**Aspergillus oryzae** AoSO Is a Novel Component of Stress Granules upon Heat Stress in Filamentous Fungi

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**Abstract**

Stress granules are a type of cytoplasmic messenger ribonucleoprotein (mRNP) granule formed in response to the inhibition of translation initiation, which typically occurs when cells are exposed to stress. Stress granules are conserved in eukaryotes; however, in filamentous fungi, including *Aspergillus oryzae*, stress granules have not yet been defined. For this reason, here we investigated the formation and localization of stress granules in *A. oryzae* cells exposed to various stresses using an EGFP fusion protein of AoPab1, a homolog of *Saccharomyces cerevisiae* Pab1p, as a stress granule marker. Localization analysis showed that AoPab1 was evenly distributed throughout the cytoplasm under normal growth conditions, and accumulated as cytoplasmic foci mainly at the hyphal tip in response to stress. AoSO, a homolog of *Neurospora crassa* SO, which is necessary for hyphal fusion, colocalized with stress granules in cells exposed to heat stress. The formation of cytoplasmic foci of AoSO was blocked by treatment with cycloheximide, a known inhibitor of stress granule formation. Deletion of the AoSO gene had effects on the formation and localization of stress granules in response to heat stress. Our results suggest that AoSO is a novel component of stress granules specific to filamentous fungi. The authors would especially like to thank Hiroyuki Nakano and Kei Saeki for generously providing experimental and insightful opinions.

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**Introduction**

The ability to sense environmental stimuli, including stress, activate signal transduction, and mount appropriate acute and adaptive responses is crucial for eukaryotic cell survival. Adaptation is achieved through the regulation of gene expression. Traditionally, transcriptional regulation has been regarded as the major determinant of gene expression. However, accumulating evidence indicates that posttranscriptional modulation of mRNA stability and translation plays a key role in the control of gene expression and provides greater plasticity, allowing cells to immediately adjust protein synthesis in response to changes in the environment [1]. Recent studies have demonstrated that one aspect of this modulation involves the remodeling of mRNAs translated from polysomes into non-translating messenger ribonucleoproteins (mRNPs), which accumulate in discrete cytoplasmic foci known as stress granules and processing bodies (P-bodies) [2–8].

Environmental stress response mechanisms in eukaryotic cells are characterized by global translational inhibition, which inhibits protein synthesis to conserve anabolic energy and also involves the reconfiguration of gene expression to effectively manage stress conditions. Global translational arrest is initiated by the activation of several stress-responsive serine/threonine kinases, including general control non-derepressible-2 (GCN2), dsRNA-dependent protein kinase R (PKR), heme-regulated inhibitor kinase (HRI), and PKR-like ER kinase (PERK), which phosphorylate the translation initiation factor eIF2α [9]. eIF2α is a subunit of eIF2 (together with eIF2β and eIF2γ), which is part of a ternary complex, consisting of eIF2, GTP, and methionyl-initiator tRNA (Met-tRNAiMet), that delivers initiator tRNA to the 40S ribosome [1]. During translation initiation, GTP is hydrolyzed to GDP, generating eIF2-GDP, which needs to be recharged to eIF2-GTP by a guanine nucleotide exchange factor following each round of initiation. Phosphorylation of eIF2 by eIF2α kinases inhibits the formation of eIF2-GTP, thereby reducing the level of the ternary complex, which ultimately limits translation initiation [1]. The stalled preinitiation complexes, together with their associated mRNAs, are routed into stress granules. However, a subset of mRNAs required for cell survival under stress conditions are not delivered to stress granules but stabilized and preferentially translated in the cytoplasm [10–13]. As stress granules are formed in response to stress, they are generally not observable under normal growth. Although the composition of stress granules is dynamic and dependent on the type of stress and cell species, they typically contain 40S ribosomal subunits, translation initiation factors (eIF1G, eIF4E, eIF4A, eIF4B, eIF3, and eIF2), and proteins involved in the regulation of mRNA function [5,14].
function of stress granules is not fully understood; however, they have been implicated in the posttranscriptional regulation processes, such as mRNA translational repression and storage, and cellular signal transduction [4,5,15,16].

