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Diffuse Decapping Enzyme DCP2 Accumulates in DCP1 Foci Under Heat Stress in *Arabidopsis thaliana*

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The decapping enzymes DCP1 and DCP2 are components of a decapping complex that degrades mRNAs. DCP2 is the catalytic core and DCP1 is an auxiliary subunit. It has been assumed that DCP1 and DCP2 are consistently co-localized in cytoplasmic RNA granules called processing bodies (P-bodies). However, it has not been confirmed whether DCP1 and DCP2 co-localize in *Arabidopsis thaliana*. In this study, we generated DCP1–green fluorescent protein (GFP) and DCP2–GFP transgenic plants that complemented *dcp1* and *dcp2* mutants, respectively, to see whether localization of DCP2 is identical to that of DCP1. DCP2 was present throughout the cytoplasm, whereas DCP1 formed P-body-like foci. Use of DCP1–GFP/DCP2–red fluorescent protein (RFP) or DCP1–RFP/DCP2–GFP plants showed that heat treatment induced DCP2 assembly into DCP1 foci. In contrast, cold treatment did not induce DCP2 assembly, while the number of DCP1 foci increased. These changes in DCP1 and DCP2 localization during heat and cold treatments occurred without changes in DCP1 and DCP2 protein abundance. Our results show that DCP1 and DCP2 respond differently to environmental changes, indicating that P-bodies have diverse DCP1 and DCP2 proportions depending on environmental conditions. The localization changes of DCP1 and DCP2 may explain how specific mRNAs are degraded during changes in environmental conditions.

Keywords: *Arabidopsis thaliana* • Cold stress • Decapping enzyme • Heat stress • Processing bodies • RNA granule.

Abbreviations: CHX, cycloheximide; GFP, green fluorescent protein; MS, Murashige and Skoog; ORF, open reading frame; P-body, processing body; RFP, red fluorescent protein.

Introduction

Plants need to adapt to changing environmental conditions such as temperature changes because of their immobile nature. Exposure to temperature changes triggers positive and negative gene regulation to enable the plant to adapt (Kotak et al. 2007, Qin et al. 2011, Mittler et al. 2012). For example, heat shock factors, such as HsfA1, are up-regulated by heat treatment (Wang et al. 2004, Yoshida et al. 2008, Yoshida et al. 2011). Additionally, exposure to low temperatures triggers the increased expression of cold-inducible proteins such as ICE1 (Yamaguchi-Shinozaki and Shinozaki 2006, Chinnusamy et al. 2007, Sasaki et al. 2008). Abiotic stresses result in altered gene expression regulation not only during transcription, but also at the post-transcriptional stage (Narsai et al. 2007, Shukla et al. 2008, Ito et al. 2011, Matsunaga et al. 2012). More than 2,000 mRNAs are down-regulated by unknown mechanisms during heat treatment (Larkindale and Vierling 2008).

Cytoplasmic granules called processing bodies (P-bodies) are believed to be sites of mRNA degradation based on the observation that non-translating mRNAs and mRNA decay machineries accumulate in P-bodies in yeast (Sheth and Parker 2003, Brengues et al. 2005, Arribere et al. 2011). It is thought that P-bodies are involved in stress adaptation because P-body formation is facilitated during various stresses in yeast and animals (Kedersha et al. 2005, Teixeira et al. 2005, Ramachandran et al. 2011). Additionally, in plants, it is suggested that certain RNA-binding proteins are localized as foci depending on exposure to abiotic stresses (Pomeranz et al. 2010, Bogamuwa and Jang 2013, Jan et al. 2013, Bogamuwa and Jang 2014, Maldonado-Bonilla et al. 2014).

The decapping reaction, where 7-methyl-guanosine 5′-diphosphate (cap) is removed from the 5′ end of mRNAs, is a critical step in eukaryotic mRNA degradation pathways (Parker and Sheth 2007, Franks and Lykke-Andersen 2008). DCP1 and DCP2 form a holoenzyme decapping complex in P-bodies and possibly trigger 5′–3′ mRNA degradation in eukaryotes (LaGrandeur and Parker 1998, Coller and Parker 2004, She et al. 2008, Borja et al. 2011). Previous in vitro studies showed that DCP2 is the decapping reaction core subunit and DCP1 is an auxiliary subunit that activates the reaction (Xu et al. 2006, Deshmukh et al. 2008, Chang et al. 2014). The *dcp1* and *dcp2* null mutants of *Arabidopsis thaliana* show seedling-lethal phenotypes, suggesting the importance of mRNA decay during plant development; this is similar to what has been observed in other RNA degradation mutants (Iwasaki et al. 2007, Chiba and Green 2009, Riehs-Kearnan et al. 2012, Hirayama et al. 2013, Kumakura et al. 2013).

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DCP1 and DCP2 have been used as P-body markers in plants and yeast to facilitate observation of their localization as cytoplasmic foci (Xu et al. 2006, Iwasaki et al. 2007, Maldonado-Bonilla 2014). Because the number of plant DCP1 foci increases with stress treatment (e.g. heat), it is believed that DCP1 and DCP2 in plants behave similarly to the orthologous proteins in yeast (Weber et al. 2008, Pomeranz et al. 2010). Previous studies in plants used green fluorescent protein (GFP)-fused DCP1 or DCP2 driven by a constitutive promoter such as the 35S promoter (Xu et al. 2006, Goeres et al. 2007, Iwasaki et al. 2007). We previously reported co-localization of transiently overexpressed DCP1 and DCP2 in *Nicotiana benthamiana* (Iwasaki et al. 2007). However, DCP1 or DCP2 overexpression caused an abnormal reproductive growth phenotype (Iwasaki et al. 2007). Therefore, a detailed parallel analysis of DCP1 and DCP2 foci in plant tissues has not been completed, and a number of uncertainties should be clarified.

