHOC1 and PTD interact

SHOC1 and *PTD* belong to the same epistatic group and show similarity with XPF and ERCC1 proteins, respectively. We thus tested whether the two proteins interact in a yeast two-hybrid assay. We cloned the full-length PTD CDS and a fragment of the *SHOC1* cDNA (encoding aa 850–1209), which contains the predicted XPF-related nuclease–$(HhH)^2$ domain combination, into yeast expression vectors. Two-hybrid assays revealed that neither protein was capable of homodimerisation. However, a robust interaction between PTD and SHOC1 was observed in both directions (Fig. 3), suggesting that these two proteins can act as a heterodimer, similarly to XPF and ERCC1.

Epistatic relationships between class I and class II CO genes

We propose that the SHOC1–PTD complex acts in the class I CO formation pathway, whereas another XPF–ERCC1-related heterodimer, MUS81–EME1, is involved in the class II CO pathway. Thus, disruption of *MUS81* in the *shoc1* mutant background is expected to further reduce the CO frequency, whereas disruption of another component of the class I pathway in *shoc1* should not. We constructed a series of double mutants: *shoc1/mus81*, *shoc1/mer3*, *shoc1/msh4*, *shoc/mlh3*, *mer3/msh4* and *mlh3/msh5*, and compared them to the previously described *shoc1/msh5* and *msh5/msh4* strains (Higgins et al., 2008a; Macaisne et al., 2008). In all double mutants, except *mus81/shoc1*, the CO level was comparable to those of the single *shoc1*, *msh5* or *msh4* mutants (Student–Newman–Keuls multiple range test; P>0.05) (Fig. 1B), showing that *MSH4*, *MSH5*, *MLH3* and *MER3* act in the same pathway as *SHOC1*. Interestingly, whereas the decrease in chiasma frequency was lower in *mlh3* and *mer3* than in *msh4*, *msh5*, *shoc1*, *ptd* and *zip4* (3.64 for *mlh3*, 2.25 for *mer3*, versus ~1.5 for all the others), the frequency in *mlh3/msh5*, *mlh3/shoc1*, *mer3/msh4* and *mer3/shoc1* dropped to ~1.5 (Fig. 1B). This confirms that *MLH3* and *MER3* are involved exclusively in class I CO formation, although they are not absolutely essential for this pathway (Jackson et al., 2006; Mercier et al., 2005). In *shoc1/mus81*, the CO level was significantly reduced compared with *shoc1*, showing that *SHOC1* and *MUS81* act in independent pathways. The CO level in *mus81/shoc1* was two-thirds of the value observed in *shoc1* (0.85 vs 1.27) (Fig. 1B), which is consistent with the reduction seen in *msh4/mus81* relative to *msh4* (0.85 vs 1.25) (Higgins et al., 2008b). This suggests that MUS81 accounts for approximately one-third of the ZMM independent crossovers, at least in a *zmm* mutant situation.

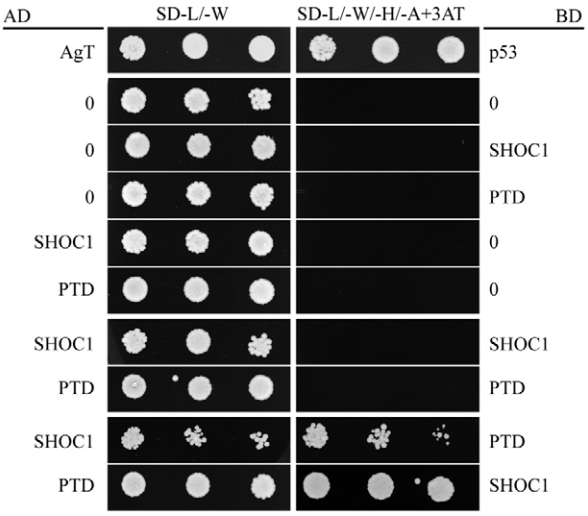


Fig. 3. PTD interacts with the XPF-related domain of SHOC1. PTD and SHOC1 correspond to the full-length protein and to the XPF-related domain (aa 850–1209), respectively. AgT represents the SV40 large-T antigen. The AH109 strain was co-transformed with the constructs indicated, carrying a binding domain (BD) and an activation domain (AD), and grown on synthetic drop-out (SD) medium lacking leucine and tryptophan (SD-L/-W) or leucine, tryptophan, histidine and adenine (SD-L/-W/-H/-A+3AT). Serial 1:10 dilutions of diploid strains were performed before spotting on medium. Yeast containing both vectors grew on SD-L/-W; positive interactions appear as white spots on SD-L/-W/-H/-A+3AT.