Eukaryotic mRNA degradation is generally initiated by the deadenylation of poly(A) tails, which triggers either decapping and 5’ to 3’ exonucleolyis or exosome-dependent 3’ to 5’ degradation [17–20]. As the inhibition of translation initiation increases the rate of deadenylation and decapping [21–24], the rate of mRNA degradation and translation initiation are often inversely related [7,25]. The cellular components involved in mRNA decapping and degradation, such as decapping enzyme complex Dcp1p/Dcp2p, the activators of decapping and/or repressors of translational factors, Pab1p, as a stress granule marker. Moreover, subcellular localization studies showed that AoSO protein accumulated at the septal pore and also in cytoplasmic foci at the hyphal tip when cells were exposed to heat stress. The cytoplasmic AoSO foci at the septal pore and also in cytoplasmic foci at the hyphal tip were cycloheximide sensitive and colocalized with stress granule marker, Dcp2p, respectively. The center entry clones were individually mixed with 5’ entry clone pg5’PaB, 3’ entry clone pg3’PaB, and destination vector pgDN [40] for the LR clonase reaction, generating plasmids pgDPapab1E, pgDPapab1E and pgDPadcp2E, respectively. For the expression of Aopab1-mDsRed, pgEpab1 was mixed with 5’ entry clone pg5’PaB, 3’ entry clone pg3’DRM-CF, and destination vector pgDSO [42] for the LR clonase reaction, generating plasmid pgCPapab1DR.

To generate the template DNA fragment for disruption of Aopab1, the 5’- and 3’-flanking regions of Aopab1 were amplified by PCR using genomic DNA of wild-type strain RIB40 as a template, and introduced into plasmids pgDONR P4-P1R and pgDONR P2-P3 by the BP clonase reaction, generating the 5’ entry clone pg5’PaB1up and 3’ entry clone pg3’PaB1dw, respectively. Plasmids pg5’PaB1up and pg3’PaB1dw were mixed with center entry clone pgEpG and destination vector pDEST R4-R3 for the LR clonase reaction to generate plasmid pgdPub1.

A. oryzae strains and transformation

The A. oryzae strains used in this study are listed in Table 1. Transformation of A. oryzae was carried out according to the standard method described previously [43,44]. To generate EGFP-expressing strains, plasmids pgPapab1E and pgPapab1E were introduced into strain NSRKu70-1-1A [42]; while plasmid pgPapab1E was introduced into strain NS4 [45]. The resulting transformants were selected using Czapex-Doxy (CD) medium (0.3% NaNO3, 0.2% KCl, 0.1% KH2PO4, 0.05% MgSO4.7H2O, 0.002% FeSO4·7H2O, and 2% glucose, pH 5.5) supplemented with 0.15% methionine (CD + Met). To generate a strain co-expressing AoDcp2-EGFP and Aopab1-mDsRed, plasmid pgCPapab1DR was introduced into strain S-Dcp2E, and positive transformants were selected on CD medium. To generate a strain co-expressing AosSO-EGFP and Aopab1-mDsRed, plasmid pgCPapab1DR was introduced into strain NSK-ASG1 [39], and positive transformants were selected on CD medium. To express Aopab1-EGFP in an Aoso deletion background, plasmid pgPapab1E was introduced into ΔAoso strain (NSK-SO11) [39], and positive transformants were selected on CD+Met medium.

To generate an Aopab1 disruptant, a DNA fragment containing the 5′-flanking region of Aopab1, pyrG marker, and 3′-flanking region of Aopab1, was amplified by PCR from plasmid pgPapab1, purified and then introduced into A. oryzae strain NSPID1 [46]. Deletion of Aopab1 was confirmed by Southern blot analysis. Briefly, genomic DNA of the strain was digested with the restriction enzymes BglII and BamHI (Takara, Otsu, Japan), and was separated in a 0.8% gel by electrophoresis. The DNA was then transferred onto a Hybond N+ membrane (GE Healthcare, Buckinghamshire, UK) and detected with specific probes using the ECL Detection kit (GE Healthcare) and a LAS-4000 mini-EPUV luminescent image analyzer (Fujifilm, Tokyo, Japan). The 1.5-kb downstream flanking regions of Aopab1 amplified from pgdPub1 were used as probes.