In this study, we used DCP1–GFP and DCP2–GFP plants constructed previously (Motomura et al. 2012). We also generated DCP1–GFP/DCP2–red fluorescent protein (RFP) and DCP1–RFP/DCP2–GFP plants to observe whether DCP1 and DCP2 co-localize. Furthermore, to understand DCP1 and DCP2 responses to abiotic stresses, we subjected these plants to heat or cold treatment, and found that DCP1 and DCP2 responded differently to temperature changes.

Results

DCP2 localization differs from DCP1 localization

We generated transgenic plants that express DCP1–GFP or DCP2–GFP using the native promoter in *dcp1-1* or *dcp2-1* mutants. It was assumed that the expressed GFP-fused proteins were functional because the phenotypes (including vegetative growth) of these mutants were similar to that of the wild-type plant.

We observed the localization of DCP1–GFP in root elongation zones using three-dimensional optical sections with confocal laser scanning microscopy. DCP1–GFP presented as foci in cytoplasm (**Fig. 1A**, left-hand panel). Next, we observed the localization of DCP2–GFP and found that DCP2–GFP was spread throughout the cytoplasm (**Fig. 1C**, left-hand panel). Our observation rather unexpectedly revealed differences in localization between DCP1 and DCP2; we had assumed that the two proteins would accompany each other to enable proper functioning.

Heat treatment induces DCP2 foci formation

To observe the behavior of DCP1–GFP and DCP2–GFP at high temperature, we subjected transgenic plants that had been grown at 22°C to 40°C for 90 min. The number of DCP1–GFP foci increased during heat treatment in root elongation zones (**Fig. 1A**, center panel, **B**). Heat treatment also induced the accumulation of DCP2–GFP into foci (**Fig. 1C**, center panel) and the number of DCP2–GFP foci significantly increased in roots ($P < 0.01$) (**Fig. 1D**). We observed similar behaviors of DCP1 and DCP2 in leaf epidermal cells exposed to heat using three-dimensional optical sections (**Supplementary Fig. S1**).

After induction of DCP1–GFP or DCP2–GFP foci formation by heat treatment, transgenic plants were returned to 22°C and kept at this temperature for 3 h. Removal from heat resulted in a reduction in the number of DCP1–GFP and DCP2–GFP foci (**Fig. 1A, C**, right-hand panels). The number of DCP1–GFP foci decreased to almost the same level as that before heat exposure (**Fig. 1B**). DCP2–GFP foci disassembled and spread throughout the cytoplasm (**Fig. 1C**, right-hand panel, **D**).

Heat treatment induces DCP2 assembly into DCP1 foci

We generated a DCP1–GFP/DCP2–RFP transgenic plant in which DCP1 and DCP2 were tagged with different colored fluorescent proteins to visualize DCP1 and DCP2 simultaneously. We chose root cells to observe co-localization since they are not affected as much by autofluorescence compared with cells from aerial plant parts. To obtain strong and well-focused fluorescent signals, we concentrated on a single plane

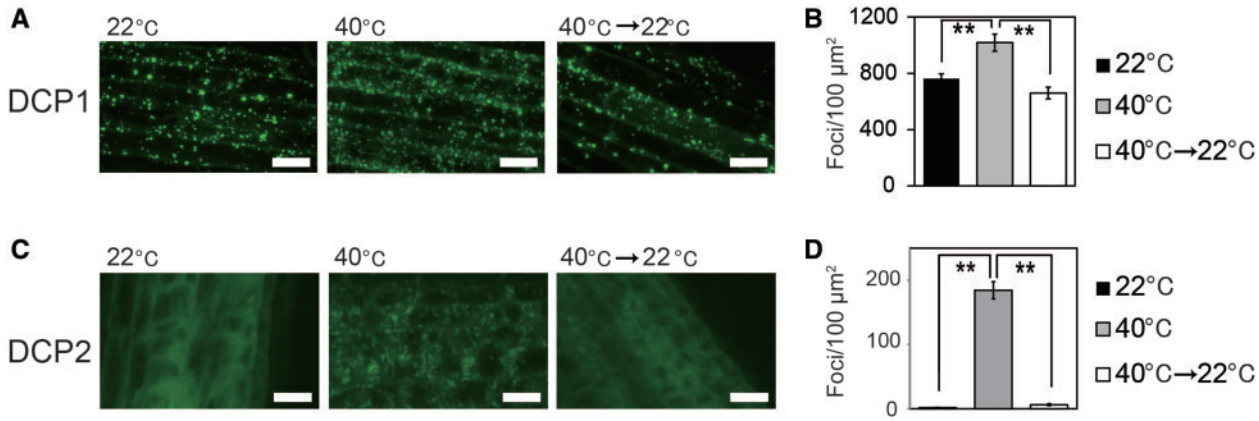

Fig. 1 Distributional changes of DCP1 and DCP2 during heat treatment in roots. (A) DCP1 localization in roots at 22°C (left), 40°C (center) and 22°C after exposure to 40°C (right). (B) The number of DCP1–GFP foci during temperature changes. (C) DCP2–GFP localization in roots at 22°C (left), 40°C (middle) and 22°C after exposure to 40°C (right). (D) The number of DCP2–GFP foci during temperature changes. Fluorescence images were obtained from root epidermal cells using three-dimensional optical sections. $^{**}P < 0.01$. Scale bars = 20 μm.

of outer cortical regions of root epidermal cells. Heat treatment increased the number of DCP1–GFP and DCP2–GFP foci at cortical regions, as determined in three-dimensional optical sections (**Supplementary Fig. S2A–D**).