In summary, we show here that: (1) PTD is specific and essential in the class I CO pathway; (2) SHOC1 promotes interfering COs, together with PTD, MSH4, MSH5, MER3 and MLH3, but not MUS81, which is involved in the class II pathway (Berchowitz et al., 2007; Higgins et al., 2008b); (3) SHOC1 and PTD interact through the SHOC1 XPF-like nuclease–$(HhH)^2$ domain combination. We propose that SHOC1 and PTD form an XPF–ERCC1-like heterodimer that is required for class I CO formation.

Identification of SHOC1 and PTD orthologs

From previous analysis, it was unclear whether *S. cerevisiae* Zip2, which is also required for class I CO formation (Borner et al., 2004), and SHOC1 are orthologs (Macaisne et al., 2008). A novel PSI-BLAST (2.2.24+, inclusion threshold 0.002) search against NCBI-nr starting with the conserved C-terminal region of *S. cerevisiae* Zip2 (NP_011265.1 500-70) converged on a set of proteins with single representative genes per species, such as SHOC1 in *Arabidopsis thaliana* (NP_200042, collected in round 5) and C9orf84 in human (NP_775792, collected in round 6). Reciprocal PSI-BLAST searches confirmed this finding. Therefore, we suggest that Zip2, SHOC1 and C9orf84 are the closest relatives in their respective species and are probably orthologs. Interestingly, no putative *C. elegans* SHOC1/Zip2 ortholog was detected,

although COs rely on the class I pathway in this species. Using a similar approach, we could not identify likely orthologs of PTD beyond the plant kingdom, confirming previous reports (Wijeratne et al., 2006). ERCC1 and FAAP24 (which forms an XPF–ERCC1-like complex with FANCM) (Ciccia et al., 2007) appear to be the closest homologs of PTD beyond plants. Zip2 and mammalian SHOC1 might act alone as a homodimer, or alternatively interact with an as yet unidentified ERCC1-related PTD-like protein. Further studies in these model organisms are needed to resolve this issue.

XPF–ERCC1-like complexes and meiotic recombination

XPF–ERCC1 complexes are involved in the recognition of Y-shaped and bubble-like DNA structures during DNA repair processes (Ciccia et al., 2008). During homologous recombination, similar structures exist, from the nascent D-loop formation to double Holliday junction (dHJ) resolution (Szostak et al., 1983). The SHOC1–PTD heterodimer might recognize, stabilize and/or participate in the resolution of these structures, notably by cleaving the dHJ. Localization of either SHOC1 or PTD during meiosis would help to understand the SHOC1–PTD complex function in the kinetics of meiosis, but unfortunately our attempts to localize these proteins by immunodetection were unsuccessful. However, the fact that the characteristic GDxnERKx3D active site motif of XPF-proteins is not conserved in SHOC1 (Macaisne et al., 2008) argues against SHOC1 having a nuclease activity required for dHJ resolution. In addition, in the *zip2* yeast mutant, one of the earliest CO-specific DNA intermediates, the single-end invasion (SEI) and dHJ are not formed (Borner et al., 2004), suggesting that Zip2/SHOC1 acts early in the CO differentiation pathway. Thus, we suggest that SHOC1 might recognize branched DNA structures to stabilize intermediates or recruit other CO-promoting factors, rather than being a dHJ resolvase component.

In addition to SHOC1–PTD, several XPF–ERCC1-like heterodimers are involved in meiotic CO formation. One of these, Mus81–Eme1, is required for the majority of COs in *Schizosaccharomyces pombe* and for a subset of COs in *S. cerevisiae*, mammals and *Arabidopsis*. Mus81–Eme1 is thought to promote CO formation by cleaving DNA intermediates other than dHJs (Cromie and Smith, 2007; Whitby, 2005). In *Drosophila*, CO formation appears to rely on a different set of proteins than in mammals, *S. cerevisiae* or plants. In particular, no ZMM homologs are found (except the synaptonemal complex element ZIP1). Instead, CO formation relies on the XPF/RAD1 homolog itself, MEI-9, which is thought to cleave double-Holliday junctions. In the *Drosophila mei-9* mutant, CO formation is reduced by 90% (Yildiz et al., 2002). This suggests that during evolution XPF–ERCC1-related complexes, carrying out various biochemical activities, were recruited several times to promote CO formation during meiosis.