Fluorescence microscopy

For microscopic analysis, approximately 10⁴ conidia of cells were inoculated into 100 µl CD or CD+Met media in a glass-bottom dish (Asahi Techno Glass, Chiba, Japan), and incubated at
30°C for 18 h before being exposed to stress conditions. For temperature stress, cells were shifted from 30°C to 4°C for 30 min or to 45°C for 10 min. For glucose deprivation, cells were washed three times with CD medium without glucose, and further incubated for 10 min in CD medium without glucose. For ER, osmotic, and oxidative stresses, culture medium was removed and replaced by medium containing 10 mM DTT, 1.2 M sorbitol, or 2 mM H₂O₂, respectively, and cells were further incubated for 30, 30, and 30 min. Small stressed cells were observed by confocal microscopy using an IX71 inverted microscope (Olympus, Tokyo, Japan) equipped with a 100X Neofluor objective lens (1.40 numerical aperture), 488-nm (Furukawa Electric, Tokyo, Japan) and 561-nm semiconductor lasers (Melles Griot, Carlsbad, CA, USA), GFP, DsRed, and DualView filters (Nippon Roper, Chiba, Japan), a CSU22 confocal scanning system (Yokogawa Electronics, Tokyo, Japan), and an Andor iXon cooled digital CCD camera (Andor Technology PLC, Belfast, UK). Images were analyzed with Andor iQ software (Andor Technology PLC) and representative images are shown.

### Results

**Stress granule formation is induced in response to stress**

The polyA-granule binding protein Pab1p, which is involved in the translational regulation and stability of mRNAs [47,48], is one of the most reliable and easily visualized components of stress granules in *S. cerevisiae* [49]. A homolog of the *S. cerevisiae PAB1* gene was found in the *A. oryzae* genome database (DOGAN; http://www.bio.nite.go.jp/dogan/Top) and designated as *Aopab1* (AO090001000353). To monitor stress granules by fluorescence microscopy, an AoPab1-EGFP fusion protein was expressed in strain SRK-Pab1E under control of the *amyB* promoter as a stress granule marker. Global translational arrest is a common environmental stress response in eukaryotes [30], and the inhibition of translation initiation leads to the formation of stress granules. Therefore, as a first step in characterizing stress granules in *A. oryzae*, several external stimuli were used to assay the induction of stress granules in cells (Figure 1). Under normal growth conditions, AoPab1-EGFP was dispersed throughout the cytoplasm (Figure 1A). Exposing cells to heat stress (45°C, 10 min) led to an induction of stress granules, as judged by the accumulation of AoPab1-EGFP as cytoplasmic foci (Figure 1A). No accumulation of EGFP was observed in the negative control strain expressing EGFP alone (Figure 1B). Similarly, stress granules were observed after cells were exposed to cold stress (4°C, 30 min), glucose deprivation (10 min), osmotic stress (1.2 M sorbitol, 30 min), ER stress (10 mM DTT, 60 min), and oxidative stress (2 mM H₂O₂, 30 min) (Figure 1C and Figure 2). In response to glucose deprivation, the accumulation of AoPab1-EGFP appeared to be short lived, as no accumulation was observed after 30 min in the majority of cells (data not shown). Small foci of AoPab1-EGFP were formed when the culture medium was replaced with medium containing 2 mM H₂O₂, and continued to increase in size until they fused together to form one large aggregate (Figure 2 and Video S1). Of the examined stresses, cold stress, glucose deprivation, and oxidative stresses, culture medium was removed and continued to increase in size until they fused together to form one large aggregate (Figure 2 and Video S1). Of the examined stresses, cold stress, glucose deprivation, and oxidative stresses, culture medium was removed and continued to increase in size until they fused together to form one large aggregate (Figure 2 and Video S1).
another well-known component of stress granules, under the same conditions. Similar results were consistently observed in cells expressing AoPub1-EGFP (see the last section of Results).