In DCP1–GFP/DCP2–RFP plants, only a fraction of DCP2–RFP signals was co-localized with DCP1–GFP at 22°C (**Fig. 2A**, arrowheads), and most DCP2–RFP signals diffused throughout the cytoplasm (**Fig. 2A**). Statistical analysis revealed that only 17.9% of DCP1–GFP foci contained DCP2–RFP signals at 22°C while >80% of DCP1–GFP foci were free of DCP2 signal (**Fig. 2A**, left- and right-hand panels, **C**). Heat treatment at 40°C for 90 min induced DCP2–RFP foci formation and altered their sizes so they were similar to DCP1–GFP foci (**Fig. 2B**). The percentage of DCP2–RFP foci that were co-localized with DCP1–GFP foci was 68.9% after exposure to heat (**Fig. 2C**). Additionally, we found that 13.2% of DCP2–RFP foci were independent of DCP1–GFP at 40°C (**Fig. 2C**).

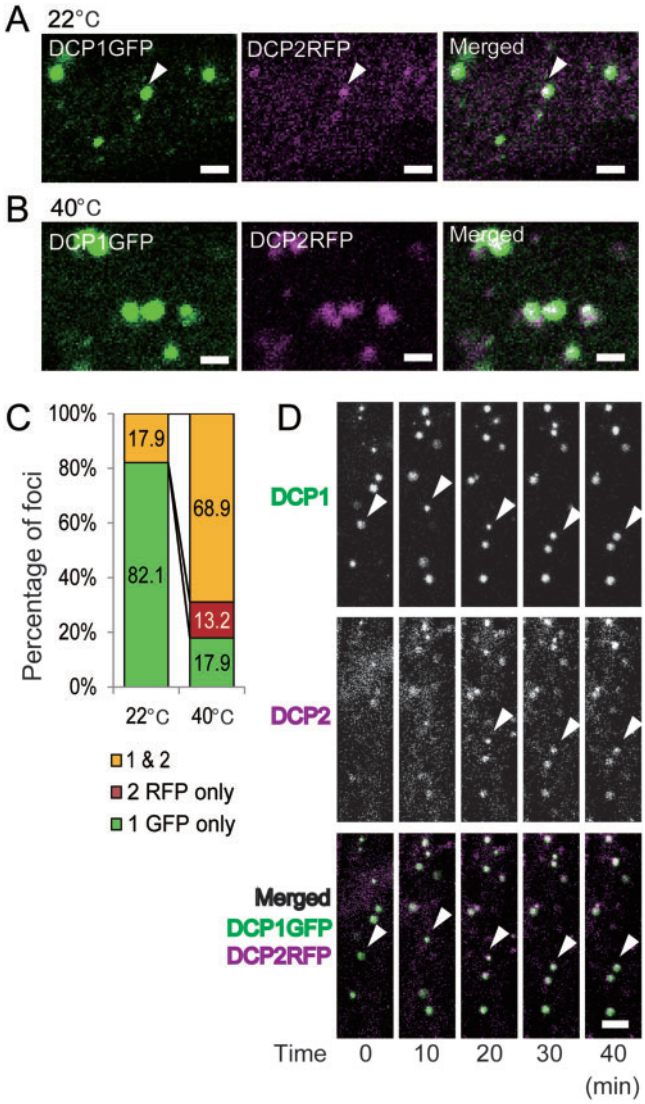

Fig. 2 Co-localization analyses of DCP1–GFP and DCP2–RFP during heat treatment. (A) Localization of DCP1–GFP (green) and DCP2–RFP (magenta) at 22°C. Arrowheads indicate co-localized foci. Scale bars = 2 µm. (B) Localization of DCP1–GFP (green) and DCP2–RFP (magenta) at 40°C. Scale bars = 2 µm. (C) Quantitative analysis of DCP1–GFP and DCP2–RFP localization. The total number of analyzed foci was 832 at 22°C and 1,311 at 40°C. (D) Time-lapse observation of DCP1–GFP and DCP2–RFP during heat treatment. DCP1–GFP and DCP2–RFP are shown in green and magenta, respectively, in a merged row. Arrowheads indicate co-localized foci. Seedlings grown at 22°C were subjected to heat treatment at 40°C for 40 min. Scale bars = 5 µm. Fluorescence images were obtained from cortical regions of the root epidermis.

To eliminate the possibility that the observed differences were caused by the differences in the properties of fluorescent proteins, we switched the fluorescent protein fused to DCP1 or DCP2, and observed DCP1 and DCP2 behavior in transgenic plants (DCP1–RFP/DCP2–GFP). Approximately 85% of DCP1–RFP foci were free of DCP2–GFP (**Supplementary Fig. S3A, C**); this was similar to what was observed in DCP1–GFP/DCP2–RFP plants (**Fig. 2A, C**). The percentage of DCP1–RFP and DCP2–GFP foci that co-localized increased with heat treatment from 14.4% at 22°C to 53.1% at 40°C (**Supplementary Fig. S3B, C**). These results revealed that heat treatment increased the percentage of co-localization. We hypothesized that DCP2 assembles with DCP1 foci during heat treatment.