Materials and Methods

Plant material, growth conditions and genotyping

Plants (Col-0) were cultivated with a 16 hours day and 8 hours night photoperiod, at 20°C. *Atmus81-3* (SALK_002176) were genotyped with N502176U (5′-CACATACGTTTTTGGTTCCC-3′) and N502176L (5′-AGTGTCCAAGTCCTGCTTTC-3′) for the wild-type allele. For the mutant allele, N502176L and LBSalk2 (5′-GCTTTCTTCCCTTCCTTTCTC-3′) were used. *Shoc1-1* (SALK_057589) (Macaisne et al., 2008), *Atmer3-1* (SALK_045941) (Mercier et al., 2005), *Atmsh5-1* (SALK_110240) (Higgins et al., 2008a), *ptd-1* (SALK_127447) (Wijeratne et al., 2006), *Atmsh4-1* (SALK_136296) (Higgins et al., 2004) and *Atmlh3-1* (SALK_015849) (Jackson et al., 2006) were genotyped as indicated in the corresponding references.

Fluorescent-tagged lines

The fluorescent-tagged lines were obtained from Gregory P. Copenhaver (Department of Biology and the Carolina Center for Genome Sciences, University of North Carolina at Chapel Hill, Chapel Hill, NC). Fluorescent-tagged T-DNAs of plants were genotyped using FTL1963U (5′-GTCTTATCTTCGGTCCGAGAA-3′) and FTL1963L (5′-TCAACAACACCTTCTCCTCG-3′) for the wild-type allele and FTL1963U and LB-FTL (5′-GGCATGCAAGCTGATAATTC-3′) for the CFP-coding allele; FTL1143U (5′-CTCTGTAGCTTTACATCCATG-3′) and FTL1143L (5′-GCAGATTCTAGTGACCGATA-3′) for wild-type allele and FTL1143U and LB-FTL for the YFP-coding allele; FTL2450U (5′-ACTGGTTCGTTCTGCAACTT-3′) and FTL2450L (5′-GCTATTCTTGGAGTTGCTGG-3′) for the wild-type allele and FTL2450L and LB-FTL for the DsRed-coding allele. The *qrt1-2* mutation was genotyped as described previously (Francis et al., 2006). Tetrad counts (NPD, PD, T) and statistical analysis were performed according to published methods (Berchowitz and Copenhaver, 2008; Malkova et al., 2004) and using Stahl Lab Online Tools.

Cytology

DAPI-stained spreads were prepared as described previously (Ross et al., 1996). Images were acquired with a LEICA DM RXA2 epifluorescence microscope equipped with a coolSNAP camera (Roper Scientific) and with OpenLab 4.0.4 software (Improvision). Wild-type fluorescent-tagged lines were analyzed using the automatic slide-scanner function of the ZEISS AxioObserver DIC FISH Apotome and its workbench. All images were processed with Adobe Photoshop CS2 (Adobe Systems).

Yeast two-hybrid assay

The *SHOC1* domain was PCR-amplified using the primers SHOC1GatewayF2 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTATAGTCTCAAAATGATAAAG-3′) and SHOC1GatewayR (5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′) and cloned in pDONR207 (Invitrogen). The pENTR223.1-sfi-PTD plasmid expressing the full-length *PTD* CDS was obtained from the Ecker Lab through the *Arabidopsis* Biological Resource Center (ref. G82309). Expression cassettes were transferred into modified versions of pGADT7 and pGBKT7 (Rossignol et al., 2007). Vectors were co-transformed into yeast strain AH109 following the protocol MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech). Co-transformants were selected on SD-LW. Interactions were tested on SD-LWAH supplemented with 20 mM 3-amino-triazole (3AT). Serial 1:10 dilutions were prepared in water and 5 µl of each dilution was used per spot. Plates were incubated at 30°C for 3 days before scoring. The SV40 antigen T (AgT), known to interact with the p53 protein, was used as a positive control (Bartel et al., 1993).

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