Stress granules colocalize with P-bodies in response to heat stress

In eukaryotic cells, non-translating mRNAs also accumulate in P-bodies, which contain a conserved core of proteins involved in translational repression and mRNA degradation. Although stress granules and P-bodies are compositionally and morphologically distinct entities, evidence suggests they are spatially and functionally linked [49,51–55]. Dcp2p is the catalytic subunit of a decapping enzyme that cleaves the 5' cap of mRNA [56,57] and is frequently found as a distinct component of P-bodies in S. cerevisiae [49]. To monitor P-bodies in A. oryzae, a homolog of S. cerevisiae Dcp2p, AoDcp2 (AO090120000363), was fused with EGFP and expressed under control of the amyB promoter. In unstressed cells, AoDcp2-EGFP was detected as discrete bright dots in the cytoplasm, in which it also showed a faint diffuse distribution (Figure S1). Under normal growth conditions, AoSO-EGFP was evenly distributed throughout the cytoplasm, but accumulated at the septal pore after cells were exposed to heat stress, as previously reported [39]. However, AoPab1-mDsRed did not accumulate at the septal pore in cells exposed to heat stress (Figure 4A) or any other of the examined stress conditions (cold stress, glucose deprivation, and ER, osmotic and oxidative stresses; data not shown). In cells exposed to heat stress, AoSO-EGFP also accumulated as cytoplasmic foci, which

stress, low temperature, and carbon deprivation (Figure S1). To determine the relative localizations of stress granules and P-bodies, an A. oryzae strain co-expressing AoDcp2-EGFP and AoPab1-mDsRed was examined after being exposed to 45°C for 10 min. Under heat stress, AoPab1-mDsRed cytoplasmic foci were colocalized with P-bodies labeled with AoDcp2-EGFP (Figure 3).

AoSO colocalizes with stress granules upon heat stress

To investigate the possibility that AoSO protein is involved in the function of stress granules, a strain co-expressing AoSO-EGFP and AoPab1-mDsRed was used to examine the cellular localizations of AoSO and stress granules in cells exposed to heat stress (Figure 4A). The functionality of AoSO-EGFP was previously confirmed by demonstrating that expression of the fusion protein complemented the phenotypes of Δao so strain [39]. Under normal growth conditions, AoSO-EGFP was evenly distributed throughout the cytoplasm, but accumulated at the septal pore after cells were exposed to heat stress, as previously reported [39]. However, AoPab1-mDsRed did not accumulate at the septal pore in cells exposed to heat stress (Figure 4A) or any other of the examined stress conditions (cold stress, glucose deprivation, and ER, osmotic and oxidative stresses; data not shown). In cells exposed to heat stress, AoSO-EGFP also accumulated as cytoplasmic foci, which

Figure 1. Stress-induced formation of stress granules. Approximately 10⁴ conidia of cells expressing AoPab1-EGFP were grown in CD+Met medium at 30°C for 18 h before being exposed to various types of stress. (A) Subcellular localization of AoPab1-EGFP. Accumulation of AoPab1-EGFP (indicated by the arrow) was induced when cells were exposed to 45°C for 10 min. (B) Subcellular localization of EGFP. Accumulation of EGFP was not observed in cells exposed to heat stress. (C) Accumulation of AoPab1-EGFP (indicated by the arrows) was induced in cells treated with cold stress (4°C, 30 min), glucose deprivation (10 min), osmotic stress (1.2 M sorbitol, 30 min), and ER stress (10 mM DTT, 60 min). Scale bars = 5 μm.
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colocalized with stress granules labeled with AoPab1-mDsRed at the hyphal tip (Figure 4A).