To test the hypothesis that DCP2 assembles with preformed DCP1 foci during heat treatment, we performed a time-lapse analysis of DCP1 and DCP2 localization during heat treatment. Most DCP2 signals were spread throughout the cytoplasm before heat treatment (**Fig. 2D**, 0 min). After exposure to heat for 20 min, diffuse signals of DCP2 started to accumulate near DCP1–GFP foci. The greatest accumulation occurred during heat exposure for 40 min (**Fig. 2D**, 40 min). DCP1–GFP foci moved throughout the cytoplasm before heat exposure, but gradually became immobile. Some DCP1–GFP foci appeared to fuse with each other (**Supplementary Video S1**). We accordingly found that the size of DCP1–GFP foci increased during heat treatment (**Supplementary Fig. S2E**). These results supported the hypothesis that heat treatment induces DCP2 assembly into pre-existing DCP1–GFP foci.

Cycloheximide inhibits the assembly of DCP1 and DCP2 triggered by heat treatment

Cycloheximide (CHX) treatment leads to a decrease in the number of P-bodies in eukaryotes because CHX blocks mRNA release from polysomal complexes (Ferraiuolo et al. 2005, Franks and Lykke-Andersen 2007, Swetloff et al. 2009). We examined whether CHX treatment inhibits DCP1 and DCP2 foci accumulation before and after heat treatment. As in previous reports (Goeres et al. 2007, Weber et al. 2008, Perea-Resa et al. 2012), CHX treatment significantly reduced the number of DCP1–GFP foci at 22°C ($P < 0.01$) (**Fig. 3A, C**). Furthermore, plants treated with CHX at 40°C had significantly fewer DCP1–GFP foci compared with untreated plants both at 22°C and at 40°C ($P < 0.01$) (**Fig. 3A–C**).

There were relatively few DCP2–GFP foci at 22°C, as stated above. CHX treatment had no effect at this temperature (**Fig. 3D, F**). However, CHX treatment significantly inhibited heat-induced DCP2–GFP foci accumulation at 40°C (**Fig. 3E, F**). These results suggest that heat-induced DCP1–GFP and DCP2–GFP foci are RNA granules encompassing

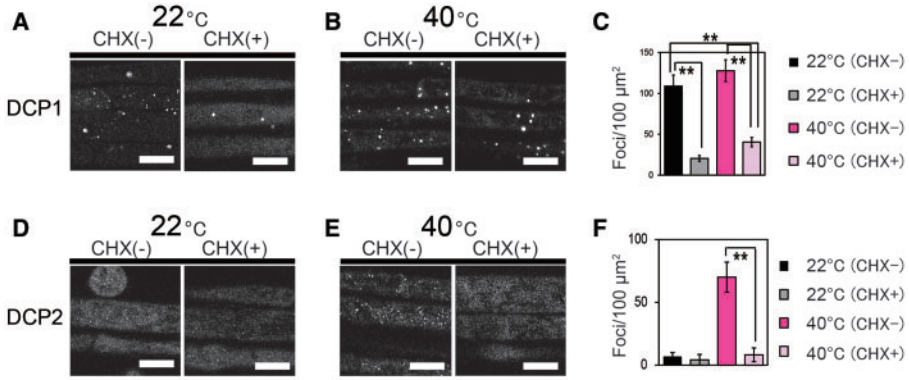

Fig. 3 Effect of CHX on DCP1 and DCP2 assembly. (A) DCP1 localization in cortical regions at 22°C in the absence (left) or presence of CHX (right). (B) DCP1 localization in cortical regions at 40°C in the absence (left) or presence of CHX (right). (C) The number of DCP1–GFP foci during temperature changes in the absence or presence of CHX. (D) DCP2 localization in cortical regions at 22°C in the absence (left) or presence of CHX (right). (E) DCP2 localization in cortical regions at 40°C in the absence (left) or presence of CHX (right). (F) The number of DCP2–GFP foci during temperature changes in the absence or presence of CHX. Fluorescence images were obtained from cortical regions of the root epidermis. $^{**}P < 0.01$. Scale bars = 20 μm.

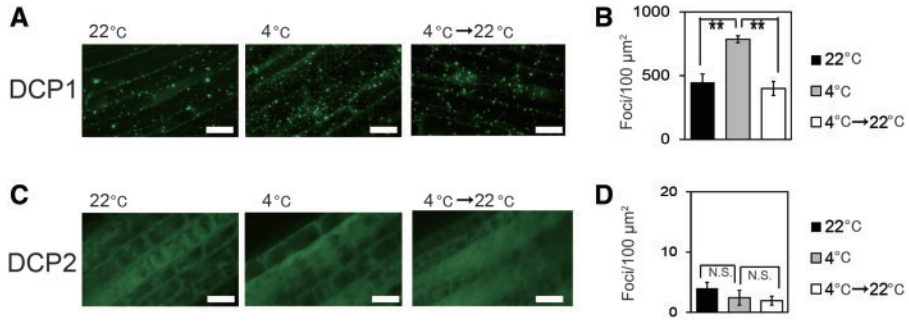

Fig. 4 Distributional changes of DCP1 and DCP2 during cold treatment in roots. (A) DCP1 localization in roots at 22°C (left), 4°C (middle) and 22°C after exposure to 4°C (right). (B) The number of DCP1–GFP during temperature changes. (C) DCP2–GFP localization in roots at 22°C (left), 4°C (middle) and 22°C after exposure to 4°C (right). (D) The number of DCP2–GFP foci during temperature changes. Fluorescence images were obtained from root epidermal cells using three-dimensional optical sections. $^{**}P < 0.01$. N.S., not significant. Scale bars = 20 μm.

certain mRNAs; they are similar to P-bodies reported in yeast and animals.