To further determine the physical association of AoSO with the stress granule component AoPab1, a strain co-expressing AoSO-EGFP and AoPab1-3HA was used in co-immunoprecipitation experiments. The interaction between AoSO-EGFP and AoPab1-3HA was confirmed by co-immunoprecipitation (Figure S2). This association is not mediated via the EGFP portion as no association was detected in the negative control strain co-expressing EGFP and AoPab1-3HA. However, the association between AoSO-EGFP and AoPab1-3HA was not induced or increased after cells were exposed to heat stress.

To clarify if the aggregation of AoSO requires the presence of non-translating mRNAs, cycloheximide, which blocks translation elongation and traps mRNAs in polysomes, was used to deplete the pool of non-translating mRNAs [49,53,60] (Figure 4A and 4B). The strain co-expressing AoSO-EGFP and AoPab1-mDsRed was treated with 200 μg/ml cycloheximide for 30 min before being exposed to heat stress. The formation of stress granules labeled with AoPab1-mDsRed was sensitive to the cycloheximide treatment, confirming they are typical mRNP granules, as previously reported [49,53,60]. In addition, the heat stress-induced formation of cytoplasmic AoSO foci at the hyphal tip was greatly impaired by cycloheximide (Figure 4A and 4B), suggesting that cytoplasmic AoSO foci require a pool of free mRNAs for their aggregation. However, cycloheximide did not affect the accumulation of AoSO at the septal pore. Overall, these results suggest that AoSO is a novel component of mRNP granules in the filamentous fungus A. oryzae.

**Deletion of Aoso affects the formation and localization of stress granules**

To gain a better understanding of the role of AoSO in stress granules, the effect of Aoso deletion on stress granule formation was examined. Compared to 100% formation of stress granules in wild-type cells exposed to heat stress, the heat stress-induced formation of stress granules in the Aoso-deletion strain was decreased to 87.7 ± 1.34 % (hyphae = 50; n = 7; p < 0.005) (Figure 5A). No obvious change in the size of stress granules was observed in the Aoso-deletion strain. The movement of stress granules labeled with AoPab1-EGFP in stressed cells was monitored by live-cell imaging, which revealed that in contrast to other types of stress where the stress granules were highly dynamic (Video S2 for oxidative stress and data not shown), the heat stress-induced stress granules were nearly stationary (Video S3). By taking advantage of this feature, the effect of Aoso deletion on stress granules was further evaluated by measuring the distance between the hyphal tip and stress granules. We found that the distribution of the largest stress granules labeled with AoPab1-EGFP was less concentrated and more distant from the hyphal tip in the Aoso-deletion strain (Figure 5B). Additionally, we observed that in a small portion of hyphae, heat stress-induced stress granules were more dynamic in the Aoso-deletion strain (Video S4). However, the motility of heat-stress induced stress granules in the Aoso-deletion strain was different from that of all the other stress conditions examined in this study with a long distance movement (Video S2), but moved around in a confined region (Video S4). We have examined the growth test of the Aoso deletion strain in the stress conditions used in Figure 6; however, no growth difference between wild-type and the Aoso deletion strain was observed.

**Figure 2. Time-lapse observation of stress granule formation upon oxidative stress.** Approximately 10^4 conidia of cells expressing AoPab1-EGFP were grown in CD+Met medium at 30°C for 18 h before being exposed to oxidative stress (2 mM H_2O_2). Accumulation of AoPab1-EGFP in cells was observed in a time-lapse manner. Scale bar = 5 μm.

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The ability to form stress granules is correlated with the survival of cells exposed to stress [5]. The C-terminal regions of TIA-1 and TIAR (mammalian homologs of AoPub1), and their orthologs, contain a glutamine/asparagine (Q/N)-rich prion-like domain, which have a strong tendency to self-aggregate and promote stress granule formation [60–63]. To investigate whether the formation of stress granules influences cell survival against stress, an AoPub1 disruptant was constructed and cultured on PD plates containing 10 mM DTT, 2 mM H$_2$O$_2$, or 1.2 M sorbitol to examine sensitivity to these three types of stress. The induction of stress granules was confirmed in the AoPub1-EGFP expressing strain under each of the stress conditions (Figure 6A). The phenotype of the AoPub1 disruptant was characterized by a slower growth rate compared to wild-type cells, and a severe impairment in the formation of conidia (Figure 6B). The growth retardation of the AoPub1 disruptant was more severe under all examined stress conditions (Figure 6C). Colony sizes of AoPub1 disruptant compared with those of wild-type strain were reduced by 16.5%, 21.1%, 24.7%, and 33.2% in the control, 10 mM DTT, 2 mM H$_2$O$_2$, and 1.2 M sorbitol conditions, respectively.

**Discussion**

**Stress granules are conserved in A. oryzae**

Stress granules are widely observed in eukaryotes; however, prior to the present study, these mRNP granules had not yet been defined in filamentous fungi. We used EGFP-fused AoPab1 as a stress granule marker, and observed that AoPab1 accumulated as cytoplasmic foci at the hyphal tip of cells exposed to various stresses. This finding indicates that the formation of stress granules is a general phenomenon in response to external stress. To our knowledge, the present study is the first to identify stress granules in a filamentous fungus, which suggests that stress-induced reprogramming of mRNAs to enter stress granules occurs in *A. oryzae*.

Although P-bodies and stress granules are compositionally and functionally distinct structures, accumulating evidence suggests they are spatially and functionally associated [49,51–55]. Here, we showed that AoPab1-mDsRed, which is the core component of stress granules, colocalizes with P-bodies labeled with AoDcp2-EGFP. The spatial relation of stress granules and P-bodies is also seen in mammalian cells [51] and *S. cerevisiae* [49,53]. The stress-induced formation of stress granules, in which mRNAs are not degraded, but maintained in a nontranslating state, is thought to make mRNAs available for rapid reinitiation when cells recover from stress. In agreement with this speculation, stress-induced stabilization of viable mRNAs has been reported [64–66], suggesting that certain components of the mRNA degradation pathway are impaired in response to stress. This finding may explain why the number and size of P-bodies were increased coordinately in *A. oryzae* cells under stress (Figure 3 and Figure S1).

It is noteworthy that in mammalian and yeast cells, numerous stress granules are distributed throughout the cytoplasm following exposure to stress; however, only a few stress granules that were located predominately at the hyphal tip were found in *A. oryzae*. Although the underlying reason for this observation is not yet clear, the polarized localization of stress granules suggests a spatial specificity of the posttranscriptional regulation of gene expression in *A. oryzae*. It is well known that filamentous fungi have a highly polarized cell structure in which secretory vesicles, cytoskeletal elements, and related components are concentrated at the hyphal tip as a well-organized cluster that determines hyphal growth and polarity [67,68]. The localization of ribosomes at the hyphal tip supports the idea that mRNA translation actively occurs in this region [69]. Based on these findings, we speculate that asymmetrical localization of mRNA at the hyphal tip results in the spatially restricted formation of stress granules or that mRNA at the hyphal tip is preferentially routed into stress granules as an acute response to stress.