Cold treatment induces only DCP1 assembly

It was reported that cold treatment triggers some responses such as change of gene expression (Yamaguchi-Shinozaki and Shinozaki 2006), but no one has investigated P-body response to cold in plants. Therefore, we exposed DCP1–GFP and DCP2–GFP plants to 4°C and observed the behaviors of DCP1 and DCP2 under cold stress. First, we examined DCP1–GFP in root elongation zones using three-dimensional optical sections. The number of DCP1–GFP foci significantly increased during cold treatment ($P < 0.01$; **Fig. 4A**, center panel, **B**). The increase in the number of DCP1–GFP foci during cold treatment was also observed in leaf epidermal cells (**Supplementary Fig. S4A, B**). After induction of DCP1–GFP foci formation by cold treatment, DCP1–GFP plants were returned to 22°C and kept at this temperature for 5 h. Removal from the cold induced a significant decrease in the number of DCP1–GFP foci ($P < 0.01$; **Fig. 4A**, right-hand panel, **B**) to almost the same level as that before cold treatment.

In contrast, cold treatment did not induce accumulation of DCP2–GFP into foci. Instead, exposure to cold resulted in a slight decrease in the number of DCP2–GFP foci in roots (**Fig. 4C**, center panel, **D**) and in leaves according to three-dimensional optimal sections (**Supplementary Fig. S4C, D**), although this decrease was not significant. Returning the cold-treated plants to 22°C did not result in any noticeable changes (**Fig. 4C**, right-hand panel, **D**). We also confirmed that the number of DCP1–GFP foci significantly increased in cortical regions of root epidermal cells following cold treatment ($P < 0.001$; **Supplementary Fig. S5A, B**), while the number of DCP2–GFP foci significantly decreased ($P < 0.05$; **Supplementary Fig. S5C, D**).

When we subjected DCP1–GFP/DCP2–RFP plants to cold treatment, we observed that a few DCP2–RFP signals were co-localized with DCP1–GFP at 22°C in cortical regions of root epidermal cells (**Fig. 5A**, arrowhead). We found that 25.1% of foci included both DCP1–GFP and DCP2–RFP signals (**Fig. 5C**). Cold treatment produced more diffuse DCP2–RFP signals (**Fig. 5B**). It also lowered the percentage of DCP1–GFP and DCP2–RFP signal co-localization to only 10.9 % (**Fig. 5C**); this decrease resulted from the increase in the number of

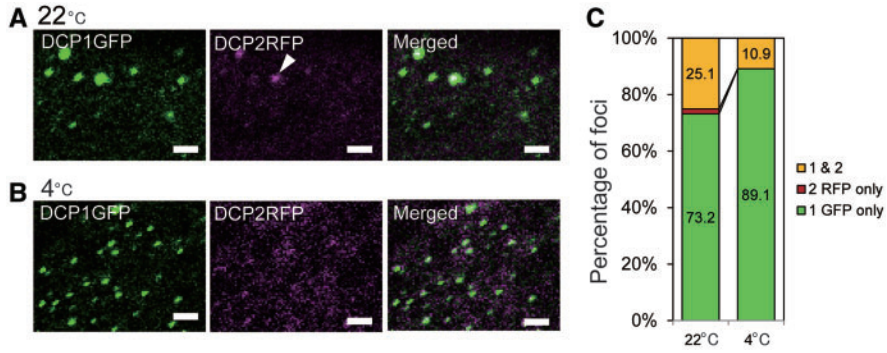

Fig. 5 Co-localization analyses of DCP1–GFP and DCP2–RFP during cold treatment. (A) Localization of DCP1–GFP (green) and DCP2–RFP (magenta) at 22°C. (B) Localization of DCP1–GFP (green) and DCP2–RFP (magenta) at 4°C. (C) Quantitative analysis of DCP1–GFP and DCP2–RFP localization. The total number of analyzed foci was 2,074 at 22°C and 1,087 at 4°C. Fluorescence images were obtained from cortical regions of the root epidermis. Scale bars = 2 μm.

DCP1–GFP foci and the decrease in the number of DCP2–RFP foci. An increase in the number of DCP1 foci during cold treatment was also observed in DCP1–RFP/DCP2–GFP plants (**Supplementary Fig. S6**).

DCP1 and DCP2 protein levels are not changed by heat or cold treatment

There are two possible explanations for the observed changes in the number or size of foci during exposure to high or low temperatures. One is that DCP1/DCP2 proteins underwent altered localization, which led to assembly/disassembly of foci in the cytoplasm. The other is that de novo DCP1/DCP2 synthesis or degradation occurred. To assess which of these possibilities was responsible for our observations, we performed Western blot analyses to determine in planta DCP1 and DCP2 protein levels before and after heat or cold treatments.

We detected two different DCP1 bands (**Fig. 6A**); one is the phosphorylated form of the protein and the other is the non-phosphorylated form. Whether the proteins were phosphorylated was confirmed using an established phosphatase treatment (Xu and Chua 2012) (data not shown). Similar double bands were observed in previous studies of eukaryotes (Beelman et al. 1996, Rzeczkowski et al. 2011, Aizer et al. 2013, Chiang et al. 2013) and plants (Xu and Chua 2012). The total DCP1 protein level was not altered by temperature changes from 22 to 40°C and back to 22°C (**Fig. 6A**). Similarly, DCP2 was detected at almost the same level at 22, 40 or at 22°C following exposure to 40°C (**Fig. 6B**).