**AoSO is a component of stress granules**

Deletion of the so gene in *N. crassa* results in a pleiotropic phenotype characterized by a lack of hyphal anastomoses, reduced aerial hyphae, slower growth rate, altered conidiation pattern, and female sterility [37]. Mutation of the conserved WW domain in SO, which is predicted to mediate protein-protein interactions,
does not affect SO localization to the septal pore; however, the phenotypic defects of the so disruptant are not fully complemented [35]. Clearly, SO is a multi-function protein, and a plugging of the septal pore is not sufficient to explain the multiple phenotypic defects observed in the so-deletion strain. The molecular function of the SO protein remains largely unknown. Localization analysis has revealed that the N. crassa SO homolog S. macrospora Pro40 partially associates with Woronin bodies [38], and that N. crassa SO contributes to the sealing efficiency of pores plugged by Woronin bodies after hyphal injury [35]. Additionally, cell-cell signaling and tropic growth of N. crassa germlings involve the unusual subcellular dynamics of SO and the MAP kinase (MAPK-2), which are recruited to the plasma membrane of cell tips of interacting germlings in an oscillating and alternating manner [36]. In the present study, we found that AoSO accumulates not only at the septal pore, but also at the hyphal tip, in cells exposed to heat stress. In addition, cytoplasmic AoSO foci colocalized with AoPab1-mDsRed-labeled stress granules at the hyphal tip and were sensitive to cycloheximide treatment (Figure 4), suggesting that cytoplasmic AoSO foci are mRNP granules, and that AoSO therefore may participate in the posttranscriptional regulation of mRNA in response to heat stress. The physical association between AoSO-EGFP and AoPab1-3HA was further confirmed by co-immunoprecipitation (Figure S2); however, this association was not induced or increased after cells were exposed to heat stress. An inconsistence between the results of co-immunoprecipitation and colocalization analysis may be explained by the different culture conditions (DPY complete medium in submerged culture and CD+Met minimal medium in stationary culture). Orthologs of the so gene have only been identified in the genomes of Pezizomycotina species [37], therefore we presume that AoSO is a novel component of stress granules specific to Pezizomycotina. The effect of Aoso deletion on the function of stress granules revealed that AoSO is not indispensable for stress granule formation; however, the formation and localization of stress granules were influenced in the absence of AoSO (Figure 5). Protein-protein interaction has been implicated in the assembly of mRNP granules, including stress granules. One mechanism of assembly is mediated through the glutamine/asparagine (Q/N)-rich prion-like domain, which has a strong tendency to self-aggregate and is found in many components of mRNP granules, including TIA-1 and TIAR (mammalian homologs of AoPub1) [60–63]. Moreover, the long, intrinsically disordered domains identified in septal pore-associated (SPA) proteins show an inherent tendency to aggregate [70]. The N-terminal domain of AoSO is also predicted to be disordered (data not shown), suggesting that it has the potential to form aggregates of mRNP granules, although N. crassa SO, which contains a disordered domain that is enriched in glutamine, fails to form aggregates in vitro [70]. In consistence with this property, deletion of Aoso resulted in an 12.3 ± 1.34 % reduction in the number of tip cells displaying stress granules labeled with AoPab1-EGFP under the

![Figure 4. Subcellular localizations of AoSO-EGFP and AoPab1-mDsRed in response to heat stress.](image-url)
heat stress condition. We also observed that in a small portion of hyphae, heat stress-induced stress granules were more dynamic in the \(Aoso\)-deletion strain (Video S4). It remains unclear how AoSO influences the localization and motility of stress granules. AoSO contains a conserved WW domain, as well as a proline-rich domain predicted to mediate protein-protein interactions. The localization and dynamics of stress granules may be indirectly influenced through protein-protein interactions of AoSO with other proteins (Figure 5 and Video S4). Our present findings, in addition to the known functions of AoSO and its homologs in hyphal fusion, sexual reproduction, and septal plugging [37–39], raise the possibility that AoSO participates in the posttranscriptional regulation of mRNA in response to heat stress. However, the localization of mRNAs in AoSO cytoplasmic foci remains to be conclusively demonstrated.

**Aopub1** disruptant has defects in the conidia formation and displays more severe growth retardation in stress conditions