When we subjected plants to 4°C, the total abundance of DCP1 and DCP2 proteins did not change during temperature changes from 22 to 4°C and back to 22°C (**Fig. 6C, D**). These results supported the notion that cytoplasmic localization changes of DCP1 and DCP2 proteins are responsible for DCP1 and DCP2 foci formation or dissociation during changes in environmental conditions.

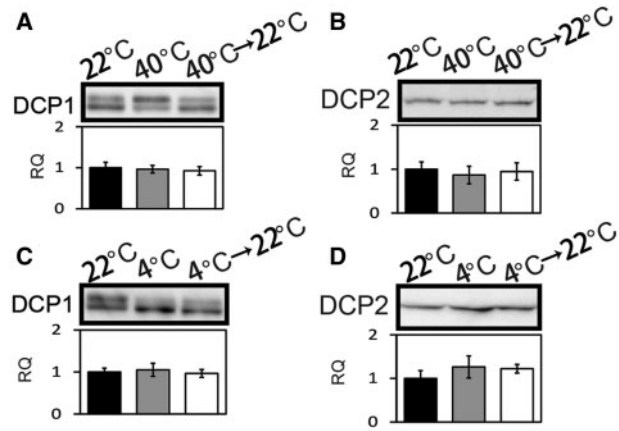

Fig. 6 Western blot analyses of DCP1 and DCP2 during heat or cold treatment. (A) DCP1 protein levels in plants at 22°C (left), 40°C (middle) and 22°C after exposure to 40°C (right). (B) DCP2 protein levels in plants at 22°C (left), 40°C (middle) and 22°C after exposure to 40°C (right). (C) DCP1 protein levels in plants at 22°C (left), 4°C (middle) and 22°C after exposure to 4°C (right). (D) DCP2 protein levels in plants at 22°C (left), 4°C (middle) and 22°C after exposure to 4°C (right). Total DCP1 abundance was calculated based on the sum of the intensities of the two DCP1 bands. RQ, relative quantity.

Discussion

It was generally thought that DCP1 and DCP2 consistently form complexes. Thus, many researchers have used DCP1 and DCP2 proteins as P-body markers in plants without differentiating between the two proteins (Xu and Chua 2011). Our observations revealed that DCP2 disperses throughout the cytoplasm at 22°C, but assembles into distinct foci at 40°C (**Fig. 1C**). In contrast, DCP1 forms distinct foci at both 22 and 40°C (**Fig. 1A**). Co-localization analyses in this study indicated that we cannot discriminate between foci that differ in DCP1 and DCP2 composition if we focus only on DCP1 or DCP2. We define foci as P-bodies if they include DCP1 and/or DCP2; this definition is based on earlier studies (Parker and Sheth 2007, Xu and Chua 2011). We refer to these possible P-bodies with different compositions as DCP1 bodies, DCP2 bodies and DCP1/2 bodies. For example, DCP2 accumulates in DCP1 during heat treatment, and this complex becomes a DCP1/2 body (**Fig. 2D**).

Fig. 7 presents our model showing possible events occurring in a cell and is based on our results. At 22°C, most DCP1 form

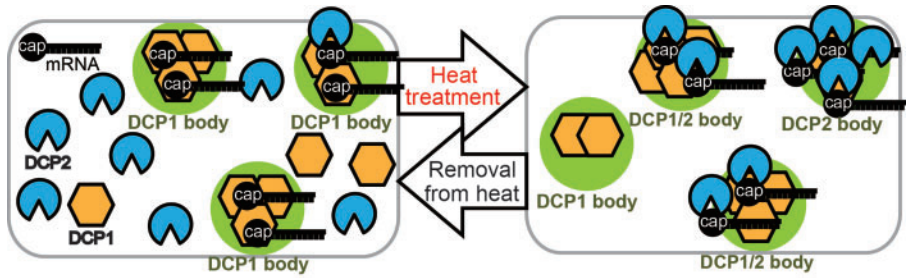

Fig. 7 Diagram of proposed P-body formation during heat treatment. At 22°C, most DCP2 are present throughout the cytoplasm, but DCP1 is localized as cytoplasmic foci (DCP1 bodies) along with mRNA (left part). Once subjected to 40°C, DCP2 is assembled with DCP1 bodies to form DCP1/2 bodies. DCP1 also assembles with other DCP1 foci or forms new DCP1 bodies after exposure to 40°C (right part). Decapping enzymes and heat stress.

DCP1 bodies possibly encompassing certain mRNAs. Most DCP2 spread throughout the cytoplasm (**Fig. 7**, left). Once subjected to 40°C, DCP2 assemble with DCP1 bodies, resulting in DCP1/2 bodies (co-localization form). DCP1 proteins also assemble with each other, leading to an increase in the number of DCP1 bodies (**Fig. 7**, right).

Our observation that DCP2 was localized throughout the cytoplasm was inconsistent with previous studies that indicated that overexpressed DCP2 formed foci as did DCP1 (Xu et al. 2006, Goeres et al. 2007). This inconsistency may result from the differences in the promoters used; we used the DCP2 native promoter, while constitutive promoters such as the 35S promoter were used in previous studies.

It was believed that DCP1 and DCP2 behaved similarly in response to stress. In this study, when plants were subjected to 4°C, the number of DCP1 bodies increased (**Fig. 4A**). In contrast, there were very few DCP2 bodies, if any, at 22°C, and none in cells of plants exposed to 4°C (**Fig. 4C**; **Supplementary Fig. S5C**). Such differences in behavior of DCP1 and DCP2 have never been described in other eukaryotes. The results suggest that DCP1 can form DCP1 bodies independently of DCP2 and that these proteins behave differently in response to cold. Interestingly, heat and cold treatments resulted in completely opposite changes in DCP2 bodies.

Although some reports have suggested the importance of P-body components for stress responses, their exact role during stress is currently unknown, (Xiong et al. 2001, Xu and Chua 2012). Recently, it was reported that heat stress possibly promotes 5′–3′ mRNA degradation (Merret et al. 2013). DCP2, which is the catalytic core of the decapping reaction, assembles with DCP1 bodies during heat treatment, suggesting that DCP1/2 body formation enhances mRNA decay through decapping of a group of mRNAs that is not beneficial for heat adaptation. The down-regulation of many transcripts during heat stress supports this hypothesis (Larkindale and Vierling 2008). A fraction of DCP2 signals is co-localized with DCP1 bodies at 22°C, suggesting that a certain level of mRNA degradation occurs even at this temperature (**Fig. 2A**, arrowheads).

In contrast, cold-induced DCP1 bodies may have functions in addition to or other than mRNA degradation, thus explaining the increase in the number of DCP1 bodies with few DCP2 during cold treatment. It is possible that DCP1 bodies repress translation and induce mRNA storage during cold stress until the plant returns to a normal ambient temperature where mRNAs can be translated again. In fact, it has been reported in yeast that P-body mRNAs are translationally inactive during exposure to stress (Brengues et al. 2005) and that a subset of mRNA exits the P-bodies to be translated after the stress is removed (Arribere et al. 2011). Cold treatment leads to stabilized mRNAs in *A. thaliana* (Chiba et al. 2013). These results support a hypothesis that cold-induced DCP1 bodies take mRNAs from ribosomes, protect them from RNases and store them so they are ready for prompt translation at a later time. DCP2 bodies at 40°C may also have functions in addition to mRNA degradation.

Taken together, our results show that changes in DCP1 and DCP2 localization occur during temperature changes; these proteins do not consistently form complexes. We cannot discriminate DCP1 bodies, DCP2 bodies and DCP1/2 bodies from other RNA granules such as stress granules. It is possible that P-body components and stress granules co-localize in some conditions; P-bodies and stress granules share several protein components in mammalian cells (Kedersha et al. 2005). The identification of each P-body component will help to uncover the roles of different types of P-bodies in stress adaptation in plants. How the RNA degradation pathway can degrade specific mRNAs in particular environmental conditions is not known. It is possible that some protein modifications trigger P-body formation because, during heat treatment, DCP1 was phosphorylated concomitantly with DCP2 assembly. Additionally, a more detailed examination of DCP1 and DCP2 will help to clarify the mechanism mediating the possible selective degradation of mRNA.

Materials and Methods

Plants and growth condition

We used heterozygotes and homozygotes of *A. thaliana* mutant lines, *dcp1-1* (GABI_844B03) and *dcp2-1* (SALK_000519). Plants were grown in pots with soil at 22°C under continuous light. Plants for microscopic or protein analyses were grown on agar-solidified half-strength Murashige and Skoog (MS) medium at 22°C under continuous light as described (Kumakura et al. 2013).

Plasmid construction

We constructed pGWB-DCP1-GFP and pGWB-DCP2-GFP vectors to generate DCP1–GFP- and DCP2–GFP-expressing transgenic plants, respectively, as

described (Motomura et al. 2012). For pGWB-DCP1-tagRFP, we amplified a DNA fragment containing the DCP1 promoter region (2,063 bp) and the open reading frame (ORF) from *A. thaliana* Col-0 genomic DNA using the caccDCP1 Left border and the caccDCP1 Right border primers (**Supplementary Table S1**). For pGWB-DCP2-tagRFP, a DNA fragment containing the DCP2 promoter region (2,555 bp) and the DCP2 ORF from Col-0 genomic DNA were amplified using the caccDCP2 Left border and the caccDCP2 Right border primers (**Supplementary Table S1**). We introduced these PCR products into pENTR/D-TOPO (Invitrogen). The DNA fragments were then transferred into pGWB459 and pGWB559 by in vitro recombination (Nakagawa et al. 2007) using LR Clonase (Invitrogen) to construct pGWB-DCP1-tagRFP and pGWB-DCP2-tagRFP.

Plant transformations and crosses

We introduced pGWB-DCP1-tagRFP and pGWB-DCP2-tagRFP into *Agrobacterium tumefaciens* strain GV3101 and then transformed them into *dcp1-1* heterozygotes and DCP1–GFP plants, respectively, by the floral dip method as previously described (Tsuzuki et al. 2014). Harvested T_1 and T_2 seeds were selected on half-strength MS medium supplemented with kanamycin (20 μg ml^{-1}) or hygromycin B (50 μg ml^{-1}). The selected plants were then subjected to genotyping using the dcp1-1 LP and DCP1_3′UTR-Rv primers (**Supplementary Table S1**) for *dcp1-1* and observed by microscopic analysis. The selected plants were named DCP1–RFP and DCP1–GFP/DCP2–RFP. We then crossed DCP1–RFP and DCP2–GFP plants to create DCP1–RFP/DCP2–GFP plants. Harvested T_2 plants were selected by microscopic analysis. Selected DCP1–GFP/DCP2–RFP and DCP1–RFP/DCP2–GFP plants were used to count cytoplasmic foci. The DCP1–RFP and DCP2–RFP constructs phenotypically rescued the *dcp1-1* and *dcp2-1* homozygote lethal phenotype, respectively. These complemented plants could maintain normal vegetative growth.