The formation of stress granules in response to stress is widely observed in eukaryotes. Although relatively little is known about the mechanisms regulating the formation of stress granules, their assembly seems to be mediated through a self-assembly process that involves the Q/N-rich prion-like domains of a number of protein components associated with stress granules, including TIA-1, a mammalian homolog of AoPub1 [7,61,62,71,72]. As Pab1p is an essential protein in *S. cerevisiae* and is not required for stress granule formation [73], we investigated whether deletion of the *Aopub1* gene confers cellular sensitivity to stress. The *Aopub1* disruptant displayed a slower growth rate compared to wild-type cells, and the growth retardation was more severe when cells were cultured under stress conditions, which included ER, oxidative, and osmotic stresses. Although we lack direct evidence that the formation of stress granules is impaired in the *Aopub1* disruptant, a \(\Delta pub1\) strain of *S. cerevisiae* displays a dramatic decrease in the average number of stress granules, as judged by Pab1p localization [49,74]. The data presented here suggest that the integrity of stress granules is important for the survival of *A. oryzae* cells exposed to stress.
Figure S1 P-bodies under normal growth condition and in response to stresses. Approximately $10^4$ conidia of cells expressing AoDcp2-EGFP were grown in CD+Met medium at 30°C for 18 h before being exposed to various stresses. For temperature stress, cells were shifted from 30°C to 4°C for 30 min or to 45°C for 10 min. For glucose deprivation, cells were washed three times with CD medium without glucose, and further incubated for 10 min in CD medium without glucose. Scale bar = 5 μm. (TIF)

Figure S2 Co-immunoprecipitation of AoSO-EGFP and AoPab1-3HA. (A) Approximately $10^7$ conidia of cells expressing AoDcp2-EGFP were grown in CD+Met medium at 30°C for 18 h before being exposed to various stresses. For temperature stress, cells were shifted from 30°C to 4°C for 30 min or to 45°C for 10 min. For glucose deprivation, cells were washed three times with CD medium without glucose, and further incubated for 10 min in CD medium without glucose. Scale bar = 5 μm. (TIF)

Supporting Information

Figure S1 P-bodies under normal growth condition and in response to stresses. Approximately $10^4$ conidia of cells expressing AoDcp2-EGFP were grown in CD+Met medium at 30°C for 18 h before being exposed to various stresses. For temperature stress, cells were shifted from 30°C to 4°C for 30 min or to 45°C for 10 min. For glucose deprivation, cells were washed three times with CD medium without glucose, and further incubated for 10 min in CD medium without glucose. Scale bar = 5 μm. (TIF)

Video S1 Time-lapse capture of the subcellular movements of AoPab1-EGFP revealed the fusion of stress granules. Approximately $10^4$ conidia of AoPab1-EGFP expressing cell were grown in CD+Met medium at 30°C for 18 h before being exposed to oxidative stress (2 mM H$_2$O$_2$). Single focal planes were used to track the location and subcellular movements of stress granules. The experiment was repeated three times with similar results. (TIF)
were captured at 550 ms intervals (total 32 frames). The video is presented at 5 frames/s. (AVI)

**Video S2** Time-lapse capture of the subcellular movements of AoPab1-EGFP in the wild-type strain after cells were exposed to oxidative stress for 10 min. Note that the stress granule labeled with AoPab1-EGFP at the hyphal tip moved toward to the subapical region with a long distance. Cells were cultured and examined under the same condition described in the Video S1. Single focal planes were captured at 550 ms intervals (total 100 frames). The video is presented at 5 frames/s. (AVI)

**Video S3** Time-lapse capture of the subcellular movements of AoPab1-EGFP in the wild-type strain revealed that the heat stress-induced stress granule labeled with AoPab1-EGFP at the hyphal tip was nearly stationary. Approximately 10^4 conidia of cell were grown in CD+Met medium at 30°C for 18 h before being exposed to heat stress. Single focal planes were captured at 550 ms intervals (total 100 frames). The video is presented at 5 frames/s. (AVI)

**Video S4** Time-lapse capture of the subcellular movements of AoPab1-EGFP in the Aoso stain revealed that a confined movement of the heat stress-induced stress granule. Note the stress granule labeled with AoPab1-EGFP at the subapical region. Cells were cultured and examined under the same condition described in the Video S3. Single focal planes were captured at 550 ms intervals (total 100 frames). The video is presented at 5 frames/s. (AVI)

**Author Contributions**

Conceived and designed the experiments: HH JM KK. Performed the experiments: HH. Analyzed the data: HH. Wrote the paper: HH JM KK.

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