Microscopic analyses and stress treatments

We observed 10-day-old plants using three-dimensional optical section images from a confocal fluorescence microscope (LSM510META, ZEISS) (**Figs. 1**, **4**; **Supplementary Figs. S1**, **S4**). Cortical regions of root epidermal cells were observed with a confocal fluorescence microscope (Ti-E, Nikon) equipped with a confocal unit (C2, Nikon) with a perfect focus system (Optical Insight) (**Figs. 2**, **3**, **5**; **Supplementary Figs. S2**, **S3**, **S5**, **S6**). Images were taken with NIS-elements software (Nikon) and analyzed with ImageJ (NIH; http://rsbweb.nih.gov/ij/). We merged three-dimensional optical section images for digital counting. Foci in the images were counted using Analyze Particles or the KbiCountPeaks plugin from the KBI plugins package (http://hasezawa.ib.k.u-tokyo.ac.jp/zp/Kbi/ImageJKbiPlugins). We measured image areas and calculated the number of foci per unit area on each image. For the percentage of co-localization, two separate images were merged using Image Calculator. We confirmed that there was no difference in responses between the aerial and underground plant parts in high and low temperature conditions. We chose to observe root cells more closely as they are not affected by autofluorescence as much as the cells from aerial parts. It would be difficult to distinguish autofluorescence from fluorescence in small foci. Therefore, we counted foci over 2 μm^2 to distinguish the foci from autofluorescence in leaves. Eight independent plants were observed in each condition in **Fig. 1** ($n = 8$). Twelve (22°C) or 23 (40°C) independent plants were observed in **Fig. 2A–C**. Nineteen independent DCP1–GFP plants were observed in each condition in **Fig. 3** ($n = 19$). Nine independent DCP2–GFP plants were observed in each condition in **Fig. 3** ($n = 9$). Seven independent plants were observed in each condition in **Fig. 4** ($n = 7$). Eighteen (22°C) or 13 (4°C) independent plants were observed in **Fig. 5**. We used Welch's *t*-test to analyze results from experiments in which two conditions were compared. Analysis of variance (ANOVA) and Tukey's test were performed in experiments in which multiple comparisons were made. Standard errors were represented by error bars in all experiments.

For heat treatment, we subjected plants on agar-solidified half-strength MS medium at 22°C to 40°C for 90 min in the dark (for three-dimensional optical sections) or under light (for cortical regions). These treatments were similar to those from previous studies (Larkindale and Vierling 2008, Weber et al. 2008, Perea-Resa et al. 2012, Merret et al. 2013, Rasmussen et al. 2013). To enable plants to recover from heat treatment, plants on agar-solidified half-strength MS medium exposed to 40°C for 2 h were maintained at 22°C for 3 h. For time-lapse analysis, DCP1–GFP/DCP2–RFP plants were mounted and observed with a confocal microscope while being subjected to 40°C for 60 min.

To assess the effects of cold treatment, we exposed plants on agar-solidified half-strength MS medium to 4°C for 20 h in the dark. To observe plant responses during recovery from cold stress, plants on agar-solidified half-strength MS medium were maintained at 22°C after exposure to 4°C for 20 h.

For CHX treatment, we grew seedlings in liquid half-strength MS medium supplemented or not with 300 μg ml^{-1} CHX for 3 h at 22°C. For heat treatment, seedlings were incubated for 2 h at 40°C after 1 h incubation at 22°C in the presence of CHX.

Protein extraction and Western blot analysis

Ten-day-old control (grown at 22°C) and treated (heat or cold stressed) DCP1–GFP and DCP2–GFP plants were frozen with liquid nitrogen and ground to a fine powder. We extracted total protein with extraction buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Triton-X100, 5% glycerol, 5 mM diuthiothreitol (DTT) and complete protease inhibitor cocktail (Roche)]. Extracts were centrifuged twice (20,400 × *g* for 5 min) and we analyzed the supernatants by SDS–PAGE. Separated proteins were transferred onto an Immobilon-P Transfer Membrane (Merck Millipore) with transfer buffer [25 mM Tris–HCl (pH 8.0), 200 mM glycine and 15% (v/v) methanol]. After incubating the membrane in blocking buffer [1% skim milk, 1 × phosphate-buffered saline (PBS)–Tween] for 1 h, we incubated the membrane in a 1 : 1,000 dilution (in blocking buffer) of anti-GFP (Roche) overnight at 4°C. The membrane was washed with wash buffer (1 × PBS–Tween) three times and then incubated in a 1 : 5,000 dilution (in blocking buffer) of anti-mouse IgG horseradish peroxidase (HRP; GE Healthcare) for 1 h. We washed the membrane with wash buffer and then incubated it with Luminata Forte Western HRP substrate (Merck Millipore). Chemiluminescence was detected using the LAS-3000 Luminescent Image Analyzer (Fujifilm) and we quantified bands with ImageJ. Three biological replicates, in which the samples were independently collected, were separately analyzed by Western blotting. Standard errors were represented by error bars in all experiments.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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